INTERNATIONAL JOURNAL OF FERTILITY AND STERILITY (Int J Fertil Steril)

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Printing Company: Jurband Ghareprint Co.
NO. 5, Jalil khoob alley, Nirou Havaei Street, Tehran, Iran
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An Update on Ovarian Aging and Ovarian Reserve Tests

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Abstract
Ovaries are the female organs that age more quickly than other tissues such as the uterus, the pituitary gland or pancreas. Different from males, an interesting question is why and how the females lose fertility so rapidly. During the aging process, both the number and quality of the oocytes in the ovaries decrease and reach a point beyond that no more viable offspring may be produced and the associated cyclic endocrinological activities cease, entering the menopause in females at an average age of 50 years. Females who delayed childbearing with or without their willing until their 30 years or 40 years constitute the largest portion of the total infertility population. Ovarian reserve tests (ORTs) provide an indirect estimate of a female’s diminishing ovarian reserve or remaining follicular pool. This article briefly reviews recent progresses in relation to ovarian aging and ORTs.

Keywords: Ovarian Aging, Infertility, Menopause, ROC Curve, Anti-Mullerian Hormone

Introduction
Physiologic ovarian aging is defined by age-specific declines of functional ovarian reserve within expected ranges. Scientific reports have long indicated that female ovarian reserve declines progressively with increasing chronological age. Fecundity, in both natural and stimulated ovarian cycles, reduces with maternal age that is why optimal fertility is accepted to be between 20 and 30 years old (1-3). In relation to this issue, the common concept of female reproductive aging assumes that the decline of reproductive potential or quantity and quality of oocyte/follicle pool estimates an age-dependent loss of female fertility. Because ovaries undergo much more serious efforts of aging than any other tissues of the female body, offspring has been demonstrated to be inversely proportional to age as shown in figure 1.

Usually healthy female possesses ~400,000 primordial follicles at the beginning of puberty, each of which contains an immature ovum. About 300 to 400 follicles reach maturity during the reproductive life span of an adult female. The rest of the follicles are lost with apoptosis, which continue approximately for seven months during periods when there is no ovulation, such as pregnancy, breastfeeding or use of oral contraceptives. The most of oocytes are lost via apoptosis which is a more accelerated process in the last 10-15 years before menopause (4). The age-related decline of female fertility is frequently associated with the reduced monthly likelihood of conception and the increased probability that a pregnancy will terminate (e.g. the loss of embryo, pregnancy, fetal, and spontaneous abortion) sooner or later after conception or implantation between the ages of 35-45. In addition to these, scientific reports confirmed that the probability of achieving a pregnancy within one year was significantly higher in women <30 years than those in women >35 years (5, 6). When a female reaches the mean age of 45, follicle pool usually decreases below a critical value of ~1000 or less follicles and irregular cyclic changes exist as the first clinical sign of ovarian aging (1, 6). Along with these statements, female reproductive aging is nearly associated with a dysregulation
of the gonadotrophin releasing hormone (GnRH) pulse generator in the hypothalamus due to a progressive lack of neuro-endocrine control from other brain parts, resulting in changes in the regular GnRH pulse pattern. The first sign of this change is the early elevation of follicle stimulating hormone (FSH) leading to acceleration of follicle depletion (1, 6, 7). Age-related changes in neuroendocrine response also contribute to the decline in reproductive function. There are two major theories on the origin of ovarian aging. One theory is that it is driven by the ovary itself. The rise of FSH is only secondary to loss of ovarian follicles and reduction of inhibin level. The other theory is that dysregulation of hypothalamic GnRH production, leading to rise in FSH levels and increase loss of follicles, causing ovarian aging.

There is a large individual variability that exists in the age at which the various reproductive events occur in the context of normal reproductive female ovarian aging. In relation to issue, various gynecologic disorders or diseases and treatments, environmental and genetic factors contribute to biological ovarian aging and ovarian reserve decreasing. These factors include ovarian toxicants, cigarette smoking, alcohol abuse or chronic alcoholism, nutritional deficiencies, oxidative stress, some metabolic disorders, autoimmunity, long term stress-depression, iatrogenic treatments (pelvic surgeries, chemotherapy and radiotherapy), ovary inflammation and pelvic infection or tubal disease, severe endometriosis, meiotic division errors, chromosomal abnormalities, gene and mitochondrial DNA mutations in oocytes, and family history of early menopause in connection with the ovary aging and depletion of ovarian follicles and reduced ability to produce oocytes competent for fertilization and further development as well as infertility (3, 8-15).

The probability of spontaneous conception (fecundity) and infertility treatment success depend on functional ovarian reserve particularly and total ovarian reserve. As both parameters decline with advancing age, pregnancy chances, thereupon, decrease in parallel (16-26). Age is obviously known to be the most important factor determining the pregnancy potential in normally cycling females. Therefore, chronological age alone has a limited value in predicting individual responses. Age is the main determinant of the chance of successful pregnancy. Moreover, the quantitative response to ovarian stimulation with gonadotrophins depends on the ovarian reserve. This depends on age, genetic and some environmental factors. It means that 95% of reproductive aging is determined by age and genetics, and less than 5% is determined by environmental factors (6, 27). The decline rate of ovarian reserve varies among females, making it a challenge to estimate an individual female’s remaining reproductive function. Therefore, it is necessary to evaluate females’ reproductive potential prior to infertility treatment in order to succeed (11). Hence, ultrasonography, various biochemical and histo-pathological markers (tests) have become popular in the last years in assessment of ovarian reserve. The aim of these tests is to contribute to counseling and treatment of infertile couples (1, 2, 17, 28).

Ovarian reserve tests (ORTs)

Anti-mullerian hormone (AMH)

AMH is a dimeric glycoprotein expressed by granulose cells of pre-antral and early antral follicles of ovary during the female reproductive
Ovarian Aging and ORTs

life span. AMH level in blood is considerably low before puberty, but after puberty, it reaches a maximum level and then its concentration progressively declines as a sign of exhaustion of total follicular reserve throughout reproductive life, reaching undetectable values by menopause (14, 18). Studies have shown that AMH is a better marker than antral follicle count (AFC), baseline FSH, estradiol (E$_2$) and inhibin B in estimating ovarian reserve. Age-related decrease in the number of oocytes leads to a decrease in E$_2$ and inhibin B levels, as a result of which FSH rises. In addition, AMH levels correlate strictly with AFC and age. Recent reports have indicated that AMH, indeed, can be used as a test for ovarian aging and reserve as it can be measured in blood during any phase of the menstrual cycle (17-20). AMH appears to be the best biochemical-endocrine marker in assessing the age-related decline of the ovarian pool and predicting the ovarian response of induced patients including poor and hyper-responses of in vitro fertilization (21).

**Antral follicle count**

Ovarian volume and AFC, evaluated by transvaginal ultrasonography, provide direct ovarian assessments. The latter, defined as the number of follicles smaller than 10 mm in diameter in the early follicular phase, is considered to have the best discriminating potential for a poor ovarian response compared to the total ovarian volume and basal serum values of FSH, E$_2$ and inhibin B on 3rd day of the cycle. Therefore, it is accepted to be predictor of the number of oocytes remaining. AFC also reflects the ovarian reserve better than ovarian volume in infertile patients (2, 14, 17).

**Basal follicle stimulating hormone**

Elevation in basal FSH level, occurring usually at the ages of 35-40, is the first sign of ovarian aging that can be detected in females. Early follicular phase (basal) or cycle day-3 FSH level is an indirect marker of ovarian reserve and reflects the negative feedback effects of inhibin-B and E$_2$ on hypophysis (2, 4, 14). An increase in blood FSH levels occurs due to follicle depletion. In females with regular cycling, very high FSH levels may predict a poor response, thus this can be useful in screening of a small infertile group. Along with other markers, it may be used to counsel families about poor response (2).

**Basal estradiol**

Early rise in blood E$_2$ (17β-estradiol is derived almost exclusively from the ovaries, and its measurement is frequently considered sufficient to evaluate ovarian function) level is known as a consequence of the advanced follicular development and early selection of a dominant follicle observed in cycling females with increased FSH levels (22). A combination of FSH and E$_2$ in screening for declined ovarian reserve seems to be more sensitive than either test alone. However, basal E$_2$ level has little value as an ovarian reserve test and its routine use is not advised (2, 17).

**Inhibin B**

Inhibin B is a heterodimeric glycoprotein released by the granulose cells of the follicles, and its concentration peaks during the follicular phase. The inhibin B level has been used in conjunction with serum FSH and E$_2$ to assess ovarian function. A decline in inhibin B concentrations in early follicular phase may be observed before an increase in FSH level. Inhibin B seems to be a good indicator of ovarian activity, whereas it has minimal value in predicting ovarian reserve, so that its routine use is not recommended (1, 2, 17).

**Gonadotrophin releasing hormone agonist stimulation test (GAST)**

GAST is associated with the assessment of serum E$_2$ on days 2-3 of the cycle following subcutaneous application of 100 µg GnRH agonist (e.g. Triptorelin). The response of E$_2$ to GnRH agonist is an indirect indicator of ovarian reserve. As GnRH agonists may lead to decrease E$_2$ elevation when the follicular cohort is small in ovarian tissue; an increase in serum E$_2$ is considered to be indicative of good ovarian function. Although this test seems to be valuable in prediction of poor ovarian reserve, it is not superior to AMH, AFC or inhibin B in this theme (2, 14, 17).

**Exogenous follicle stimulating hormone ovarian reserve test (EFORT)**

EFORT involves the measurement of basal FSH and E$_2$ following the administration of 300 IU FSH on the 3rd day of the menstrual cycle. The change
in basal FSH and a rise in E2 levels (>30 pg/ml) 24 hours after FSH administration, may predict ovarian reserve. However, the authors did not recommend this test alone for identification of hyper-responders in assisted reproductive technologies (ART) cycles (2, 14, 17).

**Clomiphene citrate challenge test (CCCT)**

CCCT is a provocative test aimed to assess ovarian reserve. In this test, 100 mg CC is administered daily from day 5 to day 9 of the cycle. Day-3 FSH and E2 levels are measured and followed by the administration of CC from day 5 to day 9. FSH-E2 measurements are repeated on day 10 and high day-10 FSH level suggests poor ovarian reserve. CCCT effectively reflects the quantity and quality of the recruited oocytes, but its predictive value is low, while it is expensive and more time consuming. Also, meta-analysis has reported that CCCT is not better than basal FSH in predicting a clinical pregnancy (2, 17, 23).

**Ovarian biopsy**

Studies on ovarian biopsy which is done at laparotomy or laparoscopy have indicated that follicular density declines with age and is correlated with the ovarian volume in females >35 years. However, the distribution of follicles is not uniform within the ovary, so that the biopsy is not able to represent the true follicular density. Therefore, ovarian biopsy is rarely necessary and it is not recommended as an ORT (2, 17).

**Genetic markers of ovarian reserve**

With developments in molecular genetics has given hope to researchers about prediction of single nucleotide polymorphisms (SNPs) in gonadotropins and their receptor genes, BMP-15, GDF9, FMR1 gene, MCM8 gene, and the other candidate genes which identify females with a genetic predisposition to early ovarian aging (6, 24, 25). There are currently no reliable genetic markers of ovarian reserve that can be used as a routine test (screening/diagnostic).

**Conclusion**

The current literature reports and meta-analysis ROC curves indicate that AMH and AFC are currently promising predictor tests, and FSH is a screening test widely used for assessment of diminished ovarian reserve in addition to chronological age. AMH can be applied to all females to identify decreased ovarian reserve before it reaches a critical low value (Fig.2) (1, 26-28).

Increasing female education and their career levels, and participation in the labor force are important trends in freedom of females currently taking place in most countries including Turkey. A farseeing of this societal adjustment involves extraordinary changes in reproductive behavior such as consciously choosing a life without children and delays in childbearing. A number of females also decide not to have a child at younger ages, but change their minds at later ages. It is known that some tissue/cell functions and aging are different in females and males. Though oocytes/germ cells are not absolutely required for the living, reproduction is critically important for the survival of the population of the living species in the world. A healthy baby should be a nice heritage for families, so it may be suggested to females to deliver a baby or babies before 30 years because of the possible ovarian aging.

**Acknowledgements**

The authors would like to thank Dr. Emel Kurtoğlu for providing review of this article. The
authors declare no financial support and conflicts of interest in this article.

References

Oxidative Stress during Ovarian Torsion in Pediatric and Adolescent Patients: Changing The Perspective of The Disease

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Abstract

Among the different causes of gynecological acute pelvic pain, ovarian torsion represents a surgical emergency. It is a rare case in the pediatric/adolescent aged group that must be included in the differential diagnosis of any girl with abdominal pain or pelvic/abdominal mass. Current recommendations suggest that laparoscopic detorsion should be performed in order to preserve the integrity of the ovaries and fertility, although oophoropexy may be considered in case of severe necrosis. Nevertheless, maintaining the circulation of the ovary after detorsion deteriorates the tissue injury and leads to a pathologic process called ischaemia/reperfusion (I/R) injury, which is characterized by oxidative stress. During the detorsion process, an excess amount of molecular oxygen is supplied to the tissues, and reactive species of oxygen (ROS) such as superoxide radical (\(O_2^-\)), hydrogen peroxide (\(H_2O_2\)), hydroxyl radical (\(OH^*\)), as well as reactive nitrogen species (RNS) are produced in excess. ROS, RNS and their toxic products cause DNA damage and lipid peroxidation in the cellular and mitochondrial membranes, leading to cell death. In spite of attention on this topic, currently there is no shared and clear evidence about the use of anti-inflammatory and antioxidant agents to prevent I/R damage after laparoscopic ovarian detorsion. Considering this element, future research should aim to develop shared protocols for the clinical use (route of application, dosage and time of application) of antioxidants after laparoscopic management of this condition.

Keywords: Paediatrics, Adolescents, Ischaemia/Reperfusion, Oxidative Stress, Antioxidants.

Introduction

Ovarian torsion in pediatric and adolescent patients: what to do?

Among the different causes of gynecological acute pelvic pain, ovarian torsion (1) represents a surgical emergency. It occurs in 2.7% of all pediatric/adolescent population who presents with acute abdominal pain (2, 3). In this regard, torsion of the fallopian tube without torsion of the ipsilateral ovary, as known as Isolated Tubal Torsion (ITT), is an extremely rare event (4, 5) which may be caused by extremely large Morgagni hydatid (6). Furthermore, ovarian torsion must be considered in the differential diagnosis of any girl with abdominal pain or pelvic/abdominal mass (7, 8). Adnexal masses are uncommon events in the pediatric/adolescent population. The estimated incidence is approximately 2.6 per 100,000 girls younger than 18 years of age (9) and 10% of pediatric ovarian masses are found to be malignant (10-12).

According to Millar et al. (13) ovarian cysts are found in 2-5% of prepubertal females undergoing ultrasound scan, although they are mostly small (<1 cm) and insignificant. Considering only the neoplastic disease, it has been estimated that approximately 67% of ovarian cancers in childhood and adolescence are originated from germ cells (14), whereas the classic epithelial ovarian cancer occurs very rarely in pre-menarche (15). In the post-pubertal age, functional ovarian cysts could occur under several conditions: anovulation, excessive stimulation by follicle-stimulating hormone (FSH) or lack of normal luteinizing hormone (LH) peak, estrogens excess without corresponding progesterone influence (13, 16, 17).

Clinical presentation may comprise spotting of moderate vaginal bleeding and acute/chronic pelvic pain (18). In particular, the failure of luteolysis 14 days after the ovulation could cause persistence of theca-lutein cyst, and this condition could also occur in pregnancy for the high level of human chorionic gonadotropin (hCG) which cause hypertrophy of theca cells (19). About 3% of theca-lutein cysts are complicated by torsion or hemorrhage and 30% of these can cause hyperandrogenism (17). Furthermore, the other kind of ovarian cysts may be complicated with infarction and necrosis (20). Adnexal torsion in pediatric/adolescent population is most commonly (approximately 97%) caused by a benign ovarian cyst or teratoma (21). The size of an ovarian cyst has not been shown to correlate with an increased risk of ovarian torsion (22); moreover, ovarian torsions are more likely to result in the right side due to the protective effect of the sigmoid colon (23).

On ultrasound scan, an enlarged ovary and increased volume ratio in comparison to the contralateral ovary is indicative of an ovarian torsion (24). In this regard, ultrasound scan remains the most useful investigation (25-28), although in pediatric/adolescent population blood flow on Doppler examination does not exclude ovarian torsion (18, 23, 29) and sometimes computed tomography (CT) scan is needed (20). Current recommendations of treatment strongly support ovary conservation since macroscopic appearance of the ovary is not a reliable indicator of the degree of necrosis and potentiality for ovarian recovery (8). For children/adolescent with ovarian torsion, laparoscopic detorsion should be performed in order to preserve the integrity of the ovaries and fertility, although oophoropexy may be considered in case of severe necrosis (30, 31).

Nevertheless, in case of laparoscopic detorsion, it must be taken into account that the restoring of ovarian blood flow may be only partial and slow, and in addition to that, the ovarian damage could progressively increase for oxidative stress after reperfusion (32). Considering all these elements, early diagnosis and surgical intervention are essential, especially in adolescents, in order to preserve the anatomy and function of ovaries (1, 33).

Reactive oxygen species and cellular microenvironment: master and minion

Reactive oxygen species (ROS), derived from molecular oxygen, may present as free radicals or other forms; they have electronically unstable and ionized atomic structure, which interacts with biological macromolecules by capturing electrons and interfering with their biological functionality (34-37). There are several enzymatic activities involved in the generation of ROS: the reduction of dioxygen (O₂) in the mitochondria can lead to various intermediate forms of ROS; within peroxisomes, the reduction of amino acid oxidase trig-
Markers of oxidative stress: causes and effects

Since ROS are potentially harmful, the human body has evolved highly complex antioxidant defense systems, both enzymatic and non-enzymatic, which synergistically function in combination. Some antioxidants are produced directly in the intracellular microenvironment (enzymatic antioxidants), such as glutathione oxidase and peroxidase, ubiquinone (CoQ10), α-lipoic acid (ALA), SOD and superoxide catalase (49), while the non-enzymatic antioxidants consist of dietary supplements and synthetic antioxidants such as vitamin C, glutathione, taurine, hypotaurine, vitamin E, Zn, selenium (Se), betacarotene, and carotene (34, 50, 51).

Mechanism of antioxidants’ action depends on their concentration, which is variably presented in fluids and tissues. Multiple redox reactions occur within cell metabolism which can lead to oxidative stress if unbalanced in homeostatic mechanisms. Although cells have several intrinsic antioxidant mechanisms, when ROS are present in large amount, the ability to rebalance homeostasis is exceeded and as a result cellular damage may occur (34-37). An excess of ROS may cause a cascade of events such as the release of Ca²⁺, which results in mitochondrial permeability and provokes mitochondrial membrane instability and consequent cessation of adenosine triphosphate (ATP) production; the lipid peroxidation, which increase the peroxyl radicals, damage of amino acids which leads to the formation of carbonyl groups. Oxidation of the mitochondrial DNA (mtDNA) without protection of histones, does not own any repair mechanisms (43). As result of this complex mechanisms, oxidative stress finally causes DNA damage and/or apoptosis of the cell (52). The total antioxidant status (TAS) was employed to assess the general antioxidative status (53).

Accordingly, total oxidant status (TOS) is obtained to ascertain the overall oxidation status. Represented as the ratio of TOS to TAS, the oxidative stress index (OSI) considered a precise index of oxidative stress (54). As meticulously reviewed by Agarwal et al. (49) oxidative stress seems to play a key role in the physiologic processes of menstrual cycle and ovulation, embryo implant, placental framework development, menopause. Conversely, accumulating evidence suggest that a breakdown in red-ox homeostasis occurs during several reproductive disease such as endometriosis, polycystic ovary syndrome, unexplained infertility, spontaneous abortion, recurrent pregnancy loss, preeclampsia, intrauterine growth restriction.
Oxidative stress during ovarian torsion in pediatric and adolescent patients

The ovaries are organs of constantly predictable change during the reproductive cycle. Their arterial supply comes from two different vessels that anastomose in the mesovarium: the ovarian artery, branch of the abdominal aorta, and the ovarian branch of the uterine artery, that ascends the uterus tortuously to reach the mesovarium. Although ovarian torsion may occur most often in the first 3 decades in normal ovaries, it is more frequent in association with pre-existing tubal/ovarian pathologies (55) for this reason it is a condition which rarely occurs in pediatric/adolescent patients. There might be more than one pathophysiological mechanism for ovarian torsion in these patients: the most important of which are growth of ovarian volume and excessive mobility of the tube and mesosalpinx with long ovarian ligaments (56).

Furthermore, several Authors (57, 58) suggest an average age of 10 years for occurring this condition. The time of the tissue’s exposure to ischemia is critical. According to Macdougall (59), it is possible to have restitutio ad integrum of the adnexa following reperfusion within 18-24 hours after blood supply interruption. Moreover, ovarian torsion is not an isolated disease: Also unilateral torsion and ovariectomy affect the ovulation in contralateral ovaries after ipsilateral ovarian ischaemia, which may cause reduction of asynchronous bilateral torsions treated by ovarian removal and simple untwisting of the other have been published, although it could be considered a viable option in this condition (74-76). Nevertheless, maintaining the circulation of the ovary after detorsion makes the tissue injury worse and leads to a pathologic process called ischaemia/reperfusion (I/R) injury, characterized by oxidative stress (77). During the detorsion process, an excess amount of molecular oxygen is supplied to the tissues, and ROS such as superoxide radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH$^-$), and NO are produced in excess. ROS, RNS and their toxic products cause DNA damage and lipid peroxidation in the cellular and mitochondrial membranes (77-79).

As reported by Ozat et al. (80), during I/R injury a massive influx of Ca$^{++}$ into the cell occurs, caused by the action of sarcolemma and release of this cation from intracellular binding/sequestration sites (81, 82). Moreover, the excess of cytosolic Ca$^{++}$ triggers several enzymatic pathways which provokes an inflammatory status and accumulation of free arachidonic acid and leads to the depletion of Ca$^{++}$ from the endoplasmic reticulum lumen (64, 82-84). Following these events, the intracel-
lular excess Ca\(^{++}\) causes the release of cytochrome c from mitochondria and activation of caspase-dependent and caspase-independent cell death (8, 85, 86). During I/R injury, the most important mediator which is released in this process, and contribute as biomarkers of oxidative stress, is malondialdehyde (MDA) (87, 88). Till now, a number of anti-inflammatory and antioxidant agents have been used to prevent I/R injury, most of them in animal models (89, 90): SOD (91), curcumin (2, 92, 93), iloprost (a prostacyclin analogue) (80), melatonin (94), aprotinin (95), recombinant erythropoietin (rhEPO) (67, 96, 97), calcium channel blockers (98, 99) as 2-Aminoethoxydiphenyl borate (2-APB) (86, 100-102), growth hormone (GH) (103), ozone (O\(_3\)) therapy (104), dimethylsulfoxide (DMSO) (105), alpha-lipoic acid (LA, 1, 2-dithiolane-3-pentanoic acid) (106), genistin (107), marrubium cordatum (108) and amlodipine (109). Despite of the increasing attention to this topic, currently there is no shared and clear evidence about the use of anti-inflammatory and antioxidant agents to prevent I/R damage after laparoscopic ovarian detorsion. According to our literature review, future researches should focus on the correlation between I/R injury and consequent cellular/molecular events, especially by taking into account the role of the alterations in cellular immunity (110) and the apoptosis pathways (111, 112).

**Conclusion**

Current evidence suggests to performing laparoscopic ovarian detorsion, with respect to oophorectomy, in case of ovarian torsion in pediatric and adolescent patients. Although this procedure is acceptable, we must keep in mind that I/R injury can extend and make worse the ischemic and necrotic damage. Considering this element, future research should aim to develop shared protocols for the clinical use (route of application, dosage and time of application) of antioxidants after laparoscopic ovarian detorsion.

**Acknowledgements**

All Authors have no proprietary, financial, professional or other personal interest of any nature in any product, service or company. The Authors alone are responsible for the content and writing of the paper.

**References**


Epidemiology of Uterine Myomas: A Review

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Abstract

Myomas are the most common benign tumors of the genital organs in women of childbearing age, causing significant morbidity and impairing their quality of life. In our investigation, we have reviewed the epidemiological data related to the development of myomas in order to homogenize the current data. Therefore, a MEDLINE and PubMed search, for the years 1990-2013, was conducted using a combination of keywords, such as "myoma," "leiomyoma," "fibroids," "myomectomy," "lifestyle," "cigarette," "alcohol," "vitamins," "diet," and "hysterectomy". Randomized controlled studies were selected based upon the authors’ estimation. Peer-reviewed articles examining myomas were sorted by their relevance and included in this research. Additional articles were also identified from the references of the retrieved papers and included according to authors’ estimation.

Many epidemiologic factors are linked to the development of myomas; however, many are not yet fully understood. These factors include age, race, heritage, reproductive factors, sex hormones, obesity, lifestyle (diet, caffeine and alcohol consumption, smoking, physical activity and stress), environmental and other influences, such as hypertension and infection. Some of the epidemiological data is conflicting. Thus, more research is needed to understand all the risk factors that contribute to myoma formation and how they exactly influence their onset and growth.

Keywords: Uterine Myoma, Fibroid, Leiomyoma

Introduction

Myomas are the most common benign neoplasm of the reproductive organs in women of reproductive age. They could have a negative impact on the reproductive system and can be single, but are more often multiple, causing significant morbidity, and deterioration of quality of life (1, 2). According to relevant literature, 40-60% of all the hysterectomies performed are because of the presence of myomas. Myomas are the most common indication for hysterectomy in the USA and Australia (3, 4). Matthew Baille was the first to describe myomas in 1793. Myomas consist mainly of smooth muscle cells and contain different amounts of fibrous tissue (5). During its growth, a myoma compresses the surrounding structures (the myometrium and connective tissue), causing the progressive formation of a sort of pseudocapsule, rich in collagen fibers, neurofibers and blood vessels (Fig.1). Occasionally, the continuous surface of the pseudocapsule is interrupted by bridges of collagen fibers and vessels that anchor the myoma to the myometrium. This causes the formation of a clear cleavage plane between myoma and the pseudocapsule.
and between the pseudocapsule and the surrounding myometrium. This pseudocapsule causes a displacement action (which is not destructive) on the myometrium; however, the integrity and contractility of uterine structure is maintained (6, 7).

Literature data has shown that between 5.4 to 77% of women have myomas, depending on either the study population or the diagnostic techniques applied (8). Studies conducted using the ultrasound have confirmed that myoma prevalence is lower in Europe than in the United States, and this is probably due to racial differences (9, 10). Myomas are detected in 70% of uteri after hysterectomy, where multiple myomas are present in more than 80% of cases (11). Myoma prevalence was largely underestimated in previous epidemiological studies that focused mostly on symptomatic women (5, 10-12). By using more advanced non-invasive imaging techniques, such as 3D-4D ultrasonography (US) screening on the general population, epidemiological studies have become more accurate over the past two decades (1, 10). Thus, Laughlin et al. (13), reported a lower myoma prevalence of 10.7% in women screened in the first trimester of pregnancy.

The data on epidemiologic factors associated with myoma risk are either well defined or not yet fully understood (10). Those factors include age, race, body mass index (BMI), heritage, reproductive factors, sex hormones, obesity, lifestyle (diet, caffeine and alcohol consumption, smoking, physical activity and stress), environmental and other impacts like hypertension and infection (1, 10). The reported impacts of these factors in literature are conflicting (10, 12, 14). This could be attributed to bias in patient selection, given that some of the studies are based on surgical or symptomatic cases, while others on the incidental diagnosis of myomas (10).

**Fig.1:** A composed image in clockwise fashion showing: A. Transvaginal transversal scan showing a posterior corporal myoma, B. An eco Doppler transvaginal scan detecting the myoma pseudocapsule as a “ring of fire”, C. A T2 pelvic MRI showing a posterior corporal myoma enhanced by a white ring, D. Laparoscopic image showing the myoma enucleation surrounded by pseudocapsule. The arrows indicate the myoma pseudocapsule, as a fibrovascular connective network surrounding myoma and E. A laparotomic image showing a large uterine myoma surrounded by pseudocapsule during enucleating from myometrium.
Discussion

In this article, we have investigated the available epidemiological data regarding myoma development. For this purpose a MEDLINE and PubMed search, for the years 1990-2013, was conducted using a combination of keywords, such as "myoma," "leiomyoma," "fibromyoma", "leiomyofibroma", "fibroleiomyoma", "fibroid," "myomectomy," "lifestyle," "cigarette," "alcohol," "vitamins," "diet," and "hysterectomy". Randomized controlled studies were used when available; otherwise, literature that was the most relevant to the topic was used based on the authors’ estimation. Peer-reviewed articles regarding myomas, fibroids and leiomyomas were included in this paper. Additional articles were identified from the references of relevant papers. The terms "leiomyomas", "fibroids", "fibromyomas", "leiomyofibromas" and "fibroleiomyomas" can also be found in the literature describing myomas (15). In this paper, we have used the term myoma. The aim of this review is to provide information about epidemiological data regarding myoma development and make it more homogenous.

Age

During the reproductive years, the risk of myoma development increases with age (10). Myomas do not occur before puberty and their frequency decreases with menopause (16, 17). Myomas are diagnosed in 20-25% of women of reproductive age, and 30-40% of women older than 40 years (1, 4, 5, 18). Women with an earlier age of menarche have a higher risk for uterine myoma development (5, 10). It is to be expected that late-onset menopause increases risk of myoma occurrence due to longer exposure to gonadal steroids. However, the epidemiological data on this is still insufficient (10). The clinical incidence of myomas, in terms of a symptomatic disease requiring treatment, is the most frequent in perimenopause, whereas after menopause it rapidly decreases (19).

Race

Myomas are the most common in women of the black race, and the rarest in women of the Asian race (5). The data regarding racial differences other than in Caucasian and African American women are limited (10, 20). Laughlin et al. (13) determined the following prevalence: 18% in black women, 8% in white women, 10% in Hispanic women and 13% in the "others" group, consisting largely of Asian women. Black women are usually diagnosed at a younger age, with myomas that are often multiple, larger and accompanied by more severe symptoms than in other ethnic groups (10, 12, 16). Thus, black women are subjected to hysterectomies and myomectomies at an earlier age than white women (5). Myoma regression after pregnancy occurs more often in white women than in black (1). In addition, the myoma growth rate is slower as age progresses in white women than in black women (20).

The exact reasons for racial variations in the occurrence of myomas are mostly unknown. In literature, as the possible cause given for this phenomenon are the racial differences in the biosynthesis and/or metabolism of estrogens. Differences in the expression and/or function of receptors for steroid hormones among races can be considered as another possible cause of ethnic differences in myoma incidence (16). Aberrant expression of micro-RNA is another possible molecular mechanism involved in the development of myomas (16, 20). Micro-RNAs are a class of small non-coding RNAs important in the regulation of cell proliferation, differentiation and death, and their expression shows significant differences in various ethnic groups (16). Other causes analyzed in literature include heritage, lifestyle, dietary habits, and stress. However, these factors, can only somewhat explain the racial differences in myoma occurrence and their growth rates (10, 14, 20-25). By examining the data on why various races and ethnic groups have an increased risk of myoma development, new facts may be discovered regarding the etiology, formation and growth mechanisms of myomas, which could lead to new strategies for their assessment and treatment (20).

Genetics

Genetic factors can play a significant role in myoma development (5, 26). The growth of multiple myomas in the same uterus implies that heritage plays an important role in myoma development, causing some women to be more predisposed than others. The existence of the so-called "myoma families" (19, 26) described in literature proves a familial predisposition to myoma formation. Ui-mari et al. (26), in Finland, observed that in cases
of familial myomas women were diagnosed at an earlier age and more commonly with multiple myomas, so they tended to undergo hysterectomies at a younger age as well. Studies on twins have revealed a greater risk of myoma formation in monozygotic than in dizygotic twins (5, 27). The high myoma recurrence rate following myomectomy indicates that women with myomas have an inherited gene or some other genetic predisposition to myoma development. Cytogenetic analysis of the myoma cells proved the existence of tumor-specific chromosomal abnormalities in approximately 40% of the tested samples (10). Cytogenetic analysis of multiple myomas from the same uterus may show different chromosomal changes, which can mean that each myoma develops independently (5) and that certain regions of the genome may be involved in the pathogenesis of the myomas.

It is known that somatic mutations involving the gene encoding the mediator complex subunit 12 (MED12) and the gene encoding the high-mobility group AT-hook 2 (HMGA2) are associated to myoma (28). Mäkinen et al. (29) found that approximately 70% of myomas had heterozygous somatic mutations that affect MED12, transcriptional regulator complex subunit 12, a gene located on the X chromosome. The authors demonstrated that all mutations resided in exon 2 (codon 44), suggesting that the aberrant function of this region of MED12 contributes to tumorigenesis.

Since genetic analyses have supported the idea of a genetic component in myoma predisposition, Eggert et al. (30) genotyped and analyzed a genome-wide single nucleotide polymorphisms (SNP) linkage panel in 261 white myomas-affected sister-pair families from the Finding Genes for Fibroids study. All women were from two cohorts. The first was the Women’s Genome Health Study (WGHS), a prospective cohort of female North American health-care professionals representing a combination analysis at each of these loci were SNPs show wide significant associations with myomas. The 10q24.33, 22q13.1 and 11p15.5 revealed genome-wide association scans. This investigation was the first genome-wide association scan for myomas in African Americans and the first admixture mapping study of myomas in any population. In the results, the mean percentage of European ancestry was significantly lower among cases than among controls, with a stronger association in younger cases, less than 35 years old at diagnosis. Furthermore, the authors found only suggestive evidence for an association with European ancestry at specific loci (chromosomes 2, 4, and 10), with stronger results among younger and surgical cases for chromosome 2 only. This feature implied that a genetic variation for myomas differs in populations with and without African ancestry. The admixture findings further indicated that no single highly differentiated locus is responsible for the ethnic disparity in myomas, raising the possibility that multiple variants jointly contribute to the higher incidence of myomas in African Americans. Nevertheless, authors failed to replicate results from a recent GWAS in Japanese women by Cha et al. (31). In this investigation, authors reported a case-control GWAS that aimed to identify common genetic variants associated with uterine myomas. In this GWAS, the authors examined 1,612 individuals who were clinically diagnosed to have myomas at affiliated hospitals of the BioBank Japan Project and 1,428 female controls without a history of uterine myomas. They analyzed 457,044 SNPs in all patients. Three loci on chromosomes 10q24.33, 22q13.1 and 11p15.5 revealed genome-wide significant associations with myomas. The SNPs showing the most significant association in a combination analysis at each of these loci were
rs7913069, rs12484776 and rs2280543, respectively. Moreover, to assess whether these loci could be associated with clinically symptomatic myomas or with related phenotypes of the disease, authors performed subgroup analyses, founding that each marker SNP consistently showed a strong association with myoma formation regardless of presence or absence of hypermenorrhea or dysmenorrhea. These results indicated that these SNPs were associated with the development of myomas but not with the progression of disease.

After the Cho’s study, Edwards et al. (32) tested these SNPs for association with myomas in US cohorts. At patients’ enrollment, a transvaginal ultrasound was conducted to assess embryonic development and to systematically examine the uterus for presence of myomas. Patients were from a community-based pregnancy cohort that was carried out between 2001 and 2012, the Right from the Start (RFTS) cohort and the BioVU DNA repository. The authors tested 65 candidates and haplotype-tagged SNPs for association with myoma presence, and combined associated results from both cohorts using meta-analysis. Authors analyzed 1,086 European American cases and 1,549 controls. They observed strong evidence of association across several markers with transport 1 homolog (BET1L) and trinucleotide repeat containing 6B (TNRC6B), including two of the previously associated GWAS index SNPs. Meta-analyses combining evidence from RFTS, BioVU, and prior GWAS showed little heterogeneity in effect sizes studies, with meta-p values between 7.45×10^8 and 3.89×10^9, which were stronger than prior GWAS and supported associations observed for all previously identified loci. This data suggests that common variants increase risk for myomas in both European American and Japanese populations, even if further research is needed to assess the role of these genes across other racial groups.

**Reproductive factors**

The inverse association between myoma risk and parity is well known (5, 10, 12, 33) and an increasing number of term pregnancies decreases myoma risk. Both hormonal and non-hormonal mechanisms may also explain this association. Parity means decreased menstrual cycling and term pregnancies cause changes in ovarian hormones, growth factors and estrogen receptor levels, and changes in the uterine tissue (12). Thus, myomas are more common in nulliparous women, although excess weight and obesity seem to lessen the inverse association with parity (10, 12). Myoma development risk is reduced with the older age of the woman in last term pregnancy. Results from Nurses’ Health Study II have documented that myoma risk is reduced with the older age of the woman at the first birth and the last birth, and the more recent with the last birth (33). The study of Wise et al. (12), in African American women showed that time since the most recent birth is positively related to myoma risk among parous women. This observation can be explained by non-hormonal causes, such as postpartum tissue changes during uterine involution process (10). Increased risk for myomas is associated with early menarche and older age of the first term of pregnancy (5). The cause of this is thought to be increased exposure to menstrual cycles during a nulliparous woman’s lifetime, uninterrupted by pregnancy and lactation. This is also a plausible explanation for early menarche. Pregnancies that did not reach full term seem to have no influence on myoma formation risk (5, 12). Among multiparous women, the inverse association between myoma risk and exclusive breastfeeding throughout life was demonstrated by Terry et al. (33). This can be explained by the fact that lactation suppresses ovarian hormones. On the contrary, Wise et al. (12) did not find either lactation or its duration to be a protective factor in myoma development in African American women. This may be explained by the fact that breastfeeding happens only during a short period of a woman’s lifetime to have any significant impact on myoma development. It is not clear why pregnancy causes a reduction in myoma risk, but it can be that the postpartum physiological involution of the uterus eliminates myomas or reduces their size after delivery (10, 34, 35). This is confirmed by the recently published data (10).

**Endogenous hormones**

Myomas occur only during the reproductive period, which proves their dependence on ovarian steroids (36). The fact that estrogen and progesterone are significant in myoma onset and growth is evident in both clinical and experimental studies (10, 12). How they exactly influence myoma formation and growth is not yet fully understood (37). Early menarche increases the risk of myomas, due
to longer exposure to circulating ovarian steroids over a lifetime. Estrogen is believed to promote the growth of myomas (12). Recent researches have indicated that progesterone may also be important for the growth of myomas, because it acts synergistically with estrogen to stimulate myoma (10). For such reasons, selective progesterone receptor modulators (SPRMs), such as asoprisinil, ulipristal and telapristone have been researched as potential therapeutic drugs for uterine myomas (38). Ulipristal acetate (UPA) has demonstrated promising results for becoming a suitable therapeutic drug for uterine myomas. Results of international randomized controlled trials (PEARL I and PEARL II) showed that UPA decreased the size of the myomas and reduced bleeding, while increasing the red blood cell count after three months’ use of 5 mg/day (38, 39). Thus, UPA has been registered in some countries for the preoperative treatment of myomas for a period up to three months.

Myoma risk correlates with increased luteinizing hormone (LH) levels. Literature data indicate a positive association between polycystic ovary syndrome (PCOS) and myomas (5, 10, 40). A 65% higher incidence of myomas in women with PCOS compared with those without it, even after adjustment for potential confounding factors, was determined in the Black WHS (BWHS). The drawback of this study documenting the positive association between the PCOS and myomas in African American women is that the PCOS was self-reported. The LH hypothesis is also supported by the finding that the effect of PCOS is stronger among lean than in obese women. The explanations for this association are insulin resistance and elevated levels of insulin-like growth factor I (IGF-I), and hyperandrogenism (40). Still, Wise et al. (40) failed to determine that diabetes modified the association between myomas and PCOS.

**Exogenous hormone use**

The relationship between oral contraceptives and myomas has been widely researched (10, 12). Epidemiological data on the relationship between the use of oral contraceptives and myomas is inconsistent (17, 41). Oral contraceptive use may enhance diagnosis due to detection bias. Published studies show either a reduced or an absence of risk between the use of combined oral contraceptives and the occurrence of myomas (41). Thereby, according to Wise et al. (12), there is no link between the use of oral contraceptives and the risk of myoma in African American women. In this study, myoma risk was influenced by neither the ingredients of oral contraceptive nor its hormonal strength, not by duration or recency of use. A slightly higher risk is related to the age of first oral contraceptive use. This study shows a decreased risk of myomas in current users of progestin-only injectables. The reason for this is downregulation of the estrogen receptors in myomas caused by progestin (12).

The effects of IUDs with the levonorgestrel and risk of myoma development is still unknown (41, 42).

In postmenopausal women receiving hormone replacement therapy, both in women receiving estrogens only and in those receiving combined therapy, there is an increased occurrence of myoma growth (10).

Another factor that could also contribute to myoma risk is exogenous hormones in food. They could be in the form of the so-called phytoestrogens, as well as of those of artificial origin (24). Diethylstilbestrol (DES) exposure studies are influenced by reporting bias; therefore, their findings are conflicting (10). Further research in this field is needed by means of well-designed studies. This is necessary as laboratory data indicate a positive association, while clinical reports documented both positive association and absence of any association (10).

**Obesity**

The relationship between obesity and myoma development has shown to be inconsistent in literature (5, 40). Some epidemiological studies have found the increased risk of myoma development to be associated with obesity and diabetes mellitus (5, 10, 17, 40, 43). The common factor contributing to this association is insulin resistance, which is believed to be responsible for myoma risk developing in obese women, together with elevated IGF-I and androgen levels (5, 44).

A significantly higher BMI in women with myomas was documented in the Finnish twin cohort study (27). This can be explained by the presence of increased levels of circulating estrogens, caused by the aromatization of androgens by peripheral fatty tissues in obese women (44). However, most
of the circulating estrogens originate from ovaries in premenopausal women, which questions this theory (10). Certainly, what can be considered as a contributing factor in high myoma risk in these women is the decreased hepatic production of sex hormone binding globulin (SHBG), resulting in increased bioavailability of estrogens and androgens (5, 10, 44). He et al. (45) also found an increased risk of myomas in premenopausal Asian women with a high BMI. However, Chiaffarino et al. (46), in Italy, did not find any association between BMI and the risk of myomas.

In the US, obesity is prevalent among black rather than among white women. Thus, obesity is believed to be one of the reasons for the racial differences in the risk of myoma development. The results from the BWHS revealed a complex non-linear, but inverse-J shaped pattern between BMI and myoma risk (25). This connection appears to depend on parity, extent of obesity, and detection bias. There is also a positive association between myoma risk and weight gain during adulthood (10). In both white and black women, the association between the BMI of overweight women and myoma risk was found to be stronger in surgically confirmed cases (10, 25). In the US, both in white and black women, an absence of association was found between height and myomas (25).

**Lifestyle**

Lifestyle factors, such as diet, caffeine and alcohol consumption, smoking, physical activity, and stress have a potential effect on the formation of myomas and their growth (45). For easier reporting, we have divided the results of our research into subheadings.

**Diet**

The study results investigating the impact of diet on the occurrence of myomas are inconclusive, due to selection biases and the presence of confounding factors (10). Differences in diet could partly explain the racial differences in the prevalence of myomas. Therefore, in African American women myomas are more frequent, and they consume less fruit, vegetables, vitamin and mineral supplements (21, 22). Several dietary factors have been shown to contribute to the development of symptomatic myomas (45). Myoma formation risk is slightly higher in women consuming food with a higher glycemic index. Vitamins A and D are potential protective factors. Soy food was claimed to have an inverse relationship with myomas, but researches in this area have failed to find this association (22, 45). Furthermore, they have also failed to prove reduced myoma risk in populations with a high soy intake (10).

**Meat**

Current data demonstrate a positive link between a diet rich in red meat and myoma incidence (17). Chiaffarino et al. (46) conducted a case-control study of surgically confirmed cases in Italy, which demonstrated that women with myomas had a higher intake of beef, other red meat and ham and a lower intake of green vegetables, fruit and fish. Data obtained in this study are difficult to interpret due to several biases. Recently, Wise et al. (23) published the results on the relation of dietary fat intake and myoma risk in African American women, confirming an increased risk associated with the intake of long-chain omega-3 fatty acids, specifically marine fatty acids (MFA). Dark-meat fish was the main source of MFA in this study. Nevertheless, a dose-response relation for dark meat fish was not established. The overall risk of myoma has not been associated with total fat and fat subtypes intake in this study.

**Fruit and vegetables**

Wise et al. (21) validated that a diet rich in fruit and vegetables reduced the risk of myomas, especially one rich in fruits. Women who consumed a high amount of citrus fruits had a much smaller risk of myoma. The inverse association between myomas and vegetable and fruit intakes was also recorded by He et al. (45) in a study conducted in Beijing. The protective effect of a high intake of green vegetables and fruit was reported by Chiaffarino et al. (46) in Italy. They suggested that a higher intake of vegetables, fruit and fish indicates healthier dietary and lifestyle habits. The limitation of this study is the absence of total energy intake data, as information was collected only on frequency of vegetable intake, and during interviews with patients after they had been diagnosed with myoma (46).
Dairy

In a case-controlled study, Chiaffarino et al. (46) determined a null association between milk and butter consumption and myoma risk. In fact, investigations from the BWHS showed an inverse association of calcium, phosphorus and calcium-to-phosphorus ratio with myoma risk (10). The data from BWHS documented an inverse association of both low fat and high-fat milk with myoma risk. Thus, Wise et al. (22) concluded that racial differences in myoma incidence could be a result of differences in dairy intake. A subsequent paper by the same authors (24) noted that this relation could not be attributed to African ancestry.

Micronutrients

There is limited data about the effects of micronutrients on myoma formation and development, thereby the exact mechanisms involved in this association are not yet fully understood (47).

Dietary intake of vitamins C or E and folate were not found to be associated with myoma formation risk (21). Furthermore, the intake of vitamin B6, vitamin B12, folate and vitamin E were also not proven to have any association with myoma formation. Martin et al. (47) did not find vitamins A and C to reduce the risk of myoma formation either.

Vitamin D

Hypovitaminosis D, both in black and white women, is postulated as a potential risk factor in the myoma formation (48). Vitamin D is a fat-soluble steroid generated in the skin from a precursor molecule after sunlight exposure, or assumed in dietary foods (sometimes artificially enriched in vitamin D). Laboratory and animal evidence demonstrate that 1,25-dihydroxyvitamin D3 inhibits myoma growth and induces apoptosis (49). Recent research by Baird et al. (48) concluded that women with sufficient vitamin D have a reduced risk of myoma in comparison with women with vitamin D deficiency, and this was shown to be similar for both black and white women. African American women, who have a higher incidence of vitamin D deficiency, also have a higher frequency of myoma. Insufficient and inconclusive data in literature regarding this topic requires further research in this field.

Vitamin A

The data analyzing the relation between myoma and vitamin A are rare. A positive association between vitamin A and myoma formation was determined by Martin et al. (47). They have demonstrated a dose-response relationship between serum levels of vitamin A and myoma development odds. The limitations of their study are a self-reported myoma status and potential changes to the participants’ dietary habits following myoma diagnosis.

Wise et al. (21), demonstrated an inverse association of dietary vitamin A intake and myoma risk in black women. However, this association was present only when the intake of vitamin A is derived from animal products, while it was absent when the total vitamin A intake was from other sources. Thus, they concluded that the risk reduction was caused from other ingredients, rather than from the vitamin A in the food.

Carotenoids

Carotenoids are fat-soluble pigments found in many fruits and vegetables (50). They are powerful antioxidants, and some have pro-vitamin A activity, of which lycopene has the strongest antioxidant properties without any vitamin A activity. Animal studies have demonstrated that diets supplemented with lycopene reduce the number and size of myomas in a dose dependent manner (51). Literature data analyzing lycopene effect on myoma growth in humans is scarce. According to Terry et al. (50), the risk of myoma diagnosis is not associated with dietary carotenoids. The absence of association between myoma risk and carotenoid intake was also documented by Wise et al. (21).

Bioflavonoids

Myoma frequency is lower in Asian women because they consume more soy food products, which are rich in isoflavones, than other races (52). Although phytoestrogens found in soy foods were believed to reduce myoma risk, He et al. (45) did not find any relation between soy products and myoma risk in Asian women. No relation between soy intake and myomas was also confirmed in a study conducted by Nagata et al. (52) in Japan. Data consistent with those two studies were provided by Atkinson et al. (53), who did not find any connection between isoflavone urinary excretion.
and myomas in a population with a low intake of soy foods.

The results of experimental studies on myoma cell lines demonstrated that flavonoids from Scutellaria barbata D. Don induce apoptosis and inhibit cell proliferation (54). This makes flavonoids from the Asian herb possible substances for developing anti-myoma medications in the future.

Green tea extract has shown to inhibit proliferation and induce apoptosis on myoma cells in animal studies (55). Gallactocatehin gallate (EGCG), an extract (catechin) of green tea, has been proven to inhibit cell proliferation on cultured human leiomyoma cells in a dose-and time-dependent manner (56). Thus, EGCG needs to be further researched as a potential drug for myoma treatment.

### Caffeine and alcohol

Literature data indicate that both caffeine and alcohol can change endogenous hormone levels (10, 57). Alcohol consumption has been proven to increase the risk of myoma (52, 57). A positive association between alcohol consumption and risk of myomas was confirmed in Japanese women (52). In the BWHS, Wise et al. (57) found the association to be stronger in beer drinkers, rather than in wine drinkers. Chiaffarino et al. (46) in Italy did not notice any association between myoma risk and the intake of coffee, tea or total alcohol consumption. The reason for the absence of such an association could be the fact that wine accounted for more than 90% of the alcohol consumed in this study. In African American women, Wise et al. (57) did not find any association between coffee and caffeine consumption and myoma risk. More research is needed in order to determine the link between myoma risk and caffeine and alcohol consumption, given that these risk factors may be modifiable.

### Smoking

The studies showing the relation between cigarette smoke and myoma risk are overall inconsistent (57). In earlier epidemiological studies, current or former smokers had a 20-50% (10) decreased risk of myomas compared to non-smokers, which suggested a protective effect of smoking on myoma formation (5, 10, 17, 43, 58). More recent and better-designed studies have not documented such a relationship (10). Dragomir et al. (59) conducted a research on both black and white American women, which revealed a positive association between current smoking and diffuse myomas. However, this association was absent in cases with either submucosal or intramural/subserosal myomas. How smoking influences myoma formation is not entirely clear and further research is necessary (5, 10).

### Physical activity

There have been few studies investigating the effect of physical activity on the risk of myoma development. Despite this, a reduced risk of myoma formation was determined in women who take regular physical exercise and have a normal body weight (17). In women who take regular physical exercise, the risk of myoma is lower compared to women who do not exercise (10). Baird et al. (60) also demonstrated an inverse association in both black and white women regarding current physical activity and myoma development, where there is a stronger relation to myoma onset than to myoma growth. In Asian women, He et al. (45) found a marginal association between myomas and weekly physical activity non-related to women’s occupation. Women with moderate intensity of physical activity related to work had significantly lower myoma development risk. Given that this is a modifiable risk factor, more research is necessary to assess the effects of physical activity on myoma biology.

### Stress

Stress can also be a potential risk factor in myoma formation (61, 62). However, data is lacking on this topic. Stress could lead to myoma formation causing the increase of estrogen and progesterone levels, due to the effect on the hypothalamo-pituitary-adrenal gland axis activation and release of cortisol, a stress hormone (62). For example, black women who have experienced stress resulting from racial discrimination are more likely to have myomas. The potential reasons for this association are heavy alcohol consumption, poor diet, and obesity (61). The association between major life stress and myomas was also analyzed by Vines et al., who explored both the number of major life events experienced and the stress intensity associated with those events in relation to myoma pres-
ence. A positive association with myomas among black women in the high stress intensity group was shown by the cited authors (62). In the Asian population, no association between myomas and stress, depression and feelings of anxiety was documented (45).

Environmental factors

Myomas are believed to develop under the influence of environmental factors, such as irradiation. Studies have shown a significantly higher myoma incidence in women who survived the atomic explosion, the incidence being dependent on the dose of irradiation (63).

Other factors

Hypertension and diabetes

Several epidemiological studies found the increased risk of myomas in women with diabetes mellitus and arterial hypertension (5, 10, 17, 37, 43, 44). While experimental studies demonstrated stimulation by IGF-I of proliferation of myoma cells in the culture, clinical studies did not prove the association between myoma risk and plasma levels of IGF-I (10, 37). No association between circulating insulin levels and the presence of myomas was determined in both black and white women according to Baird et al. (37). Furthermore, elevated insulin was shown to be protective for large myomas, particularly among the black population. An inverse association between diabetes and myoma risk was confirmed in different studies. Wise and Laughlin-Tommaso (10) documented it in black women and Baird et al. (37) in both black and white women. Myoma development is thought to be inhibited by systemic vascular dysfunction in women with diabetes.

The coexistence of uterine myomas with hypertension was noted since the 1930s (64). Thus, hypertension has been considered as a risk factor for myoma development (20). Hypertension in women with myomas is usually chronic and requires treatment with antihypertensive drugs (64). In the study conducted by Boynton-Jarrett et al. (65) on women in the Nurses’ Health Study II cohort, an association was determined between higher diastolic pressure and myoma risk regardless of antihypertensive drug use. According to the results of this study, the duration of hypertension also increased myoma formation risk. To explain such an association, the authors suggested that hypertension may have caused cytokine release or injury to the smooth muscle of the uterus (44, 65). The results of those studies may be questioned in terms of possible screening and intervention biases, as they investigated symptomatic or surgery-confirmed cases (10, 64, 65). To further evaluate this association, it is necessary to conduct more research (10).

Infection and uterine injury

Infection or irritation causing uterine injury and followed by a disordered healing process was assumed as a possible reason for myoma formation in the first half of the twentieth century. It was suggested that uterine injury could induce changes in various growth factors causing myoma formation onset (10).

One study suggested that the use of perineal talc acting as a possible uterine irritant is associated with myoma formation. This case-controlled study documented a positive association both for frequency and duration of use (64). Another case-controlled study from Brazil showed an association between the Chagas disease and myomas in multiparous white women subjected to surgery either for myoma presence or for uterine prolapse (66). Faerstein et al. (64) showed a dose-response relation between ultrasound or surgically confirmed diagnosis of myomas and a number of physician diagnosed episodes of pelvic inflammatory disease (PID). Chlamydia infection was associated with a non-significant increase of myoma diagnosis in this study. This case-controlled study failed to establish an association between myoma and genital herpes or warts. It is necessary to conduct more studies in order to determine the relation between abnormal wound healing and myoma formation.

Conclusion

Clearly more research is necessary to determine the risk factors associated with myoma onset and growth considering that they cause significant morbidity and impair the quality of life. Clear insight into myoma epidemiology has not yet been achieved, and future research into modifiable risk factors may shed light on myoma prevention and provide new approaches to non-surgical myoma treatment.
Acknowledgements

The authors have no conflicts of interest in this study.

References

Epidemiology of Uterine Myomas


A Preliminary Report of A Low-Dose Step-Up Regimen of Recombinant Human FSH for Young Women Undergoing Ovulation Induction with IUI

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Abstract

Background: The aim of this study was to evaluate the efficacy and safety of a recombinant human follicle stimulating hormone (r-FSH) low-dose step-up regimen for controlled ovarian hyperstimulation in patients undergoing ovulation induction (OI) with intrauterine insemination (IUI).

Materials and Methods: The study was conducted in the Department of Obstetrics and Gynecology, Far Eastern Memorial Hospital, New Taipei, Taiwan. In this prospective, observational study, consecutive infertile women (20-35 years) with regular menstrual cycles and a normal baseline FSH level were prospectively enrolled between January 2010 and September 2010. A starting dose of 112.5 IU/day r-FSH was administered on day 3 and increased by 37.5 IU/day every 2 days until a follicle ≥11 mm in diameter was present. Recombinant human chorionic gonadotropin (r-hCG) was administered when a follicle ≥18 mm was noted. Monifollicular development was defined as only one follicle with a diameter ≥16 mm. Clinical pregnancy was defined as a pregnancy diagnosed by ultrasonographic visualization of one or more gestational sacs.

Results: A total of 29 women and 30 cycles were included. The mean daily dose of r-FSH to achieve a follicle of ≥11 mm in diameter was 131.3 ± 23.6 IU and the mean total dose was 1030.0 ± 383.2 IU. Approximately 41% of the cycles were monofollicular. Clinical pregnancy was observed in 9 (30.0%) cycles, and a fetal heart beat was observed in 7 (23.3%). There were no multiple pregnancies. Mild ovarian hyperstimulation syndrome, which was resolved with conservative management, was observed in 3 (10.0%) cycles.

Conclusion: This r-FSH low-dose step-up regimen seems to be a feasible and practical method for OI in younger infertile women undergoing IUI.

Keywords: Infertility, Ovarian Hyperstimulation Syndrome, Ovulation Induction


Introduction

Controlled ovarian hyperstimulation improves the cycle fecundity rate in part by increasing the number of follicles available for fertilization and correcting subtle, unpredictable ovulatory dysfunction. Intruterine insemination (IUI) is an established treatment for infertility due to cervical factor, male factor, or with an unexplained etiology. Combined with IUI, ovulation induction (OI) is recommended for many causes of infertility in pa-
tients with patent fallopian tubes (1). The overall success rate of IUI is approximately 10-15% (2, 3). Women with minor endometriosis or infertility due to unknown reasons may elect to undergo OI/IUI to increase the pregnancy rate, but the risk of multiple gestations is also increased (3). To date, no ideal stimulation protocol that provides a high pregnancy rate and low rate of complications such as ovarian hyperstimulation syndrome (OHSS) and multiple pregnancies has been identified (3, 4).

OI aims at the selection of a single follicle that will be able to reach the pre-ovulatory size and rupture. A recent retrospective cohort study indicated that induction of more than one follicle did not improve the ongoing pregnancy rate, but increased the risk of multiple pregnancies (3). Thus, it was suggested that in all IUI cycles for unexplained non-conception monofollicular growth should be sought to reduce the number of multiple pregnancies. Controlled OI with recombinant human follicle stimulating hormone (r-FSH) has been shown to be predictive of ongoing pregnancy rate, and studies have attempted to determine the r-FSH threshold (i.e. r-FSH dose on the day when a follicle is >10 mm in diameter) on the basis of pre-treatment and screening characteristics (4-6). The Gonal-f® New Generation Pre-Filled Pen (Merck Serono, Germany) is a disposable, pre-filled drug delivery system intended for the subcutaneous injection of multiple and variable doses of a liquid formulation of r-FSH. It is indicated to induce the development of multiple follicles in patients participating in an assisted reproductive technology program (7-10). Though limited data is available, studies have also shown that r-FSH seems to be at least as effective as urinary FSH preparations.

Materials and Methods

This was a single center, prospective, observational study on the use of r-FSH (Gonal-f®) in subjects undergoing OI/IUI. All patients provided written informed consent for participation in the study, and the study was approved by the Research Ethics Committee of Far Eastern Memorial Hospital, New Taipei, Taiwan.

The inclusion criteria were women between 20 and 35 years of age, regular menstrual cycles of 25-35 days, the presence of both ovaries, normal uterine cavity and patent fallopian tubes as investigated by either ultrasound scan, hysteroscopy, or hysterosalpingography, normal baseline serum FSH level (<10 μg/dL), and male partner semen analysis considered adequate for IUI in accordance to the center’s standard practice (i.e. >1×10^7 sperm/mL after sperm washing). The exclusion criteria included extrauterine pregnancy or abortion in the past 3 months, abnormal gynecologic bleeding of undetermined origin, history of OHSS, and known hypersensitivity to human r-FSH preparations.

The objective of r-FSH therapy is to develop a single mature Graafian follicle from which the ovum will be liberated after the administration of hCG. A starting dose of r-FSH 112.5 IU/day was begun on the 3rd day following the administration of hCG. Intrauterine insemination was then performed every 2 days beginning on the 7th day. If on the 7th day, a follicle had not reached 11 mm in diameter, the dose was increased to 150 IU/day (+37.5). If ultrasound on the 9th day did not show a follicle had reached 11 mm, the dose was again increased by 37.5 IU (187.5 IU/day). The same increase was made if on the 11th day, a follicle had not reached 11 mm. The maximum dose administered was 225 IU/day.

When an optimal response was obtained (dominant follicle ≥18 mm), a single subcutaneous injection of recombinant-human chorionic gonadotropin (r-hCG, 6500 IU, Ovidrel®, Merck Serono, Germany) was administered 24 hours after the last r-FSH injection. Serum estradiol (E2) was measured on the day of r-hCG. Intrauterine insemination was then performed 24 hours later. If an excessive ovarian response (i.e. E2>3500 μg/dL) occurred, treatment was stopped and r-hCG withheld. A new cycle was then initiated at a lower r-FSH dosage than that of the prior cycle, and the cycle with the hyper-response was excluded from the analysis. A maximum of three cycles (excluding those in which a hyper-response occurred) were allowed in an individual patient.

Monofollicular development was defined as only one follicle with a diameter ≥16 mm. Clinical
pregnancy was defined as a pregnancy diagnosed by ultrasonographic visualization of one or more gestational sacs based on the definition proposed by the International Committee for Monitoring Assisted Reproductive Technology (ICMART) (14).

**Statistical analysis**

The primary endpoint was the clinical pregnancy rate. The secondary endpoints were multiple pregnancy rate and occurrence of OHSS. The Shapiro-Wilk test was implemented to test whether the distributions of continuous variables met the assumption of a bell shape. Normally distributed continuous data were presented as mean ± standard deviation (SD), while categorical data were presented as number (n) and percentage (%). Non-normally distributed data were presented as median (range). Descriptive statistics were performed using Statistical Package for the Social Sciences (SPSS) version 15.0 (SPSS Inc., USA).

**Results**

Between January 2010 and September 2010, 30 consecutive women were enrolled in the study. The study was conducted in the Department of Obstetrics and Gynecology, Far Eastern Memorial Hospital, New Taipei, Taiwan. One patient with an abnormal baseline serum FSH level was excluded in the follow-up visit. Twenty-eight women underwent only one OI/IUI cycle. One woman failed to get pregnant at her first OI/IUI cycle, and then received her 2nd OI/IUI cycle. Thus, a total of 30 OI/IUI cycles were performed in this study and analyzed.

Baseline data are shown in table 1. On the day of r-hCG, all 30 cycles had follicles at least 16 mm in diameter, the median E₂ level was 898.0 pg/mL, and the mean endometrial thickness was 12.0 mm. The average total r-FSH dose was 1030.0 IU, and the average daily r-FSH dose was 122.5 IU. The average r-FSH dose when the follicular diameter was >10 mm in diameter (i.e. threshold of r-FSH) was 131.3 IU. The average length of time from the first injection of r-FSH until the day of r-hCG injection was 8.0 days. Twelve cycles met the defined criteria for monofollicular development on the day of r-hCG administration (Table 2).

Clinical pregnancy was observed in nine (30.0%, 95% confidence interval (CI)=12.6 to 47.4%) cycles, and a total of nine gestational sacs were found at follow-up. However, lack of fetal heart activity was found in two gestational sacs, thus the pregnancy rate in which a fetal heart beat was present was 23.3% (95% CI=7.3 to 39.4%). OHSS was observed in three (10.0%, 95% CI=0 to 21.4%) cycles and in all cases was grade I (mild: ascites with bilateral ovarian size less than 8 cm). In all patients, OHSS symptoms resolved within 1 week with conservative management.

There were two subjects with eight follicles ≥16 mm, and they had high estradiol levels, 3205 pg/ml and 3493 pg/ml. These two subjects had a hyper-response, but did not get pregnant.

### Table 1: Baseline patient data (n=29)

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Y)</td>
<td>31.0 ± 2.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.0 ± 2.0</td>
</tr>
<tr>
<td>Infertility</td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>19 (65.5)</td>
</tr>
<tr>
<td>Secondary</td>
<td>10 (34.5)</td>
</tr>
<tr>
<td>Duration of infertility (Y)</td>
<td>3 (1, 9)</td>
</tr>
<tr>
<td>Type of infertility</td>
<td></td>
</tr>
<tr>
<td>Female and male infertility*</td>
<td>2 (6.9)</td>
</tr>
<tr>
<td>Female infertility only</td>
<td>19 (65.5)</td>
</tr>
<tr>
<td>Male infertility only*</td>
<td>2 (6.9)</td>
</tr>
<tr>
<td>Unexplained</td>
<td>6 (20.7)</td>
</tr>
<tr>
<td>Causes of female infertility*</td>
<td></td>
</tr>
<tr>
<td>Tubal factor§</td>
<td>5 (17.2)</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>4 (13.8)</td>
</tr>
<tr>
<td>Ovulatory dysfunction</td>
<td>6 (20.7)</td>
</tr>
<tr>
<td>Others</td>
<td>17 (58.6)</td>
</tr>
<tr>
<td>Previous fertility treatment*</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1 (3.4)</td>
</tr>
<tr>
<td>Medication</td>
<td>28 (96.6)</td>
</tr>
<tr>
<td>IUI</td>
<td>14 (48.3)</td>
</tr>
<tr>
<td>Baseline FSH level (mIU/mL)*</td>
<td>6.1 ± 1.9</td>
</tr>
<tr>
<td>Baseline number of antral follicles*</td>
<td>7 (2, 31)</td>
</tr>
</tbody>
</table>

* There were four cases of male factor infertility. In these cases, the sperm concentration after washing was >1x10⁷/ml, which was considered adequate for IUI.  
\(^a\) Mean ± standard deviation, \(^b\) Number (percentage), \(^c\) Median (range), BMI; Body mass index, IUI; Intrauterine insemination, FSH; Follicle stimulating hormone, r-FSH; Recombinant FSH and §; The 5 patients with tubal factor infertility had tubal obstruction and/or adhesions which were treated prior to participation in the study.
Table 2: Clinical variables and outcomes of 30 cycles of ovulation induction and intrauterine insemination

<table>
<thead>
<tr>
<th>Variables</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>On the day of r-hCG administration</td>
<td></td>
</tr>
<tr>
<td>Number of follicles 11-15 mm in diametera</td>
<td>1 (0, 7)</td>
</tr>
<tr>
<td>Number of follicles ≥16 mm in diametera</td>
<td>2 (1, 8)</td>
</tr>
<tr>
<td>E₂ level (pg/mL)a</td>
<td>898.0 (60.1, 3493.0)</td>
</tr>
<tr>
<td>Endometrial thickness (mm)b</td>
<td>12.0 ± 2.4</td>
</tr>
<tr>
<td>Monofollicular developmentc</td>
<td>12 (41.4)</td>
</tr>
<tr>
<td>Ovarian stimulation</td>
<td></td>
</tr>
<tr>
<td>Total r-FSH dose (IU)b</td>
<td>1030.0 ± 383.2</td>
</tr>
<tr>
<td>Average daily r-FSH dose (IU)</td>
<td>122.5 ± 12.6</td>
</tr>
<tr>
<td>Threshold of r-FSH (IU)b</td>
<td>131.3 ± 23.6</td>
</tr>
<tr>
<td>Duration of r-FSH treatment (days)b</td>
<td>8.0 ± 2.5</td>
</tr>
<tr>
<td>Outcomes</td>
<td></td>
</tr>
<tr>
<td>Clinical pregnancyc</td>
<td>9 (30.0%; 12.6-47.4%)</td>
</tr>
<tr>
<td>Multiple pregnancyc</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Number of gestational sacs with fetal heart activityc</td>
<td>7 (23.3%; 7.3-39.4%)</td>
</tr>
<tr>
<td>Number of gestational sacs without fetal heart activityc</td>
<td>2 (6.7%; 0-16.1%)</td>
</tr>
<tr>
<td>OHSSc</td>
<td>3 (10.0%; 0-21.4%)</td>
</tr>
</tbody>
</table>

Monofollicular development was defined as only one follicle with a diameter ≥16 mm.
a; Median (range), b; Mean ± standard deviation, c; Number (percentage; 95% confidence interval), E₂; Estradiol, OHSS; Ovarian hyperstimulation syndrome, hCG; Human chorionic gonadotropin and r-FSH; Recombinant follicle stimulation hormone.

Discussion

The r-FSH low-dose step-up regimen described in this study was shown to be associated with a good clinical pregnancy rate (30.0%), and no multiple pregnancies were observed. In addition, though OHSS occurred in 10% of the cycles, all cases were mild and resolved with conservative management.

r-FSH, which completely lacks LH activity and extraneous human protein, has numerous advantages over prior medications (8, 10, 11). The results from a recent randomized study suggests that the use of r-FSH, as compared to urinary formulations, results in an increased clinical pregnancy rate [25.9% with follitropin alpha, 13.8% with urinary FSH and 12.5% with hepatic 3-hydroxy-3-methylglutaryl (hMG)] in IUI cycles for unexplained infertility (2). Another study, however, found that highly purified urinary FSH is as efficacious as r-FSH for ovulation induction in women with World Health Organization (WHO) group II anovulatory infertility (12) and provides a similar singleton live birth rate (15.1% with urinary FSH group vs. 15.4% with r-FSH), despite a difference in clinical pregnancy rate (17.8% with urinary FSH group vs. 21.8% with r-FSH group). Based on the clinical pregnancy rate data of the two aforementioned studies, it seems that the use of r-FSH in our study is a reasonable choice for OI/IUI.

As with prior FSH formulations, an ideal protocol of r-FSH has yet to be determined. Our protocol aimed for a monofollicular cycle and this occurred in 41.4% of the cases. It is always a challenge to determine the FSH threshold to achieve a
monofollicular cycle. The lowest dose to develop a follicle has to be determined and then the optimal dose for a monofollicular cycle is determined. In this study, we set the endpoints as clinical pregnancy rate, multiple pregnancy rate, and OHSS rate rather using an endpoint of monofollicular cycles. With a starting dose of r-FSH of 112.5 IU, a few women will be hyper-responsive. These patients may do better with a lower dose (i.e. 75 IU of r-FSH), but a dose of 75 IU r-FSH might not reach the FSH threshold for most of the patients. The two subjects with eight follicles ≥16 mm exhibited a hyper-response, but did not get pregnant. They may have other infertility problems which may be addressed by in vitro fertilization (IVF).

Despite the fact that no multiple pregnancies in our study, there were two subjects with a hyper-response. Thus, we cannot neglect the possibility of multiple pregnancies while utilizing this regimen into clinical practice.

Demirol and Gurgan (2) reported an OI protocol with a daily dose of 75 IU r-FSH if the patient’s body mass index (BMI) was <25 kg/m², and 150 IU if the patient’s BMI was ≥25 kg/m². Balen et al. (12) treated patients with the starting dose of 75 IU r-FSH daily for 7 days, and then an increase of 37.5 IU increments according to the individual response. Chung et al. (15) compared two different r-FSH doses (150 IU vs. 100 IU every other day) with 5 days of concomitant clomiphene citrate (100 mg/day) for OI/IUI, and found that the low r-FSH dose (100 IU) resulted in a lower multiple pregnancy rate (12.5%). However, the clinical pregnancy rates reported by the authors (14.5% in the 150 IU group and 20.4% in 100 IU group) were lower than the 30% found in our study. Though the starting r-FSH dose (112.5 IU) in our study was higher than that of Balen et al. (12) and equal to the average of Demirol and Gurgan (2), the non-inferior clinical pregnancy rate in our study (30.0%) as compared to those (25.9%) and Balen et al. (21.8%), lack of multiple pregnancies and low OHSS rate suggests our protocol a viable choice for OI/IUI (2, 12).

The primary limitations of this study are the small sample size and lack of control group. A randomized controlled study comparing the r-FSH low-dose step-up regimen with spontaneous/natural cycles would be beneficial. The low-dose regimen described may decrease the overall cost; however, a cost analysis was not part of the study design.

Conclusion

The r-FSH low-dose step-up regimen for OI/IUI is a practical method with a low rate of complications and low risk of multiple pregnancies for younger infertile women with good pre-treatment characteristics. Further clinical studies are required to define the optimal dose of r-FSH, and whether the same regimen can be applied in aged patients with a similar outcome.

Acknowledgements

This post-marketing study was sponsored by Merck Serono. All authors certify they have no conflict of interest.

References
10. Somkuti SG, Schertz JC, Moore M, Ferrandes L, Kelly E, Gonali-F Prefilled Pen in OI Study 24785 Group. Patient experience with follitropin alfa prefilled pen versus previ...


**Abstract**

**Background:** Therapeutic potential of *in vitro* maturation (IVM) in infertility is growing with great promise. Although significant progress is obtained in recent years, existing IVM protocols are far from favorable results. The first aim of this study was to investigate whether two step IVM manner change reactive oxygen species (ROS) and total antioxidant capacity (TAC) levels. The second aim was to find the effect of alpha lipoic acid (ALA) supplementation on oocyte maturation rate and on ROS/TAC levels during IVM.

**Materials and Methods:** In this experimental study, mouse germinal vesicle (GV) oocytes divided into cumulus denuded oocytes (DOs) and cumulus oocyte complexes (COCs) groups. GVs were matured *in vitro* in the presence or absence of ALA only for 18 hours (control) or with pre-culture of forskolin plus cilostamide for an additional 18 hours. Matured oocytes obtained following 18 and 36 hours based on experimental design. In parallel, the ROS and TAC levels were measured at different time (0, 18 and 36 hours) by 2',7'-dichlorodihydrofluorescein (DCFH) probe and ferric reducing/antioxidant power (FRAP) assay, respectively.

**Results:** Maturation rate of COCs was significantly higher than DOs in control group (P<0.05), while there was no significant difference between COCs and DOs when were pre-cultured with forskolin plus cilostamide. ROS and TAC levels was increased and decreased respectively in DOs after 18 hours while in COCs did not change at 18 hours and showed a significant increase and decrease respectively at 36 hours (P<0.05). ROS and TAC levels in the presence of ALA were significantly decreased and increased respectively after 36 hours (P<0.05) whereas, maturation rates of COCs and DOs were similar to their corresponding control groups.

**Conclusion:** ALA decreased ROS and increased TAC but could not affect maturation rate of both COCs and DOs in one or two step IVM manner.

**Keywords:** Oocytes Maturation, Oxidative Statuse, Alpha Lipoic Acid

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**Introduction**

*In vivo* maturation (IVM) of oocytes is an important issue of assisted reproduction techniques (ART) which is used as a method in treatment of infertility. The success of IVM requires a fine synchrony between nuclear maturation and cytoplasmic maturation (1). In the *in vitro* condition, cytoplasmic maturation of oocyte lags behind since cytoplasmic maturation completes gradually during folliculogenesis therefore an asynchrony is
occurred between nuclear maturation and cytoplasmic maturation (1, 2).

It has been revealed that higher cellular cyclic adenosine mono-phosphate (cAMP) level could arrest the oocyte nuclear maturation and improve oocyte development competence (3, 4) by providing an opportunity for essential modification of cellular organelles and biochemical state to sustain normal fertilization and further embryonic development (5).

Adenylyl cyclase (AC) and phosphodiesterases (PDEs) are two enzymes that control intra-oocyte level of cAMP via its synthesis and degradation, respectively (6). cAMP-dependent protein kinases through inhibiting of maturation-promoting factor (MPF) and mitogen-activating protein kinase (MAPK) arrest the meiotic division (7). Hence, it has been postulated that adding an AC activator and/or PDEs inhibitors to the oocytes maturation medium via increasing cAMP level prevents nuclear maturation and consequently, the maturation of oocyte cytoplasm and nucleus will be somewhat concurrent (3, 8-12). It has been demonstrated that cumulus cells produce cAMP and transmits it via gap junctions to oocyte which in turn supporting nuclear and cytoplasmic maturation of oocyte (13).

Higher oxygen concentration in the in vitro systems than in vivo condition lead to build up of reactive oxygen species (ROS) (14). Amongst numerous factors that may be contributed in low outcomes of IVM, production of ROS within the oocytes and culture medium lead to oxidative stress (OS) that affects IVM. However, the role of ROS in IVM of oocyte and its developmental competence remains controversial. Under optimal in vivo condition, increased generation of ROS was neutralized by both enzymatic and non-enzymatic antioxidants (15). It has been shown that, total antioxidant capacity (TAC) of ovarian follicular fluid can serve as a predictive marker of in vitro fertilization (IVF) success (16). Therefore, there is an urgent need to understand the scope of the OS-relevant factors that may affect oocyte development. In this continuum, supplementing maturation medium with different antioxidants has been reported that overcome OS and improves oocyte developmental competence (15, 17-20). In this sense, alpha lipoic acid (ALA) as a component of biological membranes and an imperative cofactor of mitochondrial dehydrogenases is well known for its antioxidative properties (21, 22). The ALA and its reduced form dihydrolipoic acid (DHLA), has been shown as potent antioxidant in both in vivo (23-25) and in vitro conditions (17). In essence, due to the unfavorable outcome of IVM and suspicious effects of OS on the oocyte IVM, the purposes of the present study were i. Assessment of the changes of ROS and TAC levels during two step IVM of oocytes with or without cumulus cells and ii. Determine whether adding ALA to maturation medium considering increasing cultivation period and possibility of excessive production of ROS in two step culture manner could modify ROS and TAC levels and improve the oocyte maturation.

Materials and Methods

Reagents

All chemicals were purchased from Sigma-Aldrich, UK unless otherwise stated and all media were prepared using Milli-Q water.

Animal subjects

In this experimental animal study, adult female (8-10 weeks old, n=25) Naval Medical Research Institute (NMRI) mice supplied from Pasteur Institute, Iran were cared for and used according to the guide for the care and use of laboratory animals of our university that fulfills and follows declaration of Helsinki as revised in Tokyo 2004. They were housed under 12 hours light: 12 hours dark regimen (light on at 7:00 am), at temperature of 23 ± 3°C and relative humidity of 44 ± 2% for at least 1 week before use with free access to food and water.

Experimental design

Germinal vesicle (GV) oocytes (vide infra) were divided into two main groups: cumulus oocyte complexes (COCs) and cumulus denuded (DOs). Each main group was randomly distributed among following subgroups: i. IVM without any intervention (control) and ii. IVM in the presence of 50 µM forskolin, an AC activator, in combination with 10 µM cilostamide, a PDE3 inhibitor. In separate
experiments, each group was also cultured in the absence of ALA. In sum, 8 experimental groups were studied. IVM with meiotic inhibitors were performed in two-step manner, briefly; step i. GV oocytes were cultured with cilostamide and forskolin for 18 hours and then step ii. Meiotic inhibitors were removed and oocytes were cultured for additional 18 hours. The control group was cultured without any meiotic inhibitors only for 18 hours (one-step culture). Based on the experimental group, 18 or 36 hours after onset of cultivation, the maturational status of the oocytes in each group was examined and classified as GV, GV breakdown (GVBD) or metaphase II (MII). In parallel, biochemical assay were performed for determining of TAC and ROS levels at initial time, 18 and 36 hours based on the experimental groups.

Preparation of cilostamide, forskolin and alpha lipoic acid stock solutions

Cilostamide, forskolin and ALA were dissolved in dimethylesulphoxide (DMSO) at 100 mM concentration as stock solution, protected from light and kept at -20°C. Before using, they were immediately diluted to the appropriate concentration in maturation medium to reach the final concentrations of 10, 50 and 100 µM respectively. Final concentrations of DMSO in maturation medium were 0.001% for cilostamide, 0.005% for forskolin and 0.01% for ALA. It has been shown that, the concentration of DMSO in the medium up to 0.1%, does not have any adverse effect on oocyte (4).

Isolation of germinal vesicle oocytes

GV oocytes were obtained from 8-10 weeks old female mice based on described previously methods (26). Briefly, primed mice with an intraperitoneal injection of 7.5 IU pregnant mare’s serum gonadotropin (PMSG, Intervet, Australia) were killed after 48 hours by cervical dislocation and their ovaries were collected in HEPES-buffered TCM199 medium (Gibco, UK) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, UK), 0.23 mM sodium pyruvate, 100 IU/ml penicillin and 75 µg/ml streptomycin. The COCs were achieved by puncture of antral follicles with sterile 29 gauge needles. COCs with uniform covering of 3-5 layers of cumulus cells and homogenous cytoplasm were selected for experiments. The DOs were obtained by repeated pipetting and flushing a portion of COCs through a small fine controlled bore pipette. After washing the oocytes in fresh HEPES–buffered TCM199 medium; they were used as described in experimental design. Collections of oocytes were performed at minimum possible time prior transfer to maturation medium. Also, in order to prevent spontaneous maturation, oocytes of each experimental group were collected in the presence of meiotic inhibitors which used in the same group.

In vitro maturation of germinal vesicle oocytes

The basal maturation medium was as described previously (27). In brief, TCM199 supplemented with 0.22 g/L NaHCO₃, 100 IU/ml penicillin and 75 µg/ml streptomycin, 0.23 mM sodium pyruvate, 10% FBS, 10 ng/ml epidermal growth factor (EGF), 75 mIU/ml recombinant human follicle stimulating hormon (rhFSH) and 10 IU/ml human chorionic gonadotropin (hCG). According to the experimental design, 100 µM of ALA, 10 µM of cilostamide and 50 µM of forskolin were added to the maturation medium. Groups of five oocytes were cultured in a drop of 20 µl of maturation medium under mineral oil at 37°C, 100% humidity in 5% CO₂ for 18 or 36 hours according to experimental design. At the end of the culture period, the number of degenerated oocytes, oocytes at GV, GVBD and MII stages were counted using an inverted microscope with Hoffman modulation contrast equipment (Nikon, Japan).

Biochemical assay

The intracellular ROS production of cultured oocytes was measured as described previously with some modification (17, 18). Briefly, 15 oocytes were used at different times of culturing period, (0, 18 and 36 hours) in each study group. The oocytes were washed with phosphate buffer saline solution (PBS, Gibco, UK) and incubated in 40 mM of Tris–HCl buffer (pH=7.0) containing 5 uM of 2’,7’-dichlorodihydrofluorescin diacetate (DCHFDA, Merck, Germany) at 37°C for 30 minutes. Oocytes were then washed again with PBS and then transferred to 100 µM of Tris-HCl buffer (40 mM, pH=7.0) and sonicated at 50 W for 1 minute. Resulting mixture was centrifuged at 10000×g for 20 minutes at
4°C and fluorescent intensity of supernatant was monitored by using a spectrofluorometer at 488 nm excitation and 525 nm emission. Corrections for autofluorescence were made by including parallel blank in each experiment. Standards curves were conducted by using known amounts of H$_2$O$_2$ as described previously (28). All experiments were repeated at least four times.

The assessment of TAC levels of cultured oocytes was performed according to common ferric reducing/antioxidant power (FRAP) method (17, 18). Briefly, 15 oocytes at different times of culture period (0, 18 and 36 hours) were homogenized in 100 ul Tris–HCl buffer (40 mM, pH=7.0) and sonicated at 50 W for 1 minute then centrifuged at 10000×g for 20 minutes at 4°C. Cellular supernatant (100 µl) was added to 1 ml of freshly prepared FRAP reagent (Tripyridyltriazine, TPTZ, Merck, Germany) in a cuvette and incubated in 37°C for 10 minutes. Absorbance of blue-colored reagent was read against a blank at 593 nm.

**Statistical analysis**

All experiments were repeated at least four times.

Differences among groups in the proportion of GV, GVBD and MII oocytes and TAC and ROS levels were statistically analyzed by one-way ANOVA using SPSS (version 19, Chicago, IL, USA) software. An independent sample t test was conducted to compare the rates of meiotic stages (GV, GVBD and MII) and TAC and ROS levels of ALA-treated groups and non-ALA treated groups. Percentages of degeneration, GV, GVBD and MII were statistically analyzed after arcsine transformation. Assessment of interaction among ALA, cumulus cells and meiotic inhibitors were statistically analyzed by two-way ANOVA. When ANOVA indicated a significant difference (P<0.05), post hoc Tukey’s HSD was used.

**Results**

**Maturational status of oocytes**

Rates of GV, GVBD, MII and degenerated oocytes of COCs and DOs following one step (control) and two step IVM manner in the presence or absence of ALA are shown in table 1.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>GV (n/all) (% ± SD)</th>
<th>GVBD (n/all) (% ± SD)</th>
<th>MII (n/all) (% ± SD)</th>
<th>Degeneration (n/all) (% ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control COC</td>
<td>352</td>
<td>27/120 (22.50 ± 2.50)</td>
<td>17/120 (14.17 ± 1.44)</td>
<td>73/120 (60.83 ± 1.44)</td>
<td>138/232 (59.51 ± 1.59)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>54/232 (23.26 ± 0.55)</td>
<td>31/232 (14.17 ± 1.44)</td>
<td>18/120 (14.17 ± 1.44)</td>
<td>3/120 (2.50 ± 2.50)</td>
</tr>
<tr>
<td>DO</td>
<td>176</td>
<td>29/98 (29.59 ± 2.14)</td>
<td>20/98 (20.40 ± 1.44)</td>
<td>45/98 (45.94 ± 0.64)</td>
<td>33/98 (42.43 ± 2.25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22/78 (28.16 ± 2.07)</td>
<td>20/98 (20.40 ± 1.00)</td>
<td>45/98 (45.94 ± 0.64)</td>
<td>4/98 (5.19 ± 2.05)</td>
</tr>
<tr>
<td>Cilostamide</td>
<td>240</td>
<td>13/120 (10.83 ± 1.44)</td>
<td>7/120 (5.83 ± 1.44)</td>
<td>8/120 (6.67 ± 1.44)</td>
<td>1/120 (0.83 ± 1.44)</td>
</tr>
<tr>
<td>and forskolin</td>
<td></td>
<td>14/120 (11.67 ± 1.44)</td>
<td>7/120 (5.83 ± 1.44)</td>
<td>14/120 (11.67 ± 1.44)</td>
<td>3/120 (2.50 ± 1.00)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27/120 (22.50 ± 1.44)</td>
<td>27/120 (22.50 ± 1.44)</td>
<td>79/120 (65.83 ± 1.44)</td>
<td>77/120 (64.17 ± 1.44)</td>
</tr>
</tbody>
</table>

Different superscript letters in the same columns indicate significant differences (P<0.05). *: Indicate significant difference with respective DOs groups; #: Indicate significant difference with respective control group, GV; Germinal vesicle, GVBD; Germinal vesicle breakdown, MII; Metaphase II, COC; Cumulus oocytes complexe, DO; Cumulus denuded oocyte and ALA; Alpha lipoic acid. Two step *in vitro* maturation manner: cultured with meiotic inhibitors from 0 to 18 hours, no meiotic inhibitors from 18 to 36 hours, One step *in vitro* maturation manner (control): cultured without meiotic inhibitors only for 18 hours.
After 18 hours culture in one step IVM manner, the rates of MII oocytes (59.5%), in COCs control group were significantly higher than DOs control group (42.4%). The rates of GV (23.26%), GVBD (13.34%) and degeneration (3.88%) in the COCs control groups were also statistically lower than those of DOs control group (28.16, 20.42 and 8.97% respectively). At end of two step IVM manner, MII rate (66.7%) of COCs was significantly higher than COCs control group. In addition, 6.67% of COCs was arrested at GV stage which was significantly lower than those of COCs control group. On the contrary, the rate of GVBD (25.83%) for two step in vitro matured COCs groups was significantly higher than those of COCs control group. The percentages of degenerated oocytes were not statistically different among groups (P>0.05).

The rate of MII oocytes in cilostamide and forskolin treated DOs groups was 64.2% which was significantly higher than that of respective control group. The rates of oocytes that arrested at the GV stage after 36 hours culture in cilostamide and forskolin treated DOs group (11.67%) was significantly lower than that of control DOs group. There was no significant difference between GVBD rate of pre-matured DOs group with combination of cilostamide and forskolin (20.63%) and that of control DOs group (P>0.05). There was no significant difference in the presence or absence of ALA in all experiment groups (P>0.05).

There were no significant differences in the rates of MII oocytes, of COCs groups after pre-maturation with forskolin and cilostamide with those of DOs groups (P>0.05) while, the rates of MII oocytes of COCs control groups were statistically higher than those of DOs control groups. There was a significant interaction between the effect of pre-maturation with meiotic inhibitors and the presence and absence of cumulus cells on the rates of MII oocytes. They were more effective on the DOs than COCs groups. Also, there was no interaction between ALA and meiotic inhibitors and presence or absence of cumulus cells (P>0.05).

### Oxidative status

ROS concentration and TAC of COCs (4.27 ± 0.13 µM and 87.5 ± 2.78 µmol/µl respectively) and DOs (4.07 ± 0.4 µM and 90.77 ± 2.11 µmol/µl respectively) at initial time of culture period were not significantly different among groups (P>0.05, Fig.1). The ROS concentrations of COCs did not increase significantly at 18 hours (4.27 ± 0.47 µM, Table 2), while ROS production in DOs (5.4 ± 0.36 µM) significantly increased (Fig.1A, Table 2). In two step culture condition a significant increase of ROS level were observed in both COCs and DOs from 18 hours (4.13 ± 0.6 µM and 5.6 ± 0.26 µM respectively, Table 3) to 36 hours (5.5 ± 0.3 µM and 6.47 ± 0.35 µM respectively, Fig.1). Also, the ROS levels of DOs at 18 and 36 hours of culture (5.6 ± 0.26 µM and 6.47 ± 0.35 µM respectively, Table 3) were significantly higher than those of COCs (4.13 ± 0.6 µM and 5.5 ± 0.3 µM respectively, Fig.1A).

In the presence of ALA, ROS production of COCs was significantly decreased from initial time (4.03 ± 0.42 µM) to 18 hours (2.53 ± 0.42 µM, Table 3) and remained without change, up to 36 hours (2.8 ± 0.26 µM) in two step culture condition (P>0.05, Fig.2A). ROS levels of ALA treated DOs did not significantly change during culture period up to 36 hours (initial time: 3.57 ± 0.38 µM, 18 hours: 4.03 ± 0.21 µM, 36: 4.37 ± 0.4 µM, P>0.05, Fig.2B).

As showed in table 3, maximum TAC levels in COCs was observed at the initial time (87.5 ± 2.8 µmol/µl) and 18 hours (86.7 ± 4.2 µmol/µl), while, significantly decreased up to 36 hours (75.0 ± 3.0 µmol/µl) in two step in vitro condition (P>0.05). TAC levels of ALA treated COCs increased significantly after 18 hours (103.0 ± 4.6 µmol/µl, Table 3) and remained constant without significant difference up to 36 hours later (102.7 ± 4.7 µmol/µl, Fig.3A). In DOs group, TAC levels were significantly decreased at 18 hours (76.0 ± 4.0 µmol/µl) and 36 hours (63.3 ± 3.1 µmol/µl) in comparison with initial time (89.5 ± 1.0 µmol/µl, Fig.3B). While, there was no significant difference in the TAC levels of ALA treated DOs between the end (91.7 ± 3.1 µmol/µl, Table 3) and beginning (90.3 ± 1.9 µmol/µl) of the culture period (P>0.05, Fig.3B, Table 3).
**Fig 1:** A. ROS and B. TAC concentrations of one and two step *in vitro* cultured oocytes without pre-treatment of ALA.

In all cases 4 experimental replicates were performed. Different superscripts (a, b, c) reflect different levels of significant differences at different times of cultivation period within the same group (P<0.05).

*: Indicate significant differences with COC groups at same times of cultivation period (P<0.05), ROS; Reactive oxygen species, TAC; Total antioxidant capacity, DO; Denuded oocytes and COC; Cumulus oocyte complex.

Two step *in vitro* maturation manner: cultured with meiotic inhibitors from 0 to 18 hours, no meiotic inhibitors from 18 to 36 hours. One step *in vitro* maturation manner (control): cultured without meiotic inhibitors only for 18 hours. Data were presented as mean ± SE.

**Table 2:** ROS concentrations as µM H$_2$O$_2$ of one and two step *in vitro* cultured oocytes with or without pre-treatment of ALA

<table>
<thead>
<tr>
<th>Groups</th>
<th>Initial time</th>
<th>18 hours</th>
<th>36 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALA-</td>
<td>ALA+</td>
<td>ALA-</td>
</tr>
<tr>
<td>Control</td>
<td>COC</td>
<td>4.05 ± 0.13</td>
<td>4.27 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>DO</td>
<td>3.60 ± 0.44</td>
<td>4.07 ± 0.4</td>
</tr>
<tr>
<td>Cilostamide and forskolin</td>
<td>COC</td>
<td>4.03 ± 0.42</td>
<td>4.23 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>DO</td>
<td>3.57 ± 0.38</td>
<td>3.97 ± 0.4</td>
</tr>
</tbody>
</table>

In all cases 4 experimental replicates were performed, $^*$: Reflect significant differences with initial time within the same group (P<0.05), $^*$: Indicate significant differences with respective ALA treated groups at same times of cultivation period (P<0.05), $^*$: Indicate significant differences with COC groups at same times of cultivation period (P<0.05), ROS; Reactive oxygen species, DO; Denuded oocytes, COC; Cumulus oocyte complex and ALA; Alpha lipoic acid.

Two step *in vitro* maturation manner: cultured with meiotic inhibitors from 0 to 18 hours, no meiotic inhibitors from 18 to 36 hours. One step *in vitro* maturation manner (control): cultured without meiotic inhibitors only for 18 hours. Data were presented as mean ± SE.

**Table 3:** TAC concentrations as µM/µL of one and two step *in vitro* cultured oocytes with or without pre-treatment of ALA

<table>
<thead>
<tr>
<th>Groups</th>
<th>Initial time</th>
<th>18 hours</th>
<th>36 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALA-</td>
<td>ALA+</td>
<td>ALA-</td>
</tr>
<tr>
<td>Control</td>
<td>COC</td>
<td>88.90 ± 2.54</td>
<td>87.50 ± 2.78</td>
</tr>
<tr>
<td></td>
<td>DO</td>
<td>90.07 ± 4.61</td>
<td>90.77 ± 2.11</td>
</tr>
<tr>
<td>Cilostamide and forskolin</td>
<td>COC</td>
<td>90.93 ± 1.1</td>
<td>87.67 ± 1.26</td>
</tr>
<tr>
<td></td>
<td>DO</td>
<td>90.33 ± 1.9</td>
<td>89.50 ± 1.04</td>
</tr>
</tbody>
</table>

In all cases 4 experimental replicates were performed, $^*$: Reflect significant differences with initial time within the same group (P<0.05), $^*$: Indicate significant differences with respective ALA treated groups at same times of cultivation period (P<0.05), $^*$: Indicate significant differences with COC groups at same times of cultivation period (P<0.05), TAC; Total antioxidant capacity, DO; Denuded oocytes, COC; Cumulus oocyte complex and ALA; Alpha lipoic acid.

Two step *in vitro* maturation manner: cultured with meiotic inhibitors from 0 to 18 hours, no meiotic inhibitors from 18 to 36 hours. One step *in vitro* maturation manner (control): cultured without meiotic inhibitors only for 18 hours. Data were presented as mean ± SE.
Fig. 2: A. ROS concentrations of one and two step in vitro cultured COCs and B. DOs with or without pre-treatment of ALA. In all cases 4 experimental replicates were performed. Different superscripts (a, b, c) reflect different levels of significant differences at different times of cultivation period within the same group (P<0.05).

*; Indicate significant differences with respective ALA treated groups at same times of cultivation period (P<0.05), ROS; Reactive oxygen species, DO; Denuded oocytes, COC; Cumulus oocyte complexe and ALA; Alpha lipoic acid.

Two step in vitro maturation manner: cultured with meiotic inhibitors from 0 to 18 hours, no meiotic inhibitors from 18 to 36 hours. One step in vitro maturation manner (control): cultured without meiotic inhibitors only for 18 hours. Data were presented as mean ± SE.

Fig. 3: A. TAC level of one and two step in vitro cultured COCs and B. DOs with or without pre-treatment of ALA in all cases 4 experimental replicates were performed. Different superscripts (a, b, c) reflect different levels of significant differences at different times of cultivation period within the same group (P<0.05).

*; Indicate significant differences with respective non-ALA treated groups at same times of cultivation period (P<0.05), TAC; Total antioxidant capacity, DO; Denuded oocytes, COC; Cumulus oocyte complexe and ALA; Alpha lipoic acid.

Two step in vitro maturation manner: cultured with meiotic inhibitors from 0 to 18 hours, no meiotic inhibitors from 18 to 36 hours. One step in vitro maturation manner (control): cultured without meiotic inhibitors only for 18 hours. Data were presented as mean ± SE.
Discussion

In this investigation, cilostamide and forskolin have been used in the oocyte maturation medium to coincide maturation of nuclear and cytoplasm of mouse oocytes. In the recent years, many advantages have been achieved in the oocyte IVM, although oocyte IVM is always associated with many challenges. One of the predicaments is absence of critical cytoplasmic biochemical and molecular events which are essential for acquisition of developmental competence of oocyte (13). Reversible cessation of oocyte meiotic division using cAMP elevating agents in order to improve oocyte developmental competence via synchrony of nuclear and cytoplasmic maturation has been shown previously (3, 4, 8, 9, 11, 12). In this study, a combination of forskolin and cilostamide was used because it was demonstrated that there is a synergistic effect between those on the oocyte maturation, fertilization and subsequent embryonic development (12). The role of delayed in vitro oocyte nuclear maturation in improvement of developmental competence remains controversial. Some studies confirmed that postponement of oocyte nuclear maturation leads to increase developmental failure (29) while others believe that it progressed developmental competence through improvement of oocyte cytoplasmic and nuclear maturation (3, 9, 11, 12). However, our findings indicate that combination of forskolin and cilostamide will improve developmental competence of mouse oocytes.

This study showed higher maturation rate of COCs than DOs in one-step IVM manner while there were no significant difference between maturation rates of COCs and DOs in biphasic IVM manner. In this line, several studies have showed the decisive role of cumulus cells in the oocyte developmental competence (23, 30). These studies indicate that meiosis inhibitors were more effective on the DOs than COCs in two-step IVM manner. It seems that exposure to gonadotrophin in the absence of cumulus cells accelerate meiotic progression as a non-physiological condition of oocyte maturation (11).

In the present study, EGF was used in maturation medium with or without forskoline and cilostamide. Although, EGF is one of the most relevant factors play a role in oocyte meiotic maturation, its meiosis-stimulating effects on the mouse oocyte is associated with minimal changes in cAMP production and is independent of cumulus expansion. Therefore, it seems that EGF cannot induce oocyte maturation by overcome tyrosine kinase inhibitors pathways of cAMP (31).

An increase of ROS and a decrease of TAC during in vitro culture in both DOs and COCs up to 36 hours have been detected in the present study. This is in agreement with other investigations which indicated OS was increased during cultivation period (14, 15). However, ROS production of in vitro matured COC did not change up to 18 hours. It seems that activity of cumulus cells have been changed during IVM process of COCs, which in turn, led to conserve ROS concentration at the basal level. With respect to increase the number of cumulus cells during IVM of COCs, it is appeared that ROS production per cumulus cell has been declined. On the other hand, ROS concentration of in vitro matured DOs increased at 18 hours which is inconsistent with results of Cetica et al. (19), who showed ROS production did not change during bovine DOs IVM. It seems that, the conflicting result was due to differences in experimental strategies and animal species.

Results of this study indicated that TAC level decreased in DOs after 18 hours of culture period while it did not change in COCs groups. Oocytes have their own enzymatic antioxidant activity which was attributed to expressed genes encoding antioxidant enzymes. Although, the COCs antioxidant capacity mostly dependent on cumulus cells (19, 32). It is not known that this system of cumulus cells and oocytes could remain constant until the end of biphasic cultivation period since TAC (18) levels decreased at 36 hours in both in vitro cultured DOs and COCs.

In order to reduce OS, supplementing culture medium with antioxidants has been broadly used (17, 18, 33), although the advantages of adding antioxidants in oocyte maturation medium still remains controversial (15). The results of the present study showed that ALA as a potent antioxidant could not affect the maturation rates of both COCs and DOs in one and two steps in vitro culture manner. However, in the ALA treated groups, ROS and TAC levels were decreased and increased, respectively. It was demonstrated that supplemented medium with non-enzymatic and enzymatic antioxidant
dant improves the developmental competence of *in vitro* matured bovine oocyte (33). Whereas, it was shown that antioxidants restrain resumption of meiosis, which may show a role of ROS in oocyte maturation (34). On the other hand, it was indicated that OS induces meiotic arrest (35). The inconsistent results may be due to the dose and types of antioxidants and animal species have been used. For example, supplemented maturation medium with cysteamine, cysteine, and β-mercaptoethanol could improve the rate of porcine embryo production (36, 37), while β-mercaptoethanol, superoxide dismutase, or ascorbic acid had no optimistic effect on subsequent development on bovine oocytes maturation medium (38). It has been shown that ALA improves developmental competence of mouse isolated pre-antral follicles somewhat via decreasing and increasing ROS and TAC levels, respectively (17), however, in the present study ALA treated groups in one or two step culture manner did not show any significant difference with untreated respective groups. It is appeared that, these inconsistency results are related to the duration of culture period because duration of cultivation period of isolated pre-antral follicle was 12 days while maximum duration of oocyte *in vitro* culture was 36 hours. In this regard, it has been shown that ROS production was increased during cultivation period (17, 18). Also, regarding to ability of oocytes and cumulus cells to express genes encoding antioxidant enzymes (19, 32) may explains why adding an antioxidant could not improve IVM rates of both COCs and DOs. In addition, it was revealed that certain levels of ROS during IVM may be beneficial and play a crucial role in the induction of oocyte maturation which in turn improve embryo production rates (39).

Conclusion

ALA supplemented maturation medium did not had a significant effect on IVM rates of both COCs and DOs, although it decreased ROS and increased TAC levels in two *step in vitro* matured oocytes. Furthermore, two step IVM manner with meiotic inhibitors was more effective on improvement of DOs maturation rates than COCs.

Acknowledgements

This study was supported by the Grants from school of biology of Damghan University and institute of biological sciences of Damghan University. The authors are grateful to Mrs. Leili Hosseinpour and Mrs. Rada Dehghan for their technical assistance. There is no conflict of interest in this article.

References

Increased Length of Awareness of Assisted Reproductive Technologies Fosters Positive Attitudes and Acceptance among Women

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Abstract

**Background:** The field of infertility medicine has witnessed a surge of scientific developments in recent years, but research on public attitudes towards infertility treatments has remained minimal. This study examined the social and demographic factors that affect women’s attitudes towards assisted reproductive technology (ART) in general, as well as their opinions of specific issues related to ART.

**Materials and Methods:** This cross-sectional study was conducted from March 2011 to April 2011 by means of an online survey administered to a sample of 287 women.

**Results:** Women with a longer length of awareness of ART had significantly greater attitudinal favorability towards ART. Political affiliation was also significantly related to general attitudes, as well as several specific aspects of ART issues.

**Conclusion:** The results of this study suggest that several factors influence attitudes that women hold in regards to ART. Identifying some of these factors serves as a crucial starting point for devising strategies to increase public acceptance of ART.

**Keywords:** Assisted Reproductive Technology (ART), Attitudes, Awareness, Infertility, In Vitro Fertilization (IVF)

**Citation:** Fortin C, Abele S. Increased length of awareness of assisted reproductive technologies fosters positive attitudes and acceptance among women. Int J Fertil Steril. 2016; 9(4): 452-464.

Introduction

Over the past half-century, societal changes in the United States have markedly altered typical childbearing patterns. For a number of reasons, including the penalties associated with taking time off work and the enormous expense of raising a child, an increasing number of women have chosen to wait to have children. Each year, women are having their first child later than ever before. Whereas birth rates for U.S. women who are in their twenties have steadily declined each year, the rate has soared for those in their thirties and forties (1). As more and more women choose to delay motherhood, the frequency of women experiencing infertility issues has continued to rise. Today, a shocking one in six U.S. couples is affected by infertility. In 1982, 6.6 million women in the U.S. received infertility treatments and in 2002, this number increased to 7.3 million (2). Globally, it has been estimated that as many as 48.5 million couples worldwide are infertile (3).

Since the first child conceived through in vitro fertilization (IVF) was born in 1978 (4), there has been an explosion of advancements in the area of assisted reproductive technology (ART). ART is the collective term used to refer to the medical procedures involving the laboratory manipulation of eggs and sperm that increase the chances that a woman will achieve pregnan-
cy. The most commonly performed ART procedure is IVF, but the term also includes preimplantation genetic diagnosis (PGD) and the use of donor eggs (5).

The recent advancements in ART have expanded the choices available to both physicians and patients, and have simultaneously created new ethical issues. Some of these issues concern the medical risks associated with infertility techniques, such as the high incidence of multiple pregnancies—which increases the risk of cesarean section, preterm labor and delivery, low birth weight and death (6). Others involve redefining what constitutes a family and whether there are negative implications for children brought into a family by means of certain ART techniques. Recently, many of these issues have revolved around questions of patient access to infertility treatments.

Empirical research on these newly conceived ethical issues has not kept pace with this rapidly evolving field of medicine. Published series have mainly focused on the obstetric outcome and development of children conceived via infertility treatments. There is limited research on the public’s perceptions of these treatments, yet it is essential that health care providers are aware of these perceptions, so that they can practice ART in a fashion that is acceptable to the public.

ART has always been controversial on religious grounds, and thus, religion is likely a factor that influences women’s attitudes towards this subject. Research suggests that highly religious individuals tend to hold more traditional views on marriage and family patterns (7). Those with strong religious beliefs also tend to hold more conservative views towards genetic testing (8) and have ethical concerns with ART procedures (9, 10). Surprisingly, however, the Catholic Church remains the only major world religion that explicitly forbids the use of IVF (11).

Past research suggests that political affiliation influences attitudes towards ART and other reproductive health issues. In fact, a recent study found that political affiliation was one of the strongest predictors of approval of IVF use for nontraditional women (e.g. single women, homosexuals), with approval being higher amongst Democrats than Republicans (12). Shreffler et al. (9) demonstrated that those with liberal social-political views are less likely than their conservative counterparts to have ethical concerns with ART. Similar findings have been documented in the realm of embryonic stem cell research (13), abortion (14) reproductive genetic testing (15) and posthumous reproduction (16). These findings parallel the core values that divide party lines: Republicans tend to place more value on the traditional family structure, whereas Democrats are often more liberal in regards to reproductive health issues (17-20).

To our knowledge, no previous research has explored a possible link between attitudes and women’s length of awareness of infertility treatments. It is reasonable to expect a relationship to exist between these variables based on a concept known as the mere exposure effect, a phenomenon in which the mere repetition of an individual’s exposure to a stimulus enhances his/her attitude towards the stimulus (21). This idea that familiarity leads to liking has been demonstrated across a variety of stimulus domains—including attitudes towards mental illness (22), organ transplantation (23), assisted living (24), newborn screening programs (25), biotech foods (26) and epilepsy (27)—but heretofore not in the realm of infertility medicine.

The goal of this study was to investigate the social and demographic factors related to women’s attitudes towards ART, including political affiliation, education, ethnicity, religion, income, and age. Other factors more specific to the study of infertility treatments, such as length of awareness of ART, having participated in ART techniques, knowing someone else who has undergone ART, exposure to ART through the media, and current health status (fertile or infertile), were also examined. Finally, women’s knowledge of infertility treatments was examined to identify any potential misconceptions of ART.

**Materials and Methods**

The Institutional Review Board for the Use of Human Subjects in Research at Miami University approved the research plan and the survey content on March 17, 2011. Participants were all recruited through Qualtrics, an online professional survey firm (www.qualtrics.com). The recruitment pool is managed by Qualtrics’. 
panel partner, Clearvoice Research, which comprises a census-representative panel of over one million members around the world. We recruited only panelists from the United States for this study. Past medical research has demonstrated the effective use of this company to recruit participants (28-30).

The company pulls a sample in quota groups and then uses simple randomization to produce a representative sample. The average panelist response rate (determined by clicks per invitation sent) is 20%. Many procedures are in place to confirm the identity of respondents, including verification of United States Postal Services (USPS) postal addresses, using flash cookies, and tracking internet protocol (IP) addresses. The research company maintains full records on panelist activity and limits panelists to one completed survey every ten days. Survey respondents are rewarded with a cash value amount, ranging from $1.00 to $20.00, based on the length of the survey and the target audience. This reward is then credited to the respondent’s account. Once the respondent’s account value exceeds $10.00, he/she can redeem for his/her selection of gift certificates or prepaid debit cards.

This cross-sectional study excluded men in an attempt to thoroughly examine many factors that impact attitudes towards ART rather than looking at any gender differences that might exist. Of the 341 women that were invited to take the survey and subsequently clicked the invitation, 324 agreed to the consent form and completed the survey (response rate: 95%). Data from 37 respondents were eliminated because they either did not fit into the specified age group and gender, they did not complete the entire survey, or they completed the survey too quickly for their results to be considered reliable (i.e. under five minutes). Thus the final sample size was 287. Table 1 summarizes the demographic and clinical characteristics of respondents.

All of the women surveyed fell into one of three age groups: 24 to 29 (n=84), 34 to 39 (n=106), and 44 to 49 (n=97). These age groups were chosen, so that distinctive differences between age groups could be identified. Aside from these age and gender restrictions, no other qualifiers were used, and qualifying participants were selected at random. All participants provided consent before being able to access the survey, and were debriefed upon completion of the survey.

After obtaining approval from the Institutional Review Board at Miami University in Oxford, OH, the survey was developed and pilot-tested. The survey instrument was formulated on the basis of a review of the literature related to attitudes towards ART. The survey was pilot-tested on ten undergraduate students in March 2011 at Miami University. The subjects were asked to complete the survey and provide feedback on the questions. They were also asked to record the amount of time required to complete the survey. The feedback obtained was used to develop the final version of the survey; however, no results were obtained and/or used.

The finalized survey was then made available on the Qualtrics online survey system from March to April 2011. It consisted of three main sections: questions pertaining to attitudes towards ART, demographics, and knowledge of ART. All questions, with the exception of one question ("Where did you hear about these treatments?"), were mutually exclusive; in other words, participants were only allowed to select one answer of those available. The survey took participants approximately 15 minutes to complete.

A series of 36 attitudinal questions were used to measure respondent opinions on the ethical aspects of ART. Participants were instructed to gauge their opinion on a nine-point Likert-type scale, with responses ranging from "strongly disagree" (1) to "strongly agree" (9). A subset of six of the attitudinal questions assessed respondents’ general attitudes towards infertility treatments. Six additional subscales were constructed to examine attitudes towards specific details of ART. For each subscale, items were selected for inclusion based on content analysis and subsequent factor reliability analysis. Table 2 depicts the composition of each subscale, as well as the Cronbach’s alpha (α), which is a measure of inter-item reliability.
Table 1: Demographics and clinical characteristics of participants

<table>
<thead>
<tr>
<th>Demographic</th>
<th>n</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td><strong>Age (Y)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24-29</td>
<td>84</td>
<td>29</td>
</tr>
<tr>
<td>34-39</td>
<td>106</td>
<td>37</td>
</tr>
<tr>
<td>44-49</td>
<td>97</td>
<td>34</td>
</tr>
<tr>
<td><strong>Educational attainment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High school or lower</td>
<td>56</td>
<td>20</td>
</tr>
<tr>
<td>Some college</td>
<td>84</td>
<td>29</td>
</tr>
<tr>
<td>Associates degree</td>
<td>39</td>
<td>14</td>
</tr>
<tr>
<td>Bachelor’s degree</td>
<td>73</td>
<td>25</td>
</tr>
<tr>
<td>Master’s degree</td>
<td>25</td>
<td>9</td>
</tr>
<tr>
<td>Doctoral degree</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td><strong>Religion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muslim</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Christian (non-Catholic)</td>
<td>143</td>
<td>50</td>
</tr>
<tr>
<td>Roman Catholic</td>
<td>76</td>
<td>27</td>
</tr>
<tr>
<td>Jewish</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Hindu</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Buddhist</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>None</td>
<td>29</td>
<td>10</td>
</tr>
<tr>
<td>Other</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td><strong>Frequency of church attendance</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>115</td>
<td>40</td>
</tr>
<tr>
<td>Religious holidays only</td>
<td>78</td>
<td>27</td>
</tr>
<tr>
<td>Monthly</td>
<td>35</td>
<td>12</td>
</tr>
<tr>
<td>Weekly</td>
<td>51</td>
<td>18</td>
</tr>
<tr>
<td>Daily</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td><strong>Annual household income</strong></td>
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<td></td>
</tr>
<tr>
<td>Less than $25,000</td>
<td>55</td>
<td>19</td>
</tr>
<tr>
<td>$25,000-$50,000</td>
<td>85</td>
<td>30</td>
</tr>
<tr>
<td>$50,000-$100,000</td>
<td>101</td>
<td>35</td>
</tr>
<tr>
<td>Above $100,000</td>
<td>31</td>
<td>11</td>
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**Table 1: Continued**

<table>
<thead>
<tr>
<th>Demographic</th>
<th>n</th>
<th>%</th>
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</thead>
<tbody>
<tr>
<td>Prefer not to answer</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>236</td>
<td>82</td>
</tr>
<tr>
<td>African American</td>
<td>15</td>
<td>5</td>
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<tr>
<td>Hispanic</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>Asian</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>Other</td>
<td>6</td>
<td>2</td>
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<tr>
<td>Political affiliation</td>
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<td></td>
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<tr>
<td>Democrat</td>
<td>102</td>
<td>36</td>
</tr>
<tr>
<td>Republican</td>
<td>67</td>
<td>23</td>
</tr>
<tr>
<td>Independent</td>
<td>63</td>
<td>22</td>
</tr>
<tr>
<td>Other</td>
<td>42</td>
<td>2</td>
</tr>
<tr>
<td>Prefer not to answer</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Length of awareness of ART</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Less than one year</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>One year</td>
<td>19</td>
<td>7</td>
</tr>
<tr>
<td>Five years</td>
<td>85</td>
<td>30</td>
</tr>
<tr>
<td>Ten years or longer</td>
<td>172</td>
<td>60</td>
</tr>
<tr>
<td>Current health status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fertile</td>
<td>136</td>
<td>47</td>
</tr>
<tr>
<td>Infertile</td>
<td>62</td>
<td>22</td>
</tr>
<tr>
<td>Unknown</td>
<td>89</td>
<td>31</td>
</tr>
<tr>
<td>Recipient of ART</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>No</td>
<td>271</td>
<td>94</td>
</tr>
<tr>
<td>Know recipient of ART</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>116</td>
<td>60</td>
</tr>
<tr>
<td>No</td>
<td>171</td>
<td>40</td>
</tr>
<tr>
<td>Heard of Octomom?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>187</td>
<td>35</td>
</tr>
<tr>
<td>No</td>
<td>100</td>
<td>65</td>
</tr>
<tr>
<td>Heard of Frieda Birnbaum?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>109</td>
<td>62</td>
</tr>
<tr>
<td>No</td>
<td>178</td>
<td>38</td>
</tr>
</tbody>
</table>

ART; Assisted reproductive technology and IVF; *in vitro* fertilization.
## Table 2: Attitudinal subscales

<table>
<thead>
<tr>
<th>Subscale</th>
<th>Items</th>
<th>Cronbach’s alpha (α)</th>
</tr>
</thead>
<tbody>
<tr>
<td>General</td>
<td>I am in favor of infertility treatments in general</td>
<td>0.859</td>
</tr>
<tr>
<td></td>
<td>Infertility treatments are tampering with nature*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infertility treatments are tampering with nature…; and therefore, make me uneasy so I would not consider them for myself*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infertility treatments are tampering with nature…; and therefore, are unethical and should not be performed*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>The benefits of infertility treatments outweigh the risks</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infertility treatments carry unknown consequences*</td>
<td></td>
</tr>
<tr>
<td>Sperm donation</td>
<td>If a man’s sperm are not viable, it is acceptable for him to use sperm donation</td>
<td>0.804</td>
</tr>
<tr>
<td></td>
<td>It is acceptable for a young, healthy man to donate his sperm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sperm donor bank are acceptable for homosexuals who want to have a child</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sperm donor banks are acceptable for parents to choose a father who is particularly intelligent</td>
<td></td>
</tr>
<tr>
<td>Egg donation</td>
<td>If a woman’s eggs are not viable, it is acceptable for her to use egg donation</td>
<td>0.844</td>
</tr>
<tr>
<td></td>
<td>It is acceptable for a young, healthy woman to donate her eggs</td>
<td></td>
</tr>
<tr>
<td>IVF</td>
<td>IVF is an acceptable treatment for couples with infertility problems</td>
<td>0.703</td>
</tr>
<tr>
<td></td>
<td>Preimplantation genetic diagnosis is a procedure of genetic testing performed on an embryo prior to implantation. I believe that this is an acceptable procedure in order to select a healthy, compatible embryo that can cure a sibling suffering from some disease</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IVF is an acceptable option for couples with serious genetic diseases to select embryos that do not carry the defective gene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>For fertile couples, it is acceptable to use IVF to choose the sex of their child</td>
<td></td>
</tr>
<tr>
<td>Selective embryo reduction</td>
<td>Selective embryo reduction is a procedure in which the number of fetuses is reduced in a pregnancy involving more than one fetus. I believe that this practice is appropriate</td>
<td>0.613</td>
</tr>
<tr>
<td></td>
<td>Selective embryo reduction is appropriate if the baby and/or mother are threatened</td>
<td></td>
</tr>
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</table>
Table 2: Continued

<table>
<thead>
<tr>
<th>Subscale</th>
<th>Items</th>
<th>Cronbach’s alpha (α)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulation of ART</td>
<td>I trust those in charge of new developments to act in society’s interests in regards to infertility treatments</td>
<td>0.573</td>
</tr>
<tr>
<td></td>
<td>I trust the regulatory system for infertility treatments to keep pace with scientific advancements</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Regulations on infertility treatments are too relaxed*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>The rules governing infertility treatments are well enforced</td>
<td></td>
</tr>
<tr>
<td>Accessibility of ART</td>
<td>There should be an age limit for infertility treatments*</td>
<td>0.771</td>
</tr>
<tr>
<td></td>
<td>Single women should have access to infertility treatments</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Individuals with criminal charges or a history of sexual offense should have access to infertility treatments</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Individuals with diseases/disabilities that may interfere with their ability to parent a child should have access to infertility treatments</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sperm donor banks are acceptable for homosexuals who want to have a child</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IVF is an acceptable option for couples with serious genetic diseases to select embryos that do not carry the defective gene</td>
<td></td>
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<tr>
<td></td>
<td>For fertile couples, it is acceptable to use IVF to choose the sex of their child</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sperm donor banks are acceptable for parents to choose a father who is particularly intelligent</td>
<td></td>
</tr>
</tbody>
</table>

ART; Assisted reproductive technology, IVF; In vitro fertilization and *; Reverse scored.

Respondent knowledge of infertility treatments was measured with 22 multiple-choice items. Each question had one correct response. These questions tested knowledge of a variety of aspects of ART, such as procedural information, the incidence of infertility, and the financial burden of treatment (see supplemental section at www.IJFS. ir). The remaining questions pertained to demographics and other clinical factors specific to ART (Table 1).

Following data collection, a series of statistical analyses were run using the Statistical Package for the Social Sciences (SPSS) program, version 18 (SPSS Inc., Chicago IL). An analysis of variance (ANOVA) was run to examine associations between each demographic variable and attitudes on the ethics of ART, as well as knowledge of ART. The paired samples t test was used to identify relationships between women’s attitudes of sperm and egg donation. A series of analysis of covariance (ANCOVA) was run to determine if any other factors covaried with respondent ethnicity and also to determine whether or not the two factors, political affiliation and length of awareness, covaried with each other. Finally, we studied the relationship between length of awareness and general attitudes by means of a general linear regression.

Results
The measure of general attitudes towards
ART was significantly related to two factors. First, a simple linear regression using length of awareness as a predictor and general attitudes as the dependent variable revealed a significant regression weight (b=0.188) for the length of awareness [t (285)=3.23, P<0.001]. Hence, participants’ general attitudes towards ART became progressively more positive as their length of awareness of ART increased (Fig. 1). As depicted in table 3, political affiliation was also significantly related to general attitudes towards ART [F (2, 229)=7.24, P=0.001]. In general, all affiliations were relatively supportive of ART (M>5 in all three groups); however, Democratic women were the most supportive and Republican women were the least supportive.

Fig. 1: General attitude toward ART depending on length of awareness. *; Measured on a 9-point Likert-type scale (1=lowest; 9=highest) and ART; Assisted reproductive technology.

Political affiliation and length of awareness were unrelated [F<1, n.s.]. An ANCOVA, using political affiliation as a factor and length of awareness as a covariate, yielded a significant result of the covariate, length of awareness [F (1,228)=8.08, P<0.005], and a significant result of political affiliation [F (2,228)=8.15, P<0.000]. Therefore, while both length of awareness and political affiliation affect women’s general attitude towards ART, both of these effects are parallel, and the effect of political affiliation cannot be explained by length of awareness.

A series of ANOVAs were run to examine the relationship between each individual attitudinal subscale and each of the demographic/clinical factors listed in table 1. Many of the factors varied significantly within specific attitudinal subscales. Table 3 shows the mean attitudinal ratings corresponding to the statistically significant ANOVAs. Significant F values indicate that the average attitudinal rating for a specific subscale differed significantly across the individual categories of that particular demographic-clinical factor. The remaining ANOVAs not shown in table 3 were not significant. We wished to further analyze women’s attitudes towards specific aspects of the regulation of ART, which is depicted in table 4.

The attitudinal questions regarding gamete donation revealed significant differences in women’s opinions of sperm and egg donation. Sperm donors were rated as significantly more likely to donate only for the money [t(286)=8.38, P=0.000], but egg donors were rated as significantly more likely to later regret their decision to donate for both psychological and medical reasons [t(286)=6.88, P=0.000 and t(286)=7.06, P=0.000, respectively]. Also, for both egg and sperm donation, women were significantly more supportive of an individual donating their gametes than they were of an individual utilizing donated material [t(286)=2.06, P=0.040 and t(286)=3.33, P=0.001, respectively] (Table 5).

Over four-fifths (83%) of respondents who had heard of IVF (n=263) underestimated the incidence of twins with IVF. When asked what fraction of infertility patients eventually has a baby after treatment, only 25% of participants correctly selected 70% of patients. Only 17% of women knew that the level of malformations is higher in ART children. Similarly, over half (54%) of participants overestimated the percentage of infertility cases due to female factors as compared to the percentage due to male factors. Among those who had heard of IVF, 95% overestimated the frequency of IVF as the treatment of choice for infertility. And finally, respondents who underestimated the price of a single cycle of IVF (M=5.93, SD=2.42) were significantly less likely to agree that the cost of infertility treatments is unreasonable than those who did not underestimate this value (M=6.80, SD=1.98) [ F(1,261)=4.71, P=0.031].
Fortin and Abele

Table 3: ANOVA results of mean (SD) attitudinal ratings within factors influencing attitudes towards ART

<table>
<thead>
<tr>
<th>Attitudes toward</th>
<th>Political affiliation*</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Republican</td>
<td>Democrat</td>
<td>Independent</td>
</tr>
<tr>
<td>General attitudes</td>
<td>5.4 (1.9)</td>
<td>6.4 (1.6)</td>
<td>6.1 (1.6)</td>
</tr>
<tr>
<td>Sperm donation</td>
<td>5.3 (2.2)</td>
<td>6.5 (1.7)</td>
<td>6.0 (2.0)</td>
</tr>
<tr>
<td>Egg donation</td>
<td>6.3 (2.3)</td>
<td>7.1 (2.0)</td>
<td>7.1 (2.0)</td>
</tr>
<tr>
<td>IVF</td>
<td>4.8 (1.8)</td>
<td>5.8 (1.6)</td>
<td>5.4 (1.8)</td>
</tr>
<tr>
<td>Regulation of ART</td>
<td>4.4 (1.7)</td>
<td>5.4 (1.4)</td>
<td>5.0 (1.6)</td>
</tr>
<tr>
<td>Accessibility of ART</td>
<td>3.9 (1.7)</td>
<td>5.0 (1.4)</td>
<td>4.6 (1.6)</td>
</tr>
</tbody>
</table>

Frequency of church attendanceb

<table>
<thead>
<tr>
<th></th>
<th>Frequently</th>
<th>Infrequently</th>
<th>Never</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm donation</td>
<td>5.0 (2.3)</td>
<td>6.0 (1.9)</td>
<td>6.3 (1.9)</td>
<td>8.79</td>
<td>0.000</td>
</tr>
<tr>
<td>Egg donation</td>
<td>4.8 (2.6)</td>
<td>5.4 (1.9)</td>
<td>5.5 (1.9)</td>
<td>7.33</td>
<td>0.001</td>
</tr>
<tr>
<td>IVF</td>
<td>4.8 (2.0)</td>
<td>5.4 (1.5)</td>
<td>5.5 (1.7)</td>
<td>3.93</td>
<td>0.021</td>
</tr>
<tr>
<td>Embryo reduction</td>
<td>4.9 (2.4)</td>
<td>5.8 (2.0)</td>
<td>5.9 (1.8)</td>
<td>5.29</td>
<td>0.006</td>
</tr>
<tr>
<td>Accessibility of ART</td>
<td>3.9 (1.9)</td>
<td>4.5 (1.5)</td>
<td>4.8 (1.4)</td>
<td>7.10</td>
<td>0.000</td>
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</table>

Religionc

<table>
<thead>
<tr>
<th></th>
<th>Catholic</th>
<th>Non-Catholic Christian</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo reduction</td>
<td>6.0 (1.8)</td>
<td>5.4 (2.2)</td>
<td>4.28</td>
<td>0.040</td>
</tr>
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</table>

Know participant of ART?

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulation of ART</td>
<td>4.6 (1.6)</td>
<td>5.1 (1.5)</td>
<td>7.01</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Participant of ART?d

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulation of ART</td>
<td>3.8 (4.9)</td>
<td>2.0 (1.6)</td>
<td>13.66</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Ethnicitye, f

<table>
<thead>
<tr>
<th></th>
<th>Caucasian</th>
<th>Non-Caucasian</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulation of ART</td>
<td>4.9 (1.6)</td>
<td>5.3 (1.6)</td>
<td>4.26</td>
<td>0.040</td>
</tr>
<tr>
<td>Accessibility of ART</td>
<td>4.4 (1.6)</td>
<td>4.9 (1.5)</td>
<td>4.73</td>
<td>0.030</td>
</tr>
</tbody>
</table>

* The categories "Other" and "Prefer not to answer" and "None" were excluded from analysis. b; For analysis purposes, women who reported attending monthly or only on religious holidays were considered to have "infrequent" attendance. Those who said that they attend either daily or weekly were classified as having "frequent" attendance. c; Religion was dichotomized into Catholic vs. non-Catholic Christian due to insufficient participants in other religious groups. d; The number of women who reported being a participant in ART (n=12) is too low to make any solid conclusions. e; Women were divided into two ethnic groups-Caucasian and non-Caucasian due to insufficient participants in other ethnic groups. f; A series of ANCOVAs was run to determine if any other factors covaried with ethnicity [between-subjects factor: ethnicity (Caucasian, non-Caucasian); covariates: education, religion, political affiliation, frequency of church attendance, and length of awareness of ART] revealed that none of these measures could explain the effect of ethnicity (all P>.20). ART; Assisted reproductive technology and IVF; in vitro fertilization.
**Table 4: Attitudes towards regulation of ART**

<table>
<thead>
<tr>
<th>Attitudes toward</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>I trust those in charge of new developments to act in society’s interests in</td>
<td>4.97</td>
<td>2.21</td>
</tr>
<tr>
<td>regards to infertility treatments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I trust the regulatory system for infertility treatments to keep pace with</td>
<td>5.15</td>
<td>2.13</td>
</tr>
<tr>
<td>scientific advancements</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regulations on infertility treatments are too relaxed*</td>
<td>4.95</td>
<td>2.04</td>
</tr>
<tr>
<td>The rules governing infertility treatments are well enforced</td>
<td>4.69</td>
<td>1.81</td>
</tr>
</tbody>
</table>

*; Reverse scored and ART; Assisted reproductive technology.

**Table 5: Attitudes towards gamete donation**

<table>
<thead>
<tr>
<th>Attitudes toward</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>If a woman’s eggs are not viable, it is acceptable for her to use egg donation</td>
<td>287</td>
<td>6.71</td>
<td>2.31</td>
<td>1.39</td>
<td>0.167</td>
</tr>
<tr>
<td>If a man’s sperm are not viable, it is acceptable for him to use sperm donation</td>
<td>297</td>
<td>6.60</td>
<td>2.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>It is acceptable for a young, healthy woman to donate her eggs</td>
<td>287</td>
<td>6.91</td>
<td>2.21</td>
<td>-0.15</td>
<td>0.878</td>
</tr>
<tr>
<td>It is acceptable for a young, healthy man to donate his sperm</td>
<td>287</td>
<td>6.92</td>
<td>2.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Most egg donors only donate their eggs for the money</td>
<td>287</td>
<td>5.46</td>
<td>2.27</td>
<td>-8.38</td>
<td>0.000</td>
</tr>
<tr>
<td>Most sperm donors only donate their sperm for the money</td>
<td>287</td>
<td>6.29</td>
<td>2.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>It is likely that an egg donor would later regret her decision to donate her</td>
<td>287</td>
<td>4.46</td>
<td>2.20</td>
<td>6.88</td>
<td>0.000</td>
</tr>
<tr>
<td>eggs for psychological reasons</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>It is likely that a sperm donor would later regret his decision to donate his</td>
<td>287</td>
<td>3.63</td>
<td>2.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sperm for psychological reasons</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>It is likely that an egg donor would later regret her decision to donate her</td>
<td>287</td>
<td>4.07</td>
<td>2.20</td>
<td>7.06</td>
<td>0.000</td>
</tr>
<tr>
<td>eggs for medical reasons</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>It is likely that a sperm donor would regret his decision to donate his sperm</td>
<td>287</td>
<td>3.32</td>
<td>2.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>for medical reasons</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Discussion

The overall general attitude towards ART was significantly related to two demographic factors: length of awareness of ART and political affiliation. This study is the first to identify a significant tie between the length of time that individuals are aware of ART and their attitudes towards these treatments. It is possible that this observed outcome is a manifestation of the mere exposure effect; that merely being exposed to infertility treatments is enough to increase acceptance and augment positive attitudes towards these treatments (21).

But why does this connection between length of awareness of ART and favorability of ART exist in the first place? Several researchers have attempted to explain the reasoning behind the link between familiarity and acceptance. Diamantopoulos et al. (27) studied attitudes towards epileptic individuals and concluded that people tend to be fearful of the things that they do not know or understand, so being familiar with a disorder naturally increases the degree of tolerance towards it. Perhaps those who have not been exposed to the topic of infertility treatments feel uneasy because they lack a basic understanding of these treatments, which hinders any opportunity for acceptance.

A similar theory proposes that familiarity influences stigma (25). Based on this model, those who have been aware of ART for a longer length of time might be less likely to endorse stigmatizing attitudes towards ART. This highlights the importance of informing the public of these treatments, so that stigma can be reduced and public acceptance facilitated.

The finding that political affiliation was significantly related to the general measure of attitudes towards ART as well as five of the specific attitudinal subscales indicate that this demographic factor is a major predictor of attitudes towards ART. In each of these instances, Republican women were less favorable towards ART than both Democratic and Independent women—results that further validate previous findings (8, 9, 14). This finding might reflect the effect of Republican women holding more conservative views generally, rather than being specifically induced by categorizing oneself as Republican. Regardless, it is important to discover what changes can be made to the presentation of ART, so that it is accepted by all political parties.

The observed attitudinal differences between Catholics and non-Catholic Christians were not congruent with what might be expected based on traditional religious orthodoxy. It was surprising to find that Catholic women were actually significantly more supportive towards many of the specific aspects of ART than non-Catholic Christian women. As has been previously documented, however, inconsistencies do exist between official religious discourse and the individual beliefs of followers (31, 32). Thus, perhaps the more appropriate indicator of one’s religiosity is frequency of church attendance. When viewed in this manner, it becomes apparent that there is a significant inverse relationship between religiosity and several of the specific measures of attitudes towards ART—namely, attitudes towards sperm donation, egg donation, IVF, selective embryo reduction, and accessibility of ART.

The observation that there were significant differences in participant attitudes towards egg and sperm donation indicates that women do not view these two procedures as equal. The apparent gender discrepancy may indicate that social stigmas affect women’s opinions on these issues. Women also hold differing opinions towards gamete donation depending on whether an individual is donating or receiving a gamete. Again, it is possible that using donated gametes—but not being a donor of gametes—is a procedure that is stigmatized. Both of these findings are research questions that should be explored further.

Age was not significantly related to any of the attitudinal subscales. This finding was not unexpected, as previous studies of this association have been varied and inconsistent. A large survey on the public’s perceptions of infertility treatments conducted in six European countries, the USA, and Australia reported that opinions varied little among age groups (33). Similarly, Sigillo et al. (12) found no association between age and attitudes towards IVF for nontraditional women. On the other hand, Shreffler et al. (9) found that women under age 30 and women beyond 40 had higher ethical concerns than women in their thirties.

The lack of sufficient participants in certain categories (e.g. religious affiliation, ethnicity and being a participant of ART) is a limitation in our study.
A large portion of our respondents were Caucasian and well-educated. A larger, more diverse pool of participants would likely yield data that permit a clearer evaluation of national opinion. Our study is also limited due to the fact that the survey was administered online, which renders it prone to the limitations associated with internet research, including technical difficulties and uncertain representativeness of selected samples.

Because of the socioeconomic and ethical issues raised by ART, an awareness of the various public attitudes surrounding ART has important implications for many specific sectors. Medical professionals must be especially cognizant of these attitudes in light of the public’s concern for the ethics of medicine. A comprehensive understanding of public perceptions of ART is essential for all medical professionals, but particularly for those who practice reproductive techniques.

Our research highlights a need to inform the public in the realm of infertility medicine, so that any misconceptions can be eliminated or prevented—an undertaking that can only be realized with the support of the medical field. The high likelihood of overestimating and underestimating on many of the knowledge items indicates that the public is rather misinformed on some aspects of ART. The finding that those who underestimated the cost of IVF were less likely to agree that the cost of IVF is unreasonable illustrates the direct influence that misconceptions can have on one’s attitudes. This research should help healthcare professionals to educate the public about ART, so that misunderstandings do not hinder public acceptance of these treatments.

This line of research also has practical implications for legislators, and should help direct them towards making informed decisions about future ART policies. Our results revealed that women are not extremely trusting of the regulatory system, and tend to disagree that the rules governing infertility treatments are well enforced. Furthermore, it appears that those who are more familiar with, and have had more exposure to, ART are actually less supportive of the regulatory system (e.g. those who have undergone infertility treatments, those who know a participant of infertility treatments, and those who are infertile). It is essential that authorities continually gauge public opinion to uphold the public’s endorsement of a field that is constantly evolving.

Conclusion

Our study identified key factors that influence the attitudes that women hold in regards to ART. For the first time, a link has been established between the length of time that a woman has been aware of ART, and her general attitudes towards ART. Age did not appear to be a significant factor; however, political affiliation and religion were significantly associated with women’s attitudes towards ART. Identifying some of the factors associated with decreased approval of infertility treatments serves as a crucial starting point for formulating strategies for wider public understanding.

Acknowledgements

We would like to thank the Miami University Senate Committee and the Miami University Honors College for funding this project. Additionally, we express our appreciation to Dr. Michael Diamond, Dr. Gary Stasser, Dr. Jennifer Green, Dr. Matthew Groebe, and Dr. Christopher Chartier for their assistance with editing our manuscript. None of the authors have any conflicts of interest to disclose.

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Sexual Satisfaction and Sexual Reactivity in Infertile Women: The Contribution of The Dyadic Functioning and Clinical Variables

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Faculty of Psychology, University of Finance and Management in Warsaw, Warsaw, Poland

Abstract

Background: Infertility is a factor which has been linked to higher prevalence of sexual dysfunctions in women; however, ambiguous results have been reported about the impact of infertility on women’s sexual satisfaction. The purpose of this study was to compare sexual and dyadic functioning in infertile and fertile women. Furthermore, the associations between sexual variables and clinical variables (depressive symptoms, period trying to conceive, and treatment period) were assessed in infertile women sample.

Materials and Methods: The cross-sectional study involved 50 women with the history of infertility and 50 fertile women recruited from the general population. The Sexual Satisfaction Scale (SSS), Mell-Krat Scale (women’s version), Family Assessment Measure (FAM-III), and Beck Depression Inventory (BDI) were administered to all participants.

Results: Infertile women reported lower sexual satisfaction and more maladaptive patterns of dyadic functioning in comparison to the control group. As many as 45 (90%) of infertile women, compared to 13 (26%) of the control group, reported the scores on the Mell-Krat Scale indicative of the presence of dysfunctions in sexual reactivity (P≤0.001). Infertile women reported significantly higher levels of depressive symptoms than the women from the control group (P≤0.001). Negative correlations were observed between sexual satisfaction and dyadic functioning in both groups (P≤0.05); however, the patterns of these associations were different in infertile and fertile women. For example, negative correlations were found between satisfaction with control and role performance, affective involvement, and values and norms in infertile women. However, these relationships were not observed in the control group. No correlations were revealed between sexual reactivity and dyadic functioning in infertile women and the control group. Negative correlations were observed between satisfaction with control and relationship duration as well as between sexual reactivity and period of trying to conceive. Multiple regression analyses also revealed different predictors of sexual satisfaction in both groups: affective involvement (P≤0.05) and relationship duration (P≤0.05) in infertile women, whereas communication (P≤0.05), affective expression (P≤0.05) and depressive symptoms (P≤0.05) in the control group.

Conclusion: Infertility is an important factor affecting sexual and dyadic functioning and is linked to higher depressive symptoms in infertile women.

Keywords: Infertility, Female, Sexuality, Sexual

Introduction

Infertility is a socio-medical problem affecting couples all over the world. Infertility is defined as ‘the inability to conceive after 12 months of regular unprotected sexual intercourse’ (1). The number of infertile women is constantly increasing. It has been estimated that approximately 72.4 million women worldwide are infertile (2). The prevalence rate of infertility in Polish women varies from 10 to 15% (3), whereas 13-17% of women in Finland are affected by infertility (4). Similarly, high prevalence rates of infertility have been reported in other countries such as Turkey (10%), USA (10%) and China (7-10%) (5). Taking into account the high prevalence of infertility and its psychological, social and economic consequences, it is important to realize that infertility is not only a common medical problem, but also an acute psychosocial issue (6).

Hence, numerous studies have been devoted to investigating causes of infertility (7-9) and its psychological implications (10, 11). For instance, it has been demonstrated that learning about one’s infertility is one of the most stressful experiences, comparable to those associated with the diagnosis of cancer, hypertension and human immunodeficiency virus (HIV)-positive status (12). Parenthood is believed to be not only a stage of life but also one of the most important developmental tasks with numerous implications for the relationships in the couple and within the whole family system. Therefore, unwanted infertility can be the cause of severe distress in infertile couples and the futile efforts to conceive exert long-term effects on human functioning.

Tao et al. (13) reported that infertility may have a negative influence on sexual behavior; however, this effect depends on gender, cause of infertility, duration of infertility and treatment success. For example, men revealed lower sexual satisfaction than women, especially when the male factor infertility was involved, whereas women had lower sexual satisfaction after an in vitro fertilization treatment that had been unsuccessful. Similarly, another study carried out among 200 infertile couples and 200 fertile couples indicated that infertile couples demonstrated lower self-esteem, marital satisfaction and sexual satisfaction (14) and it is supported by other findings indicating that infertility lasting for 3-6 years was linked to the highest relationship instability and the lowest sexual satisfaction (3). Lower sexual satisfaction may be caused by a decreased frequency of intercourse and it can be associated with sexual dysfunctions such as problems with sexual arousal (in infertile women), premature ejaculation and erection dysfunction (in infertile men) (13). The findings obtained in another study conducted among 308 infertile women and 308 fertile women indicated that 61.7% of infertile women had sexual dysfunctions compared to 26.55% of those without infertility. The dysfunctions involved desire, arousal and orgasm problems (5).

Moreover, it has been demonstrated that stress can play a detrimental role in the functioning and longevity of a close relationship (15). For instance, Bodenmann et al. (16) proposed the stress-divorce-model, in which he suggested that chronic stress has an impact on marital satisfaction by decreasing the time that partners spend together, which in turn results in reduction of shared experiences, decreasing the quality of communication, increasing the likelihood that problematic personality traits will be expressed between partners (rigidity, anxiety, and hostility) and increasing the risk of psychological and physical problems, such as sleep disorders, sexual dysfunctions and mood disturbances.

Indeed, previous studies indicated that infertile couples have a higher level of depression than fertile couples (17). Likewise, childless women had an increased risk of dysthymia and anxiety disorders compared to women with children. Interestingly, women who currently have a child but experienced infertility in the past have a higher risk of developing a panic disorder (4). Among variables which may contribute to the development of depression and anxiety in infertile women, researchers found such factors as duration of infertility, cause of infertility, educational level (18), age, male factor infertility (19) and pressure from family (20). High rates of depressive symptoms among infertile women may also have an important impact on sexual activity and satisfaction. It has been demonstrated extensively that depressive symptoms are associated with impairments of sexual function and satisfaction (21) by inhibited sexual arousal, inhibited orgasm and less pleasure experienced during intercourse (22).

Taking into account the inconsistency of the findings from previous studies, it is essential to
continue research on sexual functioning among infertile women.

Thus, in this study, we concentrated on comparison of infertile and fertile women with regard to sexual and dyadic functioning. Additionally, the associations between sexual satisfaction and dyadic functioning in infertile and control group as well as relationships between sexual satisfaction and clinical variables (depressive symptoms, period trying to conceive and treatment period) in infertile women sample were evaluated.

Thus, the present study aimed at i. Comparing sexual and dyadic functioning in infertile and fertile women samples and ii. Determining the relationship between sexual satisfaction/reactivity and relational and clinical variables in Polish infertile women.

Materials and Methods

The study had a cross-sectional design and was conducted between November 2011 and February 2012. One-hundred women were recruited to participate in the study, including 50 infertile women and 50 women without known infertility diagnosis (control group). All the infertile women participated in the reproductive treatment in a private infertility outpatient clinic in Warsaw, Poland. The inclusion criteria for participation were: i. A confirmed diagnosis of infertility on the part of the women made by a gynecologist, ii. Ineffective efforts to conceive undertaken for a period of at least one year prior to the study, iii. Age between 18 and 40 years, iv. No children and v. Staying in an intimate relationship with a partner. Exclusion criteria included: i. The co-occurrence of serious chronic somatic diseases (such as diabetes, hyperthyroidism), ii. The co-occurrence of psychiatric disorders involving delusions or hallucinations and iii. Lack of consent to participate. The women fulfilling these criteria were approached by their treating doctor with the invitation to enroll into the study during their pre-scheduled visits in the clinic. A total of 79 women were initially approached, out of whom 50 agreed and gave their written informed consent to participate in the study. The control group consisted of 50 women recruited from the general population, who reported they did not have infertility-related problems. The women from the control group were recruited through announcements made by a local university newspaper and through the Internet. The women from the control group were offered an incentive in the form of a book. Initially, 92 women responded to the announcements, out of whom 50 fulfilled the required inclusion/exclusion criteria and showed up for a scheduled appointment to complete the questionnaires. After thorough explanation of the objective and nature of the study, written informed consent was obtained from all participants. The project of the study was approved by the University Ethical Committee, University of Finance and Management in Warsaw, ul. Pawia, Poland.

All participants completed a battery of questionnaires measuring sexual satisfaction, sexual reactivity, depressive symptoms and the qualities of dyadic functioning in their close relationship. All the instruments were standard psychological or sexological measures which had previously been validated in the Polish population. Additionally, socio-demographic and clinical data were collected by means of the Personal Data Sheet, designed for the purpose of this study. The infertile women filled in the questionnaires individually, in a separate room in the clinic, supervised by one of the researchers, a qualified psychologist. The women from the control group were invited to come to the University and completed the questionnaires individually in a separate room at the University during a pre-scheduled appointment, supervised by the same researcher.

The following instruments were used in the study:

Sexual Satisfaction Scale (SSS) is a self-report questionnaire developed by Davis et al. (23) to assess sexual satisfaction. The SSS consists of 21 items designed. All items are affirmative statements in the first person singular and they constitute three subscales: physical satisfaction, emotional satisfaction and satisfaction with control. The physical satisfaction subscale measures satisfaction with fulfillment of sexual needs, quality of the couple’s sexual contacts and the partners’ sexual abilities. The emotional satisfaction subscale is designed to evaluate satisfaction with the feelings towards the partner and his/her sexual behaviors. The satisfaction with control subscale serves to assess the control over one’s own sexual
performance and frequency and timing of sexual intercourses. The responses are given on a 5-point Likert scale anchored with: I definitely disagree, I don’t agree, I am not sure/It is difficult to decide, I agree, I definitely agree. The scores are computed for each subscale. Higher scores indicate higher sexual satisfaction. High or satisfactory reliability coefficients (Cronbach’s α) were reported for the instrument: 0.85 for physical satisfaction, 0.84 for emotional satisfaction and 0.75 for satisfaction with control.

Mell-Krat Scale (the version for women) is a self-report questionnaire measuring sexual reactivity (24, 25). Mell-Krat Scale consists of 20 multiple-choice items measuring a range of psychophysiological characteristics related to sexual reactivity like orgasm frequency, libido, intercourse orgasm frequency, pre-copitus arousal, vagina contractions during orgasm, etc. Each item describes five qualitatively different states related to a given aspect of sexual reactivity, ranging from highly disordered to fully satisfactory states. The items are scored on a 5-point scale, from 0 to 4. The total score is computed by summing up the scores for all items. The theoretical range of the scores is from 0 to 80, with lower scores interpreted as indicative of problems in sexual reactivity. The score of 55 was proposed as the cut-off for the recognition of sexual disorders. The reliability coefficient (Cronbach’s α) of 0.92 was reported for the Scale.

Family Assessment Measure (FAM-III) is a complex measure developed by Skinner et al. (26, 27). The Polish adaptation of the instrument was performed by Beauvale et al. (28). FAM-III consists of three questionnaires (the general scale, the self-rating scale and the dyadic relationship scale) used to evaluate family functioning. The general scale captures the family in terms of a system and it generally serves to examine general family functioning and its overall health. The self-rating scale is used to evaluate one’s own functioning within the family system. In the present study, only the scores from the third scale—the dyadic relationship—were used for further statistical analyses. This scale consists of 28 items designed to assess a particular relationship within the family system, for instance, an individual’s relationship with another family member. The items of this scale pertain to seven dimensions of family functioning: task accomplishment, role performance, communication, affective expression, affective involvement, control, and values and norms. The task accomplishment is the most important dimension in the process model as it allows to achieve biological, psychological and social goals by task fulfillment within the family system. The role performance dimension grasps the couple’s functioning, especially when the couple is faced with the need of tackling a common task. To facilitate task accomplishment and role performance, two other processes (communication and affective expression) are used. The affective involvement dimension refers to the degree and quality of the couple’s mutual concern and interest. The control dimension is designed to cover various interpersonal strategies and techniques, which can be used by couples to impact the partner’s behavior. Finally, the values and norms influence all aspects of the couple’s functioning and determine what is acceptable within the dyad. The items are rated on a 4-point scale, ranging from 0 (‘strongly agree’) to 3 (‘strongly disagree’). The scores are computed separately for the seven subscales by adding up the points for each item. Scoring for some items is reversed due to negative wording. The theoretical range of the scores for each dimension is from 0 to 12. Higher scores indicate more maladaptive patterns of a given dimension of family functioning. The authors reported internal reliability coefficients (alphas) of 0.86-0.95, and test-retest reliabilities of 0.57-0.66 (26, 27).

Beck Depression Inventory (BDI) is a self-report measure designed to measure severity of depressive symptoms as experienced over an indicated period of time (29). The Polish adaptation of the original version of this instrument was used in the study, previously validated in Polish samples (30). BDI contains 21 multiple-choice items, each scored on a 4-point scale, from 0 to 3. The total score ranges from 0 to 63 with higher scores implying more severe depressive symptoms (29, 30). Several proposals of the BDI threshold for depression were suggested; in this study, we assumed the score of 12 as a cut-off for clinical depression (31). Most studies reported Cronbach’s alpha reliability coefficients higher than 0.75, the mean alpha coefficient of 0.88 was reported for psychiatric samples, and 0.82 for non-psychiatric samples (32).

Personal Data Sheet was developed for the purpose of this study. It was used in two versions - for infertile and fertile women and it served to collect selected socio-demographic data, including age, place of residence, educational level, and the relationship status and duration. The version for infertile women contained additional questions referring to clinical variables pertaining to infertility,
such as the length of period the couples have been trying to conceive, treatment period, and a possible cause of infertility.

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS; SPSS Inc., USA) for Windows 12.0. Descriptive statistics were calculated as frequencies/means and standard deviations for each variable. Based on the features of the data distribution, appropriate analyses were performed, including Pearson’s r correlation coefficients or Spearman’s rank correlation to analyze the relationships between the variables measured on continuous scales. Student’s t tests and Pearson’s chi-squared tests were used to test the between-the group differences for continuous and categorical variables, respectively. Multiple regression analysis was applied to identify statistically significant predictors of sexual satisfaction and sexual reactivity. P≤0.05 was accepted as the level of statistical significance.

Results

Participants’ characteristics

The socio-demographic characteristics and variables associated with infertility in the sample are shown in table 1. The cause of infertility was known in 41 women (82%) from the clinical group, and in nine cases, it remained unrecognized. The treatment duration was as follows: less than 6 months (48%), between 6 months to 12 months (10%), between 13 to 24 months (28%), and more than 24 months (14%).

<table>
<thead>
<tr>
<th>Table 1: Socio-demographic characteristics and variables associated with infertility in the sample</th>
<th>Infertile women</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Age (Y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤20</td>
<td>1</td>
<td>2.0</td>
</tr>
<tr>
<td>21-30</td>
<td>34</td>
<td>68.0</td>
</tr>
<tr>
<td>31-40</td>
<td>15</td>
<td>30.0</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary</td>
<td>13</td>
<td>26.0</td>
</tr>
<tr>
<td>Higher</td>
<td>37</td>
<td>74.0</td>
</tr>
<tr>
<td>Origin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urban</td>
<td>32</td>
<td>64.0</td>
</tr>
<tr>
<td>Rural</td>
<td>18</td>
<td>36.0</td>
</tr>
<tr>
<td>Relationship duration (Y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5-1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2-4</td>
<td>25</td>
<td>50.0</td>
</tr>
<tr>
<td>≥5</td>
<td>25</td>
<td>50.0</td>
</tr>
<tr>
<td>Time trying to conceive (Y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-2</td>
<td>21</td>
<td>42.0</td>
</tr>
<tr>
<td>3-4</td>
<td>20</td>
<td>40.0</td>
</tr>
<tr>
<td>≥5</td>
<td>9</td>
<td>18.0</td>
</tr>
<tr>
<td>Infertility reason</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Known</td>
<td>41</td>
<td>82.0</td>
</tr>
<tr>
<td>Unknown</td>
<td>9</td>
<td>18.0</td>
</tr>
<tr>
<td>Treatment duration (Y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤0.5</td>
<td>24</td>
<td>48.0</td>
</tr>
<tr>
<td>0.5-1</td>
<td>5</td>
<td>10.0</td>
</tr>
<tr>
<td>1-2</td>
<td>14</td>
<td>28.0</td>
</tr>
<tr>
<td>&gt;2</td>
<td>7</td>
<td>14.0</td>
</tr>
</tbody>
</table>
Comparison of infertile women and the control group on psychological measures

Statistically significant differences were found between infertile women and fertile women with respect to sexual satisfaction and sexual reactivity. Significantly lower mean scores on all dimensions of sexual satisfaction (physical satisfaction, emotional satisfaction and satisfaction with control, \(P \leq 0.001\)) were found in infertile women as compared to the control group. The index of sexual reactivity (the total score on Mell-Krat Scale) was significantly lower (indicating more problems with sexual reactivity) in the sample of infertile women as compared to fertile women (\(P \leq 0.001\)). Ninety percent of infertile women and 26% of fertile women obtained the scores lower than the Mell-Krat cut-off score of 55, which might imply an elevated risk of dysfunctions in sexual reactivity. These results are presented in table 2.

With regard to the dyadic relationships, infertile women differed significantly from fertile women in the scores on six FAM-III subscales (task accomplishment, role performance, communication, affective expression, affective involvement, and values and norms). Infertile women were found to report higher scores on each of these subscales, compared to the control women. No statistically significant difference was observed with regard to the control subscale of FAM-III (\(P=0.30\), Table 2).

A significant difference was observed between the infertile women and the control group with respect to severity of depressive symptoms. The mean BDI score was significantly higher in the group of infertile women indicating that they were more severely depressed (Table 2). Based on the BDI threshold score of 12, 39 (78%) of infertile respondents were within the range for depression [including 38 (76%) for moderate and 1 (2%) for severe depressive symptoms]. Only 1 (2%) woman from the control sample obtained the scores within the range for depression, and the difference between the groups was statistically significant \((\chi^2=60.26, df=3, P<0.001)\).

<table>
<thead>
<tr>
<th>Table 2: Comparison of the mean scores on the SSS subscales, Mell-Krat Scale, FAM-III subscales and BDI in infertile women (n=50) and in the control group (n=50)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Infertile women</strong></td>
</tr>
<tr>
<td>Physical satisfaction (SSS)</td>
</tr>
<tr>
<td>34.36</td>
</tr>
<tr>
<td>Emotional satisfaction (SSS)</td>
</tr>
<tr>
<td>Satisfaction with control (SSS)</td>
</tr>
<tr>
<td>Sexual reactivity (Mell-Krat Scale)</td>
</tr>
<tr>
<td>Task accomplishment (FAM-III)</td>
</tr>
<tr>
<td>Role performance (FAM-III)</td>
</tr>
<tr>
<td>Communication (FAM-III)</td>
</tr>
<tr>
<td>Affective expression (FAM-III)</td>
</tr>
<tr>
<td>Affective involvement (FAM-III)</td>
</tr>
<tr>
<td>Control (FAM-III)</td>
</tr>
<tr>
<td>Values and norms (FAM-III)</td>
</tr>
<tr>
<td>Depressive symptoms (BDI)</td>
</tr>
</tbody>
</table>

The comparisons were made by means of Student’s t tests, with Cochran-Cox correction for heterogeneous variances. M; Mean, t; Statistic of Student’s t test, P; Significance level, SSS; Sexual satisfaction scale, FAM-III; Family assessment measure and BDI; Beck depression inventory.
Psychological correlates of sexual satisfaction and sexual reactivity in infertile women and in the control group

In both samples, a pattern of negative correlations was found between physical sexual satisfaction and the qualities of the dyadic relationship, as measured by FAM-III (Table 3), indicating that higher physical sexual satisfaction was associated with better dyadic functioning (P≤0.05). However, in the sample of infertile women, the correlations of physical satisfaction with affective expression and control were non-significant, and overall the correlations were slightly lower than in the control group. Emotional sexual satisfaction was significantly associated with affective involvement (P≤0.001) and values and norms (P≤0.05) in infertile women, whereas with role performance and communication (P≤0.001) in the control group. Satisfaction with control over sexual life was unrelated to the dyadic functioning in the control group, and significantly associated with task accomplishment, role performance, affective involvement and values and norms (P≤0.001) in infertile women. Negative correlations were found between all three indexes of sexual satisfaction and depressive symptoms in the control group. Interestingly, these associations were not statistically significant in the sample of infertile women. There was no correlation between sexual reactivity and the aspects of the dyadic relationship or between sexual reactivity and depressive symptoms in either sample (Table 3).

Table 3: Pearson’s r correlation coefficients between sexual satisfaction and sexual reactivity with FAM-III subscales and the BDI total score in the samples of infertile (I) and fertile (F) women

<table>
<thead>
<tr>
<th></th>
<th>Physical satisfaction</th>
<th>Emotional satisfaction</th>
<th>Satisfaction with control</th>
<th>Sexual reactivity Mell-Krat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>F</td>
<td>I</td>
<td>F</td>
</tr>
<tr>
<td>Task accomplishment</td>
<td>-0.32*</td>
<td>-0.48**</td>
<td>-0.25</td>
<td>-0.23</td>
</tr>
<tr>
<td>Role performance</td>
<td>-0.32*</td>
<td>-0.47**</td>
<td>-0.20</td>
<td>-0.38**</td>
</tr>
<tr>
<td>Communication</td>
<td>-0.31*</td>
<td>-0.54**</td>
<td>-0.08</td>
<td>-0.40**</td>
</tr>
<tr>
<td>Affective expression</td>
<td>-0.23</td>
<td>-0.51**</td>
<td>-0.06</td>
<td>-0.14</td>
</tr>
<tr>
<td>Affective involvement</td>
<td>-0.43**</td>
<td>-0.51**</td>
<td>-0.37**</td>
<td>-0.17</td>
</tr>
<tr>
<td>Control</td>
<td>-0.15</td>
<td>-0.49**</td>
<td>-0.03</td>
<td>-0.12</td>
</tr>
<tr>
<td>Values and norms</td>
<td>-0.34**</td>
<td>-0.31**</td>
<td>-0.25*</td>
<td>-0.12</td>
</tr>
<tr>
<td>Depressive symptoms (BDI)</td>
<td>-0.11</td>
<td>-0.35**</td>
<td>-0.12</td>
<td>-0.33**</td>
</tr>
</tbody>
</table>

Higher scores on FAM-III (dyadic functioning) are indicative of more maladaptive functioning. *; P≤0.05, **; P≤0.001, FAM-III; Family assessment measure and BDI; Beck depression inventory.
Socio-clinical correlates of sexual satisfaction and sexual reactivity in infertile women

Associations between sexual satisfaction and reactivity, and the relationship duration, period of trying to conceive and treatment period were assessed in the sample of infertile women (Table 4). Out of three indexes of sexual satisfaction, statistically significant negative correlations were found only for satisfaction with control. This aspect of sexual satisfaction correlated negatively with relationship duration and treatment period (P≤0.001). A statistically significant negative correlation was also observed between sexual reactivity and period of trying to conceive (P≤0.001).

Prediction of sexual satisfaction and sexual reactivity-regression analysis

Multiple regression analysis was performed to identify the variables which are best predictors of sexual satisfaction and sexual reactivity in infertile women and in the control group. The following independent variables were entered into the regression model: socio-demographics (age, educational level, place of origin, and duration of the relationship) dyadic functioning and depressive symptoms. In the sample of infertile women, clinical variables (period trying to conceive and treatment period) were additionally entered into the regression model. Sexual satisfaction and sexual reactivity were used as dependent variables.

Regression analyses revealed different predictors of sexual satisfaction and sexual reactivity in each sample. Affective involvement and relationship duration were found to have a statistically significant predictive value for sexual satisfaction in infertile women, while communication, affective expression and depressive symptoms were significant predictors of sexual satisfaction in the control women.

Period of trying to conceive was the only significant predictor which explained approximately 15% of the variance in sexual reactivity in infertile women. None of the variables entered into the regression model were found to have a significant predictive value for sexual reactivity in the control group. The summary of the models obtained in stepwise multiple regression analysis is shown in table 5.

| Table 4: Spearman’s rank correlation coefficients between sexual satisfaction and sexual reactivity, and relationship duration, period of trying to conceive and treatment period in the sample of infertile women |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                | Physical satisfaction | Emotional satisfaction | Satisfaction with control | Sexual reactivity Mell-Krat |
| Relationship duration          | -0.22            | -0.13            | -0.40**         | 0.03            |
| Period of trying to conceive   | -0.25            | -0.05            | -0.22           | -0.32**         |
| Treatment period               | -0.19            | 0.02             | -0.31**         | -0.09           |

**; P≤0.001.

| Table 5: Results of stepwise multiple regression analyses performed for sexual satisfaction and sexual reactivity (dependent variables) in infertile women and in the control group |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                | Infertile women | Control group   | Infertile women | Control group   | Infertile women | Control group   | Infertile women | Control group   |
| Dependent variable              | Predictor       | Beta  | R²     | P    | Dependent variable | Predictor       | Beta  | R²     | P    |
| Physical satisfaction           | Affective involvement | -0.43 | 0.19   | 0.002 | Physical satisfaction | Communication | -0.36 | 0.014 |
| Emotional satisfaction          | Affective involvement | -0.37 | 0.13   | 0.009 | Emotional satisfaction | Affective expression | -0.31 | 0.35  | 0.033 |
| Satisfaction with control       | Affective involvement | -0.38 | 0.32   | 0.003 | Satisfaction with control | Communication | -0.40 | 0.16  | 0.004 |
|                                | Relationship duration | -0.38 | 0.32   | 0.003 |                                | Depressive symptoms | -0.32 | 0.10  | 0.024 |
| Sexual reactivity               | Period of trying to conceive | -0.39 | 0.15   | 0.005 |                                |                                |                |                |

For each dependent variable, only these predictors are shown in the table which reached the statistical significance. R²; Coefficient of determination and P; Significance level.
Discussion

Previous studies have indicated that infertility might have an extensive, deleterious impact on sexual satisfaction (3, 13, 14) and sexual functioning (33, 34). In our sample of infertile women, we found the evidence for an elevated risk of sexual dysfunctions: as many as 90% of infertile women, as compared to 26% of women from the control group, reported the scores on the Mell-Krat Scale indicative of dysfunctions in sexual functioning. The numbers of infertile women at risk for sexual dysfunctions we found in our study are even higher than those reported in other studies, e.g. 40% in Millheiser et al.’s (35) study or 61.7% in Oskay et al.’s (5) study. This difference may be due to different criteria for detecting sexual dysfunctions applied in the studies, as the latter studies used for this purpose Female Sexual Function Index, whereas we applied the Mell-Krat Scale which is more commonly used in Poland. The cut-off score of 55 on the Mell-Krat Scale is recommended in Polish sexological literature for detecting increased risk of sexual dysfunction. However, the rate of sexual dysfunctions in the control group from our study was consistent with the data for the general population of Polish women, in which 25% of Polish women complained of lowered sexual needs (36). Even if the prevalence of sexual dysfunctions we found in the sample of infertile women is slightly overestimated, it is nevertheless much higher than in the control group, which altogether adds to the previously reported evidence for the link between infertility and sexual dysfunctions in women.

In the present study, infertile women reported significantly lower levels of sexual satisfaction as well as more maladaptive patterns of couple functioning, compared to the control group. Infertile women scored significantly lower on all three dimensions of sexual satisfaction and significantly higher (maladaptive) on all six domains of dyadic functioning (task accomplishment, role performance, communication, affective expression, affective involvement, and values and norms). Based on these findings, one can speculate that infertility may be considered as a specific crisis, in which the quality of sexual functioning is closely associated with treatment procedures, for example timing of the intercourse around the ovulatory cycle. This ‘crisis’ may also be a stressful experience because of the long-lasting treatment and its in-conveniences, especially when the long-term treatment is unsuccessful. Infertility crisis may induce stress in affected couples, which in turn may have a deleterious impact on both sexual activity and dyadic functioning. It was previously found that experienced stress was negatively correlated with sexual behavior and satisfaction, and that higher self-reported stress in daily life was associated with lower level of relationship satisfaction (37).

The present study found significantly higher rates of depressive symptoms in infertile women, as compared to the control group. More than three-quarter of our infertile women had the scores on BDI within the range of the risk for clinical depression. The mean levels of depressive symptoms were also significantly higher in infertile women than in the control group. Other studies reported similar results, demonstrating that infertile women have elevated mean levels of depressive symptoms (38), more than three times higher odds ratios for dysthymia compared to the women without infertility (4) and increased rates of depression (18). It should be remembered, however, that our sample of infertile women contained slightly more participants aged above 30 (30%) than the control group (8%), which might also affect the higher rates of depression in the sample of infertile women.

Previous studies suggested the bidirectional relationship between depressive symptoms and sexual/marital satisfaction, so that high levels of depressive symptoms might be considered as a predictor of low sexual satisfaction (39), as well as marital dissatisfaction can be regarded as a risk factor for depressive symptoms (40). However, in the present study, a significant relationship between sexual satisfaction and depressive symptoms was found in the control group but not in the women with infertility. This suggests that while in healthy women sexual satisfaction is negatively associated with levels of depressive symptom, in infertile women depression levels (even though significantly elevated) do not relate to levels of sexual satisfaction. It is of interest that one study found infertile couples to exhibit more resilience (resistance to psychosocial stress) than fertile couples, and the levels of resilience are correlated positively with quality of life (41). If resilience works as a protective factor in infertile individuals, this can shed more light on our findings, suggesting resilience may buffer the relationship between depres-
sive symptoms and sexual satisfaction in infertile women from our sample.

It was also reported that sexual satisfaction may depend on relationship duration, with women exhibiting greater sexual satisfaction later rather than earlier in the relationship (42). On the other hand, ambiguous data were reported for the association between sexual satisfaction and treatment duration (43, 44). The results obtained in this study showed that both relationship duration and fertility treatment duration were negatively correlated with sexual satisfaction, particularly with satisfaction with control over sexual activity.

Likewise, a pattern of negative correlations was found between physical sexual satisfaction and the qualities of the dyadic relationship in both samples, indicating that sexual satisfaction is closely related to dyadic adjustment, irrespective of the fertility status. It is of note, however, that the correlations between physical sexual satisfaction and dyadic functioning were generally stronger in the control group than in the women with infertility. This suggests that infertility may be a mediator of the relationship between physical sexual satisfaction and dyadic functioning. This also means that sexual satisfaction and dyadic functioning may be less dependent on each other in infertile couples and instead be affected to a greater extent by other factors such as those related to infertility.

No correlation was observed between sexual reactivity and dimensions of dyadic functioning in infertile and control group. Negative correlations were revealed between sexual reactivity and the period of trying to conceive. It seems essential that a longer history of efforts to conceive is associated with a longer history of intercourses primarily aimed at fertilization and a longer history of frustration (failed attempts to get pregnant), which may result in decreased levels of libido and weaker precoitus arousal in infertile women (45) which may in turn lead to dysfunctions in their sexual life. These findings were partially supported by results obtained in stepwise multiple regression analysis, which was performed to better understand the relationship between sexual satisfaction and sexual reactivity, and dyadic functioning. Multiple regression analyses revealed different predictors of sexual satisfaction and sexual reactivity in each sample. Indeed, the period of trying to conceive was found to have a predictive value for sexual reactivity in infertile women, implying that a longer history of unsuccessful attempts to conceive may induce discouragement and impatience that lead to a decline in sexual functioning. Different predictors of sexual satisfaction in each group (affective involvement and relationship duration in infertile women and communication, affective expression and depressive symptoms in the control group) might reflect the differences of the life situation in both groups. Perhaps, in the face of infertility crisis, affective involvement is more important than other aspects of dyadic functioning for sexual functioning, since it helps to bind the couples emotionally, and feelings of closeness, togetherness, and fulfillment of safety and understanding needs may be especially important in the case of infertility.

In summary, the relationships between sexual satisfaction and reactivity, and dyadic functioning were evaluated in the present study. The negative pattern of correlations was found between sexual satisfaction and dyadic functioning in either sample, indicating that sexual satisfaction is closely related to partner adjustment. It seems that sexual satisfaction is an important aspect of partner functioning and it might form a bidirectional link with dyadic functioning. On the one hand, impairment of sexual satisfaction may decrease dyadic adjustment. On the other hand, lower levels of partner functioning might lead to lower sexual satisfaction. Furthermore, the treatment period and relationship duration were found to be related to sexual satisfaction, so that infertile women whose relationships lasted longer and those whose treatment had been unsuccessful for a longer period were less satisfied with their sexual relationships.

In this study, infertile women were less satisfied with their sexual relationships and they revealed more dysfunctions of sexual functioning, compared to the control group. They also reported more depressive symptoms than the control sample. These findings are supported by previous studies in which infertile women were found to reveal higher levels of depression and anxiety (18) and had more sexual dysfunctions than fertile women (5). Interestingly, different variables were found to have a predictive value for sexual satisfaction in both samples (affective involvement and relationship duration for infertile women and communication, affective expression and depressive...
symptoms for the control group), which might be attributed to the differences of the life situation in each group.

Some limitations of this study must be mentioned. First, the sample was not cross-cultural or recruited from multiple centers; therefore, the results we obtained may be specific only for the Polish cultural background from which the sample originated. Second, the control group was recruited based on the participants’ self-reports of no problems with fertility. However, since the women did not intend to get pregnant, some of them might be unaware of their fertility status. Anyway, this limitation is very difficult to avoid, if childless women are matched for the infertile women. Third, the size of the sample was relatively small, which might affect the power of some statistical tests employed. For instance, the number of the independent variables entered into regression models might be excessive for the actual sizes of the subsamples. Finally, the instruments we used also carry cultural specificity, for instance, Mell-Krat Scale is commonly used in Poland; however, is relatively unknown abroad, which makes it difficult to make comparisons with other studies. All these limitations must be taken into consideration when generalizing and interpreting the results of this study.

Conclusion

The results of this study provide evidence for a detrimental impact infertility can exert on sexual satisfaction, sexual reactivity and dyadic functioning. Moreover, it is linked to elevated depressive symptoms in infertile women sample, especially when fertilization treatment is unsuccessful for a prolonged time.

Acknowledgements

A part of this work was prepared by Dr. Janowski when he was receiving a scholarship grant from the Polish Ministry of Science and Higher Education. Authors declare no conflict of interest in this study.

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24. Lew-Starowicz M, Gellert R. The sexuality and quality of

Sexuality in Infertile Women

Menstrual Pattern following Tubal Ligation: A Historical Cohort Study

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Abstract

Background: Tubal ligation (TL) is recommended for women who have completed their family planning. The existence of the menstrual disorders following this procedure has been the subject of debate for decades. This study was conducted to identify the relationship between tubal ligation and menstrual disorders.

Materials and Methods: A historical cohort study was carried out on 140 women undergoing tubal ligation (TL group) and on 140 women using condom as the main contraceptive method (Non-TL group). They aged between 20 and 40 years and were selected from a health care center in Rudsar, Guilan Province, Iran, during 2013-2014. The two groups were comparable in demographic characteristics, obstetrical features and menstrual bleeding pattern using a routine questionnaire. A validated pictorial blood loss assessment chart (PBLAC) was also used to measure the menstrual blood loss.

Results: Women with TL had more menstrual irregularity than those without TL (24.3 vs. 10%, P=0.002). Women with TL had more polymenorrhea (9.3 vs. 1.4%, P=0.006), hypermenorrhea (12.1 vs. 2.1%, P=0.002), menorrhagia (62.9 vs. 22.1%, P<0.0001) and menometrorrhagia (15.7 vs. 3.6%, P=0.001) than those without TL. There is a significant difference in the PBLAC score between women with and without TL (P<0.0001). According to logistic regression, age odds ratio [(OR=1.08, confidence interval (CI):1.07-1.17, P=0.03)], TL (OR=5.95, CI:3.45-10.26, P<0.0001) and cesarean section (OR=2.72, CI:1.49-4.97, P=0.001) were significantly associated with menorrhagia.

Conclusion: We found significant differences in menstrual disorders between women with and without TL. Therefore, women should be informed by the health providers regarding the advantages and disadvantages of TL before the procedures.

Keywords: Historical Cohort Study, Tubal Ligation, Menstrual Disorders, Pictorial Blood Loss Assessment Chart


Introduction

Some women who have completing family planning choose tubal ligation (TL) as a method of contraception (1). Menstrual disorder is one of the problematic effects of TL, although the results of related studies have been inconsistent and inconclusive (2, 3).

The occurrence of abnormal bleeding after TL was first described by Williams et al. (4). It has been hypothesized that ligation may increase incidence of menstrual disturbances among women receiving TL. Several studies about the side-effects of TL on menstrual function have been conducted (5, 6), yet the existence of a post TL syndrome has
been debated. The term post tubal ligation syndrome (PTLS) was first reported in the early 1950s based on the results of a study in which the effect of menstrual disorders on some of somatic and psychological symptoms were evaluated (4). Although based on the conjecture, it has been hypothesized that TL may result in low blood flow to the ovaries, leading to impairment of follicular growth and altered gonadotropin signal and ovarian hormone levels, resulting in menstrual disorders (7). Abnormalities reports associated with TL surgery include the entire spectrum of menstrual disorders, such as: more frequent menstrual periods, irregular menstrual cycles, menorrhagia, metrorrhagia, spotting, dysmenorrhea and oligomenorrhea (8). However, some studies (2, 9) showed no increase in menstrual disorders in women undergoing TL as compared with a control group.

Resolving the debate about menstrual disorders after TL is important for safeguarding women’s health. Therefore, we compared the occurrence of menstrual disorders in women with and without TL. This is a pioneer study in Iran investigating type of menstrual disorders in women with TL.

Materials and Methods

For this historical cohort study, first a pilot study was conducted on 60 women. Then, using the appropriate formula with α at 0.05 and 1-β at 0.95, it was found that a sample size of 130 women was needed for each group. Therefore, 140 women undergoing TL at least a year ago, and 140 women using condom as contraceptive method at least for 3 months were assigned as TL and non-TL groups, respectively. All participants were recruited from a healthcare center in Rudsar, Guilan Province, Iran, between 2013 and 2014.

The inclusion criteria were as follows: i. Multiparous, ii. 20-40 years of age, iii. Free of chronic diseases, including diabetes, hypertension, thyroid and cardiovascular diseases, iv. Free of any gynecological diseases and v. At least three normal cycles before TL.

We compared the distribution of demographic characteristics, obstetrical features and menstrual bleeding pattern between two groups using a routine self-administered questionnaire. A validated pictorial blood loss assessment chart (PBLAC) was also used for the evaluation of menstrual blood loss (MBL) (10). This chart records the amount of daily menstrual bleeding by noting the number of clots, the amount of staining on each pad or tampon. Everyone completed their charts for one menstrual cycle. All patients used the same sanitary products.

In order to build a prediction model and to find the most important factors affecting menorrhagia, we used backward logistic regression analysis in which a p value of 0.15 was used as an entry criterion, whereas a p value of 0.10 was the threshold for a variable to stay in the model.

The outcome variable was menorrhagia. The following variables were included in the logistic regression model: age, age at menarche, parity, body mass index (BMI), education status, TL status (women with or without TL) and method of delivery.

This study was approved by the Ethics Committee of the Tarbiat Modares University. All women participated voluntarily and provided a signed informed consent.

Definitions and Terminology for Menstrual Pattern

Normal menstrual: A menstrual interval of 21-35 days and a flow duration of 7 days or less are considered normal (11).

Menstrual cycle length: The number of days from the beginning of one menstrual period to the beginning of the next one is defined as menstrual cycle length (11).

Menstrual irregularities: A menstrual interval shorter than 21 days and longer than 35 days is defined as menstrual irregularities. Amount of bleeding is varied (5).

Oligomenorrhea: Bleeding intervals longer than 35 days is defined as oligomenorrhea (12).

Polymenorrhea: A menstrual interval shorter than 21 days is defined as polymenorrhea (13).

Hypermenorrhea: Flow more than 7 days is considered as hypermenorrhea (11).

Menorrhagia: Menorrhagia is defined as vaginal bleeding occurring between the expected menstrual periods (3).
Menstrual Pattern following TL

score of ≥100 (14). Length of menstruation cycle is not important in diagnosis of menorrhagia because this definition is not valid by itself (15).

**Menometrorrhagia:** Excessive and prolonged bleeding occurring irregularly is defined as menometrorrhagia (11).

**Statistical analysis**

All statistical analyses were performed by the Statistical Package for the Social Sciences (SPSS) version 20.0 (SPSS Inc., USA). Student’s t test and chi-square test were carried out to reveal the statistical differences between the groups. We used logistic regression to determine the risk factors associated with menorrhagia. Odds ratio (OR) and 95% confidence interval was also calculated for each factor. A P value less than 0.05 was considered to be statistically significant.

**Results**

By considering the inclusion criteria, 140 tubal ligated and 140 non-tubal ligated subjects were evaluated for menstrual disorders. Table 1 gives the characteristics of TL and non-TL groups. There are no significant differences in the age, age of menarche, BMI, parity, education status and the method of delivery between women with TL compared to non-TL group. However, there is a significant difference in PBLAC score for menstrual loss between the two groups. The mean score of PBLAC is statistically significant in women with TL compared to non-TL group (137.72 ± 90.91 vs. 87.91 ± 51.06, P<0.0001, Table 2). Table 2 displays findings regarding the participants’ menstruation disorders. Women with TL had more menstrual irregularity than those without TL (24.3 vs. 10%, P=0.002). Women with TL had more polynorhea (9.3 vs. 1.4%, P=0.006), hypermenorrhea (12.1 vs. 2.1%, P=0.002), menorrhagia (62.9 vs. 22.1%, P<0.0001) and menometrorrhagia (15.7 vs. 3.6%, P<0.001) than those without TL.

The mean duration of TL was 4.6 ± 1.4 years. The duration of TL had no effect on menorrhagia. The mean duration of TL is not statistically significant in the women with menorrhagia as compared to the non-menorrhagia (4.57 ± 1.50 vs. 4.80 ± 1.45, P=0.37) (The data are not shown).

In the logistic regression model, age (OR=1.08, CI:1.07-1.17, P=0.03), TL (OR=5.95, CI: 3.45-10.26, P<0.0001) and cesarean section (OR=2.72, CI:1.49-4.97, P=0.001) are positively associated with menorrhagia (Table 3).

### Table 1: Comparison of demographic and personal characteristics between TL and non-TL groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Non-TL</th>
<th>TL</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women’s age (Y)</td>
<td>35.45 ± 4.51</td>
<td>36.22 ± 3.14</td>
<td>0.09a</td>
</tr>
<tr>
<td>Partner’s age (Y)</td>
<td>38.92 ± 4.41</td>
<td>38.15 ± 3.10</td>
<td>0.59a</td>
</tr>
<tr>
<td>Age of menarche (Y)</td>
<td>12.65 ± 1.34</td>
<td>12.73 ± 1.38</td>
<td>0.71a</td>
</tr>
<tr>
<td>Parity</td>
<td>2.21 ± 0.46</td>
<td>2.32 ± 0.53</td>
<td>0.36a</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>27.67 ± 4.53</td>
<td>28.37 ± 5.16</td>
<td>0.21a</td>
</tr>
<tr>
<td>Educational level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Under diploma</td>
<td>70 (50)</td>
<td>74 (52.9)</td>
<td>0.14a</td>
</tr>
<tr>
<td>Diploma and high school diploma</td>
<td>70 (50)</td>
<td>66 (47.1)</td>
<td></td>
</tr>
<tr>
<td>Method of delivery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal vaginal delivery</td>
<td>50 (35.7)</td>
<td>40 (28.6)</td>
<td>0.22a</td>
</tr>
<tr>
<td>Caesarian section</td>
<td>90 (64.3)</td>
<td>100 (71.4)</td>
<td></td>
</tr>
<tr>
<td>Previous contraceptive method used</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pill</td>
<td>3 (2.1)</td>
<td>5 (3.6)</td>
<td></td>
</tr>
<tr>
<td>Condom</td>
<td>127 (90.7)</td>
<td>117 (83.6)</td>
<td>0.22a</td>
</tr>
<tr>
<td>Other</td>
<td>10 (7.1)</td>
<td>18 (12.9)</td>
<td></td>
</tr>
</tbody>
</table>

TL; Tubal ligation, a; T test, b; Chi-square test, BMI; Body mass index and *; This category included withdrawal and natural family planning or the rhythm method.
### Table 2: Comparison of menstrual disorders between groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Non-TL</th>
<th>TL</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menstrual irregularities</td>
<td>14 (10)</td>
<td>34 (24.3)</td>
<td>0.002</td>
</tr>
<tr>
<td>Oligomenorrhea</td>
<td>12 (8.6)</td>
<td>21 (15)</td>
<td>0.12</td>
</tr>
<tr>
<td>Polymenorrhea</td>
<td>2 (1.4)</td>
<td>13 (9.3)</td>
<td>0.006</td>
</tr>
<tr>
<td>Hypermenorrhea</td>
<td>3 (2.1)</td>
<td>17 (12.1)</td>
<td>0.002</td>
</tr>
<tr>
<td>Metrorrhagia</td>
<td>9 (6.4)</td>
<td>12 (8.6)</td>
<td>0.64</td>
</tr>
<tr>
<td>Menorrhagia</td>
<td>31 (22.1)</td>
<td>88 (62.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Menometrorrhagia</td>
<td>5 (3.6)</td>
<td>22 (15.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>PBLAC score</td>
<td>87.91 ± 51.06</td>
<td>137.72 ± 90.91</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*; n (%), **; Values are mean ± SD, †; Chi-square test, ‡; T test, TL; Tubal ligation, and PBLAC; Pictorial blood loss assessment chart.

### Table 3: Logistic regression analysis of 280 women for menorrhagia

<table>
<thead>
<tr>
<th>Variables</th>
<th>OR (95% CI)</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.08 (1.07-1.17)</td>
<td>0.03</td>
</tr>
<tr>
<td>TL status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5.95 (3.45-10.26)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>No</td>
<td>1**</td>
<td></td>
</tr>
<tr>
<td>Method of delivery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cesarean section</td>
<td>2.72 (1.49-4.97)</td>
<td>0.001</td>
</tr>
<tr>
<td>Normal vaginal delivery</td>
<td>1**</td>
<td></td>
</tr>
<tr>
<td>Constant</td>
<td>0.007</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*; OR, CI (OR; Odds ratio, CI; Confidence interval), †; Reference category and TL; Tubal ligation.

### Discussion

Numerous investigators have evaluated the impact of TL on menstrual cycle characteristics. Although the literature on the effects of TL and menstrual disorders are comprehensive, they have been inconsistent (2, 6, 16, 17).

Our results indicated that sterilized women were more likely to experience an increase in polymenorrhea, hypermenorrhea, menorrhagia, and menometrorrhagia and to have an irregular menstrual cycle when compared with the other group.

Some studies showed a significant increase in incidence of menstrual disorder in women undergoing TL when compared with a control group (4, 16, 17). Increased duration (hypermenorrhea) and amount of bleeding (menorrhagia) have been reported by Shain et al. (18). TL has been considered as the cause of menstrual abnormalities by damaging the ovary (19), including acute increase in pressure in the utero-ovarian arterial loop (20).

Peterson et al. (5) found women undergoing TL experienced a shortened interval between menses and a decrease in volume of menstrual flow and in bleeding days as compared with related values in non-sterilized women. However, Shobeiri and
Menstrual Pattern following TL

Atashkhoii (9) concluded that TL does not cause menstrual disorders. Several other studies concluded that the duration of bleeding, volume of menstrual flow, menstrual cycle length and cycle irregularity are similar in women with and without tubal legation (2,6). Although it has been hypothesized that menstrual disorders are caused by the damaging effect of TL on ovarian function through an increase in pressure within the utero-ovarian arterial circulation or disruption of the ovarian blood supply, some researchers have not observed an alteration in ovarian function (5,6). In addition, laboratory studies comparing women before and after TL have found no constant abnormalities in ovarian function (5), indicating no difference in luteinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol (E2) levels in women undergoing TL compared with a non-TL group (6).

Menorrhagia is identified as the most common bleeding disorders (21). Several methods were used to measure menstrual blood loss, like alkaline hematin method that is a cheap, acceptable, easy and relatively accurate test (22); however, we preferred to measure indirectly the blood loss using the PBLAC (10). We found a significant increase in PBLAC score for menstrual blood loss in women undergoing TL when compared with a non-TL group. Several studies showed that there was no significantly difference regarding menorrhagia between the case and control groups (9,23). In another study by Wilcox et al. (17), they reported heavy menstrual flow (41%) after 5 years following TL.

We evaluated patient characteristics, age, TL and cesarean section as predictors of menorrhagia. Our findings showed that age, TL and cesarean section are positively associated with menorrhagia.

Some studies also indicated that age could be considered as a risk marker for menorrhagia and irregular menstrual bleeding (10,24). The most significant changes in late reproductive age include a decrease in anti-Mullerian hormone (AMH) and in early cycle inhibin B levels. A decline in inhibin B results in an increase in FSH levels (25). Burger et al. (26) showed that increasing FSH levels are associated with normal or higher E2 concentrations. Ultimately, these changes cause menstrual disorders. The mechanisms leading to menstrual disorders may involve the temporary ovarian nonresponsiveness to FSH stimulation and the critical numbers of follicles. No ovarian response may occur for several days with increasing FSH levels, but finally a follicle starts to develop, leading to a hyper-respons and higher concentration of E2(25).

The present study also assessed the relationship between method of delivery and menorrhagia. Our results indicated menorrhagia was more common in women with history of cesarean section. Harlow et al. (6) concluded that menstrual irregularity, length of menstruation, length of cycle and flow volume are similar in women with and without TL, but women with a history of cesarean section and TL experienced an increase in volume of menstrual flow compared with women who did not undergo TL. Uppal et al. (24) reported similar findings. Regnard et al. (27), however, found no relationship between the method of delivery and menstrual disorders. Osser et al. (28) have also referred to endometrial defects at cesarean scar site and the weakness of uterine contractions as a cause for menstrual disorders.

The present study shows that menstrual disorders were more common in women with TL. There are still many important questions to be investigated about probable effects of TL on menstrual disorders. This study conveys an important message that TL may influence irregular menstruation and menorrhagia. Hence, women should be informed and instructed by health providers such as midwives and gynecologists regarding the advantages and disadvantages of TL. Definitely, this database is not large enough to give precise conclusion and needs further supports for long-term follow-up for menorrhagia in patient undergoing TL.

Our findings suggest that menorrhagia and menstrual irregularities are more prevalent than previous reports about Iranian women with TL. This is a pioneer study in Iran investigating type of menstrual disorders in women with TL. The different studies have showed that the relationship between TL and menstrual disorders is a complex process influenced by multiple factors. Therefore, biological, physiological, psychological, cultural, behavioral, ethnicity, climate, and religious conditions as well as lack of knowledge of women about TL may affect the present findings.

Most of women participating in this study had no information about other types of sterilization and their side effects. Consequently, we were unable to evaluate the effect of particular method of TL.
on menstrual disorder, indicating limitation in our study. On the other hand, as our study was a historical cohort, no documents were available about surgical skills used for TL, which shows another limitation in this study.

Conclusion

Overall, this study showed that TL is a cause of menstrual disorders. However, we need more evidence based on cohort studies to confirm the results of the present study.

Acknowledgements

We are grateful to the entire colleagues of the Guilan University of Medical Sciences, Rasht, Iran for their contributions in accomplishing this project. This study was a part of dissertation for receiving Ph.D. degree in Reproductive Health (Shahideh Jahanian Sadatmahalleh). The study was financially supported by Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran. There is no conflict of interest regarding this article.

References

Performance of Circulating Placental Growth Factor as A Screening Marker for Diagnosis of Ovarian Endometriosis: A Pilot Study

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². Pelvic Endoscopy and Minimally Invasive Gynaecologic Surgery, St. Orsola Malpighi University Hospital, Bologna, Italy
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Abstract

**Background:** The aim of this study is to compare the circulating placental growth factor (PlGF) concentration in women with and without endometrioma to verify the performance of this marker to diagnose the disease.

**Materials and Methods:** In this case-control study, thirteen women with histological diagnosis of ovarian endometriosis were compared with women without endometriosis disease. PlGF plasma levels of endometriotic patients and controls were investigated using a fluorescence immunoassay technique.

**Results:** PlGF showed a direct correlation with body mass index (BMI) only in the control group (P=0.013). After adjustment for BMI values, PlGF median value in endometriosis group (14.7 pg/mL) resulted higher than in control group (13.8 pg/mL, P=0.004).

**Conclusion:** PlGF is a promising peripheral blood marker that can discriminate between patients with and without ovarian endometriosis.

**Keywords:** Endometriosis, PlGF, Blood Marker, Endometrioma


Introduction

Endometriosis is a estrogen-dependent chronic disorder often resulting in morbidity, pelvic pain and infertility (1). Although endometriosis typically appears benign on histological examination, it is characterized by a malignant tumour-like nature in that it grows, infiltrates and adheres to the surrounding tissues. The gold standard for diagnosis is laparoscopic surgery with histologic confirmation. However, every surgical procedure has potential risks for patients (2). Ultrasound should be the first-line imaging modality for the evaluation of patients with suspected endometriosis. Its accuracy has greatly improved over recent years, but its performance is heavily operator-dependent (3). Detection of simple and non-invasive diagnostic test is one of the priorities in endometriosis research.

Most of the proposed non-invasive diagnosis methods are based on the identification of biomarkers believed to be involved in the pathophysiology of the disease and differentially expressed in the peripheral blood of patients as compared to
health subjects. The increasing interest in angiogenic factors as putative peripheral blood markers for endometriosis is not surprising, since several lines of evidence suggest that the angiogenic factors are involved in the establishment of neovascularization requirement for development and maintenance of endometriotic lesion (4-8).

Vascular endothelial growth factor (VEGF) is the most widely studied angiogenic factor. Although several authors have evaluated serum or peripheral blood levels of VEGF in endometriosis patients, contradictory results have been reported and the validity of using VEGF in endometriosis diagnosis has not been definitely attested (9-17).

Placental growth factor (PlGF) is a member of the proangiogenic vascular endothelial growth factor family (18, 19). PlGF presented some similarities to the structure of VEGF-A with a 42% amino acid sequence identity. Nevertheless, they have significant functional differences. PlGF was originally identified in the placenta, where it has been proposed to control trophoblast growth, differentiation and invasion (20-22). Its biological effect is mediated by VEGF receptor 1 (VEGFR-1), a tyrosine kinase receptor expressed on the surface of several cell types including endothelial cells, macrophages, bone marrow precursors and cancer cells (23). PlGF shows a high disease-specific activity and its contribution to the angiogenetic switch in pregnancy, wound healing, ischemic conditions and tumor growth has been well documented (24-30). In cancer, PlGF may also facilitate metastasis by increasing the motility and invasion of malignant cells, and it has been demonstrated that the levels of PlGF in plasma and serum correlate with tumor stage and poor survival in various tumors (31-37). Despite the above-mentioned angiogenic and prometastatic activities, the role of PlGF as candidate biomarker in diagnosis of endometriosis has been poorly investigated. Suzumori et al. (38) have indicated increased levels of placental growth factor in the peritoneal fluid of women with endometriosis compared with women with cystadenomas, suggesting that the production of PlGF may contribute to the pathogenesis of endometriosis by promoting neovascularization.

We aim to analyze and to compare the preoperative PlGF concentration in the peripheral blood of women with and without ovarian endometriosis in order to verify the performance of this putative marker to diagnose the disease.

Materials and Methods

Subjects

In the present case-control study, we enrolled a total of 26 women in our center of Pelvic Endoscopy and Minimally Invasive Gynaecologic Surgery, S. Orsola-Malpighi University of Bologna, Bologna, Italy, from October 2012 through September 2013. Ethics Committee approval of S. Orsola-Malpighi Hospital was obtained before starting the data collection. The approval code is PLGF 167/2012/O/Tess. All study subjects provided a written informed consent for the use of biological specimens for research purposes. Thirteen patients (group A) with a preoperative ultrasound diagnosis of ovarian endometriosis (defined as the presence of a unilocular cyst with a regular wall and homogeneous low-level echogenicity of the cyst’s content), subsequently confirmed by histological analysis (Fig.1), were included in the study. For each case, a consecutive control of same age, parity and BMI without endometriosis disease was recruited; therefore, a 1:1 match was generated. Patients of the control group (group B) were operated for leiomyoma pathology. Again, we excluded primi/pluriparae women with history of pre-eclampsia and/or intrauterine fetal growth restriction (IUGR). All patients were submitted on peripheral blood collection during the proliferative phase of the menstrual cycle and they were not on hormonal treatment since at least 3 months (Table 1).

Blood samples/measurement of placental growth factor concentration

A peripheral blood sample (10 ml) from each woman enrolled in our study was collected in sterile tubes containing ethylenediaminetetraacetic acid (EDTA) and treated for PlGF evaluation within 2 hours of being drawn. Blood samples were centrifuged at 1500 g for 10 minutes at 4°C; the obtained plasma samples were stored at –20°C until the measurement of PlGF plasma levels. PlGF quantification was performed by the Alere PlGF Test (Fig.2) using Triage® MeterPro instrument (Alere Srl, Italy), according to the manufacturer’s instructions. The test is based on a fluorescence immunoassay technique and provides a PlGF measurable range of 12 to 3,000 pg/mL.
Table 1: Demographic and clinical characteristics of the data set

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls (n=13)</th>
<th>Endometriosis Cases (n=13)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (minimum-maximum) (Y)</td>
<td>34.5 (25–46)</td>
<td>34 (26–45)</td>
<td>0.513</td>
</tr>
<tr>
<td>Previous surgery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>76.9</td>
<td>46.2</td>
<td>0.303</td>
</tr>
<tr>
<td>Laparoscopic surgery</td>
<td>0</td>
<td>7.7</td>
<td>1.00</td>
</tr>
<tr>
<td>Abdominal surgery</td>
<td>23.1</td>
<td>30.8</td>
<td>1.00</td>
</tr>
<tr>
<td>Both</td>
<td>0</td>
<td>15.4</td>
<td>0.485</td>
</tr>
<tr>
<td>Nulliparity (%)</td>
<td>91.7</td>
<td>53.8</td>
<td>0.073</td>
</tr>
<tr>
<td>Cysts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right side % (median mm)</td>
<td>-</td>
<td>7.7 (28.5 mm)</td>
<td>-</td>
</tr>
<tr>
<td>Left side % (median mm)</td>
<td>-</td>
<td>53.8 (36.0 mm)</td>
<td>-</td>
</tr>
<tr>
<td>Bilaterality (%)</td>
<td>-</td>
<td>38.5</td>
<td>-</td>
</tr>
<tr>
<td>Median BMI (minimum-maximum)</td>
<td>22.1 (18.8-29.7)</td>
<td>22.3 (19.6-31.1)</td>
<td>0.572</td>
</tr>
<tr>
<td>Median PlGF (minimum-maximum) (pg/mL)</td>
<td>13.8 (13.7-18.6)</td>
<td>14.7 (14.5-21.0)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

*; Mann-Whitney U test or Fisher exact test, BMI; Body mass index and PlGF; Placental growth factor.

Fig.1: Histology of an ovarian endometriosis cyst wall showing endometrial tissue in the muscular layer with granulation tissue. A. Haematoxylin and eosin (H&E) staining method (×4 magnification) and B. The cyst wall is lined by cylindrical endometrial-type epithelium. (H&E) staining method (×10 magnification).

Fig.2: Placental growth factor (PIGF) measurement instrument.
Statistical analysis

Descriptive statistics was performed by means of routine analysis. Adjustment for possible confounding variables was performed by means of a general linear model (GLM) having PlGF as dependent variable plotted versus any possible correlated variable. Mann-Whitney U test and Chi square or Fisher exact test were used to compare the two generated groups of patients. Finally a non-parametric Receiver Operator Characteristics (ROC) curve was generated in order to calculate the sensitivity of adjusted PlGF values for endometriosis at fixed rate of false positive. A two tails $P<0.05$ was considered statistically significant.

Results

PlGF showed a direct correlation with body mass index (BMI), but only in the control group ($P=0.013$). For endometriosis group, in fact, PlGF lost its significant correlation with BMI ($P=0.178$) as showed in figure 3. After adjustment for BMI values and using the parameters of regression model quoted for the controls, the PlGF in endometriosis group resulted in a slight higher median value when compared to that of the control group ($P=0.004$) as reported in table 1. A non-significant direct correlation was found between PlGF and parity and it was excluded from the final model. There was no difference for all the other variables considered in the data set as reported in table 1.

ROC curve yielded a sensitivity of PlGF for endometriosis of $80\%$ at a fixed false positive rate (FPR) of $20\%$ about, with an area under the curve (AUC) = 0.834 (95%CI=0.649-1.020) and a $P=0.004$ (Fig.4).

**Fig.3:** Log-Linear regression of BMI vs. PlGF in controls and cases. As shown significant association has been found only for controls ($P=0.013$). For endometriosis cases, PlGF lost its significant correlation with BMI ($P=0.178$). BMI; Body mass index and PlGF; Placental growth factor.

**Fig.4:** Receiver operator characteristics (ROC) curve for detection of endometriosis using placental growth factor (PlGF) as explorative variable. The sensitivity of PlGF for endometriosis was 80% at a fixed false positive rate (FPR) of 20% about, with an area under the curve (AUC)=0.834 (95%CI=0.649-1.020) and a $P=0.004$. 
Discussion

To date, the gold standard for diagnosis of endometriosis is laparoscopic surgery (2). This limitation, together with the long delay between the onset of symptoms and diagnosis of endometriosis, is the main reasons for the urgent require of a non-invasive diagnosis method.

Ultrasound is also accurate, but only if performed by an expert operator (3). Detection of simple and non-invasive diagnostic test is one of the priorities in endometriosis research.

The identification of peripheral blood markers, capable of diagnosing or excluding endometriosis, could avoid the need for an invasive procedure (39, 40) or at least allow symptomatic women to be screened. Nevertheless, a biomarker with high sensitivity, specificity and clinical relevance useful for non-invasive diagnosis of endometriosis, is still unidentified.

From the pathophysiological point of view, it has been demonstrated that the ectopic implants of endometrial cells are rich in angiogenetic growth factors and it is well known that the establishment of a new blood supply is crucial for the development of endometriotic lesions. Taking into account the importance of angiogenesis in the pathogenesis of the disease (7), in this study, we assessed the role of PlGF, a member of the proangiogenic VEGF family, as putative circulating biomarkers of endometriosis.

We demonstrated that PlGF correlates with BMI in controls as a possible biological epiphenomenon of some tissue release. Whereas in endometriosis patients, this direct association is lost probably for a secondary confounding effect due to the presence of the disease. After adjustment for BMI, in fact, the PlGF resulted higher in ovarian endometriosis patients. Again, even if the PlGF increase in endometrioma is quite small, it is statistically significant by means of non-parametric analysis. Several authors have previously reported a direct association between BMI and both VEGF and PlGF plasma levels (41-44). In endometriosis patients, both actual PlGF values and correlation with BMI described in control subjects seems altered. Regardless of the mechanistic link between BMI and increased circulating angiogenetic factors, which has not been clarified yet, the impairment of the relationship between BMI and circulating PlGF suggests the involvement of this proangiogenic factor in endometriosis. It may be considered an indicator of the disease.

Despite the small differences in PlGF values, a ROC curve yielded a significant AUC with a sensitivity of 80% about at FPR of 20%. The associated PlGF cut-off was 15 pg/mL. For the linear regression, given a sample size of 13 subjects, a power of 83% at a 5% of type I error is reached for a R2=0.6. Given the R2 found in this study (0.579 and 0.292 for controls and cases, respectively), the sample size required to reach a proper power was 17 and 87. Our samples reached instead a power of 64 and 17 at a 5% of type I error. For the ROC curve, instead, a sample size of 26 cases (13+13) and an AUC of 0.834 yielded a power of 95% at a type I error of 5%. Even if this is a small series of data, the results seem encouraging for a possible use of PlGF in ovarian endometriosis evaluation in prospective studies. An increasing number of reports has documented that PlGF activity does not affect quiescent vessels in healthy tissues, but it has a role in vessel stabilization under pathological conditions (24-27). This disease-restricted activity of PlGF is an attractive property that could help to discriminate between pathological and health conditions. However, the involvement of PlGF in many other angiogenetic diseases raises the question whether PlGF could be specific enough to be proposed as a marker of endometriosis and further explorations are needed to clarify this issue.

A noteworthy feature of our study is that it employs a highly reproducible and easily-applied technique of PlGF quantification. This method is readily amenable, employs no toxic reagents and is very fast. These features make the procedure feasible in terms of clinical management and/or large-scale screening.

Conclusion

Our study identifies PlGF level as a promising biological indicator that could help to discriminate between patients with ovarian endometriosis and healthy subjects. Further investigations are needed to explore PlGF specificity degree and to confirm its prognostic/diagnostic value in clinical practice. Nevertheless, our results support the possibility of finding an easily detectable peripheral blood marker that alone or within a panel of others biomarkers could improve the diagnosis of endometriosis in
symptomatic women.

Acknowledgements

This work was financially supported by "Ricerca Fondamentale Orientata" (RFO 2010 to C Zucchini). The authors did not report any potential conflicts of interest.

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13. 501.
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Metabolic Syndrome in Patients with Polycystic Ovary Syndrome in Iran

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Abstract

Background: The prevalence of metabolic syndrome (MetS) in polycystic ovary syndrome (PCOS) has been studied in different populations, but their results were so controversial regarding Iranian women. These controversial data indicated the need for more investigation of MetS characteristics in PCOS patients in our population. So this study aimed to evaluate the clinical and laboratory characteristics and metabolic features of patients with PCOS in Rasht.

Materials and Methods: This prospective cross sectional study was conducted on 215 PCOS women who lived in Rasht, north of Iran, from March 2010 to July 2012. The participants were then divided into two groups of women with MetS (n=62) and women without MetS (n=153). The diagnosis of PCOS and MetS were based on the Rotterdam 2003 criteria and the Adult Treatment Panel III (ATP III) criteria, respectively. Demographic characteristics, fertility characteristics, family history and laboratory findings were assessed.

Results: The prevalence of MetS in women with PCOS was 28.8%. In PCOS women of both groups, the waist circumference (WC) exceeded 88cm in 72.6%, hypertension [systolic blood pressure (SBP) and/or diastolic blood pressure (DBP) ≥130/85mm Hg] was prevalent in 9.3%, fasting blood sugar (FBS) level was ≥110 mg/dl in 6%, triglycerides (Tg) level were ≥150 mg/dl in 47%, and high-density lipoprotein (HDL) level was <50 mg/dl in 86%. The values of WC, SBP, DBP, body mass index (BMI), ovarian size, Tg, cholesterol, FBS, 2-hour blood sugar, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were significantly greater in PCOS women with MetS than women without MetS. Also HDL and luteinizing hormone (LH) levels in women with MetS were significantly lower than women without MetS.

Conclusion: Prevalence of MetS in PCOS women was 28.8%, indicating that this value is higher than other studies conducted on PCOS women in Iran and other studies conducted on general population in Iran. PCOS women are considered as a high-risk population for MetS. The special strategies are required to prevent MetS and its associated complications in PCOS women.

Keywords: Polycystic Ovary Syndrome, Metabolic Syndrome, Prevalence

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common gynecological endocrinopathy among reproductive-aged women (1, 2). The prevalence of PCOS has been reported from 2.2 to 26% in different studies conducted in various countries, depending on sampling method, the criteria used for its definition and the method used to define each criterion (1-3). The clinical and biochemical features of PCOS may vary according to race, ethnicity and the diagnostic criteria used (4). In a study by Tehrani et al. (3), the prevalence of PCOS in a community sample of Iranian population was 7.1% using the National Institute of Health (NIH) definition, 11.7% by the Androgen Excess Society (AES) criteria and 14.6% using the Rotterdam consensus definition. The classic form of PCOS is characterized by chronic anovulation (oligomenorrhea or amenorrhea), hyperandrogenism, infertility, hirsutism, obesity and enlarged bilateral ovaries with cysts (5-7). The pathogenesis of PCOS is not fully understood, although genetic, metabolic and neuroendocrine interactions as well as environmental factors were discussed elsewhere (8-11). Alterations in several metabolic pathways such as steroid hormone regulation and insulin signaling pathway abnormalities were discussed in the pathophysiology of PCOS (12-14). More than 50 percent of women with PCOS are obese or overweight that may predispose them to metabolic disorders (15).

The metabolic syndrome (MetS) is one type of endocrine disturbance that consists of insulin resistance, dyslipidemia, obesity, central adiposity, and hypertension that has been shown to be associated with a two-fold increased risk of cardiovascular disease and a five-fold increased risk of type 2 diabetes (16). The prevalence of MetS in PCOS has been studied in different populations (16-19). In a study conducted in the USA that prevalence of MetS in PCOS women was approximately 43 to 46%, while for aged-matched women in the general population, it was nearly 2-fold higher (16). However, the findings of several studies conducted in Iran regarding the prevalence of MetS in PCOS women were controversial (18-20). Hosseinpanah et al. (18) showed that MetS was less frequent in patients with PCOS. However, in other studies, MetS was noted in younger PCOS patients in comparison with older PCOS women (19, 20). These controversial data indicate the need for more investigation of MetS characteristics in PCOS patients in our population, as it may help in planning screening strategies to prevent long-term effects (17). Therefore, this study aimed to evaluate the clinical and laboratory characteristics and metabolic features of patients with PCOS in Rasht, North of Iran.

Materials and Methods

This prospective cross sectional study was conducted on 215 PCOS women aged 15-35 years who were referred to private and public gynecological endocrinology clinics, Rasht, north of Iran, from March 2010 to July 2012. The exclusion criteria were as follows: lactating and pregnant women, previous history of ovarian surgery, use of steroid hormone drugs such as oral contraceptive pill and progesterone for past 6 months, use of dyslipidemia drugs for last 3 months, and use of any medications known to affect glucose metabolism or BP. Furthermore women with following conditions were excluded: hypothyroidism, hyperprolactinemia, congenital adrenal hyperplasia, androgen-producing tumor, and Cushing’s syndrome that were diagnosed by physical examination and laboratory testing using serum levels of thyroid stimulating hormone (TSH), prolactin (PRL), and 17α-hydroxyprogesterone (17α-OHP). All patients provided a written informed consent before entering the study. The participants were then divided into two groups of women with MetS (n=62) and women without MetS (n=153). The study protocol was approved by the Ethics Committee of Guilan University of medical sciences, Rasht, Iran.

The diagnosis of PCOS was based on the Rotterdam 2003 criteria, in which any two of the following three conditions need to be fulfilled for the inclusion: i. Oligo- and/or anovulation (i.e. less than 9 menstrual periods in a year or menstrual cycles more than 35 days in length), ii. Clinical hyperandrogenism (i.e. acne or hirsutism; modified Ferriman-Gallwey scores ≥8) or biochemical hyperandrogenism [i.e. free testosterone (FT) ≥7.0 pg/ml], and iii. Ultrasoundographic findings of polycystic ovarian morphology (presence of ≥12 follicles in each ovary measuring 2-9 mm in diameter). Based on these criteria, four phenotypes were formed as follows: type 1 including irregular menstruation+PCO using ultrasonographic
examination + hyperandrogenism (IM+PCO+HA), type 2 including irregular menstruation + PCO using ultrasonographic examination (IM+PCO), type 3 including irregular menstruation + hyperandrogenism (IM+HA) and type 4 including PCO using ultrasonographic examination + hyperandrogenism (PCO+HA).

MetS was defined according to the Adult Treatment Panel III (ATP III) criteria as the co-occurrence of three or more of the following risk factors: i. Central obesity with waist circumference (WC) ≥88 cm in women, ii. Elevated systolic blood pressure (SBP) and/or diastolic blood pressure (DBP) of ≥130/85 mmHg, iii. Impaired level of fasting blood sugar (FBS) ≥110 mg/dL, iv. Elevated level of fasting serum triglycerides (Tg) ≥150 mg/dL and v. Fasting high-density lipoprotein (HDL) level <50 mg/dL.

Anthropometric measurements included height in centimeters, weight in kilograms, and hip and waist circumference in centimeters according to World Health Organization (WHO) categories. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters (kg/m²). Underweight was defined as less than 18.5 (normal range between 18.5 and 24.9), overweight between 25.0 and 29.9 and obese as 30.0 or higher. Sitting blood pressure was measured after a 5-minute rest using a standard sphygmomanometer.

After 12 hours fasting during days 3-5 of menstrual cycle, 10 cc of blood sample was obtained. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels were measured using Access Immunoassay System (Beckman Coulter, Fullerton, California, USA); FT, dehydroepiandrosterone sulfate (DHEAS), and 17a-OHP levels using radio-immunometric assay (RIA) kit (Siemens, USA); as well as TSH level using a chemiluminescence immunometric assay (Immulite 2000 Analyzer, CPC, USA). Also fasting blood sample was used to measure FBS level using Hitachi 7600 analyzer, Hitachi, Japan, while the levels of cholesterol (Chol), Tg, aspartate aminotransferase (AST), alanine aminotransferase (ALT), HDL, and low-density lipoprotein (LDL) were measured using enzymatic calorimetric method (Hitachi 7600). In addition a 75-gram oral glucose tolerance test was measured.

Demographic variables including age, education, occupation, and inhabitant area; reproductive characteristics including parity, history of infertility, type of menstrual irregularity such as oligomenorrhea, amenorrhea, and menometrorrhagia; and family history of diabetes mellitus were collected.

Statistical analysis

All data were analyzed using the Statistical Package for the Social Sciences (SPSS, SPSS Inc., USA) software version 16. Continuous data were shown as mean ± standard deviation (SD). Kolmogorov-Smirnov test was used to assess the normality of continuous variable. Normal distribution of quantitative variables was analyzed by two-tailed independent t test and non-normally distributed variables by Mann-Whitney U test. Categorical data were shown as number (percentage). Fisher’s exact tests and chi-square test were used to compare the groups. Statistical significance was considered as P<0.05.

Results

Prevalence of MetS in women with PCOS was 28.8%. In PCOS women of both groups (15-35 years of age), the mean age was 25.63 ± 5.17. In this study, 72 patients were single and 123 were married, but in the analysis of reproductive characteristic, investigators did not assess single patients.

Most of women (83.8%) lived in urban area. Majority (79.7%) of women were housewife and some (38.4%) of women had a university degree. Mean infertility duration was 36.50 ± 41.26. About 54.9, 10.6, and 39.3% of married patients showed a history for infertility, abortion, and diabetes in their families, respectively. Twenty two percent of married women were multiparous. Oligomenorrhea was reported in 85.1% of women. Except family history of diabetes (P=0.043), there were no significant differences regarding demographic and fertility characteristics between PCOS women with MetS and without MetS (Table 1).

The findings of both groups showed that the WC exceeded 88 cm in 72.6%, hypertension (SBP/DBP ≥130/85 mm Hg) in 9.3%, FBS level was 110 mg/dl or greater in 6%, Tg level was 150 mg/dl or greater in 47%, and HDL level was less than 50 mg/dl in 86%. Individual components of the MetS in two groups of PCOS women (with MetS and without MetS) are shown in table 2.
Table 1: Comparison of demographic and fertility characteristics between polycystic ovary syndrome women with and without metabolic syndrome (MetS)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total (n=215)</th>
<th>With MetS (n=62)</th>
<th>Without MetS (n=153)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Y)</td>
<td>25.63 ± 5.17</td>
<td>26.81 ± 6.07</td>
<td>25.15 ± 4.69</td>
<td>0.057</td>
</tr>
<tr>
<td>Inhabitation area</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rural</td>
<td>34 (16.2)</td>
<td>9 (14.5)</td>
<td>25 (16.9)</td>
<td>0.838</td>
</tr>
<tr>
<td>Urban</td>
<td>176 (83.8)</td>
<td>53 (85.5)</td>
<td>123 (83.1)</td>
<td></td>
</tr>
<tr>
<td>Job</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Employed</td>
<td>32 (20.3)</td>
<td>11 (24.4)</td>
<td>21 (18.6)</td>
<td>0.511</td>
</tr>
<tr>
<td>Housewife</td>
<td>126 (79.7)</td>
<td>34 (75.6)</td>
<td>92 (81.4)</td>
<td></td>
</tr>
<tr>
<td>Educational level</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Under diploma</td>
<td>42 (26.4)</td>
<td>12 (27.3)</td>
<td>30 (26.1)</td>
<td>0.950</td>
</tr>
<tr>
<td>Diploma</td>
<td>56 (35.2)</td>
<td>16 (36.4)</td>
<td>40 (34.8)</td>
<td></td>
</tr>
<tr>
<td>University</td>
<td>61 (38.4)</td>
<td>16 (36.4)</td>
<td>45 (39.1)</td>
<td></td>
</tr>
<tr>
<td>Duration of infertility</td>
<td>36.50 ± 41.26</td>
<td>30.39 ± 24.04</td>
<td>39.42 ± 47.21</td>
<td>0.705</td>
</tr>
<tr>
<td>History of infertility</td>
<td>95 (54.9)</td>
<td>30 (58.8)</td>
<td>65 (68.4)</td>
<td>0.615</td>
</tr>
<tr>
<td>History of abortion</td>
<td>13 (10.6)</td>
<td>5 (13.5)</td>
<td>8 (9.0)</td>
<td>0.523</td>
</tr>
<tr>
<td>Family history of diabetes</td>
<td>83 (39.3)</td>
<td>31 (50.8)</td>
<td>52 (34.7)</td>
<td>0.043</td>
</tr>
<tr>
<td>History of parity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nulliparous</td>
<td>96 (78)</td>
<td>24 (75)</td>
<td>59 (77.6)</td>
<td>0.760</td>
</tr>
<tr>
<td>Multiparous</td>
<td>27 (22)</td>
<td>8 (25)</td>
<td>17 (22.4)</td>
<td></td>
</tr>
<tr>
<td>Oligomenorrhea</td>
<td>183 (87.6)</td>
<td>52 (89.7)</td>
<td>131 (86.8)</td>
<td>0.647</td>
</tr>
<tr>
<td>Amenorrhea</td>
<td>20 (9.3)</td>
<td>6 (10.3)</td>
<td>14 (9.3)</td>
<td>0.797</td>
</tr>
<tr>
<td>Polymenorrhae</td>
<td>6 (2.8)</td>
<td>0</td>
<td>6 (4)</td>
<td>0.190</td>
</tr>
<tr>
<td>Normal</td>
<td>6 (2.8)</td>
<td>4 (6.5)</td>
<td>2 (1.3)</td>
<td>0.059</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD or numbers (%).

Table 2: Comparison of prevalence of individual components of the metabolic syndrome (MetS) between polycystic ovary syndrome women with and without MetS

<table>
<thead>
<tr>
<th>Components of the MetS</th>
<th>Total (n=215)</th>
<th>With MetS (n=62)</th>
<th>Without MetS (n=153)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WC ≥88 cm</td>
<td>156 (72.6%)</td>
<td>59 (95.2)</td>
<td>97 (63.4)</td>
</tr>
<tr>
<td>Hypertension (SBP ≥130 mm Hg or DBP ≥85 mm Hg)</td>
<td>20 (9.3)</td>
<td>17 (27.4)</td>
<td>3 (2.0)</td>
</tr>
<tr>
<td>FBS ≥110 mg/dl</td>
<td>13 (6.0)</td>
<td>12 (19.4)</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>Tg ≥150 mg/dl</td>
<td>101 (47.0)</td>
<td>55 (88.7)</td>
<td>46 (30.1)</td>
</tr>
<tr>
<td>HDL &lt;50 mg/dl</td>
<td>185 (86.0)</td>
<td>61 (98.4)</td>
<td>124 (81.0)</td>
</tr>
</tbody>
</table>

Data are presented as numbers (%). WC; Waist circumference, SBP; Systolic blood pressure, DBP; Diastolic blood pressure, Tg; Triglycerides, FBS; Fasting blood sugar and HDL; high-density lipoprotein.
In all patients, mean values of WC, SBP, DBP, and BMI were 87.37 ± 12.38, 110.32 ± 14.72, 69.90 ± 9.64, and 28.98 ± 11.19, respectively. Mean levels of Tg, FBS, and HDL were 152.39 ± 74.29, 93.02 ± 17.79, and 41.33 ± 8.64, respectively. Mean values of WC, SBP, DBP, and BMI in patients with MetS were significantly higher than women without MetS (Table 3).

Some laboratory findings including Tg, Chol, FBS, 2-hBS, AST, and ALT were significantly greater in PCOS women with MetS than women without MetS. Also HDL and LH in women with MetS were significantly lower than women without MetS. In other laboratory findings, such as serum levels of LDL, PRL, DHEAS, T, FSH, FSH/LH, 17OHP, and TSH, differences between two groups were not significant (Table 4).

Prevalence of MetS in PCO+HA phenotype was highest, but this difference was not statistically significant among four PCOS subtypes (Table 5).

**Table 3**: Comparison of anthropometric and BP measurements between polycystic ovary syndrome women with and without metabolic syndrome (MetS)

<table>
<thead>
<tr>
<th>Components of the MetS</th>
<th>Total (n=215)</th>
<th>With MetS (n=62)</th>
<th>Without MetS (n=153)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WC (cm)</td>
<td>87.37 ± 12.38</td>
<td>97.24 ± 11.14</td>
<td>83.37 ± 10.51</td>
<td>0.0001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.98 ± 11.19</td>
<td>32.92 ± 10.80</td>
<td>27.37 ± 10.94</td>
<td>0.001</td>
</tr>
<tr>
<td>BMI ≥30</td>
<td>77 (36)</td>
<td>42 (67.7)</td>
<td>35 (23)</td>
<td>0.0001</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>110.32 ± 14.72</td>
<td>116.62 ± 15.64</td>
<td>107.77 ± 13.56</td>
<td>0.0001</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>69.90 ± 9.64</td>
<td>72.42 ± 10.93</td>
<td>68.88 ± 8.91</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. WC: Waist circumference, BMI: Body mass index, SBP: Systolic blood pressure and DBP: Diastolic blood pressure.

**Table 4**: Comparison of laboratory findings between polycystic ovary syndrome women with and without metabolic syndrome (MetS)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total (n=215)</th>
<th>With MetS (n=62)</th>
<th>Without MetS (n=137)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg (mg/dl)</td>
<td>152.39 ± 74.29</td>
<td>202.76 ± 90.69</td>
<td>132.0 ± 54.93</td>
<td>0.0001</td>
</tr>
<tr>
<td>Chol (mg/dl)</td>
<td>180.37 ± 37.08</td>
<td>195.18 ± 36.27</td>
<td>174.33 ± 35.80</td>
<td>0.0001</td>
</tr>
<tr>
<td>FBS (mg/dl)</td>
<td>93.02 ± 17.79</td>
<td>99.97 ± 29.37</td>
<td>90.20 ± 8.46</td>
<td>0.012</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>111.72 ± 28.00</td>
<td>117.42 ± 30.59</td>
<td>109.40 ± 26.63</td>
<td>0.057</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>41.33 ± 8.64</td>
<td>37.13 ± 6.31</td>
<td>43.03 ± 8.88</td>
<td>0.0001</td>
</tr>
<tr>
<td>2-h BS (mmol/l)</td>
<td>112.90 ± 37.79</td>
<td>134.28 ± 54.21</td>
<td>104.50 ± 24.53</td>
<td>0.001</td>
</tr>
<tr>
<td>AST (mg/dl)</td>
<td>22.47 ± 10.57</td>
<td>25.76 ± 10.23</td>
<td>21.12 ± 10.45</td>
<td>0.003</td>
</tr>
<tr>
<td>ALT (mg/dl)</td>
<td>20.42 ± 12.94</td>
<td>23.53 ± 9.94</td>
<td>19.15 ± 13.81</td>
<td>0.024</td>
</tr>
<tr>
<td>PRL (ng/mL)</td>
<td>19.33 ± 11.44</td>
<td>20.47 ± 14.52</td>
<td>18.85 ± 9.94</td>
<td>0.349</td>
</tr>
<tr>
<td>DHEAS (mg/dl)</td>
<td>237.45 ± 136.88</td>
<td>228.04 ± 145.99</td>
<td>241.20 ± 133.38</td>
<td>0.527</td>
</tr>
<tr>
<td>FT (nmol/l)</td>
<td>1.72 ± 1.59</td>
<td>1.89 ± 2.42</td>
<td>1.65 ± 1.10</td>
<td>0.467</td>
</tr>
<tr>
<td>FSH (mg/dl)</td>
<td>6.17 ± 1.77</td>
<td>6.01 ± 2.08</td>
<td>6.22 ± 1.65</td>
<td>0.494</td>
</tr>
<tr>
<td>LH (mg/dl)</td>
<td>7.89 ± 4.12</td>
<td>6.79 ± 1.64</td>
<td>8.29 ± 4.66</td>
<td>0.002</td>
</tr>
<tr>
<td>LH/FSH</td>
<td>1.34 ± 0.66</td>
<td>1.26 ± 0.53</td>
<td>1.38 ± 0.70</td>
<td>0.251</td>
</tr>
<tr>
<td>17OHP</td>
<td>1.91 ± 14.31</td>
<td>0.85 ± 0.57</td>
<td>2.34 ± 16.91</td>
<td>0.494</td>
</tr>
<tr>
<td>TSH</td>
<td>3.43 ± 7.75</td>
<td>4.95 ± 13.67</td>
<td>2.82 ± 2.91</td>
<td>0.228</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. Tg: Triglycerides, Chol: Cholesterol, FBS: Fasting blood sugar, LDL: Low-density lipoprotein, HDL: High-density lipoprotein, 2-h BS: 2-hour blood sugar, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, PRL: Prolactin, DHEAS: Dehydroepiandrosterone sulfate, FT: Free testosterone, FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, 17OHP: 17hydroxy-progesterone and TSH: Thyroid stimulating hormone.
Discussion

PCOS is one of the most important endocrine diseases in women. Many PCOS patients with several metabolic abnormalities are at increased risk of MetS. The prevalence of MetS differs in various populations which is mainly due to definition of PCOS or MetS, sampling methods, selecting controls, as well as age, race and weight of participants. Insulin resistance (IR) plays a crucial role in the pathophysiology of MetS. On the other hand, IR is well recognized to play a major role in the etiology of PCOS (21-23).

As expressed in previous reports, the prevalence of MetS in patients with PCOS is higher than general population (16, 18, 24), but its prevalence is not the same in different ethnic groups. Increased waist circumferences, elevated Tg level and reduced HDL level, known as important components of MetS, are associated with genetic factors and lifestyle characteristics (16, 24). The finding of an increased risk of MetS in PCOS women has raised further interest in identifying the predictors for MetS in these women (25). This study examined the prevalence and related factors of MetS in PCOS patients living in north of Iran.

In this study, the prevalence of MetS in PCOS women was 28.8%. This finding is higher than other studies in Iran (4, 18, 24), but lower than studies in United States (26, 27). Mehrabian et al. (4) showed that the prevalence of MetS were 24.9% among Iranian women diagnosed with different phenotypic subgroups of PCOS, based on the Rotterdam criteria. Moini et al. (24) who conducted a study in Tehran, Iran, reported that prevalence of MetS in PCOS women was 22.7%. In another large-scale population-based study in Tehran, the prevalence of MetS in PCOS subjects was 18.5% (18). This different prevalence may be related to population characteristics, diagnostic criteria and sample size.

This study showed that values of WC, SBP, DBP, BMI, obesity, and ovarian size were significantly greater in patients with MetS than patients without MetS. In a report by Mandrelle et al. (17), age and central obesity (waist-hip ratio/waist circumference) were considered as better predictors of MetS in PCOS women as compared to other parameters including BMI in this group of women.

This study showed that the values of biochemical parameters such as Tg, Chol, FBS, 2h BS, AST, and ALT were significantly greater in patients with MetS than without MetS. Also HDL and LH levels in women with MetS were significantly lower than women without MetS. Soares et al. (16) reported that the occurrence of low HDL was the most frequent individual component of MetS among Brazilian women with PCOS, followed by increased serum Tg.

For study limitation, our findings may be influenced by the criteria, by which PCOS and MetS were diagnosed. We used different equipment and different assessors during our study period that may affect our assessment reliability. Also this study was a cross sectional study with small sample size. So it is suggested to do more cohort or case control studies with greater sample size in future.

Conclusion

Based on our findings, prevalence of MetS in PCOS women was 28.8% that was higher than related values of other studies conducted on both PCOS women and general population in Iran. Among individual components of MetS, WC>88 cm, HDL>50, and Tg ≥150 were prevalent in more than 88% of PCOS women. PCOS women are high
risk population for MetS. The special strategies is required for prevention of MetS and its related complications in PCOS women.

Acknowledgements

We appreciate the Vice Chancellor of Research of Guilan University of Medical Sciences for funding this project. The authors sincerely thank Mr. Davoud Pourmarzi for his help to edit manuscript. The authors state that they have no conflict of interest.

References

Comparison of Pregnancy Outcome between Ultrasound-Guided Tubal Recanalization and Office-Based Microhysteroscopic Ostial Dilatation in Patients with Proximal Blocked Tubes

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Abstract

Background: The current research to the best of my knowledge is the first to compare the pregnancy outcome between ultrasound-guided tubal recanalization (UGTR) using a special fallopian tubal catheter, and office-based micrhysteroscopic ostial dilatation (MHOD) using the same tubal catheter in infertile women with previously diagnosed bilateral proximal tubal obstruction (PTO).

Materials and Methods: This prospective study reported the pregnancy outcomes for 200 women in private infertility care center in Arafa hospital in Fayoum and in El Minya University Hospital in the period between January 2010 and October 2013 treated as outpatients for their bilateral PTO after the routine hysterosalpingography (HSG). A Cook’s catheter, special fallopian tubal catheter, were used to recanalize the blocked tubes in 100 women (group A) under UGTR, and the same Cook’s tubal catheter was used through 2mm microhysteroscope to cannulate both ostia using MHOD in another 100 women (group B). Pregnancy outcome was determined after the procedures for a 12-month period follow-up.

Results: The number of the recanalization of PTO was not significantly different between two groups. As of the 200 blocked fallopian tubes in group A, 140 tubes (70%) were successfully recanalized by passing the ultrasound-guided special cannula, while 150 tubes (75%) were successfully recanalized in group B, using the same tubal catheter through a 2mm microhysteroscope. The cumulative pregnancy rate after the two procedures was not statistically different between two groups. It was 25.9% in group A, while it was 26.3% in group B, after a 12-month period follow-up.

Conclusion: UGTR is highly recommended as the first step to manage infertile women due to PTO, as it is easier procedure; however, there is possible to obtain nearly similar results after MHOD.

Keywords: Fallopian Tube, Fertility, Hysteroscopy


Introduction

Approximately 15% of couples are unable to conceive within a year of unprotected intercourse. A tubal condition known as proximal tubal obstruction (PTO) has been reported in 30% of infertile women. Fluoroscopically guided hysteroscopic tubal cannulation technique has been developed to improve diagnosis and treatment of tubal disease. For PTO, simpler cannulation technique has been also developed, guided by ultrasound or hysteroscopy (1-3).
Recent advances in fiber optics have resulted in the development of transcervical tubal catheterization procedures with improved diagnostic and treatment accuracy of tubal disease with reduced risks, costs and morbidity. Fallopian tube recanalization can be performed with catheters or flexible guidewires under endoscopic, sonographic, or tactile guidance. Falloposcopy provides a possibility to visualize and grade endotubal disease, to characterize and document endotubal lesions, as well as to identify the segmental location of tubal pathology without any complications (4-8).

Advances in hysteroscopy, including the introduction of small-caliber endoscopes, the flexible steerable hysteroscope and the use of video systems in monitoring hysteroscopic evaluations, have supported the application of office-based hysteroscopy for tubal cannulation both for diagnosing and treating cornual obstruction. Initial attempts with hysteroscopic proximal tube catheterization and balloon dilatation for recanalization have been proved intraoperatively successful in more than 80% of the cases (9-11).

Sonographically-guided transcervical tubal catheterization and transcervical balloon tuboplasty may be successfully performed to diagnose and treat patients with PTO. It may be aided with color Doppler ultrasound-guided cannulation and transcervical wire tuboplasty, hysteroscopic/laparoscopic insertion of small intraluminal ultrasound transducers inserted into catheters with diameters of 3.5 F and 5 F during transcervical fallopian tube catheterization, and transvaginal sonography-guided trans-uterine cannulation of the tubes with the Jansen-Anderson embryo catheter (Cook, Australia) and injection of sterile fluid (2, 4, 8, 12).

The aim of the current study is to compare the pregnancy outcome in infertile women who were previously diagnosed with PTO using routine hysterosalpingography (HSG) between ultrasound-guided tubal recanalization (UGTR) and office-based micrhystroscopic ostial dilatation (MHOD) procedures.

**Materials and Methods**

For this prospective study, 200 infertile women with a history of primary or secondary infertility were evaluated in private infertility care center in Arafat hospital in Fayoum and in El Minya University Hospital in the period between January 2010 and October 2013. The study protocol was approved by the ethics committee and institutional review board. The inclusion criteria were delayed conception for more than a year and bilateral PTO confirmed with routine HSG testing. Patients with unilateral PTO and other causes of infertility rather than PTO were excluded from the study. Recruited women were divided into two following groups: group A (n=100) using UGTR and group B (n=100) using the same tubal catheter through MHOD.

**Ultrasound guided tubal recanalization procedure**

Recanalization was performed during the early follicular phase of the menstrual cycle. The women were instructed to take one gram of azithromycin orally (Amoun, Egypt) before the procedure as an antibiotic prophylaxis, and 75 mg of diclofenac (Pharco, Egypt) was given intramuscularly on the day of recanalization, as an intra-operative analgesia. The women undergoing UGTR were placed in the lithotomy position with a partially full bladder in order to straighten the uterus. The cervix was exposed and the entire vagina was cleaned with betadine and draped. A Cook catheter (Cook South East Asia Pte Ltd., Singapore) was applied as in figure 1.

![Fig.1: Two soft malleable fiberoptic microhysteroscopes with diameter of 2-mm used. The upper part is a telescope with operative channel of 0-degree and diameter of 2-mm. The lower part is a telescope with operative channel of 30-degree and diameter of 2-mm. The middle part includes the diagnostic sheath of the microhysteroscopic telescope.](image-url)
vaginal ultrasound scanning, the flexible guiding cannula was pushed forward into the uterus without dilation of the cervical canal and gently advanced to reach the cornua. The guiding cannula was then fixed to the retinaculum at a 45°C and the tubal catheter was inserted in the cannula and advanced into the ostium until the obstruction was felt.

If there was resistance to the catheter advancement, the guidewire was threaded through the tubal catheter and the obstruction of proximal fallopian tube was removed. Tubal patency was then assessed by hydrotubation. Confirmation of tubal patency was achieved with observation of fluid accumulation in the cul-de-sac during the ultrasound scanning (Fig.2).

The microlaparoscopic system consists of a light source, a high speed pneumoperitoneum device and full HD video camera (Sony, Japan). Furthermore a malleable fiberoptic scope, grasping forceps, scissors, and irrigator-aspirator (Olympus, Japan) with 2 mm in diameter were used. The instruments could be used by specially designed trocar (access needle, Ethicon, OH, USA).

Premedication consisting of 0.5 mg of atropine sulfate and 1 mg/kg of midazolam (Cairo medical industry, Egypt) was given intramuscularly. One mg/kg of fentanyl (Cairo medical industry, Egypt) followed with 1.5 mg/kg of ketamine was intravenously administered through a drip infusion (Fig.1). The patient was place in a lithotomy position. An access needle was inserted through a small incision created in the subumbilical region using the closed method, after being locally infiltrated with xylocaine (Sigma, Egypt), a local anesthesia.

Pneumoperitoneum was introduced with carbon dioxide gas. Other access instruments were then inserted into both sides of the hypogastric regions under microlaparoscopic guidance. Four ml/port of 0.25% bupivacaine (Sigma, Egypt) were locally injected at the trocar insertion sites in advance. Subsequently, a micrograsper was used to expose a fimbrial tube at the time of hydrotubation. Four ml of 0.5% lidocaine was sprayed over the diaphragmatic vault. After the removal of the trocars, 5 mL of bupivacaine was then injected into the subcutaneous (SC) tissue of the insertion sites.

Fig.2: The cannula used and the ultrasound images of the ultrasound-guided tubal recanalization of proximal tubal obstruction (PTO): A. Cook’s catheter loaded with the guide wire, B. Cook’s catheter after exposure of the loaded guide wire, C. Ultrasound image of successful passage of the catheter within the right tube, D. Ultrasound image of successful passage of the catheter within the left tube and E. Ultrasound image of hydrotubation using saline and air after successfully recanalizing the proximally blocked tube.
Office microhysteroscopic ostial dilatation

All office-based microhysteroscopies were performed using a malleable fiberoptic microhysteroscope with operative channel of 30-degrees and diameter of 2-mm for the use of 5-6 F Cook’s catheter (Cook, Australia) (Fig.1). The catheter was placed through the built in operative channel and proceeded in order to be visualized at the tip of the hysteroscope. After visualization of both ostia, the catheter was pushed slowly with moderate degree of pressure relative to the resistance faced. Confirmation of successful recanalization would be made with the concomitant use of 2-mm microlaparoscope. Then methylene blue (Naser Biomedical Company, Egypt) was used for chromopertubation as a definite proof of tubal recanalization. Typically, less than 1 liter of normal saline was used as the distention media for procedures (Fig.3).

Follow-up of pregnancy outcome after both procedures

Women with successful recanalized tubes were followed up for the cumulative pregnancy rate (CPR) for a 12-month period for each group of women studied. Pregnancy outcome was correlated to the unilaterality or bilaterality of the tubes recanalized. Pregnancy complications were also evaluated, especially the incidence of developing ectopic pregnancy. Women with unsuccessful recanalized tubes who never sought another treatment for their blocked tubes were also followed up for the same period.

Fig.3: These images are produced after the combination of microhysteroscopic and microlaparoscopic ostial recanalization for proximal tubal obstruction (PTO): A. Tip of the ureteric catheter directs to the visualized ostial opening. B. Catheter successfully cannulates within the tubal lumen. C. Microlaparoscopic image shows the picture of the cannulated tube and D. Microlaparoscopic image shows the appearance of the tip of the Cook’s catheter beyond the fimbrial ostium after successful recanalization.
Recanalization of Proximally Blocked Fallopian Tubes

Results

Of the 200 women diagnosed by HSG as having bilateral PTO, 40 women were younger than 20 years, 141 were aged between 21 and 30 years, and 19 were aged between 31 and 40 years, while 76 (76%) had primary infertility and 48 (24%) had secondary infertility.

In between recruited women for UGTR in group A, 140 (70%) of the 200 tubes were recanalized successfully. Successful recanalization was bilateral in 20 women [n=40 (20%)] and unilateral in 65 women [n=65 (65%)]. Recanalization failed in the remaining 15 women [n=30(15%)] (Table 1). While use of MHOD in group B succeeded to canalize 150 (75%) tubes, which was bilateral in 20 women [n=40 (20%)] and unilateral in 70 women [n=140 (70%)] (Table 1).

In group A, the women with one or two successful recanalized tubes were followed up for CPR for 12 months, where 17 (20%) conceived within 6 months and 22 (25%) after another 6 months. While, in group B, 20 conceived (22.2%) after 6 months and 25 (27.7%) after another 6 months. Therefore, CPR was higher after two tubes recanalized (25%) in group A versus (32%) in group B, while CPR was 25% in group A versus 29% in group B after only one tube recanalized (Table 2). Ten and five of these conceptions were spontaneous in group B and group A, respectively, while the other conceptions occurred after ovulation induction and monitoring. Two and three ectopic pregnancies occurred after complicated UGTR and MHOD, respectively. Follow-up of cases failing to be recanalized showed another case of ectopic pregnancy. No serious complications were encountered.

Group B gained more benefits from combined office-based microlaparoscopy and microhysteroscopy procedures. Congenitally absent, rudimentary or highly suspended tubes were diagnosed in 5 cases. Peritubal and peri-ovarian adhesions were observed in 6 cases and managed in the same session. Mild to moderate degree of pelvic endometriosis were diagnosed in 3 cases, and managed in the same session. Both tubes failed to be recanalized in the previously diagnosed pelvic abnormalities. Microhysterectomy were diagnosed in 3 cases with endometrial polyps, of which two and three cases had polypoid endometrium and intrauterine adhesion, respectively. All those abnormalities were managed in the same session.

Table 1: Numbers of successful and unsuccessful recanalized tubes after the two studies procedures

<table>
<thead>
<tr>
<th>Procedure</th>
<th>UGTR (n=100)</th>
<th>MHOC (n=100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubes successfully recanalized</td>
<td>140 (70%)</td>
<td>150 (75%)</td>
</tr>
<tr>
<td>Bilaterally</td>
<td>40 (23.5%)</td>
<td>50 (26.3%)</td>
</tr>
<tr>
<td>Unilaterally</td>
<td>130 (65%)</td>
<td>140 (70%)</td>
</tr>
<tr>
<td>Tubes unsuccessfully recanalized</td>
<td>30 (15%)</td>
<td>10 (5%)</td>
</tr>
</tbody>
</table>

UGTR; Ultrasound guided tubal recanalization and MHOC; Microhysteroscopic ostial cannulation.

Table 2: Comparison of pregnancy outcome between both studied procedures for tubal recanalization

<table>
<thead>
<tr>
<th>Pregnancy Outcome</th>
<th>UGTR</th>
<th>MHOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPR after 6 months</td>
<td>20% (n=17)</td>
<td>21% (n=20)</td>
</tr>
<tr>
<td>CPR after 12 months</td>
<td>25.9% (n=22)</td>
<td>26.3% (n=25)</td>
</tr>
<tr>
<td>CPR after bilateral TR</td>
<td>25% (n=5)</td>
<td>32% (n=8)</td>
</tr>
<tr>
<td>CPR after unilateral TR</td>
<td>26.1% (n=17)</td>
<td>29.3% (n=23)</td>
</tr>
<tr>
<td>Spontaneous pregnancy</td>
<td>22% (n=5)</td>
<td>40% (n=10)</td>
</tr>
<tr>
<td>Ectopic pregnancy</td>
<td>13% (n=3)</td>
<td>8% (n=2)</td>
</tr>
</tbody>
</table>

CPR; Cumulative pregnancy rate, UGTR; Ultrasound guided tubal recanalization, MHOD; Micrhysteroscopic ostial dilatation and TR; Tubal recanalization.
Statistical analysis

The accuracy of HSG for PTO was assessed by the chromopertubation under microlaparoscopy. The success rate of recanalization, the pregnancy rate (PR) following both procedures, and the time taken to achieve pregnancy were investigated. Student’s t test and the Mann-Whitney U test were used. A value of P<0.05 was considered statistically significant.

Discussion

Tubal disease is the cause of subfertility in approximately 30% of women and 25% of these are due to PTO. PTO has been a diagnostic and therapeutic dilemma since its recognition more than 100 years ago. It can occur in either the intra-mural segment or the uterotubal junction, and is the result of tubal spasm or transient occlusion by mucus plugs in up to 40% of women. Proximal, distal and peritubal damage can be caused by a number of pathologic processes, such as inflammation, endometriosis and surgical trauma (13-16).

Obliterative fibrosis has been observed as the most common histologic tubal abnormality by both Wiedemann et al. (16) and Fortier and Haney (17), followed by salpingitis isthmic nodosa. The highest incidence of salpingitis isthmica nodosa (SIN) has been reported 60% by Papaioannou et al. (5, 6), while chronic tubal inflammation was 70.59% by Zhang et al. (15). On the basis of their observations regarding the pathologic spectrum of uterotubal junction (UTJ), Fortier and Haney (17) demonstrated that there are multiple distinct histologic patterns and intra-abdominal findings that do not predict the histology of the UTJ pathology (18-20).

Minimally invasive transcervical tubal catheterization procedures provide an excellent alternative to invasive and expensive surgical procedures and assisted reproductive technology (ART) for the diagnosis of tubal disease and treatment of minimally diseased proximal fallopian tubes. Fallopian tube recanalization (FTR) can be performed with catheters, flexible atraumatic guidewires or balloon systems under endoscopic (falloposcopic/hysteroscopy/laparoscopy), sonographic, fluoroscopic or even with tactile guidance (21, 22).

The current work aimed to evaluate the efficacy of a simple, easy, and reliable tool for recanalization of PTO in group A and to compare it to a more complicated procedure in group B. Therefore, the main points worthy of attention are that there are insignificant differences in success level for recanalizing the blocked tubes and developing PR after the two procedures. Moreover the minimal degree of intervention and the absence of different types of anesthesia were observed in this procedure.

Initial attempts with hysteroscopic proximal tube catheterization and balloon dilatation for recanalization proved intraoperatively successful in more than 80% of the cases. Under laparoscopic guidance, the hysteroscopic approach enables tubal cannulation and evaluation of the entire pelvis. Treatment of additional problems affecting the fallopian tubes, particularly adhesions and endometriosis is possible. Moreover laparoscopy helps to monitor the procedure and offers to assess tubal patency, leading to the ability to observe the utero-tubal junctions (UTJs) directly by hysteroscopy and to provide an excellent approach for tubal cannulation, but there is still a need for anesthesia (11, 15, 23-25).

In the current work, extra findings diagnosed (e.g. endometrial polypi, adhesions, and infection) during the concomitant office-based micrhyteroscopy and microlaparoscopy procedures in group B never added much to the final prognosis of the case of PTO, as the tubes in those women failed to be recanalized after the combined procedures. So, those cases with extra findings never affected the final pregnancy outcome. We recommend shortlisting the cases with PTO who failed to be recanalized after UGTR to be recruited for the combined office-based micrhyteroscopy and microlaparoscopy procedures, which can be considered as the second recommended step of management protocol.

Li et al. (9) described a soft and rigid operating fiberoptic hysteroscope [operative channel diameter (OD) 4.8 mm] that can be used clinically for transcervical tubo-cornual recanalization for the management of cornual occlusion. The functional part of the telescope consists of three sections: a soft, flexible front section; a rigid rotating middle section; and a semi-rigid, self-retaining rear section offering advantages of an easy, close and direct approach to the intrauterine target, usually with no cervical dilation or anesthesia, with the operator in a comfortable position and without reported complications. This new hysteroscope has proved to be a very useful tool for the treatment...
of intrauterine lesions in the theater or an office setting. Clinical results in 1503 women who underwent this panoramic, televised fiberoptic hysteroscopy without cervical dilation suggest that the soft and rigid structure of the diagnostic fiberoptic hysteroscope offers advantages over rigid scopes or conventional fiberscopes with full-length soft, malleable parts (15, 18, 20, 26).

Additional findings diagnosed during the microhysteroscopic procedure in group B could be also checked and diagnosed in group A, meaning that the use of an electrolyte solution, like regular saline for uterine inflation during the tubal recanalization procedure, which is added for confirmation of successful tubal recanalization in order to visualize the hydrotubated recanalized tube/tubes in addition to test of the accumulated fluid in the Douglas pouch during the same ultrasound session. So it is of importance to consider office-based combined microhysteroscopy and microlaparoscopy procedures in order to treat those abnormalities mentioned before, as a second line of management.

Combined hysteroscopic tubal cannulation with selective salpingography under fluoroscopic guidance has been previously reported as a safe and simple diagnostic method that has also been used to identify and to treat successfully the interstitial fallopian tube obstruction. However, compared with the other hysteroscopic cannulation techniques, the addition of selective salpingography under fluoroscopic guidance to hysteroscopic tubal cannulation appears to yield the lowest patency and PRs. A systematic review of observational studies showed that hysteroscopic tubal cannulation was associated with a higher PR (49%) than salpingography and tubal cauterezation (21%) in women with PTO (20, 22, 27).

The relatively higher success rate of recanalizing tubes after the second procedure in group B could be justified to the more accurate visualization of both ostia before passing the Cook’s catheter, which definitely facilitated the successful cannulation, in contrast to the ultrasound guided cannula which pushed to the direction of the ostia, without definitely visualizing both ostia. Moreover increased intrauterine pressure, developed from the uterine distension media used during the MHOD procedure, added more to the higher success in recanalizing the proximally blocked tubes in group B. In group A, the previously mentioned optional hydrohysterography could also increase the success of recanalizing the PTO during UGTR.

The relatively higher PR developed after the second procedure in group B could be due to more than a factor. Firstly the successful recanalization of the blocked tubes without creating a new false tract was observed more in the second procedure, which allowed for proper visualization of both ostia in contrast to the first procedure. Secondly microhysteroscopic uterine irrigation with the distension fluid media studied before was showed to lead to an increase in the PR after hysteroscopy. Thirdly the ability to manage intrauterine abnormalities during the microhysteroscopic session could also lead to this relatively higher PR.

Combined laparo-hysteroscopic tubal cannulation with or without guidewire cannulation in previous studies has yielded an average recanalization success rate of 76%, with an average intrauterine PR as high as 39%. Using combined laparoscopy and hysteroscopic tubal cannulation, Das et al. (1) concluded that hysteroscopic cannulation of the fallopian tube is a safe diagnostic procedure that can be used to identify those patients with true proximal occlusion, and may also serve as a therapeutic procedure in some of these patients. However, conception in their study was achieved after tubal cannulation and adjunctive distal tubal surgery, confounding the results. Zhang et al. (15) performed combined laparo-hysteroscopic cannulation of the proximal oviduct with a flexible guidewire to evaluate and treat intramural fallopian tube obstruction, and also concluded that this procedure is an effective method to manage PTO (10, 15, 22, 28).

In this study, another tool for tube recanalization was applied, like falloposcopy, which provides a unique possibility to accurately visualize, characterize and grade endotubal disease; identifies the segmental location of tubal pathology without complications; objectively classifies the cause of proximal tubal obstruction; as well as guides future patient management in contrast to laparoscopy and HSG that are often associated with poor or misdiagnosis of proximal tubal obstruction; however, these procedures require higher advanced instrumentation and more expert personnel. Non-hysteroscopic transuterine falloposcopy, using the linear eversion catheter, is a well-tolerated technique that can be performed in an outpatient clinic with high
rates of luminal cannulation and visualization and a good predictive value for future fertility (8, 12-16, 20-26).

Guidewire cannulation in group A yielded much lower PRs as compared with the study of Zhang et al. (15), in which they used different catheter techniques, whereas their findings showed similar tubal patency rates. Considering the associated risk factors into account, tubal recanalization procedures are contraindicated in presence of florid infections, genital tuberculosis, obliterator fibrosis, long tubal obliterations that are difficult to bypass with the catheter, severe tubal damage, male subfertility and previously performed tubal surgery. Distal tubal obstruction is not amenable to catheter recanalization techniques, while tuberculosis, salpingitis isthmica nodosa, isthmic occlusion with club-changed terminal, ampullar or fimbrial occlusion and tubal fibrosis have been cited as reasons for recanalization failure (29).

Ultrasound-guided cannulation is essentially a development of the selective fluoroscopic fallopian tube cannulation method. Ultrasound replaces fluoroscopy as a mean to observe tubal flushing and makes repeated cannulation possible. Although the ultrasound-guided method requires expert handling of the catheter and guidewire during cannulation, it has several advantages over its fluoroscopic precursor. It is less expensive, more readily available, applicable for outpatient basis, noninvasive, diagnostic, therapeutic, as well as less painful. Coaxial catheter systems have long flushing and makes repeated cannulation possible. Expert handling of the catheter and guidewire during cannulation, it has several advantages over its fluoroscopic precursor. It is less expensive, more readily available, applicable for outpatient basis, noninvasive, diagnostic, therapeutic, as well as less painful. Coaxial catheter systems have long, with consistent success, for the transcervical cannulation of fallopian tubes under hysteroscopy, fluoroscopy, ultrasonography, or tactile sensation (17, 26, 28).

Conclusion

UGTR is a simple, easy, feasible, and effective procedure to recanalize PTO with significantly low costs as compared to MHOD, which although had higher success in recanalizing PTO that was followed with higher pregnancy outcome, in addition to the extra findings diagnosed and managed in the same session; however, this difference is still not significant, while an operative intervention, like anesthesia, is necessary. Those facts could support the recommendation to consider UGTR as the first step in the management protocol of PTO before any further advanced surgical or ART. Still future studies are required to assess the risk factors associated with PTO, the relative success of tubal recanalization, and subsequent pregnancy outcome after the procedure.

Acknowledgements

The authors are quite grateful for the couples who kindly agreed to participate in this study and to comply nicely and for the patients who were followed up for 12 months after the procedure, without looking for another management pathway for their delayed conception. We have also to thank all colleagues in the private fertility care center, in both Arafa hospital in Fayou, and in El Minya University hospital who greatly supported the work and helped to finalize the work in the planned time. None of the authors had any conflict of interest before. The authors had financially covered all costs of this research work.

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Recanalization of Proximally Blocked Fallopian Tubes

Correlation of Serum CA-125 and Progesterone Levels with Ultrasound Markers in The Prediction of Pregnancy Outcome in Threatened Miscarriage

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Abstract

Background: The aim of this study was to evaluate the relationship between ultrasonographic findings and serum progesterone and cancer antigen-125 (CA-125) levels in threatened miscarriage and to predict pregnancy outcome.

Materials and Methods: In a prospective comparative case-control study, serum CA-125 and progesterone levels were measured for 100 pregnant women with threatened miscarriage who attended the outpatient clinic or the causality department of Obstetrics and Gynecology at Kasr El-Aini Hospital, Giza, Egypt, during the period from March 2013 to October 2013. Ultrasound was performed for fetal viability, crown-rump length (CRL), gestational sac diameter (GSD) and fetal heart rate (FHR). The patients were followed up and divided into two groups based on the outcome: 20 women who miscarried (group 1), and 80 women who continued pregnancy (group 2). The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall accuracy were tested for CA-125 and progesterone levels in prediction of the pregnancy outcome. Correlation of these chemical markers with the ultrasonic markers was also examined.

Results: In the group that miscarried, CA-125 level was significantly higher (P<0.001) and serum progesterone level was significantly lower (P<0.001). For prediction of the outcome of pregnancy, the cut-off limit of 31.2 IU/ml for CA-125 level yielded sensitivity, specificity and an overall accuracy of 96.2, 100 and 99.4% respectively. The cut-off limit of 11.5 ng/ml for progesterone level yielded sensitivity, specificity and an overall accuracy of 97.5, 100 and 99.8% respectively. CA-125 level had a negative correlation with progesterone level and FHR levels (r=-0.716, P<0.001) and (r=-0.414, P<0.001) respectively. Serum progesterone level correlated with GSD (r=0.521, P<0.001) and with CRL (r=0.407, P<0.001) and with FHR (r=0.363, P<0.001). CA-125 level was significantly higher in the group that showed hematoma as compared with the group without hematoma (P<0.001). Also, serum progesterone level was significantly lower in the group that showed hematoma as compared with the group without hematoma (P=0.017).

Conclusion: Serum CA-125 and progesterone levels are valid early predictors of the outcome of pregnancy in women with threatened miscarriage. They are correlated with some ultrasonographic markers (GSD, CRL, and FHR).

Keywords: First Trimester, Ultrasound, CA-125, Progesterone, Threatened Miscarriage
Introduction

First-trimester bleeding is one of the most common obstetric complications, occurring in 25% of all pregnancies (1). More than 80 percent of abortions occur in the first 12 weeks of pregnancy and at least half result from chromosomal anomalies. After the first trimester, both the abortion rate and the incidence of chromosomal anomalies decrease (2). The clinical diagnosis of threatened miscarriage is presumed when bloody vaginal discharge or bleeding appears through a closed cervical os during the first half of pregnancy (3). Ultrasonography, serial serum quantitative assessment of B-subunit of human chorionic gonadotropins (B-hCG), serum cancer antigen -125 (CA-125) and serum progesterone values measured alone or in various combinations, have proven helpful in ascertaining if a live intrauterine pregnancy is present (2). Maternal serum biochemistry has also been proposed as a predictor. La Marca et al. (4) reported that the presence of low concentrations of hCG in women with threatened abortion suggests a negative outcome for the pregnancy. Progesterone concentrations show a narrow variation in the first trimester. The lowest serum progesterone concentration associated with a viable first trimester pregnancy is 5.1 ng/ml and a single serum progesterone measurement of at least 25 ng/ml carries a 97% likelihood for viable intrauterine pregnancy, being more sensitive than two serial hCG measurements (5).

In this study we examined the diagnostic accuracy of serum CA-125 and progesterone levels in the prediction of the outcome of pregnancy in patients with threatened miscarriage, as well as the correlation between these chemical markers and ultrasound markers.

Materials and Methods

Patients

A prospective comparative case-control study was set up to determine the accuracy of biochemical markers in the prediction of the pregnancy outcome, as well as their correlation with each other and with the ultrasound markers of pregnancy outcome. The hospital ethical committee approval was attained before beginning the study. A total of 105 patients with clinical diagnosis of first trimester miscarriage were recruited in this study. They attended the outpatient clinic or the causality department of Obstetrics and Gynecology, in Kasr El-Aini Hospital, Giza, Egypt during the period from March 2013 to October 2013. Ethical committee approval of the Department of Obstetrics and Gynecology of Cairo University was obtained. All participants gave an informed consent and had preoperative clinical evaluation. Five patients did not complete their follow up program with us, and hence were considered as drop out cases leaving 100 patients who were eligible for analysis.

We included the patients that were diagnosed by 1st trimester threatened miscarriage. The patients had a singleton spontaneous pregnancy and were presenting with vaginal bleeding or spotting. The pregnancy was confirmed by a visible gestational sac of a living embryo, verified by cardiac activity visualized on real time ultrasound. The maternal age should range between 20-40 years and the gestational age should range between 7-13 weeks (calculated from the 1st day of the last normal menstrual period, preceded by 3 regular menstrual cycles, and correlating with ultrasound measurements).

We excluded patients with history of general medical disease e.g. diabetes or thyroid disease, presence of local (gynecological) disease e.g. fibroid or adnexal masses verified by normal appearance of the uterus and ovaries by ultrasound, presence of uterine malformations e.g hypoplastic uterus or septate uterus. Patients with history of recurrent miscarriages were excluded from the study; also we excluded patients with history of any maternal disease that would cause an increase in CA-125 level such as chronic pelvic infection and endometriosis. We excluded abnormal findings in the dating scan as blighted ovum or missed miscarriage. All the patients underwent vaginal examination to assess if there is any cervical dilatation as well as the amount of bleeding. They all underwent ultrasonographic and biochemical studies.

Ultrasound studies

Each participant in the study underwent preliminary ultrasound examination. The same experienced operator performed the ultrasound examinations. Ultrasound scanning was performed to all
patients using Accuvix (Medison, Korea) scanner 4-7 MHz endovaginal probe. The scan was done to assess the gestational age and fetal viability as well as to exclude any uterine malformations.

Ultrasound parameters also included gestational sac diameter (GSD), fetal crown-rump length (CRL diameter), fetal heart rate (FHR) and the presence/absence of sub-chorionic hematoma (collection between the uterine wall and the chorionic membrane).

Biochemical studies

All venous samples (5 ml) were allowed to clot, and sera were separated by centrifugation at room temperature at 3,000 rpm for 10 minutes. Sera were stored at -80°C until they were analyzed at the end of the study. Quantification of CA-125, and progesterone was performed using the direct chemiluminometric technology using kits (ADVIA Centaur) supplied by (Bayer Health Care Diagnostics, USA). The test was performed according to the manufacturer’s instructions.

Follow up of all patients was carried out until 20 weeks of pregnancy to detect the patients who would miscarry and those who would proceed into the second trimester. Then, comparison was done between the two study groups, miscarried and continued groups, for ultrasound finding data, progesterone level and CA-125 level.

Statistical analysis

Data were statistically described in terms of range, mean ± SD, median, frequencies (number of cases) and percentages when appropriate. Comparison of numerical variables between the study groups was done using one-way ANOVA test with posthoc multiple 2-group comparisons. For comparing categorical data, Chi square ($\chi^2$) test was performed. Exact test was used instead when the expected frequency is less than 5. Accuracy was represented using the terms of sensitivity, and specificity. Receiver operator characteristic (ROC) analysis was used to determine the optimum cut off value for the studied diagnostic markers. P values less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Microsoft Excel 2007 (Microsoft Corporation, USA) and SPSS (SPSS Inc., USA) version 15 for Microsoft Windows.

Results

A total of 100 pregnant patients with vaginal bleeding between 7 and 13 weeks’ gestation in which a singleton embryo with cardiac activity was initially documented completed the study. Twenty cases ended by miscarriage (20%, group 1) and 80 cases (80%, group 2) continued till 20 weeks of gestation.

No statistically significant differences were found between both groups as regards maternal age, parity, the number of previous miscarriages, and CRL. The mean GSD was significantly lower in the group that miscarried compared to the group that continued ($P=0.023$, Table 1). The mean FHR was $156.9 \pm 20$ bpm for the continued group and $122 \pm 9$ for the aborted group, which showed a statistically significant difference ($P<0.001$).

On comparison between study cases presented by sub chronic hematoma in relation to study parameters, CA-125 level was significantly higher in the group that showed hematoma as compared with the group without hematoma (52.857 ± 29.219 vs. 23.501 ± 13.295, $P<0.001$). Also, serum progesterone level was significantly lower in the group that showed hematoma as compared with the group without hematoma (14.67 ± 7.09 vs. 23.507 ± 9.39, $P=0.017$).

The level of serum CA-125 for the threatened miscarriage (miscarried) group was 54.28 ± 11.4 IU/ml; while for the threatened miscarriage (continued) group it was 18.81 ± 8.02 IU/ml. The difference was statistically significant ($P<0.001$). The level of serum progesterone for the threatened miscarriage (miscarried) group was 8.7 ± 1.85 ng/ml; while for the threatened miscarriage (continued) group it was 26.3 ± 7.2 ng/ml, which showed a statistically significant difference ($P<0.001$, Table 1).

Using a ROC curve for CA-125 in predicting the outcome of pregnancy in threatened miscarriage cases, the cut-off limit of 31.2 IU/ml of CA-125 level achieved sensitivity of 96.2% and
specificity of 100%. CA-125 level above 31.2 IU/ml predicted occurrence of miscarriage with an overall accuracy of 99.4%.

Using a ROC curve for progesterone level in predicting the outcome of pregnancy in threatened miscarriage cases, the cut-off limit of 11.5 ng/ml of progesterone level achieved sensitivity of 97.5 % and specificity of 100%. A progesterone level of <11.5 ng/ml predicted the occurrence of miscarriage with an overall accuracy of 99.8%.

CA-125 level showed a strong significant negative correlation with progesterone level ($r=-0.71$, $P<0.001$), and a significant negative correlation with the FHR ($r=-0.41$, $P<0.001$). Serum progesterone level showed a correlation with GSD ($r=0.52$, $P<0.001$), CRL ($r=0.407$, $P<0.001$) and FHR ($r=0.363$, $P<0.001$, Table 2).

### Table 1: Ultrasonographic and biochemical markers in the miscarried and continued pregnancy groups

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (miscarried)</th>
<th>Group 2 (continued)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age (weeks)</td>
<td>8.8</td>
<td>9.2</td>
<td>0.304</td>
</tr>
<tr>
<td>GSD (mm)</td>
<td>30.03</td>
<td>37.7</td>
<td>0.023*</td>
</tr>
<tr>
<td>CRL (mm)</td>
<td>24</td>
<td>28.5</td>
<td>0.317</td>
</tr>
<tr>
<td>FHR (bpm)</td>
<td>122</td>
<td>156</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Presence of SCH</td>
<td>5</td>
<td>2</td>
<td>0.002*</td>
</tr>
<tr>
<td>Serum CA-125 level (IU/ml)</td>
<td>54.280</td>
<td>18.81</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Serum progesterone level (ng/ml)</td>
<td>8.716</td>
<td>26.317</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

*; Significant difference ($P<0.05$), GSD; Gestational sac diameter, CRL; Crown-rump length, FHR; Fetal heart rate, SCH; Subchorionic hematoma and CA-125; Cancer antigen-125.

### Table 2: Correlation between CA-125 and progesterone levels to the other study parameters

<table>
<thead>
<tr>
<th></th>
<th>CA-125 level</th>
<th>Progesterone level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>P value</td>
</tr>
<tr>
<td>Age</td>
<td>-0.029</td>
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</tr>
<tr>
<td>Gestational age</td>
<td>0.072</td>
<td>0.475</td>
</tr>
<tr>
<td>CRL</td>
<td>0.035</td>
<td>0.739</td>
</tr>
<tr>
<td>GSD</td>
<td>-0.042</td>
<td>0.680</td>
</tr>
<tr>
<td>FHR</td>
<td>-0.414</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

*; Significant difference ($P<0.05$), CRL; Crown-rump length, GSD, Gestational sac diameter, FHR; Fetal heart rate and CA-125; Cancer antigen-125.
Discussion

The present study aimed to evaluate the prognostic value of serum progesterone level and serum CA-125 level at the time of initial presentation with pregnancy outcome in patients with first trimester threatened miscarriage.

As regards GSD, the GSD of the group that continued pregnancy was significantly higher than that of the group that miscarried. These results are also in agreement with the study by Falco et al. (6) who evaluated the outcome and prognostic criteria of pregnancies with first-trimester bleeding and a gestational sac \( \leq 16 \) mm. They found that of 50 patients, 32 (64\%) underwent miscarriage. The size of GSD a high level of statistical significance.

However, these results are not in agreement with the study by Oh et al. (7) who found that the mean diameter of the gestational sac at 28-42 days from the last menstrual period among normal pregnancies did not differ significantly from that in those that subsequently miscarried (2.6 vs. 2.7 mm). This difference can be attributed to the difference in the range of gestational age at which ultrasound was done, 4-6 weeks in their study and 7-13 weeks in our study.

In this study, the CRL was not significantly different between the group that continued pregnancy and the group ended by miscarriage (\( P=0.06 \)), which was inconsistent with Reljic (8) who studied 310 singleton pregnancies with live fetuses, presenting with threatened miscarriage before 13 weeks of gestation. He reported that in fetuses with CRL<18 mm, there was a significant positive association between deficit in the CRL for gestation and the incidence of subsequent spontaneous miscarriage. The smaller number of women in our study may explain this difference.

In this study, there was a significant difference between women who miscarried and women who continued regarding the presence of sub-chorionic hematoma (SCH) (\( P=0.002 \)). These results are in agreement with many studies that showed that SCH was associated with high incidence of 1st trimester miscarriage (9, 10). However, our results are not in quite agreement with Pearlstone and Baxi’s findings (9). They reviewed the English literature on SCH. Fourteen studies were reviewed. The incidence of SCH varied greatly among studies from 4 to 48 per cent. They concluded that small SCH tend to be more common in the first trimester and appear to pose no added risk to the ongoing pregnancy but this could be challenged by how small the hematoma needed to be so that to have no adverse effects. Also we didn’t correlate the size and site of the hematoma with the outcome, which is a limitation of our study.

In this study, the FHR was significantly different between the two groups (the miscarried and the continued groups). Our results are in agreement with Doubilet and Benson’s findings (11). However, when the embryonic heart rate is within the normal range for gestation, the outcome remains uncertain, as in another study done by Tannirandorn et al. (12).

The concentrations of CA-125 in the pregnant women who subsequently miscarried were higher than those who did not, thus suggesting that the serum CA-125 levels are not so important in main-taining successful pregnancy (13). CA-125 might have a role in the preparation of the endometrium for successful implantation (14). More trophoblastic damage is associated with higher levels of CA125 and lactate dehydrogenase (LDH) (15). CA-125 can be used as a prognostic factor to the outcome of pregnancy as it might be related to the extent of trophoblastic destruction.

In the present study, serum CA-125 levels showed a significant difference between the group of women that continued and the group of women that miscarried (\( P<0.001 \)). These results are consistent with other studies (14-16). There was a highly significant increase in serum CA-125 level in women who miscarried. They stated that serum CA-125 level might be developed as a cheap, sensitive and specific predictor of outcome in cases of threatened miscarriage, whereas Mahdi (16) found that there was no statistically significant difference in CA-125 level of patients who miscarried compared with those women that continued pregnancies in spite of its higher level. Their study showed that serum CA-125 level are not predictive of spontaneous miscarriage in the first trimester and failed to discriminate among threatened miscarriages and normal pregnancies.

Several cut-off values were suggested in other studies in order to predict pregnancy outcome in early viable pregnancies complicated by vaginal
bleeding or to discriminate between viable and non-viable gestations at the time of vaginal bleeding. In this study, a cut-off limit of 31.2 IU/ml of CA-125 level was suggested, with a sensitivity of 96.2% and specificity of 100%. Fiegler et al. (17) used a cut-off value of 66.5 IU/ml with a sensitivity of 55%. Schmidt et al. (18) used 65 IU/ml as a cut-off value and reported a sensitivity of 50% for this level. Azougi et al. (19) used a 125 IU/ml as a cut-off value and reported a 100% sensitivity and specificity.

The present study evaluated the possible role of serum progesterone measurement in the prognosis of first trimester miscarriage. According to the statistical analysis, there was a significant difference between the group of women that continued and the group of women that miscarried (P<0.001). This was in accordance with the study of Edwar et al. (20) who studied 78 pregnant women presented by vaginal bleeding. 44 continued till 13th week of pregnancy and 34 ended with spontaneous miscarriage. Serum progesterone level was 5.7 ± 10.9 in continuing pregnancy and 6.7 ± 4.8 in spontaneous abortion. The difference in progesterone level was highly significant.

**Conclusion**

The use of certain maternal serum markers (CA-125 and progesterone) in the first trimester represent non invasive, early and fast methods that can be considered as a good predictor for the outcome of pregnancy in cases with threatened abortion. Larger clinical trials are still needed to support this recommendation.

**Acknowledgements**

We appreciate the role of the Cairo University Teaching Hospitals in supporting the study. The authors declare that there was no conflict of interest in this study.

**References**

11. Doubilet PM, Benson CB. Outcome of first-trimester pregnancies with slow embryonic heart rate at 6-7 weeks gestation and normal heart rate by 8 weeks at US. Radiology. 2011; 236(2): 643-646.
Comparison of Toxicity of CdSe: ZnS Quantum Dots on Male Reproductive System in Different Stages of Development in Mice


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Abstract
Background: Quantum dots (QDs) are new types of fluorescent materials for biological labeling. QDs toxicity study is an essential requirement for future clinical applications. Therefore, this study aimed to evaluate cytotoxic effects of CdSe: ZnS QDs on male reproductive system.

Materials and Methods: In this experimental study, the different concentrations of CdSe: ZnS QDs (10, 20 and 40 mg/kg) were injected to 32 male mice (adult group) and 24 pregnant mice (embryo group) on day 8 of gestation. The histological changes of testis and epididymis were studied by a light microscopy, and the number of seminiferous tubules between two groups was compared. One-way analysis of variance (one-way Anova) using the Statistical Package for the Social Sciences (SPSS, SPSS Inc., USA) version 16 were performed for statistical analysis.

Results: In adult group, histological studies of testis tissues showed a high toxicity of CdSe: ZnS in 40 mg/kg dose followed by a decrease in lamina propria; destruction in interstitial tissues; deformation of seminiferous tubules; and a reduction in number of spermatogonia, spermatocytes, and spermatids. However, there was an interesting result in fetal testis development, meaning there was no significant effect on morphology and structure of the seminiferous tubules and number of sperm stem cells. Also histological study of epididymis tissues in both groups (adult and embryo groups) showed no significant effect on morphology and structure of tubule and epithelial cells, but there was a considerable reduction in number of spermatozoa in the lumen of the epididymal duct in 40 mg/kg dose of adult group.

Conclusion: The toxicity of QDs on testicular tissue of the mice embryo and adult are different before and after puberty. Due to lack of research in this field, this study can be an introduction to evaluate the toxicity of QDs on male reproduction system in different stages of development.

Keywords: Quantum Dots, Male Sexual, Development, Toxicity

Introduction
Although organic dyes are sensitive to physiological changes and photobleached under normal imaging conditions, they have been widely used for fluorophores imaging and detection of abnormalities. It has been shown that the organic dyes are not applicable for multicolor imaging due to two following properties: i. Presence of signal overlap because of...
relatively broad emission spectra and ii. Presence of a certain narrow wavelength range in order to be suitably excited (1, 2). However, semiconductor quantum dots (QDs) as tiny light-emitting particles have been applied as new class of fluorescent labels for biology and medicine (3-5).

As compared with organic dyes and fluorescent proteins, inorganic quantum dots have shown an external efficiency of 20-80% quantum, while it is stable under relative harsh environments, with the continuous absorption and the narrow emission spectra (1, 6). Furthermore QDs always emit the same lights according to excitation-emission matrix (EEM), indicating when using one laser execution source, the entire different emission colors from QDs will be observed at the same time. Due to their excellent levels, QDs are also used to determine nucleic acid or protein sequences, so the relative changes in emission intensity are considered as a variant. The long-term multiplexed imaging has recently attracted much attention (2, 7). Therefore, semiconductor QDs are applied for the development of photovoltaic devices (8).

Successful use of QDs has been reported in various medical fields, but the important point is the high toxicity of their core compounds which are composed of heavy metals such as cadmium and thallium (3-5, 9). In recent years, much attention has been paid to the toxic effect of QDs due to its wide use in medical field (10, 11). If it is determined that the combination of heavy metal has a minor role in the cytotoxicity of QDs, there will be a good possibility to limit the use of QDs as contrast agents in clinical applications (5).

Due to lack of in vivo studies in this category, this study aimed to evaluate cytotoxic effect of CdSe: ZnS QDs for first time on male reproductive system before and after puberty.

Materials and Methods

Method of producing CdSe: ZnS quantum dots

Nanoparticles were synthesized by chemical precipitation method. For this purpose, three solutions of cadmium chloride (CdCl₂.4H₂O), mercaptoethanol (ME) and sodium selenite (Na₂SeO₃.5H₂O) were prepared in the distilled deionized water, under vigorous stirring (all chemicals were purchased from Merck Chemical Co., USA). At first, CdCl₂ solution was poured into a three spout balloon container that was followed by adding ME solution and sodium selenite solution, respectively, to the same balloon under controlled atmospheric condition with nitrogen (N₂). The resulting solution was mixed with deionized water and centrifuged in order to remove any impurities. Then, the precipitated sample was dried at room temperature. All processes were done at room temperature (12).

The crystal structure and optical properties of QDs were characterized by X-ray diffraction (XRD) pattern using Cu Kα radiation (λ= 0.154 nm) by a Bruker D8 advance XRD machine (Karlsruhe, Germany) and UV-2600 ultraviolet visible spectrophotometer (Shimadzu, Japan). A scanning tunneling microscope (STM, Natsico, Iran) was also used for investigation of particle size distribution.

Breeding and treatment of animals

In this experimental study, male (n=32) and female (n=24) BALB/c mice weighing 24-30 g with 60-70 days of age were obtained from the Department of Histology, School of Medicine, Shahrekord University. Animals were housed in plastic cages and kept for 10 days under 12-hour light/dark conditions, temperature of 22-24°C, humidity of 50-60%, and free access to food and water in order to adapt their life cycle to new environment. Then, 32 adult male mice were divided randomly into four groups (n=8) as follows: control group and three treatment groups receiving 10, 20 and 40 mg/kg CdSe: ZnS QDs, respectively. In embryo group, 24 female mice were included. The mice were mated and pregnancy was determined by detection of vaginal plug. The pregnant mice were divided randomly into four following groups (n=6): control group and three treatment groups receiving 10, 20 and 40 mg/kg CdSe: ZnS QDs, respectively. In this study, work with laboratory animals was approved by the Ethics Committee of the Shahrekord University.

Study design

In adult group, CdSe: ZnS nanoparticles were prepared in normal saline solution and a single-dose of 10, 20, and 40 mg/kg was injected intraperitoneally to three treatment groups, respectively. Only saline was injected to the control group. Also in embryo group, CdSe: ZnS nanoparticles were prepared in saline and a single-dose of 10, 20, and 40 mg/kg was injected intraperitoneally to the pregnant mice of three treatment groups on day 8 of gestation, respectively, because the blood-placenta barrier and gonad development begin after days 5 to 7 of gesta-
Gestation begins with the sign of a vaginal plug as evidence of copulation or gestation day 0.

Tissue preparing
Ten days after CdSe: ZnS injection, following measurement of body weight, mice were dissected under mild anesthesia, while epididymis and testis organs were rapidly cut, weighted, and immersion-fixed in paraformaldehyde. Five micron sections were prepared, dehydrated and embedded in paraffin. The sections were stained using hematoxylin and eosin (H&E) and subsequently processed for histopathological examination under a light microscope. The morphological structure of seminiferous tubules and mean number of spermatogonia, spermatocytes and spermatids were studied in testis. Epithelial height, connective tissue, smooth muscle and sperm density were also studied in epididymis.

Statistical analysis
Data were analyzed using one-way analysis of variance (one-way ANOVA) by the Statistical Package for the Social Sciences (SPSS, SPSS Inc., USA) version 16. Data were represented as means ± SD. Differences were considered significant at P<0.001.

Results

The results of X-ray diffraction and scanning tunneling microscope
The structure of the QDs was investigated by XRD. The sample had a single phase and also a cubic crystal structure. The mean size of the particles was determined by Debye-Scherrer equation that was equal to 2.4 nm for QDs. Also the size was determined around 3 nm using STM (12).

Histological study of testis in adult and embryo groups
In adult group, mice of control group and treatment groups receiving 10 and 20 mg/kg CdSe: ZnS QDs showed normal testicular architecture with an orderly arrangement of germinal, and the seminiferous tubules showed normal spermatogenesis pattern, whereas mice of group administered 40 mg/kg CdSe: ZnS QDs showed several tissue alterations of the seminiferous tubules. Testis sections of group given 40 mg/kg CdSe: ZnS QDs depicted moderate to severely damaged seminiferous tubules including the abnormal and disorganization of spermatogenesis cells and destruction of most spermatogenesis’ layers that was clearly recognized in seminiferous tubules. In addition degeneration of the interstitial tissue, blood vessels, widening of the spaces between seminiferous tubules, as well as deformed and atrophic seminiferous tubules were seen (Fig.1). According to histopathology results of testis in adult group, table 1 shows a significant reduction (one-way ANOVA) in mean number of spermatogonia, spermatocytes I and spermatids in group treated with 40 mg/kg CdSe: ZnS QDs. But in embryo group, qualitative studies using an optical microscope showed that morphological structure of seminiferous tubules were similar in treatment and control groups (Fig.2). Also the average numbers of spermatogonia, spermatocytes, spermatids were similar in treatment and control groups (Table 2).

Histological study of epididymis in adult and embryonic groups
Qualitative studies of epididymal tissues using an optical microscope in embryo treatment groups (receiving 10, 20 and 40 mg/kg CdSe: ZnS) and in adult treatment groups (treated with 10 and 20 mg/kg CdSe: ZnS) showed that epididymal epithelium, interstitial tissue and sperm volume in lumen of epididymal duct were similar in treatment and control groups. But in adult group, in the group treated with 40 mg/kg CdSe: ZnS, although epididymal epithelium showed a normal histological appearance, the lumen of epididymal duct was devoid of spermatozoa, indicating the toxic effect of QDs on testis tissue that led to impaired spermatogenesis (Fig.3).

Body and testis weight changes in adult and embryo groups
In adult group, the testicular weight in the groups treated with 10 and 20 mg/kg CdSe: ZnS QDs were similar to control group and no significant change was found in relative testis weight, but testis weight decreased significantly in mice receiving 40 mg/kg CdSe: ZnS QDs (Fig.2) that was parallel with histological changes in mice testis in this group. The body weight did not change significantly in any of the treatment groups (Table 3). In embryo group, no significant difference was observed in testis weight of treatment groups as compared with the relative value of the control. Also there was no significant difference regarding body weight between the treatment and control groups (Table 4).
CdSe: ZnS Toxicity and Male Reproductive System

Fig. 1: Microscopic images of testis slides of adult group 10 days after injection (H & E, ×400). A-D. Control and treatment groups receiving 10, 20, and 40 mg/kg CdSe: ZnS. Sz; Spermatozoa, Lc; Leydig cells, Lp; Lamina propria, Spg; Spermatogoni, Spc; Spermatocytes and Spt; Spermatids.

Table 1: Comparison of mean numbers of sperm in one tubule in adult group after injection

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>10 mg/kg</th>
<th>20 mg/kg</th>
<th>40 mg/kg</th>
</tr>
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<tr>
<td>Spermatogonia</td>
<td>34.55 ± 6.39</td>
<td>33.6 ± 8.94</td>
<td>32.80 ± 6.67</td>
<td>18.85* ± 6.94</td>
</tr>
<tr>
<td>Spermatocyte I</td>
<td>44.15 ± 9.35</td>
<td>45.25 ± 6.21</td>
<td>43.80 ± 8.43</td>
<td>29.60* ± 6.86</td>
</tr>
<tr>
<td>Spermatid</td>
<td>111.95 ± 33.63</td>
<td>113.65 ± 23.29</td>
<td>109.15 ± 20.72</td>
<td>83.00* ± 23.44</td>
</tr>
</tbody>
</table>

All data are presented as mean ± SD. *; P<0.05.
Fig. 2: Microscopic images of testis slides of embryo groups (H & E, ×400). A-D. Control and treatment groups receiving 10, 20, and 40 mg/kg CdSe: ZnS. Sz; Spermatozoa, Lc; Leydig cells, Lp; Lamina propria, Spg; Spermatogoni, Spc; Spermatocytes and Spt; Spermatids.

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>10 mg/kg</th>
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</thead>
<tbody>
<tr>
<td>Spermatogonia</td>
<td>34.15 ± 8.39</td>
<td>34.75 ± 8.96</td>
<td>32.80 ± 9.51</td>
<td>33.90 ± 8.71</td>
</tr>
<tr>
<td>Spermatocyte I</td>
<td>44.85 ± 10.55</td>
<td>43.94 ± 7.21</td>
<td>41.10 ± 10.87</td>
<td>44.75 ± 8.59</td>
</tr>
<tr>
<td>Spermatid</td>
<td>111.65 ± 20.01</td>
<td>116.55 ± 14.86</td>
<td>120.90 ± 22.50</td>
<td>110.05 ± 18.77</td>
</tr>
</tbody>
</table>

All data are presented as mean ± SD.
Fig. 3: Microscopic images of A. Epididymis in adult and E. Embryo groups after injection of CdSe:ZnS (H & E, ×400), A, B-A, C-A, D-A. Control and treatment groups in adult group and B-E, C-E, D-E. As well as treatment groups in embryo group. L.c.t; Loose connective tissue, E.l; Principal cells, P.se; Pseudostratified stereociliated epithelium, S.m.; Smooth muscle, S.p.m; Sperm mass, E.l; Epididymal lumen, E.d.; Epididymal duct, S.c; Stereocilia.
Table 3: Comparison of mean values of testis and body weight in adult group 10 days after injection

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>10 mg/kg</th>
<th>20 mg/kg</th>
<th>40 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>27.50 ± 1.37</td>
<td>27.00 ± 2.89</td>
<td>29.41 ± 2.20</td>
<td>29.00 ± 1.26</td>
</tr>
<tr>
<td>Testis weight</td>
<td>0.093 ± 0.008</td>
<td>0.087 ± 0.012</td>
<td>0.106 ± 0.019</td>
<td>0.055 ± 0.013*</td>
</tr>
</tbody>
</table>

All data are presented as mean ± SD. *; P<0.05.

Table 4: Comparison of mean values of testis and body weight in embryo groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>10 mg/kg</th>
<th>20 mg/kg</th>
<th>40 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>28.60 ± 1.14</td>
<td>26.00 ± 3.52</td>
<td>27.08 ± 2.58</td>
<td>27.25 ± 3.30</td>
</tr>
<tr>
<td>Testis weight</td>
<td>0.092 ± 0.010</td>
<td>0.095 ± 0.018</td>
<td>0.090 ± 0.008</td>
<td>0.097 ± 0.025</td>
</tr>
</tbody>
</table>

All data are presented as mean ± SD.

Discussion

QDs are very effective for long-term fluorescence imaging; however, the potential toxicity of QDs limits their clinical applications. Due to the presence of Cd\(^{2+}\) ions in QDs, they are highly toxic (5, 13). In recent years, cytotoxicity of these particles has been considered highly due to their use in medical field (14). Although the possible toxic effects of nanoparticles on the reproductive system, placenta translocation, and fetus development are still unknown, some researchers have suggested the reproductive toxicity of nanoparticles (15-17). Our study was the first one conducted on toxicity of QDs on the reproductive system. Chan showed that CdSe-core QD induced apoptosis in mouse blastocysts in a dose-dependent manner. Some studies also showed when blastocysts are pretreatment with CdSe-core QD, cell proliferation is inhibited. Furthermore they revealed that CdSe-core QD inhibited post-implantation embryonic development, meaning that they prevented blastocysts to reach the later stages of development as compared to the controls, while the pre-implantation development of morulas into blastocysts was also inhibited by CdSe-core QD. Also CdSe-core QD with concentration of 500 nmol/L caused resorption of post-implantation blastocysts, leading to a decrease in fetal weight. Also the cytotoxicity of CdSe QD in embryonic development was significantly reduced by the addition of a ZnS coating (18). Other studies showed a significant reduction in the rates of oocyte maturation, fertilization, and in vitro embryo development that was inducted by the CdSe-core QDs, but there was no reduction when using ZnS-coated CdSe QDs. Treatment of oocytes with CdSe-core QDs with concentration of 500 nM during in vitro maturation (IVM) resulted in an increase in resorption of postimplantation embryos and a decrease in placental and fetal weights. It is noteworthy that CdSe-core QDs effectively prevented this cytotoxicity after modification of its surface with ZnS (19).

However, there are some studies regarding toxicity of other nanoparticles on the reproductive system. For example, Yoshida et al. (19) showed C60 (Carbon) nanoparticles administered intratracheally induced adverse effects on the mouse male reproductive function. Also another study showed fetal carbon black nanoparticles (CB-NPs) exposure significantly reduced daily sperm production.
The administration of CB-NPs to adult mice resulted in significant changes in fetal exposure to diesel exhaust (DE) lowered the DSP of male offspring (16). Other research has shown that fetal DE exposure may cause a decrease in sperm weight in mice (21). Furthermore, in vivo studies have revealed that intrauterine exposure to Ag nanoparticles in male offspring due to particulate matters in DE, particularly CB. Also in the testis of male offspring, intercellular adherions of seminiferous epithelium and seminiferous tubules damage were observed (22). In addition, in vitro studies showed cytotoxic effects of titanium dioxide (TiO\textsubscript{2}) on living power of mice Leydig cells. They also revealed that gold nanoparticles decreased movement of mature sperms, and silver and aluminum nanoparticles were toxic for rat spermatogonia stem cells (21, 22). Also, Sleiman et al. (23) showed the impairment in spermatogenesis and a lower sperm count in male Wistar rats that was caused by prepubertal exposure to AgNP. Mathias et al. (24) revealed that Ag nanoparticles reduced the acrosome, plasma membrane integrities, and the mitochondrial activity as well as increased the abnormalities of the sperm. However, there were no changes in sexual behavior, serum hormone concentrations and body growth were. In an experimental study, Ag nanoparticles solution with concentration of 1mg/kg was injected intravenously into male mice over 12 days. No changes were reported in body and testis weights, sperm concentration, motility, fertility indices, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) serum concentrations. However, there were significant changes in serum and intratesticular testosterone concentrations 15 days after initial treatment. Furthermore, significant changes in epithelium morphology, germ cell apoptosis and Leydig cell size were observed using a histologic evaluation. Gene expression analysis revealed a significant upregulation in Cyp11a1 and Hsd3b1 in treated animals (25).

In current study, CdSe: ZnS QDs with 2-3 nm size was synthesized by chemical sedimentation method and the cytotoxic effects on male reproductive system was evaluated. Histopathological studies of testis tissues in adult treatment group receiving 10 and 20 mg/kg CdSe: ZnS and in all embryo treatment groups showed no toxicity. According to our findings, the mean numbers of spermatogonia, spermatocytes, spermatids, as well as matured sperms in seminiferous tubules were similar in above-mentioned treatment groups and control. However, in adult group, our findings revealed that a decrease in testis weight of group receiving 40 mg/kg CdSe: ZnS QDs. Also histological studies of testis tissue showed a high toxicity of CdSe: ZnS in 40 mg/kg dose. Although in this study, cytotoxic effect of CdSe: ZnS QDs on epididymis tissue, tests, and body weight in both adult and embryo groups were studied for the first time, further studies are necessary in this field in order to identify effective background mechanism of QDs cytotoxicity.

**Conclusion**

Our findings showed that CdSe: ZnS QDs in dose of 40 mg/kg induced the toxicity in adult mice, although an in vitro study has shown that Cd\textsuperscript{2+} as main reason of QDs toxicity can be effectively prevented by surface modification of CdSe-core QDs with ZnS. It seems that other mechanisms causing QDs toxicity can be detected by quantum dot stability and time between exposure and toxicity. Also comparison of toxicity of the CdSe: ZnS QDs between adults and embryo groups showed that response of organs is different in various development stages, indicating the complicated process of QDs in vivo causes their toxicity, in spite of the obvious advantages in medicine.

**Acknowledgements**

This work was supported by Iran Nanotechnology Initiative Council. There is no conflict of interest in this study.

**References**


Effects of Adding Sodium Nitroprusside to Semen Diluents on Motility, Viability and Lipid Peroxidation of Sperm in Holstein Bulls

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2. Department of Animal Physiology, Faculty of Agriculture and Natural Resources, Science and Research Branch, Islamic Azad University, Tehran, Iran

Abstract

**Background:** Nitric oxide (NO) that plays important role in all sexual activities of animals is made from the amino acid L-arginine by the enzymatic action of NO synthase (NOS). NO makes a band with sulfur-iron complexes, but due to production of steroid sexual hormones related to the enzymes involved in this complex, NO can change the activity of these enzymes. NO affects many cells including vein endothelial cells, macrophages and mast cells. These cells are also found in Leydig cells; therefore, they are important source of NO in testis tissue. Therefore, minimizing damages to sperm at the time of freezing thawing process are really important. The aim of this study was to determine the appropriate NO concentration to be added to the freezing extender to improve the quality of thawed sperm.

**Materials and Methods:** In this experimental randomized study, sperms of four Holstein bulls with an average age of 4 were collected twice a week for 3 weeks. They received sodium nitroprusside (SNP) in concentrations of 0, 10, 50 and 100 nmol/ml. Data analysis was performed using the special issue and static (SAS) 98 software. Also, mean comparison was done using Duncan’s multiple ranges test (P<0.05). This research was conducted at the laboratory of Science and Research Branch, Islamic Azad University, Tehran at spring and summer of 2013.

**Results:** All concentrations of SNP used was found to increase motility and viability of spermatozoa at 1, 2 and 3 hours after thawing, significantly (P<0.05), but there was no significant difference at zero time. Different concentrations of SNP reduced the membrane lipid peroxidation level of sperm and increased acrosome membranes integrity, implying that SNP generally improved samples membranes, especially in 50 and 100 nmol/ml concentrations.

**Conclusion:** According to the obtained results, addition of SNP to semen diluents increases motility and viability of spermatozoa. Also, it reduces membrane lipid peroxidation level that leads to improved sperm function.

**Keywords:** Sperm Motility, Nitric Oxide, Lipid Peroxidation, Spermatozoa


Introduction

Fertility is very important in livestock, especially in bulls, because obtained sperm are used in insemination of other cows. Freezing thawing process causes sperm damage, like loss of lipid membrane integrity, mitochondria activity, and acrosome membrane integrity, leading to a reduction in motility, viability and fertility of sperm (1).

Therefore, the most important factor in freezing thawing process is to minimize the sperm damages. Freezing-thawing process causes physi-
ochemical stresses on sperm membrane, leading to decrease quality, motility, viability and fertility of sperm (2). Two types of damages are as follows: Production of lots of free radicals and occurrence of peroxidation of phospholipids in sperm membrane which increases the level of fatty acid oxidation including malondiealdehyde (MDA) (3-5). Nitric oxide (NO) as an active non organic molecule is spreadable and free and non stable which is considered as endothelium-derived relaxing factor in veins. It is made in body from the amino acid L-arginine by the enzymatic action of NO synthase (NOS). After making band with sulfur-iron complexes NO changes the activity of these enzymes. NO is an important transmitter molecule in mammalian cells including human and plays a main role in physiological and pathological processes (6, 7). Effect of NO has been observed on many physiological activities of organs, especially male sexual system, like sperm motility, acrosomic reaction, chemotaxis, ability of sperm to bind to the egg, spermatogenesis and balancing the action of hypothalamic-pituitary-gonadal axis (8).

There are few studies about the effects of NO on quality of bulls’ sperm, especially on measurement of membrane damage via measuring MDA level. The aim of this study was to determine the appropriate NO concentration to be added to the freezing extender in order to improve the quality of thawed sperm.

Materials and Methods

This research was conducted at the laboratory of Science and Research Branch, Islamic Azad University, Tehran at spring and summer of 2013. In this experimental randomized study, four Holstein bulls with a mean age of four years and appropriate quantitative and qualitative characteristics of sperm were selected one month before the test. Bulls (jahed research center) were kept in separate boxes and fed according to the National Research Council (NRC, 2000) (9).

Sperm method

Sperm was collected twice a week for 3 weeks. Then samples were transferred to laboratory rapidly and incubated at 37°C in a bain-marie. After semen volume, population and sperm motility of each bull were determined, samples were mixed, divided in four aliquots, and diluted using Bioxell (Bioxell Inc., USA) containing various concentrations of sodium nitroprusside (SNP). So, four SNP treatments including 0 (control), 10, 50 and 100 nmol/ml were studied for 6 weeks (replications). After dilution and packing in 0.5 cc pivots, 2 pivots of each sample were transferred to laboratory, and quality and quantity of sperms were analyzed using computer-aided system analysis (CASA). Samples were then frozen using digital freezer and kept in nitrogen tanks. At least after 24 hours, thawed samples were incubated and motility percentage, viability, acrosome status and MDA level were analyzed at 0, 1, 2, and 3 hours being kept at 37°C. Freezing or cry therapy is a process of long-term preservation of cells and tissues at very low temperatures. To perform the insemination, the frozen sperms should reach the proper temperature. Payout melting process was performed in a water bath of 32-35°C.

Measuring the motility of spermatozoa

Samples were placed in warm water bath at 37°C for 3 minutes, 5 μl was transferred on a slide, and covered with a covers lip of 18×18 mm. Sperm analysis was done using CASA at 0, 1, 2 and 3 hours after thawing.

Measuring viability percentage of sperm

This was carried out using eosin staining method, while nigrosin was used here as background color.

Measuring the level of membrane lipid peroxidation

MDA level was measured by Esterbauer and Cheeseman method (1990) using a spectrophotometer (spectrophotometer uv-varian-CARY50 scan visible, Thermo, USA) at 532 nm wavelength. This method is carried out on the bases of reaction of MDA with thiobarbituric acid (TBA), leading to elimination of double water molecules.

Measuring acrosome integrity of sperm

In fertility, determining the acrosome percentage is a morphological method for measuring viability of sperm after thawing. For this, sperm motility is inhibited initially by mixing semen with Glutaraldehyde 2% in phosphate buffer (NJ, USA). This buffer fixes the membrane and prohibits its deterioration. About 0.5 ml of semen sample was placed
on a slide and was mixed with one drop of buffer. This mixture was scattered on slide, and slide was observed using a contrast phase microscope with $\times 1000$ to $\times 3000$ magnifications.

**Measuring sperm membrane functionality**

About 250 ml of diluted semen was incubated in 1 ml of hypo-osmotic swelling test (HOST) solution with osmolality of 100 m Osm/kg for 40 to 60 minutes and analyzed around 400 spermatozoa using a contrast phase microscope with $\times 1000$ to $\times 3000$ magnifications.

**Statistical analysis**

Data were analyzed using the special issue and static (SAS) 98 software. ANOVA was used to compare the mean values, while Duncan’s multiple range test was carried out to analyze the difference between control and treatment groups. The significance level was set at $P<0.05$.

### Results

#### Total motility and progressive motility of spermatozoa

According to results, SNP increased total motility of sperms significantly ($P<0.05$) in comparison with control group (Table 1). It increased progressive motility in 1, 2 and 3 hours after thawing, but at zero hour (immediately after thawing), motility increased only by 100 nmol/ml, indicating that it is not significant ($P>0.05$). Increase in mobility by SNP was also not significant before freezing ($P>0.05$). Our findings showed that there is no significant difference in 100-nmol treatment regarding total motility of spermatozoa ($P>0.05$) (Table 1).

In case of progressive motility of spermatozoa, the first and second hours after thawing showed the best results, while among SNP concentrations, 100 nmol showed a significant difference compared to the control (Table 2).

### Table 1: The effects of different concentration of SNP on total motility of spermatozoa at various thawing times

<table>
<thead>
<tr>
<th>Treatment (SNP)</th>
<th>0 nmol/ml</th>
<th>10 nmol/ml</th>
<th>50 nmol/ml</th>
<th>100 nmol/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before freezing</td>
<td>74.08 ± 1.08$^a$</td>
<td>77.08 ± 1.80$^a$</td>
<td>76.5 ± 1.08$^a$</td>
<td>79.75 ± 1.85$^a$</td>
</tr>
<tr>
<td>Immediately after thawing</td>
<td>57.00 ± 2.06$^a$</td>
<td>50.16 ± 1.08$^a$</td>
<td>49.25 ± 1.08$^b$</td>
<td>56.08 ± 2.02$^a$</td>
</tr>
<tr>
<td>1 hour after thawing</td>
<td>45.33 ± 1.09$^a$</td>
<td>54.08 ± 2.8$^b$</td>
<td>53.75 ± 2.8$^b$</td>
<td>58.41 ± 2.10$^a$</td>
</tr>
<tr>
<td>2 hours after thawing</td>
<td>41.42 ± 1.02$^a$</td>
<td>48.33 ± 2.8$^b$</td>
<td>46.83 ± 2.3$^b$</td>
<td>50.66 ± 2.10$^a$</td>
</tr>
<tr>
<td>3 hours after thawing</td>
<td>33.83 ± 1.03$^a$</td>
<td>41.66 ± 1.06$^b$</td>
<td>39.58 ± 1.05$^b$</td>
<td>45.08 ± 1.09$^a$</td>
</tr>
</tbody>
</table>

The data are presented as mean ± SD. SNP; Sodium nitroprusside. Common letters in each row indicate no significant difference ($P>0.05$).

### Table 2: The effects of different concentration of SNP on progressive motility of spermatozoa at various thawing times

<table>
<thead>
<tr>
<th>Treatment (SNP)</th>
<th>0 nmol/ml</th>
<th>10 nmol/ml</th>
<th>50 nmol/ml</th>
<th>100 nmol/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before freezing</td>
<td>39.75 ± 2.07$^a$</td>
<td>42.41 ± 2.6$^a$</td>
<td>45.91 ± 2.73$^ab$</td>
<td>47.25 ± 2.8$^b$</td>
</tr>
<tr>
<td>Immediately after thawing</td>
<td>37.08 ± 1.93$^a$</td>
<td>34.25 ± 1.09$^a$</td>
<td>34.75 ± 1.09$^a$</td>
<td>40.08 ± 2.1$^a$</td>
</tr>
<tr>
<td>1 hour after thawing</td>
<td>27.41 ± 1.07$^a$</td>
<td>36.41 ± 1.7$^b$</td>
<td>35.83 ± 1.7$^b$</td>
<td>40.33 ± 2.5$^b$</td>
</tr>
<tr>
<td>2 hours after thawing</td>
<td>24.66 ± 1.04$^a$</td>
<td>29.91 ± 1.35$^b$</td>
<td>30.08 ± 1.35$^b$</td>
<td>35.33 ± 1.49$^a$</td>
</tr>
<tr>
<td>3 hours after thawing</td>
<td>20.91 ± 1.03$^a$</td>
<td>26.08 ± 1.30$^b$</td>
<td>27 ± 1.32$^b$</td>
<td>27.58 ± 1.36$^a$</td>
</tr>
</tbody>
</table>

The data are presented as mean ± SD. SNP; Sodium nitroprusside. Common letters in each row indicate no significant difference ($P>0.05$).
Sperm viability

SNP affected sperm viability and improved this parameter 1 hour after thawing. All concentrations of SNP increased viability significantly (P<0.05). This increase was observed in second hour only in 10 and 50 nmol/ml concentrations. Three hour after thawing, all concentrations increased spermatozoa viability significantly (P<0.05). However, none of the used treatments could increase viability in zero treatment (Table 3).

Lipid peroxidation

Different treatments of SNP reduced this parameter significantly (P<0.05) and damaged sperm membrane at 1 and 2 hours after thawing, but in zero time only 50 nmol caused a significant (P<0.05) reduction (Table 4).

Acrosome integrity of sperm

Variance analysis of data showed that before freezing, SNP increased this parameter significantly (P<0.05) only in 100 nmol/ml treatment. Also immediately after thawing, 100 nmol treatment increased acrosome integrity significantly (P<0.05). In first hour after thawing, 50- and 100- nmol treatments increased this parameter, while all concentrations at second and third hours increased this parameter compared to the control group. The highest ratio of healthy spermatozoa was observed in 100-nmol treatment (Table 5).

Sperm membrane functionality

SNP at all different hours after thawing and even before freezing could reduce the membrane damage significantly (P<0.05) in all treatments, while 100 nmol/ml treatment demonstrated the best results during the first hour after thawing (Table 6).

<table>
<thead>
<tr>
<th>Table 3: The effects of different concentration of SNP on viability of spermatozoa at various thawing times</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (SNP)</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Before freezing</td>
</tr>
<tr>
<td>Immediately after thawing</td>
</tr>
<tr>
<td>1 hour after thawing</td>
</tr>
<tr>
<td>2 hours after thawing</td>
</tr>
<tr>
<td>3 hours after thawing</td>
</tr>
</tbody>
</table>

The data are presented as mean ± SD. SNP; Sodium nitroprusside. Common letters in each row indicate no significant difference (P>0.05).

<table>
<thead>
<tr>
<th>Table 4: The effects of different concentration of SNP on level of membrane lipid peroxidation at various thawing times</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (SNP)</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Before freezing</td>
</tr>
<tr>
<td>Immediately after thawing</td>
</tr>
<tr>
<td>1 hour after thawing</td>
</tr>
</tbody>
</table>

The data are presented as mean ± SD. SNP; Sodium nitroprusside. Common letters in each row indicate no significant difference (P>0.05).
**Table 5:** The effects of different concentrations of SNP on acrosome integrity of spermatozoa at various thawing times

<table>
<thead>
<tr>
<th>Treatment (SNP)</th>
<th>0 nmol/ml</th>
<th>10 nmol/ml</th>
<th>50 nmol/ml</th>
<th>100 nmol/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before freezing</td>
<td>75.5 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74.16 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>72.83 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>78.66 ± 1.05&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Immediately after thawing</td>
<td>55.66 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50.00 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47.16 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>56.83 ± 0.02&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 hour after thawing</td>
<td>38.33 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.16 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.33 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>46.33 ± 0.08&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 hours after thawing</td>
<td>29.08 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.00 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38.33 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38.33 ± 0.08&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>3 hours after thawing</td>
<td>20.25 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.16 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.16 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>27.00 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The data are presented as mean ± SD. SNP; Sodium nitroprusside. Common letters in each row indicate no significant difference (P>0.05).

**Table 6:** The effects of different concentrations of SNP on membrane functionality at various thawing times

<table>
<thead>
<tr>
<th>Treatment (SNP)</th>
<th>0 nmol/ml</th>
<th>10 nmol/ml</th>
<th>50 nmol/ml</th>
<th>100 nmol/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before freezing</td>
<td>74.79 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80.79 ± 0.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>83.87 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>87.83 ± 0.61&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Immediately after thawing</td>
<td>68.54 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>76.45 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>79.54 ± 0.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>82.25 ± 0.75&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 hour after thawing</td>
<td>67.29 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74.37 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>79.75 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>84.08 ± 0.57&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 hours after thawing</td>
<td>69.37 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75.00 ± 0.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>79.37 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>83.66 ± 0.73&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>3 hours after thawing</td>
<td>72.00 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75.83 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>78.12 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>83.95 ± 0.57&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The data are presented as mean ± SD. SNP; Sodium nitroprusside. Common letters in each row indicate no significant difference (P>0.05).

**Discussion**

NO is an important biological molecule which plays critical role in sperm physiology like sperm chemotaxis, sperm motility, and spermatogenesis (8). Several studies have reported sperm motility in low concentrations of NO in mice (10), sheep and human (11, 12). Direct and significant correlation was seen between NO concentrations and sedentary sperm numbers (13, 14). Some researchers have shown that NO is the main activator of guanylate cyclase (ubiquitous enzymes). It is noteworthy to mention that considerable amount of cyclic guanosine monophosphate (cGMP) obtained from this enzyme causes acrosome reaction, chemotaxis and sperm-egg reaction (8, 15). In this study, increasing effects of SNP on some parameters like total motility and progressive motility were in agreement with some studies (15-17), whereas some other studies have reported different results which is due to various SNP concentrations (11, 18-20). Previous studies have shown that concentrations higher than nmol per liter can reduce sperm activity due to toxicity of NO, but NO in low concentrations increases quality parameters of sperm. The concentrations of 50 and 100 nmol/ml are ideal which are reported by Sharma and Aqarwal (16). SNP increased sperm viability and improved sperm parameters which are consistent with some studies (16, 17) and not consistent with others (11, 19, 20). Membrane lipid peroxidation and acrosome integrity are considered as the best indications of a healthy sperm membrane. Our findings showed that different concentrations of SNP increased HOST and incubation times, while reduced MDA level in MDA test indicating controlling effect of NO on lipid per oxidation. Other studies also confirmed these results (17, 21). By studying lipid per oxidation process, we found that membrane injures resulted in reduction in motility and death of sperm. Disorders in balance of oxygen free radicals and in difference mechanism eliminating these radicals lead to reactive oxygen species (ROS) accumulation and induction of oxidative stress that results in damage of proteins,
membrane lipids and other cell components (22). It has been reported that role of NO in prohibition of lipid per oxidation is associated with its ability in reaction with alkoxy lipid radicals (LX), lipid peroxy (LOO) and chain-breaking oxidation (23, 24), which is in agreement with results of this study. Furthermore our results indicated that SNP improved the health of acrosome integrity compared to control, while the best effect was achieved for 100 nmol/ml treatment, which was in agreement with other studies (15). Researchers have emphasized mostly on morphological changes of sperm like twisted tail, injured membranes and injured acrosomes (25). Apparently, injured sperm in freezing process increases ROS production which causes damage to other normal sperms, but SNP reduces acrosome injury via decreasing oxidative stress.

Conclusion

Based on the obtained results, SNP may improve motility, viability, acrosome and plasma membrane integrity during freezing-thawing process. Addition of SNP before freezing provides better results for bovine semen cryopreservation than its inclusion in the thawing extender.

Acknowledgements

Research group greatly appreciate the support and help of Dr. Bahraini and Mr. Toutuonchi of Islamic Azad University, Golpayegan Branch Golpayegan, Iran. There is no conflict of interest in this study.

References

Accuracy Evaluation of The Depth of Six Kinds of Sperm Counting Chambers for both Manual and Computer-Aided Semen Analyses

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Abstract

Background: Although the depth of the counting chamber is an important factor influencing sperm counting, no research has yet been reported on the measurement and comparison of the depth of the chamber. We measured the exact depths of six kinds of sperm counting chambers and evaluated their accuracy.

Materials and Methods: In this prospective study, the depths of six kinds of sperm counting chambers for both manual and computer-aided semen analyses, including Makler (n=24), Macro (n=32), Geoffrey (n=34), GoldCyto (n=20), Leja (n=20) and Cell-VU (n=20), were measured with the Filmetrics F20 Spectral Reflectance Thin-Film Measurement System, then the mean depth, the range and the coefficient of variation (CV) of each chamber, and the mean depth, relative deviation and acceptability of each kind of chamber were calculated by the closeness to the nominal value. Among the 24 Makler chambers, 5 were new and 19 were used, and the other five kinds were all new chambers.

Results: The depths (mean ± SD, μm) of Makler (new), Macro and Geoffrey chambers were 11.07 ± 0.41, 10.19 ± 0.48 and 10.00 ± 0.28, respectively, while those of GoldCyto, Leja and Cell-VU chambers were 23.76 ± 2.15, 20.49 ± 0.22 and 24.22 ± 2.58, respectively. The acceptability of Geoffrey chambers was the highest (94.12%), followed by Macro (65.63%), Leja (35%) and Makler (20%), while that of the other two kinds and the used Makler chamber was zero.

Conclusion: There existed some difference between the actual depth and the corresponding nominal value for sperm counting chambers, and the overall acceptability was very low. Moreover, the abrasion caused by the long use, as of Makler chamber, for example, may result in unacceptable of the chamber. In order to ensure the accuracy and repeatability of sperm concentration results, the depth of the sperm counting chamber must be checked regularly.

Keywords: Depth, Measurement, Sperm Counting Chamber

Introduction

Semen analysis counts significantly among laboratory examinations in andrology, and sperm concentration is one of the basic parameters of routine semen analysis. Although the world health organization (WHO) laboratory manual for the examination and processing of human semen recommends the Neubauer haemocytometer chamber as a sperm counting chamber, and provides methods for quality control of sperm counting (1), many other sperm counting chambers, including DROP, Standard Count, Cell Vision, MicroCell, 2X-CEL, Makler, JCD, Burker, Cell-VU, Leja, Macro, GoldCyto and Geoffrey, a newly designed chamber from the Geoffrey laboratory, have
also been introduced into andrology laboratories. The precision and accuracy of these sperm counting chambers have been widely evaluated and compared (2-13), and most researches have demonstrated wide differences in the results of counting between different sperm counting chambers.

Many factors contribute to the differences in the results of sperm counting, such as sample mixing, loading, environmental temperature, etc. (14-16). However, the depth of the counting chamber is an important factor influencing sperm counting and motility (17, 18). A significant positive correlation was found between the depth of the sperm chamber and bead concentration (r=0.997, P<0.01) as well as between an unacceptable sperm chamber and incorrect result of sperm concentration, and the error was directly proportional to that of the sperm chamber depth (17). If the depth of the sperm counting chamber used for semen analysis does not meet the requirements of permitted error, the accuracy of the results of sperm concentration for all semen samples in the laboratory will be inevitably affected, which is intolerable for both patients and clinicians. Even if a brand is chosen as the standard counting chamber, different batches of products by the same manufacturer might differ significantly in their depths (19). Moreover, the existing researches have raised little doubt about and paid little attention to the accuracy of the chamber’s depth. Extensive literature retrieval has failed to identify any reported studies on the measurement and comparison of the depth of sperm counting chambers. Therefore, we measured the exact depths of six kinds of sperm counting chambers which are widely used for both manual and computer-aided semen analyses (12), and evaluated their accuracy. The detailed report is as follows.

Materials and Methods

Materials

The Filmetrics F20 Spectral Reflectance Thin-Film Measurement System was provided by the DYM Ek Company, USA, which has a measurable range from 15 nm to 70 μm and the lowest detectable limit of 1 nm. The system can be used to measure the thickness of the thin-film by analyzing the reflected light off its two surfaces and then calculate the spectral reflectance at a range of wavelength. A reflectance calculator gives the thickness value of the thin-film based on the complex-matrix form of the Fresnel equations. A film of standard thickness (serial number: National Institute of Standards and Technology (NIST)-12016), SiO₂ on Si Standard Thickness=(725.9 ± 1.1) Å, was certified by the NIST, USA. Five 10 μm-deep Makler sperm chambers were purchased from Sefi Medical Instruments, Israel. Thirty-two Macro sperm counting chambers and 34 Geoffrey chambers, both of 10 μm depth, were provided by Jiangsu Rich Life Science Instrument Co., Ltd (Xuzhou, China). Twenty Gold-Cyto chambers (Microptic s.l. 321 6° 4*-08029, Barcelona, Spain) and 20 Leja chambers (Leja Products B.V. Luzemestraat 10 2153 GN, Nieuw Vennep, The Netherlands), both 20 μm in depth, were bought from Guangdong Youning Trade Co., Ltd (Guangzhou, China). Twenty Cell-VU chambers of 20 μm in depth (Millennium Sciences Inc., USA) were purchased from Nanjing Yu’an Instrument Co., Ltd (Nanjing, China). In addition, nineteen 10 μm-deep Makler sperm counting chambers (Sefi Medical Instruments, Israel), which had been used for several months or years, were obtained from different andrology laboratories in China. Makler, Macro and Geoffrey chambers are manufactured with the base glass and separate cover glass and can be used repeatedly. GoldCyto and Leja chambers belong to the disposable kind with fixed depth. Cell-VU chamber, also made from the base glass and separate cover glass, can be used repeatedly or as a disposable chamber.

Methods

This was a prospective study. First, the Filmetrics F20 Spectral Reflectance Thin-Film Measurement System was calibrated with the standard film according to the operating instruction. Then, six kinds of sperm counting chamber, including Makler, Cell-VU, Leja, Macro, GoldCyto and Geoffrey, were numbered randomly, cleansed with a solution (ether: alcohol, 7:3), and confirmed to be devoid of impurity under a microscope. Finally, the depths of the upper, lower, left, right and central parts of each chamber were measured, and the average depth, SD and the range of depth (maximum value minus minimum value) of each chamber was calculated automatically, all with the Filmetrics F20 System according to the operating instructions. All the sperm counting chambers were measured by one technician, and the supervision and verification of the results were conducted by another.

Statistical analysis

All the data obtained were put into an Excel table for calculation of the mean depth, the range of depth, and coefficient of variation (CV) of each
chamber. For those with double chambers, the paired t test was used to compare the difference between the two; for those with more than two chambers, the comparison was made by the LSD-t test, and the new and used Makler chambers were compared by the independent-sample t test. The SPSS (SPSS Inc., USA) version 11.0 software was used for analyses, and statistically significant difference was designed to be P<0.05. For a 5% permitted error, the chambers with a measured depth of 10 ± 0.5 μm or 20 ± 1 μm were judged as compatible with a nominal value of 10 μm or 20 μm, respectively, followed by assessment of the acceptability of different sperm counting chambers.

Results

The depths of all the six kinds of sperm counting chambers were measured. The results are shown in table 1.

The average depth of the 32 Macro sperm counting chambers (with a single chamber and nominal depth of 10 μm) was 10.19 μm, and CV was 4.71%. The range of the mean and CV of the five depth measurements of Macro chambers were 0.14 μm and 0.58%, respectively, and 65.63% of the 32 chambers were within acceptable limits.

The Makler sperm counting chamber is also a single chamber unit with depth of 10 μm. The mean depth of the five new Makler chambers was 11.07 μm, with a relative deviation of 10.7%, exceeding the allowable range of 5%, and that of the 19 used Makler chambers was 12.72 μm, with a relative deviation of 27.2%, far exceeding the allowable range. Moreover, the CV (8.49%) for all used chambers and the mean range (0.43 μm) and CV (1.33%) of the depth between the 5 points were obviously higher than those of the Macro chambers. The acceptability was 20% for the new Makler chambers, and zero for the used ones.

A Cell-VU sperm counting chamber includes two 20 μm-deep chambers. The mean depth and relative deviation of the 20 left chambers were 23.94 μm and 19.7%, and those of the 20 right ones were 24.49 μm and 22.45%, respectively. Although there was no significant difference in depth between the left and right chambers (t=1.231, P=0.233), the CVs (12.36 vs. 10.41%) for all the chambers on either side and the mean ranges (1.64 μm vs. 2.21 μm) and CVs (2.66 vs. 3.47%) of the depth between the 5 points in each chamber were very high. The acceptability was 5% (1/20) for both the left and right chambers. If it was required that the errors of the two chambers in one set to be within the allowable range, the acceptability of the Cell-VU chamber was zero.

A Geoffrey sperm counting chamber contains two 10 μm-deep chambers. The mean depth of the 34 left chambers was 10.01 μm and that of the 34 right ones was 9.99 μm, with no significant difference between them (t=0.801, P=0.429). The CVs (2.90 vs. 2.80%) for all the chambers on either side, and the mean ranges (0.11 vs. 0.11 μm) and CVs (0.45 vs. 0.47%) of the depth between the 5 points in each chamber were all very low. The acceptability values of the left and right chambers were 100 and 94.12%, respectively. If it was required that the errors of the two chambers in one set to be within the allowable range, the acceptability of the Geoffrey chamber was 94.12%.

A GoldCyto sperm counting chamber contains four 20 μm-deep chambers (A-D). The mean depths of chambers A, B, C and D of the 20 GoldCyto sets were 22.19, 24.79, 24.96 and 23.11 μm, respectively. The corresponding relative deviations were 10.95, 23.95, 24.8 and 15.55%, respectively, with a total deviation of 18.8%, all far exceeding the allowable range of 5%. However, the depths of chambers B and C were significantly greater than those of A and D (P<0.05), although there was no significant differences in depth either between B and C or between A and D. The acceptability values of chambers A, B, C, and D were 30, 5, 5 and 10%, respectively. If one of the chambers was unacceptable, the whole unit was appraised as unacceptable and the acceptability of the GoldCyto sperm counting chamber was zero.

A Leja sperm counting chamber consists of eight 20 μm-deep chambers (A-H). The mean depths of chambers A, B, C, D, E, F, G, and H of the 20 Leja chambers were 20.99, 20.37, 20.30, 20.34, 20.88, 20.31, 20.17 and 20.55 μm, respectively. The corresponding relative deviations were 4.95, 1.85, 1.7, 1.7, 4.4, 1.55, 0.85 and 2.75%, respectively, indicating that all were within the allowable range of 5%. However, the depths of chambers A and E were significantly greater than those of B, C, D, F, G, and H (P<0.05), and the depth of H was greater than that of G (P<0.05). The acceptability values of chambers A, B, C, D, E, F, G, and H were 65, 95, 95, 100, 65, 100, 95 and 95%, respectively. However, if one of the chambers was unacceptable, the whole unit was appraised as unacceptable and the acceptability of the Leja sperm counting chamber was 35%.
<table>
<thead>
<tr>
<th>Sperm counting chamber</th>
<th>n</th>
<th>Depth of chamber (range) (μm)</th>
<th>CV between chambers (%)</th>
<th>Variation of depth between 5 points (Max-Min, μm)</th>
<th>CV between five points (%)</th>
<th>Acceptability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macro</td>
<td>32</td>
<td>10.19 ± 0.48 (9.25-10.95)</td>
<td>4.71</td>
<td>0.14</td>
<td>0.58</td>
<td>65.63</td>
</tr>
<tr>
<td>Makler (New)</td>
<td>5</td>
<td>11.07 ± 0.41 (10.50-11.60)</td>
<td>3.70</td>
<td>0.086</td>
<td>0.30</td>
<td>20</td>
</tr>
<tr>
<td>Makler (Be used)</td>
<td>19</td>
<td>12.72 ± 1.08 (11.32-14.64)</td>
<td>8.49</td>
<td>0.43</td>
<td>1.33</td>
<td>0</td>
</tr>
<tr>
<td>Cell-VU</td>
<td>20</td>
<td>24.22 ± 2.58 (18.21-26.83)</td>
<td>10.65</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Left</td>
<td>20</td>
<td>23.94 ± 2.96 (17.01-28.34)</td>
<td>12.36</td>
<td>1.64</td>
<td>2.66</td>
<td>5</td>
</tr>
<tr>
<td>Right</td>
<td>20</td>
<td>24.49 ± 2.55 (18.68-27.83)</td>
<td>10.41</td>
<td>2.21</td>
<td>3.47</td>
<td>5</td>
</tr>
<tr>
<td>Geoffrey</td>
<td>34</td>
<td>10.00 ± 0.28 (9.54-10.62)</td>
<td>2.80</td>
<td></td>
<td></td>
<td>94.12</td>
</tr>
<tr>
<td>GoldCyto A</td>
<td>20</td>
<td>23.76 ± 2.15 (18.43-26.63)</td>
<td>9.06</td>
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<td></td>
<td>0</td>
</tr>
<tr>
<td>GoldCyto B</td>
<td>20</td>
<td>22.19 ± 2.62 (17.68-27.68)</td>
<td>11.81</td>
<td>1.81</td>
<td>3.27</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(19.30-29.31)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>20</td>
<td>24.79 ± 2.52 (19.30-29.31)*</td>
<td>10.17</td>
<td>1.99</td>
<td>3.23</td>
<td>5</td>
</tr>
</tbody>
</table>
### Table 1: Continued

<table>
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<tr>
<th>Sperm counting chamber</th>
<th>n</th>
<th>Depth of chamber (range) (μm)</th>
<th>CV between chambers (%)</th>
<th>Variation of depth between 5 points (Max-Min, μm)</th>
<th>CV between five points (%)</th>
<th>Acceptability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>20</td>
<td>24.96 ± 3.10</td>
<td>12.42</td>
<td>1.79</td>
<td>2.96</td>
<td>5</td>
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<tr>
<td></td>
<td></td>
<td>(18.79-29.67)</td>
<td>(0.37-3.90)</td>
<td>(0.69-5.96)</td>
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<td></td>
</tr>
<tr>
<td>D</td>
<td>20</td>
<td>23.11 ± 2.89</td>
<td>12.51</td>
<td>1.93</td>
<td>3.22</td>
<td>10</td>
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<tr>
<td></td>
<td></td>
<td>(17.97-27.81)</td>
<td>(0.15-4.29)</td>
<td>(0.24-6.67)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leja</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.27-21.34)</td>
</tr>
<tr>
<td>A</td>
<td>20</td>
<td>20.99 ± 0.39</td>
<td>1.86</td>
<td>0.45</td>
<td>0.85</td>
<td>65</td>
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<td></td>
<td>(20.53-21.71)</td>
<td>(0.13-0.80)</td>
<td>(0.25-1.67)</td>
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<tr>
<td>B</td>
<td>20</td>
<td>20.37 ± 0.58</td>
<td>2.85</td>
<td>0.17</td>
<td>0.33</td>
<td>95</td>
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<td></td>
<td></td>
<td>(19.99-22.68)</td>
<td>(0.05-0.42)</td>
<td>(0.10-0.85)</td>
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<tr>
<td>C</td>
<td>20</td>
<td>20.30 ± 0.34</td>
<td>1.67</td>
<td>0.15</td>
<td>0.29</td>
<td>95</td>
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<td>(19.99-21.67)</td>
<td>(0.03-0.44)</td>
<td>(0.05-0.87)</td>
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<td></td>
</tr>
<tr>
<td>D</td>
<td>20</td>
<td>20.34 ± 0.14</td>
<td>0.69</td>
<td>0.18</td>
<td>0.35</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(20.15-20.65)</td>
<td>(0.04-0.42)</td>
<td>(0.07-0.96)</td>
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<tr>
<td>E</td>
<td>20</td>
<td>20.88 ± 0.42</td>
<td>2.01</td>
<td>0.31</td>
<td>0.59</td>
<td>65</td>
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<tr>
<td></td>
<td></td>
<td>(20.28-21.64)</td>
<td>(0.11-0.60)</td>
<td>(0.21-1.29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>20</td>
<td>20.31 ± 0.26</td>
<td>1.28</td>
<td>0.21</td>
<td>0.44</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(20.00-20.92)</td>
<td>(0.07-0.38)</td>
<td>(0.14-0.86)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>20</td>
<td>20.17 ± 0.46</td>
<td>2.28</td>
<td>0.11</td>
<td>0.22</td>
<td>95</td>
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<tr>
<td></td>
<td></td>
<td>(19.89-22.07)</td>
<td>(0.03-0.4)</td>
<td>(0.04-0.69)</td>
<td></td>
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<tr>
<td>H</td>
<td>20</td>
<td>20.55 ± 0.30</td>
<td>1.46</td>
<td>0.33</td>
<td>0.63</td>
<td>95</td>
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<tr>
<td></td>
<td></td>
<td>(20.23-21.49)</td>
<td>(0.04-0.61)</td>
<td>(0.08-1.14)</td>
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</tbody>
</table>

For Cell-VU and Geoffrey chambers, the difference between the left and right chambers was compared with paired t test. There was no significant difference in depth between the left and right chambers of Cell-VU chambers (t=1.231, P=0.233) and between the left and right chambers of Geoffrey chambers (t=0.801, P=0.429). The comparison of new and used Makler chambers was analyzed with independent-samples t test, and there was significant difference between them (t=5.325, P<0.001). For GoldCyto and Leja chambers, the difference between the chambers was compared with LSD-t test. CV; Coefficient of variation; P<0.05 vs. A and D in the same group, *; P<0.05 vs. A and E in the same group and #; P<0.05 vs. G in the same group. There was no significant difference between other chambers in the same group (P>0.05).
Discussion

The WHO Manual (5th edition) emphasizes regular measurement of the depth of the sperm counting chamber, but in practice, it is never measured since its purchase till worn out. Moreover, there is a lack of methods to identify the chambers and specific measures for the implementation of quality control for the chambers.

Therefore, we measured the depth of six kinds of sperm counting chambers with the Filmetrics F20 Spectral Reflectance Thin-Film Measurement System, which has been widely used to measure the thickness of some thin film materials (20-22).

The results of measurement of the six kinds of chambers showed that no chamber, either 10 μm or 20 μm deep, was 100% acceptable. The highest acceptability was 94.12%, as exhibited by the Geoffrey sperm counting chamber, a new type developed by Jiangsu Rich Life Science Instrument Co., Ltd, China, with an optical glass plate embedded in a metal frame base and a cover plate containing a metal frame inlaid with an optical coverslip. There are four ruby spherical pillars and two independent chambers on the optical glass plate. The relatively high acceptability of Geoffrey chambers may be attributed to the fine polishing processing and calibration of each plane of the optical glass plate, strict quality control measures for the adjustment of the chamber’s depth, and precise detection of the depth of each chamber before dispatched from the factory.

The acceptability of the Macro chamber was 65.63%, and the average range between the 5 points was 0.14 μm, a little lower than that of the Geoffrey chambers. The Macro chamber, similar to the Makler chamber in design, except for its three ruby spherical pillars instead of four glass columns in the latter, has been widely used in andrology laboratories in China. Using the principle of three points defining a plane, the production of the Macro chamber may have dismissed Newton’s rings to ensure a closer contact of the cover plate with the three supporting points.

The Leja chamber comprises eight 20 μm-deep chambers. Although its overall acceptability value was only 35%, the acceptability of each chamber was high. Therefore, in order to make all chambers meet the requirements of the allowable error, the standard of the production process must be relatively high.

The acceptability of the new Makler chambers was 20%, while that of the other three kinds, including the used Makler, Cell-VU, and GoldCyto chambers, was zero, which may be attributable to the lack of strict control of chamber measurement or the use of an inaccurate measurement method at delivery inspection. In our study, the relative deviations of the left and right chambers of the Cell-VU unit were around 20%, and those of chambers B and C of the GoldCyto unit above 20%, indicating that all significantly higher than the allowable error range of 5%. The highest acceptability of all the chambers of the Makler, Cell-VU and GoldCyto was only 30%. The abrasion from long use, as of the Makler chamber, may be one of the reasons for the unacceptable chambers.

Conclusion

There exists some difference between the actual depth and the corresponding nominal value of sperm counting chambers from different manufacturers, and the difference far exceeds the acceptable (95% CI) range for most of the chambers, which inevitably results in a large variation in sperm concentration in clinical application. Therefore, in order to ensure the accuracy and repeatability of semen analysis results, the depth of the sperm counting chamber must be checked regularly, even though strictly measured at delivery inspection, and unqualified chambers must be rejected. In addition, the measurement report for each sperm counting chamber must be attached to the product for an andrology laboratory.

Acknowledgements

This study was approved and financially supported by Jiangsu Jingcheng Pharmaceuticals Co., Ltd. All authors declare no any conflict of interest in the study.

References

Evaluation of Depth of Sperm Counting Chambers


Reducing Inter-Laboratory Differences between Semen Analyses Using Z Score and Regression Transformations

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Abstract

Background: Standardization of the semen analysis may improve reproducibility. We assessed variability between laboratories in semen analyses and evaluated whether a transformation using Z scores and regression statistics was able to reduce this variability.

Materials and Methods: We performed a retrospective cohort study. We calculated between-laboratory coefficients of variation (CV) for sperm concentration and for morphology. Subsequently, we standardized the semen analysis results by calculating laboratory specific Z scores, and by using regression. We used analysis of variance for four semen parameters to assess systematic differences between laboratories before and after the transformations, both in the circulation samples and in the samples obtained in the prospective cohort study in the Netherlands between January 2002 and February 2004.

Results: The mean CV was 7% for sperm concentration (range 3 to 13%) and 32% for sperm morphology (range 18 to 51%). The differences between the laboratories were statistically significant for all semen parameters (all P<0.001). Standardization using Z scores did not reduce the differences in semen analysis results between the laboratories (all P<0.001).

Conclusion: There exists large between-laboratory variability for sperm morphology and small, but statistically significant, between-laboratory variation for sperm concentration. Standardization using Z scores does not eliminate between-laboratory variability.

Keywords: Differences, Semen Analysis, Regression, Standardization
Reducing Inter-Laboratory Differences between Semen Analyses

Introduction

Semen analysis is the cornerstone of the laboratory evaluation of the subfertile male partner (1, 2). It is well recognized that the semen analysis demonstrates large between-laboratory variability (3). As a consequence, it is difficult for doctors to interpret and compare the results of semen analyses from different laboratories and this hampers the value of the semen analysis in daily practice.

Standardization of the semen analysis might improve reproducibility. A method to standardize across laboratories is Z score transformation, expressing how many standard deviations a semen analysis result is above or below the mean. Z score transformations allow a comparison of observations from different normal distributions. A more conventional method to partition error into systematic and random error is to use regression statistics and then to use the regression coefficients to correct for the systematic error.

In this study, we assessed systematic differences between laboratories in semen analysis results. We then evaluated whether transformation with Z scores and regression statistics is able to reduce such differences.

Materials and Methods

Circulation samples

In the Netherlands, the National Foundation for Quality Control in Medical Laboratory Diagnostics [Stichting Kwaliteitsbewaking Medische Laboratoriumdiagnostiek (SKML)] sends out samples to the participating laboratories four times a year through the External Quality Assessment Scheme (EQAS). SKML processes and analyses the scores and reports these results to the laboratories. For this study, we collected data of 50 laboratories that had scored the sperm concentration and/or sperm morphology according to local protocols on samples that were regularly distributed by the SKML semen EQAS in 2003. This EQAS was organized according to the International Laboratory Accreditation Cooperation (ILAC-G13) guideline for external quality assessment schemes. All laboratories consented in participating in this study. We circulated 8 samples for sperm concentration and sperm morphology to the participating laboratories.

Circulation sample preparation

For concentration measurement, remaining semen of in vitro fertilization (IVF) treatments was mixed with Hayem medium (Boom BV, the Netherlands) and stored at 4°C. Just before a circulation, the different samples were pooled and divided over the batches. The batches were divided over a sufficient number of vials to provide each participating laboratory with a sample of each batch.

Dependent of the laboratory, sperm concentration was counted in a Makler, a Burker Turk or an improved Neubauer counting chamber. For sperm morphology, a sufficient number of semen smears was prepared from each sample on microscopic slides. The smears were air dried and distributed over the participants. Ten laboratories scored the sperm morphological parameters according to the 1999 World Health Organization (WHO) criteria and 2 laboratories according to the 1992 WHO criteria (4, 5). The procedures with respect to the use of remaining semen were approved by the local Ethical committees.

Cohort of subfertile couples

Between January 2002 and February 2004, we included 5,534 couples in a prospective cohort study performed in The Netherlands (6). The local ethics committee of all participating centers gave Institutional Review Board approval. For this analysis, we used data from 2,804 men participating in the prospective cohort in whom the semen analysis was performed by one of the laboratories participating in EQAS (7). In some hospitals, semen analyses were repeated; in our analyses only the results of the first semen analysis were used.

Data analysis

To quantify the between laboratories reproducibility in the evaluation of the circulation samples, we calculated between-laboratory coefficients of variation (CV_L) for sperm concentration and for morphology. The CV_L is defined as the ratio of the standard deviation of semen analysis results, returned by the 12 laboratories for a given sample, relative to the mean over all laboratories for that same sample (8) (Addendum 1). CV_L values close to zero indicate limited variability between laboratories, hence better reproducibility. With a CV_L of 0, all laboratories would return the same...
result for a single sample. Large \( CV_b \) indicate low reproducibility.

**Addendum 1: concept of the coefficient of variation.**

\[
CV = \frac{\sigma}{\mu} \times 100
\]

\( \sigma \): standard deviation

\( \mu \): mean

We then calculated Z scores per laboratory, based on the circulation samples (Addendum 2). The Z score represents the difference between a semen analysis result and the laboratory mean, expressed in units of the standard deviation for the results obtained in that laboratory. This Z score can be regarded as a unitless, standardized laboratory result. The Z score is negative when the result is below the mean, positive when above. As the sperm concentration and the sperm morphology were not normally distributed, we applied a natural logarithmic transformation to the sperm concentration values and a square root transformation to the sperm morphology values, before calculating Z scores. To make the Z scores comparable between laboratories, we subsequently adjusted for differences in the laboratory means.

**Addendum 2: concept of the Z score.**

\[
Z = \frac{X - \mu}{\sigma}
\]

\[
Z_i = \frac{X_{\text{original}} - \mu_{\text{laboratory}}}{\sigma_{\text{laboratory}}}
\]

\( Z_i \): Z score per laboratory

\( X_{\text{original}} \): original semen analysis result

\( \mu_{\text{laboratory}} \): mean per laboratory

\( \sigma_{\text{laboratory}} \): standard deviation per laboratory

We used linear regression as an alternative method to adjust for systematic differences between laboratories. We used the mean of the circulation samples as the dependent variable, and the laboratory results as the independent variable. With the laboratory specific regression coefficients, we then adjusted the laboratory results.

We evaluated whether the Z score transformations and the regression transformations reduced systematic differences in the circulation samples, using analysis of variance. If successful, there should be no significant differences between laboratories after standardization.

Subsequently, we standardized the semen analysis results from the men in the prospective cohort study using these Z score and regression transformations (Addendum 3). Box plots were constructed to compare semen analysis results per laboratory before and after standardization. Here also, we tested for systematic differences before and after standardization.

**Addendum 3: Standardization of the semen analysis results by Z score transformation.**

\[
X_{\text{standardized}} = \mu_{\text{original}} + \left( \frac{X_{\text{original}} - \mu_{\text{laboratory}}}{\sigma_{\text{laboratory}}} \right) \times \sigma
\]

\[
X_{\text{standardized}} = \mu_{\text{original}} + \left( \frac{X_{\text{original}} - \mu_{\text{laboratory}}}{\sigma_{\text{laboratory}}} \right) \times \frac{\sigma_{\text{original}}}{\sigma_{\text{laboratory}}}
\]

\[
X_{\text{standardized}} = X_{\text{original}} \times \frac{\sigma_{\text{original}}}{\sigma_{\text{laboratory}}} + \left( \mu_{\text{original}} - \mu_{\text{laboratory}} \right) \times \frac{\sigma_{\text{original}}}{\sigma_{\text{laboratory}}}
\]

\( X_{\text{standardized}} \): standardized semen analysis result

\( \mu_{\text{original}} \): population mean

\( X_{\text{original}} \): original semen analysis result

\( \mu_{\text{laboratory}} \): mean per laboratory

\( \sigma_{\text{laboratory}} \): standard deviation per laboratory

P values under 0.05 were interpreted to indicate statistical significance in all statistical tests. Calculations were performed using The SPSS (SPSS Inc., USA).

**Results**

The mean between-laboratory CVB for the 12 laboratories of the circulation cohort (EQAS) was 7% for concentration (range 3 to 13%) and 32% for morphology (range 18 to 51%). The CVB for sperm concentration and morphology are shown graphically in figure 1A and B, respectively.
Reducing Inter-Laboratory Differences between Semen Analyses

The mean semen analysis results of the men participating in the cohort study before and after standardization with the Z score are shown in table 1. The range for the mean semen volume was 2.6 to 3.9 mL and for the mean sperm motility 30 to 55%. When comparing the ranges for sperm concentration, the range of the mean original semen analyses was 11.6 to 42.1 and 12.1 to 40.4 after regression transformation. For sperm morphology, these ranges were respectively 4 to 31 for the mean original results, 5 to 25 for the mean results after Z score transformation and 9 to 18 for the mean results after regression transformation.

There were significant systematic differences between laboratories in the results before standardization for the circulation samples, for the four semen parameters: sperm concentration, sperm morphology semen volume and sperm motility (all P<0.001, Table 2).

We then evaluated whether the Z score transformations reduced systematic differences in the circulation samples. This was not the case (Table 2).

Transformation with the regression coefficients in the circulation cohort was valid for both semen volume and sperm morphology (Table 2).

The, in general, very minor differences for sperm concentration between the values before and after standardization are graphically depicted in figure 2A, B. There was a minimal reduction of the 25th to 75th percentile values, represented by the minimally reduced box sizes. The number of strong and weak outliers did not differ before and after transformation. The box plots for sperm morphology show more differences; the 25th to 75th percentile values are noteworthy smaller after standardization, with fewer outliers, and mean values that are more comparable between laboratories. Visual inspection of the figures showed reduction of the strong and weak outliers (Fig. 2C, D).

When we repeated the analysis of variance after standardization, we still observed significant differences between laboratories for semen volume and sperm morphology for Z score and regression transformations (all P values <0.001, Table 2).

![Fig.1: Coefficient of variation for A. Sperm concentration (%) and B. Sperm morphology (%) of 8 samples, evaluated in 12 laboratories.](image-url)
Table 1: Original results of the semen analysis stratified per hospital (n=2,804) that indicates sperm morphology with results after standardization

<table>
<thead>
<tr>
<th>Hospital</th>
<th>n</th>
<th>Semen volume (ml)</th>
<th>Sperm motility (%)</th>
<th>Sperm concentration (10⁶/l)</th>
<th>Sperm morphology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Original result</td>
<td>Original result</td>
<td>Original result</td>
<td>Original result</td>
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<tr>
<td></td>
<td></td>
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<td>Standardized result</td>
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<tr>
<td>1</td>
<td>495</td>
<td>3.1</td>
<td>35</td>
<td>40.9</td>
<td>31</td>
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<tr>
<td>2</td>
<td>544</td>
<td>2.7</td>
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<td>26</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>3.8</td>
<td>45</td>
<td>11.6</td>
<td>10</td>
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<tr>
<td>4</td>
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<td>55</td>
<td>42.1</td>
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<tr>
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<td>31.8</td>
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<td>38.5</td>
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<td>188</td>
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<tr>
<td>9</td>
<td>8</td>
<td>2.6</td>
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<td>19.9</td>
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<tr>
<td>11</td>
<td>226</td>
<td>3.7</td>
<td>30</td>
<td>34.4</td>
<td>16</td>
</tr>
<tr>
<td>12</td>
<td>204</td>
<td>3.0</td>
<td>44</td>
<td>30.9</td>
<td>26</td>
</tr>
</tbody>
</table>

Table 2: Results of tests for systematic differences in semen analysis results between laboratories in the circulation and the study cohort

<table>
<thead>
<tr>
<th></th>
<th>Circulation cohort</th>
<th>Study cohort</th>
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<tr>
<td></td>
<td>F</td>
<td>P</td>
</tr>
<tr>
<td>Sperm concentration†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>baseline differences</td>
<td>4.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>differences after correction by z score</td>
<td>6.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>differences after correction by regression</td>
<td>&lt;0.001</td>
<td>1</td>
</tr>
<tr>
<td>Sperm morphology†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>baseline differences</td>
<td>4.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>differences after correction by z score</td>
<td>&lt;0.001</td>
<td>1</td>
</tr>
<tr>
<td>differences after correction by regression</td>
<td>&lt;0.001</td>
<td>1</td>
</tr>
<tr>
<td>Semen volume</td>
<td>12.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sperm motility</td>
<td>25</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

†; Values were transformed to follow a normal distribution, F; F-statistic and P; P values.
Reducing Inter-Laboratory Differences between Semen Analyses

Fig. 2: A, B. Box plot of sperm concentration ($10^6/$mL) before and after Z score transformation. C and D. Box plot of sperm morphology (%) before and after Z score transformation. *; Strong outlier, o; Weak outlier, T; Highest value that is no outlier, □; 25th to 75th percentile, —; Median and —; Lowest value that is no outlier.

Discussion

In this study, we found systematic differences in results of semen analyses between laboratories for four semen parameters. The between-laboratory coefficient of variation for morphology was large and the between-laboratory variation for concentration was small, but still significant. Standardization of these results by Z score and regression transformations did not reduce these differences between laboratories.

In this study, we explored the concept of the Z score transformation to standardize differences between laboratories in the semen analysis results and evaluated this in a large multicenter cohort of men from subfertile couples. The Z score transformation procedure for normalizing data is a familiar statistical method in both microarray and psychological studies (9, 10), but has never been reported in the field of the semen analysis.

We used a second more conventional method for the correction of the systematic error, using regression. With the standardization method, we also observed no reduction of the differences between laboratories.

A first potential limitation of our study was that we had no data to calculate the Z score for sperm motility. A second potential limitation is that outliers in data sets that contain large differences can distort the results. A third potential limitation could be that the random error overshadowed the presence of an effect. Although all measurements are prone to random error, the source of random error in the variability of the semen analysis itself and between laboratories might be difficult
to assess. Random errors are unpredictable and scattered about the true value and tend to have null arithmetic mean when a measurement is repeated several times with the same instrument. Another explanation for the inability of both transformation methods to correct for systematic differences might be attributed to differences in populations of the laboratories. Although Z score and regression transformations have the capacity to standardize systematic differences between the laboratories, regardless the source of variability, it is not possible to standardize the effect of the random error and differences in populations.

With respect to the variability between laboratories, the results of our study are in agreement with a study of 26 semen samples of 26 men that were scored by four teams of specialists from different countries. They reported a reliable comparability for sperm concentration between the four teams, but not for sperm morphology (11).

Conclusion

Although all laboratories claimed to follow the WHO recommendations for semen analysis, we established significant differences between laboratories in semen analysis results. The data on intra-laboratory variability was limited in this study. Training and further standardization of all aspects of the semen analysis in combination with internal and external quality control schemes will have to be intensified. This may lead to substantial reductions in intra- and inter-observer variability. In the meantime, laboratories will have to remain repeating semen analyses from patients that were referred with semen analysis results from another laboratory.

Acknowledgements

There is no financially support and conflict of interest in this article.

References

Protective Effects of Thymoquinone against Methotrexate-Induced Germ Cell Apoptosis in Male Mice

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Abstract

Background: Toxic effects of anti-cancer and other drugs on the normal tissues could be reduced by the herbal plants and their fractions. This study investigated the protective effect of thymoquinone (TQ) as a fraction of Nigella sativa on methotrexate (MTX)-induced germ cell apoptosis in male mice.

Materials and Methods: In this experimental study, thirty male Balb/c mice were divided randomly into 5 groups (n=6). A single dose of MTX (20 mg/kg) and different concentrations of TQ were administrated for 4 consecutive days. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed on paraffin embedded tissue sections to analysis the occurrence of apoptosis in the testis. Reverse transcription polymerase chain reaction (RT-PCR) of apoptosis-related genes was performed with RNA extracted from testes of the mice. Statistical analysis was done using one-way ANOVA.

Results: In the MTX group, there was a significant increase in morphologic sign of germ cell degeneration of tubules (48 ± 0.6%), apoptotic index (AI; 2.3 ± 0.6%), as well as mRNA expression of p53 (P=0.008), caspase 8 (P=0.002), caspase 3 (P=0.005), caspase 9 (P=0.000), bax (P=0.004) and the ratio of bax/bcl-2 (P=0.000), whereas there was an decrease in the expression of bcl-2 (P=0.003), as compared to control group. In MTX+TQ groups, the data showed that different concentrations of TQ could improve the harmful effects caused by the MTX. The best protective effects were achieved in MTX+TQ (10 mg/kg).

Conclusion: TQ protects testicular germ cell against MTX-induced apoptosis by affecting related genes regulation.

Keywords: Apoptosis, Methotrexate, Thymoquinone, Mice, Germ Cells

Introduction

The use of methotrexate (MTX) drug, a widely used folate antagonist, has been limited based on the occurrence of short-and long-term toxicity (1-3). The genotoxic effects of MTX have been also shown in both somatic and germ cells (4). Only a single exposure to high dose of MTX induces germ cell toxicity in mice that can be transmitted to the next generation, explaining the hazardous administration of this drug (5).

Nigella sativa (black seed) plant has been investigated for its antioxidant, anti-inflammatory and anticancer activities in both in vitro and in vivo models since 1960s (6). Toxicological studies have shown that thymoquinone (TQ) as the main active
component of *N. sativa* might have a protective effect against hepatotoxicity and nephrotoxicity induced by either chemicals or diseases (7, 8). In addition, this quinone compound was found to exhibit anticancer activity through the modulation of multiple molecular targets, including *p53*, *PTEN*, *STAT3*, and *PPAR*, activation of caspases and generation of reactive oxygen species (ROS) (6).

Gkce et al. (9) suggested that TQ may decrease the destructive effects of MTX on testicular tissue. Further, Badary et al. (10) have shown that TQ has strong antioxidant activities through scavenging ability of different free radicals in an *in vitro* model.

Although germ cell toxicity of MTX and protective effects of TQ against hazardous agents have been shown previously, the involvement of apoptosis and its related genes in this issue have not been demonstrated. In this regards, this study was conducted to evaluate the protective effect of TQ against MTX-induced germ cell toxicity of mice testis. The occurrence of apoptosis in seminiferous tubules was shown using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, and the related genes were characterized by reverse transcription polymerase chain reaction (RT-PCR).

**Materials and Methods**

**Experimental design**

In this experimental study, thirty male Balb/c mice aged 10 weeks (30 ± 2 g) were obtained from a closed bred colony at Kermanshah University of Medical Sciences, Kermanshah, Iran. The animals received care as recommended by the Ethics Research Committee of the Kermanshah University of Medical Sciences (EC/KNRC/90-4) in accordance with the internationally accepted principles for laboratory animal use and care, as found in the European Community guidelines (EEC Directive of 1986; 86/609/EEC) or US guidelines (NIH publication #85-23, revised in 1985). The mice were maintained on a regular diet and water at a 12:12 hour light/dark cycle at 23°C ± 2°C. Experiment was started after one week adaptation.

The animals were divided randomly into following 5 groups (n=6): i. Control group receiving dimethyl sulfoxide (DMSO, 1:1000) in normal saline, ii. Experimental group (E1) receiving only an intraperitoneal single dose injection of MTX (20 mg/kg; Sigma Aldrich, USA), iii. Experimental groups (E2-E4) receiving an intraperitoneal injection of MTX (20 mg/kg) plus TQ (Sigma Aldrich, USA) in different concentrations of 2 mg/kg (E2), 10 mg/kg (E3), and 20 mg/kg (E4) for 4 consecutive days (8). On the day five, the mice were sacrificed by cervical dislocation.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling assay**

Apoptosis was assessed by TUNEL assay using In Situ Cell Death Detection Kit (Roche Diagnostics Deutschland GmbH, Germany). After paraffinization with xylene, 5 μm sections prepared by rotary microtome. Then, the sections were rehydrated through a series of ethanol solutions and washed in deionized water. Nuclei in the tissue sections were stripped from protein by incubating with 50 μl of proteinase K (10 mg/ml) for 20 minutes at room temperature.

After washing twice with sterile phosphate-buffered saline (PBS) for 10 minutes, the slides were incubated with TUNEL reaction mixture in a humidified chamber at 37°C for 60 minutes, followed by rinsing three times with PBS for 10 minutes. The sections were counterstained with propidium iodide (PI) solution diluted to 1 μg/ml in PBS (15 minutes), and then washed in deionized water for 5 minutes. Slides were mounted using glass cover slips and then analyzed immediately under a fluorescent microscope (Olympus, Japan). Apoptotic index (AI) was calculated by dividing the number of TUNEL positive cells to total number of the cells in randomly selected fields, and the result was multiplied by 100 (11).

**Reverse transcription–polymerase chain reaction analysis**

RNA was extracted from testes tissues using the RNAeasy Plus Mini Kit (Qiagen, Germany), including a gDNA Eliminator column to avoid DNase digestion and a RNeasy Mini Spin columns to purify RNA samples. Total RNA (≤1 μg) was reverse transcribed using a poly T tail primer included in the One Step RT-PCR Kit (Qiagen, Germany). cDNA was amplified according to the manufacturer’s instructions. Primer pairs, amplicon sizes, and annealing times are shown in table 1. Cycle conditions were as follows: denaturation at 95°C for 15 minutes that was followed by 30 cycles at 94°C for 60 seconds, annealing at 58°C to 60°C for 60 seconds, and elongation at 72°C for 60 seconds, with a final cycle at 72°C for 10 minutes. Experiments were performed...
in triplicate to ensure reproducibility.

Products were electrophoresed on a 1.5% agarose gel. Gels were stained with ethidium bromide (10 µg/ml) and photographed on an ultraviolet (UV) transilluminator (UVIdoc, Uvitec, UK). Gel images were analyzed using the UV image (UVI) band map program (Uvitec, UK). Primers characteristics are listed in table 1.

RT–PCR values were presented as a ratio of the specified gene signal divided by the glyceraldehyde-3-phosphate dehydrogenase (gapdh) signal. RT-PCR was performed as three individual replicates (12).

Statistical analysis

All data were analyzed by one-way ANOVA followed by Tukey’s test using SPSS (SPSS Inc., USA) software. Results are expressed as the mean ± SEM, and P<0.05 was considered significant.

Results

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

TUNEL assay was performed in the testis to ascertain the mode of cell death by MTX and TQ. In this regards, cross sections of seminiferous tubules were stained with TUNEL dye and analyzed. The AI was quantitatively higher in the MTX-treated group (E1, 2.3 ± 0.4) than the control group (0.2 ± 0.6, P=0.000). Treatment of TQ markedly reduced the reactivity and the number of apoptotic cells (Fig.1A, B). These data indicated that inhibition of germ cell apoptosis is the another action of TQ in basal portion of seminiferous tubules.

The expression of markers

The expression levels of markers and profiles of the relative expression levels are shown in figures 2 and 3. In the E2 group, there was a significant decrease in expression of bcl2 (P=0.003), whereas there was a significant increase in the expression levels of other markers, including p53 (P=0.008), caspase 3 (P=0.005), caspase 8 (P=0.002), caspase 9 (P=0.000), and bax (P=0.004). The ratio of bax/bcl2 also increased in E1 group (P=0.000). The expression levels of these markers in MTX+TQ groups (E2-E4) were between the related values of E1 and control groups. Furthermore, the expression level of bcl2 significantly decreased in E2 (P=0.002) and E3 groups (P=0.005).

<table>
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<th>Gene</th>
<th>Primer sequence</th>
<th>Annealing temperature</th>
<th>Size (bp)</th>
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<tbody>
<tr>
<td>gapdh</td>
<td>F: ACCCTCAACTACATGGTCTAC</td>
<td>58</td>
<td>801</td>
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<tr>
<td></td>
<td>R: TTGTCAATTGAGGCAATGCC</td>
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<td>p53</td>
<td>F: CATCATCACCGTAGAAGACTC</td>
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<td></td>
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<td>bax</td>
<td>F: GCTGATGGCAACTTCAACTG</td>
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<td>389</td>
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</tbody>
</table>
**Fig. 1:** The apoptosis inducing effect of methotrexate (MTX) (20 mg/kg) and different doses of thymoquinone (TQ) on testis of mice. A. Images by a fluorescent microscopy indicating terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of mice testicular sections that are counterstained with propidium iodide (PI). Apoptotic cells show bright fluorescence nuclei indicated by arrowheads (magnifications: ×160) and B. Percent of TUNEL positive cells (AI). The mice were grouped as Control, MTX (E1), MTX+TQ 2 mg/kg (E2), MTX+TQ 10 mg/kg (E3), MTX+TQ 20 mg/kg (E4). **; P<0.001 compare to control group, a; P <0.01 and b; P<0.05 compare to MTX group.

**Fig. 2:** Temporal expression pattern of genes in testis of mice treated with MTX plus different concentrations of TQ using by reverse transcription polymerase chain reaction (RT-PCR) in 4 experimental groups (E1-E4). MTX; Methotrexate and TQ; Thymoquinone.
**Fig. 3**: Expression of related genes of apoptosis in testis of mice treated with MTX plus different concentrations of TQ using RT-PCR in 4 experimental groups [E1-E4]. The differences between groups are presented by ANOVA. All the values are expressed as mean ± SEM. MTX; Methotrexate, TQ; Thymoquinone, RT-PCR; Reverse transcription polymerase chain reaction, **; P<0.001, *; P<0.01 compare to control group, a; P <0.01 and b; P<0.05 compare to MTX group.
Discussion

In the present study, the percentage of apoptotic cells was found to be increased after MTX treatment. It has been further reported that MTX-induced apoptosis in the hepatocytes of rats, which can be attributed to the nucleotide pool imbalance or the repression of the cJun N-terminal kinase (JNK) activity and upregulation of p53 and p21 (13). Consistent with these results, our study showed that administration of MTX induced testicular damage characterized by seminiferous tubule degeneration and apoptosis of germ cells via both p53 and Bax/Bcl-2 pathways.

The release of cytochrome c from mitochondria has been indicated to be a critical step in the activation of the caspase protease cascade. Caspases trigger a cascade of proteolytic cleavage events that are considered as central players in all apoptotic events in mammals. Bcl-2 and Bcl-x (L) inhibit apoptosis, in part by blocking the release of cytochrome c from mitochondria. On the contrary, other family members, such as bax and bad, interfere with the activity of bcl-2 by binding to them and generating a nonfunctional unit (14).

Present study showed that TQ prevented apoptosis in seminiferous tubules treated with MTX through affecting mRNA expression levels of p53, caspases 3, 8 and 9, as well as the Bax/Bcl-2 ratio. These data were confirmed by a clear decrease in the number of TUNEL positive apoptotic cells in MTX+TQ-treated mice.

These results are consistent with previous report that showed combination of TQ and conventional chemotherapeutic drugs could produce greater therapeutic effect as well as reduce the toxicity of the latter (6). These data are also in parallel with the study of Nagi et al. (15), demonstrating that cyclophosphamide-induced cardiotoxicity in rats was attenuated through activity of TQ that results in reducing oxidative and nitrosative stress, as well as preserving the activity of antioxidant enzymes. In addition, TQ was shown to protect against MTX-induced testicular injury in male mice (9).

Conclusion

Combination of TQ with chemotherapeutic agents may provide a novel therapeutic approach. However, further research in animal models is warranted to obtain more conclusive evidence for the molecular basis of TQ action. Despite its therapeutic efficacy in tumor cell lines and animal models, the data on bioavailability and other pharmacokinetic parameters of TQ are still incomplete.

Acknowledgements

We acknowledge the staff of Fertility and Infertility Research Center of Kermanshah and also Dr. Mehri Azadbakht from Department of Biology, Razi University, Kermansha, Iran. This study was supported by a grant (no 90057) from Kermanshah University of Medical Sciences. There is no conflict of interest in this article.

References


Protection against Cyclosporine-Induced Reprotoxicity by Satureja khuzestanica Essential Oil in Male Rats

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Abstract—

Background: The effects of cyclosporine (Cs), a fungal cyclic polypeptide with potent immunosuppressive activity, on fertility have assumed greater significance with the increasing numbers of transplantations being performed all over the world. Current study was undertaken to investigate the potential of Satureja khuzestanica Essential Oil (SEO) as an antioxidant to mitigate Cs-induced reprotoxicity.

Materials and Methods: In this experimental study (April-July 2012), thirty-two adult male Wistar rats were randomly divided into 4 groups of 8 animals each. Two groups of rats were administered Cs [40 mg/kg/day, per oral (p.o.)] for 45 days. One of these groups received SEO (225 mg/kg/day, p.o.) four hours after Cs administration. A vehicle-treated control group and a SEO control group were also included. Epididymal sperm characteristics, in vitro fertilizing capacity as well as embryo development were evaluated. For statistical analysis, one-way ANOVA and Tukey’s post-hoc test were used, and the value of P<0.05 was considered as the criterion for statistical significance.

Results: Sperm count and viability along with fertilization and blastocyst development rates were significantly decreased by Cs treatment. Moreover, Cs-treated group showed significant increases in DNA damage, protamine deficiency of the sperm cells and proportion of spermatozoa with cytoplasmic droplet. Notably, aforementioned parameters were improved to near normal level by SEO co-administration.

Conclusion: These results suggest that SEO has a protective action against Cs-induced reprotoxicity in a rat model.

Keywords: Cyclosporine, Sperm, In Vitro Fertilization, Satureja khuzestanica


Introduction

Cyclosporine (Cs), a cyclic polypeptide with potent immunosuppressive activity, is a metabolite isolated from Tolypocladium inflatum and Cylindrocarpon lucidum (1). Cs has been widely used in transplant medicine and it has markedly improved graft survival rates in organ transplantation (2). Additionally, this drug is utilized in the treatment of various autoimmune disorders such as idiopathic nephritic syndrome (3), uveitis (4), psoriasis (5), rheumatoid arthritis (6) and inflammatory bowel disease (7). The primary immunosuppressive action of Cs is attributed to its inhibiting feature on the interleukin-2-interleukin-2-receptor autocrine pathway (8). Although Cs has enabled the success of clinical transplantation, its therapeutic application is limited by a number of adverse effects including renal, hepatic, cardiac, alimentary, neural and reproductive toxicity (9-14). Although the precise biochemical mechanism of Cs-induced toxic-
Protection against Cs-Induced Reprotoxicity by *S. khuzestanica*

Reprotoxicity in many organs is still a matter of debate, it has been shown that generation of reactive oxygen species (ROS) and membrane lipid peroxidation are causative factors involved in Cs-induced toxicities (15-17). Furthermore, it has been demonstrated that Cs induces reproductive toxicity through direct alterations in hypothalamic–pituitary-gonadal axis (18-20) and reduction in Sertoli cell phagocytic function (1).

*Satureja khuzestanica*, belonging to the Lamiaceae (mint family), is an endemic plant of Iran that is known for its therapeutic application in folk medicine resulted from the essential oil existing in that (21). Different preparations of *Satureja khuzestanica* have been shown to have anti-inflammatory, antinociceptive, antimicrobial and antioxidant properties (22-24). Moreover, independent studies have revealed that *Satureja khuzestanica* Essential Oil (SEO) possess reproprotective and reproduction stimulatory properties (24, 25).

In view of this, since the antioxidative properties of SEO have been established, the present study was intended to evaluate the possible protective effect of SEO in experimental reprotoxicity induced in the rat model by Cs.

**Materials and Methods**

**Isolation of the essential oil**

SEO was prepared from cultivated *Satureja khuzestanica* in Khoramabad in Lorestan province, western Iran. A dried voucher specimen was deposited at the Herbarium of the Botany Department, Faculty of Science, Urmia University, Urmia, Iran. The aerial parts of the plant were collected during the flowering stage, air-dried at ambient temperature in the shade and hydro-distilled using a Clevenger apparatus (Bakhshilab Co., Iran) for 4 hours, giving yellow oil in 0.9% yield. The oil was dried over anhydrous sodium sulfate and stored at 4°C. The density of the essence was 0.94 (26).

**Animals**

For this experimental study (April-July 2012), thirty-two adult male albino rats of Wistar strain (4 months of age, 180-220 g body weight) were obtained from the Experimental Animal Production Center of the Science Faculty of Urmia University. All the animals were housed in special cages and had free access to tap water and to a pelleted commercial laboratory animal chow. Animal room temperature and relative humidity controls were set at 22 ± 2°C and 50 ± 10%, respectively. Lighting was controlled to give a 12-hour light and dark cycle. All ethical themes of studies on animals were considered carefully and the experimental protocol was approved by the Ethics Committee for Research on Laboratory Animals at Urmia University.

**Experimental design**

After an adaptation period of 7 days, the animals were randomly divided into following four groups of 8 rats each as described below according to the treatment they received: control group, SEO group, Cs group and Cs+SEO group. The two experimental groups (Cs and Cs+SEO) were gavaged Cyclosporine (Sandimmune®, Novartis Pharmaceutical Corp., Switzerland) at a dose of 40 mg/kg/day dissolved in 0.5 mL olive oil. The controls were given an equivalent amount of olive oil. The SEO group was gavaged SEO at a dose of 225 mg/kg/day dissolved in 0.5 mL olive oil. The Cs+SEO group also received the same dose of SEO four hours after Cs administration. The treatment period was 45 days. The protocol for this study, including doses and duration of treatment for Cs and SEO, were all designed according to previous studies (27-29).

**Sampling**

Animals were euthanized by cervical dislocation following anesthesia with ketamine (Alfasan, Netherlands, 75 mg/kg, IP) 24 hours after the last treatment. The abdominal cavity was opened up through a ventral midline incision and epididymides were carefully separated from the testicles under a 20-time magnification provided by a stereo zoom microscope (Olympus, Japan). Testes and epididymides were then cleared of surrounding fat and connective tissues and weighed on a Mattler Basbal scale (Delta Range, Japan).

**Epididymal sperm characteristic analysis**

**Epididymal sperm count**

Epididymal sperm concentration was determined by a standard hemocytometer (HBG, Germany) as described previously (30). Briefly, one caudal epididymis was placed in 1 ml of rat 1-cell
embryo culture medium (mR1ECM), chopped into 2-3 pieces and incubated for 10 minutes at 37°C in an atmosphere of 5% CO₂ incubator to allow sperm to swim out of the epididymal tubules. After dilution of epididymal sperm to 1:20 in mR1ECM medium, approximately 10 μl of this diluted specimen was transferred to each of the counting chambers of the hemocytometer, which was allowed to stand for 5 minutes in a humid chamber to prevent drying. The sediment cells during this time were counted with a light microscope (Olympus, Japan) at ×400. The sperm count was expressed as number of sperm per milliliter.

**Epididymal sperm viability**

A 20 μl of sperm suspension was mixed with 20 μl of 0.05% eosin-yellowish (eosin-Y, Sigma-Aldrich, USA). Slides were assessed by a bright-field microscope with ×400 magnification following 2 minutes incubation at room temperature. Dead sperms appear pink and live sperms are not stained. In each sample 200 sperms were counted and viability percentages were recorded (31).

**Epididymal sperm motility**

In order to assess the sperm motility, 10 μl of the sperm suspension was placed on a clean pre-warmed microscope slide and covered with a cover slip. At least 10 microscopic fields were examined at ×400 magnification using a light microscope equipped with a heated stage. The percentage of motile sperm was evaluated microscopically within 2-4 minutes of their isolation from the epididymes and expressed as a percentage of motile sperm of the total sperm counted (32).

**Epididymal sperm DNA denaturation**

Acridine orange (AO) assay was used to measure the susceptibility of cauda epididymal sperm DNA to acid-induced denaturation in experimental groups. To perform this assay with fluorescent microscope, thick smears were fixed in Carnoy’s fixative (methanol: acetic acid 1: 3) for at least 2 hours. The slides were stained for 5 minutes and gently rinsed with deionized water. About 200 sperms were evaluated, among which sperm heads with intact chromatin had green fluorescence, while those with denatured chromatin had orange-red staining (33).

**Epididymal sperm nuclear maturity**

The acidic aniline blue (AB) stain is used to discriminate between lysine-rich histones and arginine/cysteine-rich protamines. This technique specifically provides a positive reaction with lysine residues in nuclear histones and reveals differences in the basic nuclear protein composition of the sperm. Histone-rich nuclei of immature sperms are rich in lysine and will consequently take up the blue stain. Protamine-rich nuclei of mature sperms are rich in arginine and cysteine and contain relatively low levels of lysine bringing about negative reaction to AB. The air-dried fixed smears were stained for 7 minutes with 5% AB in in phosphate buffered saline (PBS, Sigma-Aldrich, USA). The pH was adjusted to 3.5 using acetic acid. Slides were gently rinsed in distilled water and air dried. About 200 sperms per slide were counted under a light microscope (Olympus, Japan) using a ×100 oil immersion objective to determine the percentage of sperms stained with AB (34).

**Cytoplasmic droplet count**

Spermatozoa that retained their cytoplasmic droplet were enumerated by the methodology as reported previously (35). Sperm smears were prepared on clean and grease free slides, left to air-dry overnight, stained with 1% eosin-Y/5% nigrosin and examined using an optical microscopy (Olympus, Japan). About 200 sperm cells per animal were examined to determine the percentage of sperms with cytoplasmic droplet.

**In vitro evaluation of fertility potential and embryonic development**

**Collection of oocytes**

Female rats were injected subcutaneously with 25 IU pregnant mare serum gonadotrophin (PMSG, Folligon, Netherlands). Fifty-four hours later the rats were received an intraperitoneal injection of 20 IU human chorionic gonadotropin (hCG, Folligon, Netherlands). The rats were euthanized 19 hours after hCG injection. The oviducts were removed and each ampullar portion was placed into a plastic dish containing mR1ECM medium. Under a stereo zoom microscope (Olympus, Japan), 29-gauge insulin needle was used to gently tear open the swollen ampulla, allowing the oocytes in cumulus masses
to extrude spontaneously into the medium (36).

**Sperm processing for in vitro fertilization and insemination**

Sperm suspension were prepared and processed for in vitro fertilization (IVF) as described earlier. Spermatozoa were obtained by swim-up and incubated at 37°C in an atmosphere of 5% CO₂ for 1 hour to ensure capacitation. A volume of 0.1 mL of sperm suspension was introduced into 0.9 mL fertilization drop of mR1ECM medium containing oocytes from three females. For each animal, a total of 20 oocytes were divided into 10 drops. After six hours of incubation at 37°C under 5% CO₂, the cumulus cell free fertilized oocytes were transferred to fresh drops of mR1ECM medium for culture of embryos. All medium droplets were covered with mineral oil (37).

**Evaluation of fertilization rate**

Twenty-four hours after insemination, oocytes were monitored by an inverted microscope and formation of the pronuclei and polar bodies was recorded to evaluate fertilization rate (FR).

**Blastocyst development rate determination**

Blastocyst development rate (BDR) was evaluated by determining the number of embryos that had reached the blastocyst development stage after 72 hours incubation. Both late blastocysts (blastocoeles greater than half the volume of the embryo) and expanding blastocysts (blastocoeles that are fully expanded, with a thin zona pellucida) were included in the BDR (38).

**Measurement of lipid peroxidation**

Lipid peroxidation (LPO) level in tissue homogenate was measured with the aid of a spectrophotometer. Malondialdehyde (MDA), which formed as an end product of the peroxidation of lipids, served as an index of LPO. MDA, referred to as thiobarbituric acid reactive substance (TBARS), was measured with TBA at 532 nm in a spectrophotometer (Biochrom, UK), as described previously (39). Results were expressed as nmol/g wet tissue.

**Data analysis**

All data were expressed as the mean ± standard error of mean (S.E.M.). Differences between groups were assessed by one-way ANOVA using SPSS (SPSS Inc, USA) version 18.0. Statistical significance between groups was determined by Tukey’s post hoc test and the value of P<0.05 was used as the criterion for statistical significance.

**Results**

**Reproductive organ weights**

The effects of Cs and SEO on relative testicular and epididymal weights are shown in table 1. Cs treatment produced a significant decrease in relative weights of testes and epididymides, indicating reproductive system damage, compared with the control group. These parameters rushed toward near normal levels of control in Cs+SEO-treated group.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cs</th>
<th>SEO</th>
<th>Cs+SEO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testes (weight/BW %)</td>
<td>0.89 ± 0.005</td>
<td>0.82 ± 0.003a</td>
<td>0.89 ± 0.006b</td>
<td>0.86 ± 0.003a,b</td>
</tr>
<tr>
<td>Epididymides (weight/BW %)</td>
<td>0.52 ± 0.005</td>
<td>0.45 ± 0.008a</td>
<td>0.53 ± 0.003b</td>
<td>0.49 ± 0.003a,b</td>
</tr>
<tr>
<td>Sperm count (10⁶/ml)</td>
<td>50.50 ± 1.44</td>
<td>19.50 ± 0.86a</td>
<td>49.33 ± 1.45b</td>
<td>46.00 ± 1.15b</td>
</tr>
<tr>
<td>Sperm viability (%)</td>
<td>87.00 ± 1.73</td>
<td>68.00 ± 1.73a</td>
<td>86.33 ± 1.20b</td>
<td>79.50 ± 0.86b</td>
</tr>
<tr>
<td>Sperm motility (%)</td>
<td>79.33 ± 0.88</td>
<td>56.66 ± 0.88a</td>
<td>80.00 ± 1.73b</td>
<td>64.50 ± 0.86a,b</td>
</tr>
<tr>
<td>AO-positive sperms (%)</td>
<td>6.50 ± 0.86</td>
<td>28.00 ± 1.73a</td>
<td>5.83 ± 0.60a</td>
<td>10.50 ± 1.44a</td>
</tr>
<tr>
<td>AB-positive sperms (%)</td>
<td>8.00 ± 0.57</td>
<td>19.50 ± 0.86a</td>
<td>9.16 ± 0.44a</td>
<td>11.00 ± 2.30b</td>
</tr>
</tbody>
</table>

BW; Body weight, Cs; Cyclosporine, SEO; Satureja khuzestanica essential oil, AO; Acridine orange, AB; Aniline blue. *; Statistically significant as compared with the control group at P<0.05 and ††; Statistically significant as compared with the Cs group at P<0.05. The values are expressed as mean ± SEM.
Epididymal sperm parameters

As shown in table 1, daily administration of Cs significantly reduced the mean epididymal sperm counts compared with their controls. There was also a significant decrease in the mean percentage of live and motile sperms of Cs-treated rats compared with their control counterparts (Fig.1A). However, the simultaneous administration of SEO to Cs-treated animals significantly impeded a decrease in the epididymal sperm count and the percentage of live and motile sperms.

Oral administration [per oral (p.o.)] of Cs brought about significant increase in the percentage of spermatozoa with DNA damage and chromatin abnormalities when compared to control rats (Fig.1B, C). On the other hand, co-administration of SEO caused significant decreases in sperm DNA damage and chromatin abnormalities respect to the Cs-treated group (Table 1).

The cytoplasmic droplet count for all groups is presented in figure 2. The percentage of spermatozoa that retained their cytoplasmic droplet was markedly higher in Cs-treated animals than those of the control animals, whereas co-treatment by SEO provided marked normalization in the percentage of spermatozoa having a cytoplasmic droplet when compared with the Cs group.

Fertilization rate

The effect of Cs and SEO on FR is depicted in figures 3 and 4. FR in animals exposed to Cs was significantly lower than control group, while treatment with SEO in combination with Cs increased the FR in rats compared with rats treated with Cs alone. Mean of fertile oocytes in control, Cs, SEO and Cs+SEO groups were 77.94% (152/195), 65.19% (148/227), 77.54% (145/187) and 91.80% (224/244), respectively.
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**Blastocyst development rate**

The effects of different treatments on BDR are summarized in figures 4 and 5. Treatment with Cs alone resulted in a significant decrease in BDR, indicating embryotoxicity, as compared to the control. Administration of SEO along with Cs caused a significant improvement in this parameter compared to the Cs alone group.

![Fig.4](image1)

**Fig.4:** A. Representative photomicrographs of in vitro embryo development at 72 and 120 hours of culture from control, B. *Satureja khuzestanica* essential oil (SEO), C. cyclosporine (Cs) and D. SEO+CS treated rats. Differentiation to compact morula (cm), blastocyst (b) and hatched blastocyst (h) can be observed in control and SEO-treated groups. Cs administration alone caused significant increases in the rates of unfertilized oocytes (uf) and arrested embryos (ae). SEO co-treated animals exhibited nearly normal in vitro embryo development.

![Fig.5](image2)

**Fig.5:** Effect of Cs and SEO on rat embryo development, expressed as the BDR (n=8). Cs: Cyclosporine, SEO: *Satureja khuzestanica* essential oil, BDR: Blastocyst development rate, a: Statistically significant as compared with the control group at *P*<0.05 and b: Statistically significant as compared with the Cs group at *P*<0.05.

**Testicular tissue lipid peroxidation level**

Testicular tissue LPO level, demonstrated as MDA, of all groups is given in figure 6. The MDA levels in the testicular tissue were found to be significantly higher in rats treated with Cs alone than those in the control group. The increase in MDA by Cs was significantly attenuated by SEO.

![Fig.6](image3)

**Fig.6:** Effect of Cs and SEO on rat testicular tissue LPO (n=8). Cs: Cyclosporine, SEO: *Satureja khuzestanica* essential oil, MDA: Malondialdehyde, LPO: Lipid peroxidation, a: Statistically significant as compared with the control group at *P*<0.05 and b: Statistically significant as compared with the Cs group at *P*<0.05.
Discussion

Cs as an immunosuppressive of choice in transplant surgery has improved quality of life and survival of transplant patients (40). However, its clinical utility is accompanied by numerous unwanted side-effects such as reprotoxicity (18-20). Recently, there have been increasing interests in complementary medicine to alleviate Cs-induced toxicities in different organs (41, 42).

Several reports have demonstrated that enhancement of lipid peroxidation and oxidative stress (OS) following disruption in the oxidant–antioxidant status is the most possible biochemical mechanism of Cs-induced adverse effects in reproductive system (13, 14, 43).

In the present study, reduction in weight of the testis and epididymis were indicative of Cs toxicity. Since the weight of the testes largely depends on the mass of the differentiated spermatogenic cells (44), the marked reduction in organ weight by Cs can be explained by diminished number of germ cells and a significant lower rate of spermatogenesis as confirmed by our findings.

Mammalian spermatozoon as a highly differentiated haploid cell plays a crucial role in fertilization and its impairment is considered a major contributory factor to male infertility (45). Highly specific lipidic composition of mammalian sperm plasma membrane is responsible for its flexibility and the functional abilities of spermatozoa (46). However, substantial evidence indicates that mammalian spermatozoa are markedly vulnerable to the damages induced by enhanced, pathological ROS generation due to this unusual structure of sperm membrane (47). High levels of ROS production induce lipid peroxidation, a cascade of autocatalytic chemical reactions, in sperm cell membranes which can give a rise to cell dysfunction and death (48).

In this study, Cs-treated rats showed significant decreases in epididymal sperm count and percentage of live sperm as compared to control animals. These findings are in agreement with previous reports at which it was indicated that Cs causes spermatotoxicity through peroxidation of spermatozoa plasma membranes polyunsaturated fatty acids or alterations of the intracellular redox state (14, 43).

It has been established that protamines are involved in sperm chromatin stability as well as protection of spermatozoa against a variety of endogenous or exogenous stressors (49). A growing body of evidence indicates that OS-induced defects in spermatozoon maturation process can result in prolamination disturbances leading to diminished sperm chromatin packaging which makes sperm cells more vulnerable to DNA damage (50-52). Moreover, extensive researches demonstrate that OS affects the integrity of the sperm genome by induction of high frequencies of DNA fragmentation (53-55). Consistent with above-noted findings, Cs-treated animals in this study showed a significant decrease in the prolamination of sperm chromatin as well as percentage of the sperms with double-stranded DNA in comparison to the control group.

It is known that free radicals overproduction frequently involves in defective spermiogenesis leading to release of spermatozoa from the germinal epithelium carrying excess residual cytoplasm (56). It was also found that these immature, morphologically abnormal spermatozoa are the main source of further ROS generation in semen (57). Supporting these facts, our observations revealed that Cs treatment causes a marked elevation in the proportion of spermatozoa that retained their cytoplasmic droplet via mechanisms that may be mediated by OS.

It is well documented that disturbances in the organization of the genomic material in sperm nuclei result in premature chromatin condensation leading to decreased fertilization rates and/or embryo cleavage (58-60). Additionally, earlier studies have linked high number of spermatozoa with DNA breaks and attached cytoplasmic droplets to fertilization and embryo development failures (61, 62). In view of the fact that immature and structurally deficient spermatozoa are the major source of surplus free radicals production (63), FR and BDR reduction in Cs-administered rats may be attributed to the detrimental effects of ROS-producing damaged spermatozoa during in vitro insemination of oocytes. Our findings are confirmed by the study that reported in vitro incubation of oocytes with ROS-producing spermatozoa can result in impaired embryo development (64).

Recently, there have been increasing interests in beneficial effects of antioxidants and naturally occurring substances against Cs-induced reproductive toxicity. Free radical scavengers such as ellagic acid have been shown to abate testicular damage...
and spermatotoxicity induced by Cs (43). Further, it has been reported that lycopene, an aliphatic hydrocarbon with high efficient antioxidant capacity, provides protection against Cs-induced testicular toxicity through restoration of oxidant/antioxidant balance (14). Our previous study have also demonstrated that Crataegus monogyna fruit aqueous extract with prominent antioxidant property can effectively abate Cs-induced reproductive toxicities (65).

In the present study, concomitant administration of SEO to Cs receiving rats markedly ameliorated the Cs-induced all the negative changes observed in the sperm parameters and embryo development, thereby highlighting its protective role in countering the oxidative injuries inflicted by free radicals. Consistent with our findings, prior studies have pointed out that SEO, as a strong and safe antioxidant, can protect from malathion and cyclophosphamide induced OS-mediated injurious effects (66, 29).

Conclusion

Taken together, this study projects the protection afforded by SEO against OS-related reprotoxicity induced by Cs probably on the basis of oxidant-antioxidant system management. Further investigations are needed to explore therapeutic efficacy of SEO in clinical trials.

Acknowledgements

This scientific work has been made possible by funding assistance of Urmia University and we would, therefore, like to express our gratitude to the University. The authors declare that there is no conflict of interest that would prejudice the impartiality of this study.

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Prenatal Caffeine Exposure Impairs Pregnancy in Rats

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Abstract

Background: In recent years, concerns have been raised about human reproductive disorders. Caffeine consumption is increasing by the world’s population and there is a relationship between caffeine intake and adverse reproductive outcomes. The aim of this study was to evaluate the effects of caffeine on implantation sites, number of live births, birth weight, crown-rump length (CRL) and abnormality in pregnant rats.

Materials and Methods: In this experimental study, 40 female albino rats (170-190 g) were randomly divided into two experimental and two control groups (n=10/each group). In both experimental groups, animals received caffeine intraperitoneally (IP: 150 mg/kg/day) on days 1-5 of pregnancy. In experimental group 1, treated animals were euthanized on day 7 of pregnancy and the number of implantation sites was counted. In experimental group 2, treated animals maintained pregnant and after delivery, the number of live births, birth weight, CRL and abnormality of neonates were investigated. In control group, animals received IP injections of distilled water. Data were analyzed by independent t test.

Results: Results showed that administration of caffeine significantly decreased the number of implantation sites, number of live births and CRL as compared with control group (P<0.05). There were no significant differences regarding birth weight and abnormality of neonate rats between experimental and control groups.

Conclusion: These results suggest that caffeine caused anti-fertility effect and significantly decreased CRL in neonate rats.

Keywords: Caffeine, Rat, Implantation


Introduction

Coffee, tea, chocolate, as well as certain medications are the major sources of caffeine (1-3). A variety of physiological adaptations are required to create an environment for the optimal fetal development during pregnancy. Pregnant women are concerned about what they consume during pregnancy. The widespread consumption of caffeine by pregnant women suggests that it is important to determine whether caffeine may influence maternal physiology and development of the fetus during pregnancy (1, 2).

A few of the known biological effects of caffeine are central nervous system stimulation, increased secretion of catecholamine, increased heart rate and relaxation of smooth muscle (4-6). Due to high lipid solubility and low molecular weight characteristics, caffeine crosses through the placenta easily (4). Fenster et al. (3) reported that after repeated consumption of caffeine in pregnant women, plasma caffeine levels and its biological effects are influenced by...
the metabolic characteristic of caffeine. An increase in half-life of caffeine during the last few weeks of pregnancy is caused by the elevated estrogen levels. Therefore, the caffeine blood levels in the mother and the fetus increase, but the fetus has no enzymes to metabolize it (2, 7). A number of studies have showed that caffeine may influence the reproductive outcome and fetal development (1-3).

Previous studies indicated that maternal caffeine consumption might increase the risk of an early spontaneous abortion (8, 9). Other studies showed that consumption of coffee during pregnancy might be associated with a shortened gestation and a lowered birth weight, but they did not find any relationship between risk of spontaneous abortion and caffeine metabolite (8-10).

Some investigators reported that consuming high amounts of caffeine during pregnancy may be harmful (2, 6). It is yet unknown whether consuming small amounts of caffeine during pregnancy affects fetal development. In mammals, caffeine has been shown to be teratogenic only at extremely high doses, or following a single large intraperitoneal (IP) injection. Reduced fetal body weight and delayed skeletal ossification have been observed at relatively high doses of caffeine (2, 4, 8).

Since caffeine-containing products are consumed in large quantities, its effect during gestation on the developing offspring is important (11). Although there are studies focusing on the effects of caffeine, the exact activity mechanisms of caffeine on pregnancy have been less characterized (2, 4, 8). In the present study, the effects of caffeine injection on the offspring of pregnant rats given a dose approximating to that of a high daily coffee consumption were evaluated. Furthermore we aimed to investigate the effects of caffeine on implantation sites, number of live births, birth weight, crown-rump length (CRL) and abnormality of neonate rats.

Materials and Methods

Acute toxic dose

Acute toxicity [lethal dose 50 (LD50)] of caffeine was evaluated through the IP injections into rats as described by Miller and Tainted (12). Briefly, the method involved the administration of 7 different concentrations of caffeine to 7 groups of rats (n=5/group) in pilot study. After 1 week, there were no deaths in animals receiving caffeine in concentrations of 1, 10, 50, 100, 150, 500 and 1000 mg/kg. The effects of 3 different concentrations of caffeine on fertility rate were tested. The 50 and 100 mg/kg/day showed no significant change in fertility rate; therefore, the dose was changed to 150 mg/kg/day.

Treatment

This experimental study was carried out between April to June 2012 at the Fertility and Infertility Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran. The Ethical Committee of Kermanshah University of Medical Sciences approved all procedures used in this study. In present study, 40 young female albino rats (170-190 g) were used. Prior to mating, the females were isolated for one month to rule out pre-existing pregnancy. Female rat were mated with males (3:1) in each cage, under controlled environmental conditions with a 12/12 hour light-dark cycle and free access to food. In the next morning, a positive sign of mating was confirmed by sperm-positive vaginal smears and the presence of copulatory plugs (13). The day on which a vaginal plug was found was designated as day 0 of gestation. Then, the positive vaginal smear rats were classified into two experimental (groups 1 and 2) and two control groups. Both experimental groups (n=10/each) received the IP injections of caffeine in concentration of 150 mg/kg/day on days 1-5 of pregnancy, and both control groups (n=10/each) received the IP injections of distilled water. In experimental group 1, animals were maintained on normal diet and on day 7 of pregnancy, they were euthanized. The number of implantation sites in each uterine horn was determined under a stereomicroscope (Leica, Germany) and compared with the control group. In experiment group 2, animals were maintained pregnant till delivery. After delivery, the number of live births, birth weight and CRL of neonates were investigated. Abnormality of neonates was investigated under a stereomicroscope and compared with the control group.

Statistical analysis

The data were analyzed by independent–t test using the SPSS (SPSS Inc., USA) version 15. A
value of \( P<0.05 \) was considered significant.

**Results**

The average number of the implantation sites between experimental group 1 and control group was statistically significant \( (P<0.001) \). The average number of live births between experimental group 2 and control group was statistically significant \( (P<0.001) \). There was no significant difference regarding the average birth weight between experimental group 2 and control group \( (P>0.05) \). There was no significant difference regarding abnormality between experimental group 2 and control group \( (P>0.05) \). There was significant difference regarding this parameter between experimental group 2 and control group \( [P<0.05, \text{(Figs.1-5, Table 1)}] \).

**Fig.1:** Comparison of the average number of implantation sites between experimental group 1 and control group. *\( P<0.001 \) as compared with control.

**Fig.2:** Comparison of average number of live births between experimental group 2 and control group. *\( P<0.001 \) as compared with control.

**Fig.3:** Comparison of the average birth weight between experimental group 2 and control group. There is no significant difference \( (P>0.05) \).

**Fig.4:** Comparison of the abnormality in neonates between experimental group 2 and control group. There is no significant difference \( (P>0.05) \).

**Fig.5:** Comparison of the average of CRL between experimental group 2 and control group. **\( P<0.05 \) as compared with control. CRL: Crown-rump length.
Caffeine Impairs Pregnancy in Rat

Table 1: Comparison of outcomes after administration of caffeine (150 mg/kg/day) between both experimental and control groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Number of implantation sites</th>
<th>Number of live births</th>
<th>Birth weight</th>
<th>Abnormality of neonates</th>
<th>CRL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental 1</td>
<td>10</td>
<td>2.402 ± 0.3131*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>6.9 ± 0.3027</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Experimental 2</td>
<td>10</td>
<td>-</td>
<td>2.2425 ± 0.1696*</td>
<td>4.0725 ± 0.076</td>
<td>4.32 ± 0.4509</td>
<td>9.41 ± 0.25**</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>-</td>
<td>6.11 ± 0.3756</td>
<td>4.6075 ± 0.2649</td>
<td>4.4 ± 0.2160</td>
<td>13.67 ± 1.2252</td>
</tr>
</tbody>
</table>

**P<0.05 and *P<0.001 when compared to control values. Data are presented as mean ± SD. CRL; Crown-rump length.

Discussion

In the present study, the administration of caffeine caused a significant decrease in implantation sites and number of live births. These results suggest that caffeine is likely to cause anti-fertility effect. The data also showed that caffeine consumption can decrease the birth weight of neonates that was not significant compared with the control group. Several fetuses were observed with a significant reduction in CRL.

Previous studies have shown that caffeine affects adversely human reproduction (14, 15). These effects could be very serious because most of the human populations of the world consumed caffeine-containing foods (15). The findings of caffeine effects on pregnancy outcomes and fetal development differ widely; therefore, in the current study, developmental and reproductive risks of caffeine on pregnant rats and their offspring were evaluated.

In a recent study, Dorostghoal et al. (16) showed a reduction in ovarian weight and primordial follicle population caused by consumption of high dose caffeine during pregnancy and lactation, leading to diminish fertility and reproductive ability in rat. They stated that these alterations in the ovary were associated with a significant growth retardation of the female offspring.

Savineau and Mironneau (17) showed that absence of a caffeine-sensitive calcium-release channel in the sarcoplasmic reticulum prevents spasm of rat myometrium during pregnancy. Albina et al. (2) showed that caffeine administration to pregnant mice on gestational days 0-18 at doses of 120 mg/kg/day has adverse maternal effects that are evidenced by a significant reduction in body weight gain and gravid uterine weight. In consistent with their results, in the present study, caffeine decreased the birth weight, but it was not significant as compared with control group.

The results of the current work were in agreement with the results of Gilbert and Pistey (18). They showed that repeated IP injections of caffeine (4 to 16 mg/day) to pregnant rats resulted in significant resorptions and a decrease in the birth weight of neonates, but developmental malformations were not observed in neonates. In the current study, there was no significant difference regarding abnormality between experimental group 2 and control group, confirming previous investigations.

In the study by Gilbert and Rice (1), showed that the administration of different concentration of caffeine to monkeys before, during, and after pregnancy caused a dose-related increase in stillbirths and miscarriages as well as a decrease in maternal weight gain, suggesting that increased serum caffeine levels, particularly theophylline, may affect maternal physiology in pregnant monkey.

Vik et al. (19) found that a high caffeine intake in the third trimester of pregnancy was associated with an increased risk of small for gestational age (SGA) birth among male fetuses, but not in female fetuses. In current study, several fetuses were observed with small CRL and low birth weight that can be mediated by accumulation of caffeine in fetal tissues that could influence fetal development.
Conclusion
IP injections of caffeine to pregnant rats resulted in a significant decrease in the numbers of implantation sites and live births. The findings showed that administration of caffeine induced anti-implantation activity, but the changes in abnormality and birth weight of the offspring were not significant.

Acknowledgements
This study was approved and financially supported by the Fertility and Infertility Research Center, Kermanshah University of Medical Sciences. There is no conflict of interest in this study.

References
The Effect of Prolonged Culture of Chromosomally Abnormal Human Embryos on The Rate of Diploid Cells


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Abstract

Background: A decrease in aneuploidy rate following a prolonged co-culture of human blastocysts has been reported. As co-culture is not routinely used in assisted reproductive technology, the present study aimed to evaluate the effect of the prolonged single culture on the rate of diploid cells in human embryos with aneuploidies.

Materials and Methods: In this cohort study, we used fluorescence in situ hybridization (FISH) to reanalyze surplus blastocysts undergoing preimplantation genetic diagnosis (PGD) on day 3 postfertilization. They were randomly studied on days 6 or 7 following fertilization.

Results: Of the 30 analyzed blastocysts, mosaicism was observed in 26(86.6%), while 2(6.7%) were diploid, and 2(6.7%) were triploid. Of those with mosaicism, 23(88.5%) were determined to be diploid-aneuploid and 3(11.5%) were aneuploid mosaic. The total frequency of embryos with more than 50% diploid cells was 33.3% that was lower on day 7 in comparison with the related value on day 6 (P<0.05); however, there were no differences when the embryos were classified according to maternal age, blastocyst developmental stage, total cell number on day 3, and embryo quality.

Conclusion: Although mosaicism is frequently observed in blastocysts, the prolonged single culture of blastocysts does not seem to increase the rate of normal cells.

Keywords: Aneuploidy, Blastocyst, Self-Correction, Mosaicism

on the third day post-fertilization; however, sometimes it is necessary to biopsy two blastomeres. In such cases, disagreement between the results of the two analyzed blastomeres is considered as a case of mosaicism which is identified as the presence of two or more genetically different cell lines in an embryo. Mosaicism is a highly frequent phenomenon during the cleavage stage because the majority of cell division errors in early embryos occur during this stage. Due to inactivation of the genome during human embryo fertilization, genome stability until the third cell division is mainly controlled by cytoplasmic transcriptomes of the oocyte. Degradation of mRNA in oocyte decreases fidelity of the cell division because genome activation in the human embryo mainly occurs after the third cell division (6). Therefore, preimplantation abnormalities are mainly post zygotic events that arise by error-prone cell division during inactive status of embryonic genome (7).

While clinical studies of blastocyst aneuploidy are limited, these reports have shown approximately 60% aneuploidy in blastocyst stage, of which 33% are mosaic. Of these, some are diploid-aneuploid and some aneuploid mosaic (8). Numerous reports have shown that reanalyzed embryos at the blastocyst stage, with aneuploidy on the third day of development, mostly achieve full diploidy by less than 18% (2, 3, 9-12). However, these reports have predominantly focused on day 5 blastocysts. A comparison of the aneuploidy rate in days 4, 5 and 8 of embryos co-cultured with an endometrial layer has shown an increased rate of normal cells in the analyzed embryos after increasing culture time (13). Munne et al. (14) have co-cultured aneuploid embryos with fibroblasts and analyzed these embryos on days 6 and 12. According to their results, there was an increase in the rate of normal cells to 48% by day 12. Numerous studies report derivation of normal human embryonic stem cells (hESCs) from embryos detected as aneuploid in the cleavage stage (15-18) and blastocyst stage (19). The establishment of hESC lines is routinely performed by being co-cultured with a feeder layer. While mosaicism is highly frequent in early embryos, an increase in the rate of normal cells seems to be a progressive phenomenon by additional embryonic development because of their growth advantage rather than aneuploid cells (20).

This preliminary study aimed to evaluate the effect of prolonged culture on diploidy rate. We increased the culture time to days 6 and 7 as the last days before closing of the implantation window. Numerous reports from day 5 of development in spare embryos have shown low percentage of full diploidy in analyzed blastocysts (2, 3, 9-12), while after being co-cultured for 8-12 days, there is an increase in percentage of normal cells (13, 14). As single culture is more routinely used than co-culture in assisted reproductive technology (ART), we preferred to use single culture for the embryos in order to evaluate diploidy rate.

Materials and Methods
This cohort study was approved by the Ethics Committee of Royan Institute, Tehran, Iran, and performed on spare embryos from preimplantation genetic diagnosis (PGD) candidates, who signed an informed consent. We used simple random sampling method to include the study group.

Sample preparation
In this study, inclusion criteria were as follows: stimulation by the long protocol described previously (21) and fertilization by intra-cytoplasmic sperm injection (ICSI). The embryos were cultured in sequential media (Vitrolife, Sweden) under mineral oil (Origio, Denmark). Following routine ART treatments, two pronucleate (2PN) zygotes were transferred to fresh microdrops of G-1TM V5 medium (Vitrolife, Sweden) supplemented with 10% human serum albumin (Vitrolife, Sweden). The embryo biopsy for PGD was performed 72 hours after fertilization. In order to perform an embryo biopsy on day 3, we incubated the embryos for 1-2 minutes in Ca\(^{2+}\)/Mg\(^{2+}\)-free G-PGD\(^{TM}\) biopsy medium (Vitrolife, Sweden). After the biopsy of one blastomere for PGD, the embryos were transferred to G-2\(^{TM}\) V5 medium (Vitrolife, Sweden), while those selected for freezing, either aneuploid or unsuitable, underwent a prolonged culture. We cultured 100 spare embryos of PGD candidates for 6-7 days postfertilization.

Spreading of the blastocysts
Each embryo reaching the blastocyst stage was randomly spread on day 6 or 7 of development. We performed the spreading process according to previously described procedure (22) with some modifications. Embryos were briefly washed in
Blastocysts Aneuploidy Rate in Prolonged Culture

two drops of phosphate buffer saline (PBS, Gibco, USA), then transferred to 1 mM/L HCl (Merck, USA)-1% Tween 20 (Sigma-Aldrich, USA). After 2-4 minutes, the embryos were transferred to a glass slide with less than 1 µl HCl-Tween 20. When necessary, we added additional HCl-Tween 20 to complete spreading. The slides were allowed to air dry for 45 minutes, after which they were washed in PBS for 5 minutes and dehydrated in a graded ethanol series of 70, 85 and 100%.

Fluorescence in situ hybridization

The slides were pre-treated with pepsin (Sigma-Aldrich, USA, 400 µg/ml) in 0.1N HCl at 37°C, then fixed in 10% formalin (Merck, USA) at 4°C and washed in PBS at room temperature (each step for 5 minutes), after which slides were treated by 2X standard saline citrate (SSC) for 10 minutes at 37°C. Slides were again fixed in formalin and re-washed in PBS, dehydrated in another graded series of ethanol (70, 85 and 100%), and allowed to air dry. Chromosome aneuploidies were studied in two rounds by FISH using the locus-specific identifier (LSI) 13, chromosome enumeration probe (CEP) 18, LSI 21, LSI 22, CEP 15, CEP X and CEP Y probes (Vysis, USA). Following heat denaturation of the nuclear and probes’ DNAs at 75°C for 5 minutes, the hybridization step was performed by incubation of the slides at 37°C, overnight. The next day, slides were washed in 0.4X SSC/0.3% NP-40 (Vysis, USA) at 72°C for 2 minutes that was followed by immediate washing in 2X SSC/0.1% NP-40 for 5 minutes at room temperature. After the nuclei were stained with 4’, 6-diamidino-2-phenylindole (DAPI), we analyzed only cells with interpretable signals from each blastocyst. By reanalysis of these 30 embryos, 1(4.7%) with triploidy on day 3 showed triploidy again, whereas 1(4.7%), 3(14.3%) and 16(76.2%) were diploid, aneuploid mosaic and diploid-aneuploid mosaic, respectively. Of 7 diploid embryos on day 3, only 1(14.3%) showed diploidy upon reanalysis of the blastocyst stage, while 1(14.3%) and 5(71.4%) were triploid and diploid-aneuploid mosaic, respectively.

Embryo classification

Embryos were classified according to the following characteristics: day of reanalysis (days 6 or 7 post-fertilization); stage of blastocyst reanalysis (hatched or earlier stages of the blastocyst development); numbers of total cells on day 3; maternal age (<37 or ≥37 years); indications for PGD; and quality of embryos on day 3 according to their fragmentation pattern and morphological characteristics, including blastomeres compaction, equal size, absence of vacuoles, presence of multinuclei and granularity of cytoplasm as previously described (24). Regarding very low incidence of fully diploid blastocysts, comparisons was performed between categories of more and less than 50% normal cells.

Statistical analysis

Data analysis was performed using the SPSS (version 16.0, SPSS Inc., USA) statistical software. The logistic regression models with sequential and variable selection were constructed using Hosmer–Lemeshow test (25). P<0.05 was considered significant.

Results

In this study, among 100 embryos from 19 patients, 30 reached the blastocyst stage. Table 1 presents some embryological data of these patients. The fertility rate was 70.4% and the overall maternal age was 33.9 years (range 25-40 years).

Totally, 293 nuclei from 30 blastocysts were included in data analysis; the mean number of nuclei per embryo was approximately 10 (range 3-17). It is noted that we included data regarding the cells with interpretable signals in both FISH rounds.

In primary analysis of these 30 embryos on day 3, frequencies of aneuploid and diploid embryos were 21(70%) and 7(23.3%), respectively. Two (6.7%) out of 30 embryos had no results on day 3, while they were diploid-aneuploid mosaic regarding blastocyst analysis. Of these, one was mosaic diploid-tetraploid (Fig.1A) that tetraploidy was observed in 5 out of 17 analyzed cells (29.4%, Table 2). By reanalysis of 21 aneuploid embryos, 1(4.7%) with triploidy on day 3 showed triploidy again, whereas 1(4.7%), 3(14.3%) and 16(76.2%) were diploid, aneuploid mosaic and diploid-aneuploid mosaic, respectively. Of 7 diploid embryos on day 3, only 1(14.3%) showed diploidy upon reanalysis of the blastocyst stage, while 1(14.3%) and 5(71.4%) were triploid and diploid-aneuploid mosaic, respectively.

The most frequent abnormality in the analyzed
blastocysts was mosaicism observed in 26(86.6%) embryos, of which 23(88.5%) were diploid-aneuploid mosaic. The total frequency of diploid-aneuploid mosaicism among the analyzed embryos was 76.6%. Mosaic aneuploidy was observed at a frequency of 10%, there is no diploid cell in the embryos with mosaic aneuploidy. Concordance of FISH results of all analyzed cells from each blastocyst with primary analysis on day 3 were remarkable for 4(13.3%) embryos, where 2(6.7%) were diploid and 2(6.7%) were triploid.

The total frequency of blastocysts with more than 50% diploid cells was 33.3%, 10 embryos. The distribution of embryos into categories of more and less than 50% normal cells did not show significant difference when they were classified according to total cell number on day 3, maternal age, developmental stage of the blastocyst, indications for PGD and embryo quality on day 3. The frequency of blastocysts with over 50% normal cells on day 6 was significantly more than those analyzed on day 7, 7 out of 13(53.8%) versus 3 out of 17(17.6%) (Table 3).

Although we did not find a significant difference in distribution of relatively normal embryos according to their total cell numbers on day 3, embryos lagging behind in cell divisions showed higher normalization. The frequencies of embryos with more than 50% normal cells were 62.5% (5 out of 8) versus 23.8% (5 out of 21) for embryos with 5-6 and 7-8 cells on day 3, respectively (P=0.08).

The rate of normal cells in the studied blastocysts was not different between infertile and presumed fertile patients concerning indications for PGD (Table 3).

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Oocytes</th>
<th>MII oocytes</th>
<th>2PN embryos</th>
<th>Biopsied embryos</th>
<th>Transferred embryos</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>10</td>
<td>5</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>12</td>
<td>9</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>11</td>
<td>5</td>
<td>4</td>
<td>1</td>
</tr>
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<td>*</td>
<td>*</td>
<td>8</td>
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<td>10</td>
<td>10</td>
<td>3</td>
<td>4</td>
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<td>Mean</td>
<td>10.7</td>
<td>9.8</td>
<td>6.9</td>
<td>6.1</td>
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</tr>
</tbody>
</table>

*; Missed due to using thawed embryos, MII; Metaphase II and 2PN; Two pronucleate.
Table 2: Fluorescence in situ hybridization (FISH) results of embryos in the cleavage and blastocyst stages

<table>
<thead>
<tr>
<th>Embro number</th>
<th>Patient number</th>
<th>Day 3 results of single blastomere analysis</th>
<th>Analyzed cells in blastocyst (n)</th>
<th>Aneuploidies in blastocyst cells</th>
<th>Diploid cells in blastocysts (%)</th>
<th>Blastocyst classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>Triploid</td>
<td>3</td>
<td>3N[3]</td>
<td>0</td>
<td>Triploid</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>-22 --18</td>
<td>9</td>
<td>Diploid</td>
<td>100</td>
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### Table 2: Continued

<table>
<thead>
<tr>
<th>Embryo number</th>
<th>Patient number</th>
<th>Day 3 results of single blastomere analysis</th>
<th>Analyzed cells in blastocyst (n)</th>
<th>Aneuploidies in blastocyst cells</th>
<th>Diploid cells in blastocysts (%)</th>
<th>Blastocyst classification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>++21 XO</td>
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<tr>
<td>24</td>
<td>13</td>
<td>Diploid</td>
<td>4</td>
<td>Diploid</td>
<td>100</td>
<td>Diploid</td>
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</tbody>
</table>

Digits in brackets indicate the numbers of cells that had aneuploidy mentioned before the bracket. -, +; Decrease or increase in number of chromosomes.
**Table 3:** Results of multivariable logistic regression model of relationship between 6 explanatory variables and the relatively normalization (more than 50% normal cells) in blastocysts

<table>
<thead>
<tr>
<th>Classification criteria</th>
<th>Relatively normal blastocysts</th>
<th>Total number of blastocysts</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>P value</th>
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<td></td>
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<tr>
<td>7</td>
<td>3</td>
<td>17</td>
<td>0.15</td>
<td>0.02, 0.93</td>
<td>0.04</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>13</td>
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<tr>
<td>Day 3 total cell number</td>
<td></td>
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<tr>
<td>7-8</td>
<td>5</td>
<td>21</td>
<td>0.19</td>
<td>0.03, 1.28</td>
<td>0.08</td>
</tr>
<tr>
<td>Other</td>
<td>5</td>
<td>9</td>
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<td></td>
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<tr>
<td>Stage of reanalysis</td>
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<tr>
<td>Hatched</td>
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<td>1.66</td>
<td>0.15, 17.76</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Excellent to good</td>
<td>8</td>
<td>21</td>
<td>7.60</td>
<td>0.42, 137.69</td>
<td>0.17</td>
</tr>
<tr>
<td>Fair to poor</td>
<td>2</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Indication for PGD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recurrent miscarriage</td>
<td>2</td>
<td>8</td>
<td>0.40</td>
<td>0.01, 10.07</td>
<td>0.58</td>
</tr>
<tr>
<td>Recurrent implantation failure</td>
<td>3</td>
<td>9</td>
<td>0.89</td>
<td>0.08, 9.18</td>
<td>0.92</td>
</tr>
<tr>
<td>Family Balancing</td>
<td>5</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PGD; Preimplantation genetic diagnosis.
Discussion

In the current study by reanalysis of spare embryos from PGD candidates, we found a variety of abnormalities in blastocysts that could not be diagnosed on day 3 of development analysis of single blastomeres from the same embryo. Regarding high frequency of mosaicism in reanalyzed blastocysts, it seems that the majority of them have been mosaic in cleavage stage, while they could not be diagnosed due to limitation in number of available cell for biopsy at the cleavage stage.

We used FISH as a widely applied technique, like similar research studies (2, 3, 9-12), and pre-implantation genetic screening (PGS); however, the results were in agreement with array-based studies and both approaches indicated highly frequency of mosaicism in early embryos. Of note, the majority of blastocysts in the current study were spare aneuploid embryos according to PGS-defined single blastomere from third day, while above-mentioned array-based studies (1, 5) found extensive mosaicism in good quality embryos. It has indicated that mosaicism is common in early embryos even those with good quality; however, blastocysts with aneuploidies on day 3 might contain more abnormal cells and/or more variation of abnormalities among cells. Array-based analysis has the power for analysis of all chromosomes. It is clear that analysis of all chromosomes could result in finding more abnormalities than studying of some chromosomes by FISH. However, in the current study, analysis of 7 chromosomes by FISH showed high frequency of mosaicism and no advantage for prolonged culture of the blastocysts.

We found a higher normal cell rate in embryos analyzed on day 6 compared to the related value on day 7. An increase in the rate of aneuploid cells on day 7 compared to the related value on day 6 in single culture may be caused by longer time exposure to in vitro conditions. Our first assumption for this study was to see more normalization during prolonged single culture, while by preliminary analysis of 30 blastocysts, we found a high rate of abnormalities. There are few reports about culture of embryos longer than 6 days that co-culture have been used until day 13 post-fertilization, whereas we used single culture and limited the culture prolongation to day 7 as the last day for embryo transfer before closing the implantation window (26). A recent study has searched the normal growth rate of human embryos between days 3 and 13 in either continues culture or co-culture with mouse embryonic fibroblasts. Their results have showed a higher rate of normalization in day 7 aneuploid embryos as compared with related values of days 5-6 and later up to 13. This study has concluded that normalization occurs mainly until days 7 and 8, whereas longer cultures might lead to a decrease in normalization rate (12), which is in agreement with our findings. However, we could not exactly compare our results with that study because their results are a combination of single cultured and co-cultured as well as arrested embryos.

In a similar study by Santos et al. (13) who compared days 4, 5 and 8 embryos, there was an increase in the rate of normal cells by prolongation (6% on day 4, 37% on day 5 and 58% on day 8). They studied embryos co-cultured on endometrial stromal monolayer cells. A recent study on all blastomeres of 13 good quality embryos on day 4 using array-comparative genomic hybridization showed 16-100% abnormal blastomeres in studied embryos. The authors have supposed that fully normalization might occur in later stages of development (5). This phenomenon could happen through several mechanisms for overcoming on aneuploidies, leading to an increase in the rate of diploid cells in mosaic embryos (6).

Munne et al. (14) have reported an increased rate of normal cells in embryos cultured with a fibroblast feeder layer in order to establish hESC lines from aneuploid embryos. A hypothesized reasons for derivation of normal cell lines from aneuploid embryos are the misdiagnoses by the FISH technique due to its limitation and the diagnosis of aneuploidy based on only single blastomere analysis (15). If all normal hESC lines established from day 3 aneuploid embryos have been misdiagnosed, this hypothesis could not answer the establishment of hESC lines from aneuploid blastocysts (19). Furthermore, diagnosis of aneuploidy in blastocyst stage is based on analysis of several cells. A decrease in the rate of abnormal cells might be related to the effects of co-culture of embryos with differentiated cells due to a mimic of implantation. Differentiation is known to be a barrier for the division of aneuploid cells (27). Communications between differentiated cells that have been used for co-culture and embryonic cells might induce some cellular and molecular mechanisms, leading to de-
crease in the rate of aneuploidies in the embryo. While aneuploidies are considered as an incident in early embryonic development, some aneuploid embryos would be arrested in their development to the later stages. Although aneuploidies incidence would be decreased by reaching to blastocyst stage, mosaic embryos mostly reach to blastocyst stage. Implantation is a critical stage that blastocysts should pass it after hatching. There is not any direct evidence on the effect of aneuploidies on implantation potential, but one of the main reasons for including into PGS is recurrent implantation failure. As the current study was designed for clinical benefits, we studied the embryos without being co-cultured.

The relationship between abnormal morphology on the third day of embryo development and chromosomal abnormalities has been well documented. The abnormal rate of development also correlates with aneuploidies (28). However, we found no significant association between the rate of aneuploid cells in blastocysts to their quality and total cell number on day. Maternal age as another factor for aneuploidy in the cleavage stage (8) showed no correlation with the rate of aneuploid cells in blastocysts.

Although chromosomal abnormalities are known as a cause of infertility, in our study, blastocysts from candidates for family balancing did not show higher rates of normal cells in comparison with blastocysts of infertile patients. This finding is in agreement with a recent study in presumed fertile and infertile patients (29).

A limitation for day 3 PGD is the "no result" cases, meaning that in this study, 6.7% of analyzed embryos were unable to be diagnosed on day 3 PGD, while by availability of a number of cells at the blastocyst stage, we observed decreasing the "no result" rate. Recently, array-based PGD has been more considered due to their ability to screen abnormalities in all chromosomes (5, 30, 31).

Mosaicism, in particular diploid-aneuploid, is a common phenomenon in the blastocyst stage (32). We observed a high frequency of diploid-aneuploid mosaicism in the current study. Growth advantage of diploid cells in mosaic diploid-aneuploid embryos have been speculated as one reason for overcoming on aneuploidy, because of increased death and decreased division rate in the aneuploid cells (13, 20).

There are three destinations for mosaic embryos following differentiation: abortion, birth defects or healthy newborn. We recently showed that the dominant response to DNA damage in poor-quality pre-implantation human embryos with complex aneuploidy is DNA repair rather than cell division or apoptosis (33). Self-correction could rarely occur in mosaic diploid-aneuploid embryos by advantage of diploid cells for survival and division (5).

A disadvantage for current array-based PGD in the blastocyst stage is the increased time needed to conduct an analysis using array technologies compared with FISH. With regards to the limited time for embryo transfer before closing the implantation window (26), an approach could be embryo vitrification and their transfer in the subsequent menstrual cycles. It should be mentioned that IVF outcomes may be improved by transferring frozen embryos compared with fresh embryos (34). Another concern could be survival of biopsied blastocysts after vitrification; the results of this approach indicated that the implantation rate is comparable with thawed blastocysts, without biopsy (35). Another plan would be performing a biopsy in frozen-thawed embryos prior to embryo transfer (36).

We have observed tetraploid-diploid mosaicism in 1(3.3%) embryo (embryo no.19). This event has also been reported during the blastocyst stage, as a result of synchronization of the cell divisions during this stage. This could be considered as a normal status for an embryo. Transfer of an embryo with a tetraploid karyotype on trophectoderm biopsy has resulted in a normal pregnancy (37).

Conclusion

Mosaicism is frequent in human blastocysts. Cleavage stage PGS does not show extensive aneuploidies in the embryo because of the limited number of biopsied cells. The blastocyst stage could be a good stage for aneuploidy screening by performing an analysis of several cells. Although the longer time co-culture of human embryos has been reported to decrease aneuploidy rate, we did not find any advantage in single culture of blastocysts until day 7. Of note, omission of a co-culture in the current study was to evaluate the clinical benefits of prolonged single culture. It seems for PGS, biopsy
of the embryos upon reaching to blastocyst stage and their analysis for selection of normal embryos is better than later biopsies.

**Acknowledgements**

First of all we thank the patients for donation the spare embryos. We express our appreciation to the staff of the Reproductive Genetics and Embryology Labs of Royan Institute, with particular appreciation to Najme Sadat Masoudi and Leila Karimian. This study was financially supported by Royan Institute for Reproductive Biomedicine. The authors declare no conflict of interest.

**References**

Blastocysts Aneuploidy Rate in Prolonged Culture


A Case of Bilateral Testicular Tumors Subsequently Diagnosed as Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency

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Abstract

21-hydroxylase deficiency (21-OHD) caused congenital adrenal hyperplasia (CAH) is a group of autosomal recessive genetic disorders resulting from mutations in genes involved with cortisol (CO) synthesis in the adrenal glands. Testicular adrenal rest tumors (TARTs) are rarely the presenting symptoms of CAH. Here, we describe a case of simple virilizing CAH with TARTs, in a 15-year-old boy. The patient showed physical signs of precocious puberty. The levels of blood adrenocorticotropic hormone (ACTH), urinary 17-ketone steroids (17-KS), dehydroepiandrosterone sulfate (DHEA-S), and serum progesterone (PRGE) were elevated, whereas those of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and CO were reduced. Computed tomography (CT) of the adrenal glands and magnetic resonance imaging (MRI) of the testes showed a soft tissue density (more pronounced on the right side) and an irregularly swollen mass (more pronounced on the left side), respectively. Pathological examination of a specimen of the mass indicated polygonal/circular eosinophilic cytoplasm, cord-like arrangement of interstitial cells, and lipid pigment in the cytoplasm. Immunohistochemistry results precluded a diagnosis of Leydig cell tumors. DNA sequencing revealed a hackneyed homozygous mutation, I2g, on intron 2 of the CYP21A2 gene. The patient’s symptoms improved after a three-month of dexamethasone therapy. Recent radiographic data showed reduced hyperplastic adrenal nodules and testicular tumors. A diagnosis of TART should be considered and prioritized in CAH patients with testicular tumors. Replacement therapy using a sufficient amount of dexamethasone in this case helps combat TART.

Keywords: 21-hydroxylase Deficiency, Congenital Adrenal Hyperplasia, Precocious Puberty


Introduction

Congenital adrenal hyperplasia (CAH) comprises a group of autosomal recessive genetic disorders affecting cortisol (CO) synthesis in the adrenal glands (1). As a result, there is a compensatory increase in the secretion of adrenocorticotropic hormone (ACTH), resulting in adrenal hyperplasia. Worldwide, the incidence of CAH in newborns is approximately 1/16,000–1/20,000, and approximately 1/15,000–1/16,000 in Europe and the USA. In China, the incidence of CAH is unknown due to the absence of a national screening program (2).

Deficiencies in 21-hydroxylase (21-OH), 11β2 hydroxylase, 3β2 steroid dehydrogenase, and 17α2 hydroxylase lead to CAH. Of these, 21-OH deficiency (21-OHD) is the most common, accounting for 90-95% of pediatric CAH cases. There are classic
and non-classic CAH presentations, reflecting the extent of 21-OHD (3). Classic CAH includes the simple virilizing and salt-wasting types. Among these, simple virilizing CAH is caused by partial 21-OHD, resulting in increased androgen levels, without salt wasting. Clinically, boys with this type of CAH may show pseudo-precocious puberty. Excessive androgen secretion may also inhibit the release of pituitary gonadotropin, leading to spermatogenic disorders and fertility deficits.

The formation of adrenal glands and gonads is initiated via a common primordium, which develops into different tissues during embryogenesis, with adrenal cells possibly being transferred into the XY gonad during differentiation (4). Residual adrenal cells are reported to exist in the testes of at least 15% of healthy, newborn babies (5). The presence of abnormal, residual adrenal cells leads to the development of testicular adrenal rest tumors (TARTs). Due to insufficient endocrine regulation, the compensatory secretion of ACTH from the pituitary may reach a level high enough to cause hypertrophy and hyperplasia of the testicular adrenal-derived cells. Subsequently, TARTs may worsen regulation disorders of the hypothalamic-pituitary-adrenal axis (6). In fact, TARTs are not rare in CAH cases, especially in classic CAH, and may also be found in individuals with non-classic CAH (7). However, TARTs, accompanying untreated CAH, may be somewhat difficult to diagnose and treat. Some doctors might misdiagnose them as malignancies and recommend surgical resection, potentially causing irreparable damage to the patient. Therefore, to help raise understanding of this condition and improve its management, we describe our experience with a case of bilateral TARTs, subsequently diagnosed as 21-OHD-related, simple virilizing CAH in a 15-year-old boy.

Case report

The research and report are approved by both the patient and the Ethical Committee of our hospital.

A 15-year-old boy, one of our outpatients, was diagnosed with bilateral testicular tumors. Upon presentation, his blood pressure was 117/81 mmHg, and he was 142-cm tall. His skin was deeply pigmented, and the oral mucosa, areolas, and genitalia were also pigmented; he also demonstrated convex laryngeal tuberculosis and whiskers around his lips. Physical examination revealed the length of his penis to be approximately 9.5 cm, and he had type IV pubic hair distribution, according to the Tanner grading system. A palpable lump, 2.0×1.6×1.2 cm in size, was found on his right testis (testicular volume, 12 mL) and another, 2.5×2.0×1.8 cm in size, on his left testis (testicular volume, 20 mL). Both lumps were indurated, with irregular surfaces and had normal mobility. The patient’s prostate appeared normal.

A thorough medical history revealed that our patient was born full term (birth weight, 3.40 kg), but had experienced birth trauma. The patient’s physique had always been unsatisfactory, with some secondary sexual characteristics such as a lower voice tone, larger penis/scrotum, and pubic hair developing around three years of age. Three to four years later, his physical stature had been considerably larger than that of his peers, and his secondary sexual characteristics were more pronounced. His condition was diagnosed as sexual precocity at another hospital, but it was never treated.

In order to confirm the etiology and origin of the disease, we conducted the recommended tests for diagnosing CAH. An ACS 180 SE chemiluminescence analyzer (Bayer Diagnostics, USA) was used to determine his blood levels of ACTH, CO (1), aldosterone (ALD), and a series of tumor markers [alpha-fetoprotein, carbohydrate antigen (CA), prostate antigen, and carcinoembryonic antigen (CEA)]. After the detection of ACTH and CO levels, we administered low-dose dexamethasone (0.75 mg, orally, every 6 hours, for 3 days) and re-determined his levels of ACTH and CO (Table 1).

Another chemiluminescence analyzer (Access, Beckman Coulter, USA) was used to check the patient’s levels of sex hormones, including follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), progesterone (PRGE), estradiol (E2), testosterone (T), human chorionic gonadotropin (hCG), and dehydroepiandrosterone sulfate (DHEA-S). After administering low-dose dexamethasone (0.75 mg, orally, every 6 hours, for 3 days) and re-determined his levels of ACTH and CO (Table 1).

A CX4 automatic biochemical apparatus (Beckman Coulter) was used to detect urinary levels of 17-ketone steroids (17-KS), 17-hydroxyl steroids (17-OH), and vanillalmond acid (VMA). To test liver/kidney function, the Bayer-500 (Bayer Diagnostics) urine analyzer was used to analyze the patient’s urine (Table 3).
### Table 1: Levels of adrenocorticotropic hormone (ACTH) and cortisol (CO)

<table>
<thead>
<tr>
<th>Time</th>
<th>First detection</th>
<th>Second detection (after dexamethasone suppression)</th>
<th>Reference range</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>ACTH</td>
<td>CO</td>
<td>ACTH</td>
</tr>
<tr>
<td>8:00</td>
<td>1250 pg/mL</td>
<td>79.53 nmol/L</td>
<td>0–46 pg/mL</td>
</tr>
<tr>
<td></td>
<td>CO</td>
<td>736 pg/mL</td>
<td>CO: 0–46 pg/mL</td>
</tr>
<tr>
<td></td>
<td>24.6 nmol/L</td>
<td></td>
<td>CO: 118.6–618 nmol/L</td>
</tr>
<tr>
<td>16:00</td>
<td>971 pg/mL</td>
<td>66.39 nmol/L</td>
<td>ACTH</td>
</tr>
<tr>
<td></td>
<td>CO</td>
<td>110.00 pg/mL</td>
<td>0–46 pg/mL</td>
</tr>
<tr>
<td></td>
<td>93.77 nmol/L</td>
<td></td>
<td>CO: 85.3–459.6 nmol/L</td>
</tr>
<tr>
<td>0:00 (the following day)</td>
<td>ACTH</td>
<td>423.00 pg/mL</td>
<td>ACTH</td>
</tr>
<tr>
<td></td>
<td>CO</td>
<td>39.13 nmol/L</td>
<td>0–46 pg/mL</td>
</tr>
<tr>
<td></td>
<td>51.4 pg/mL</td>
<td></td>
<td>CO: 118.6–618 nmol/L</td>
</tr>
<tr>
<td></td>
<td>2.39 nmol/L</td>
<td></td>
<td>CO: 93.77 nmol/L</td>
</tr>
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### Table 2: Levels of sex hormones

<table>
<thead>
<tr>
<th>Time</th>
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<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH</td>
<td>0.56 mIU/mL</td>
<td>1.4–18.1 mIU/mL</td>
</tr>
<tr>
<td>LH</td>
<td>0.01 mIU/mL</td>
<td>1.5–9.3 mIU/mL</td>
</tr>
<tr>
<td>PRL</td>
<td>10 ng/mL</td>
<td>2.1–17.7 ng/mL</td>
</tr>
<tr>
<td>PRGE</td>
<td>27.27 pg/mL</td>
<td>0.28–1.22 pg/mL</td>
</tr>
<tr>
<td>E2</td>
<td>9.93 pg/mL</td>
<td>0–52 pg/mL</td>
</tr>
<tr>
<td>T</td>
<td>755.84 ng/dL</td>
<td>241–827 ng/mL</td>
</tr>
<tr>
<td>hCG</td>
<td>0.0 mIU/mL</td>
<td>0–10 mIU/mL</td>
</tr>
<tr>
<td>DHEA-S</td>
<td>950.80 μg/dL</td>
<td>24–537 μg/dL</td>
</tr>
<tr>
<td>DHEA-S after dexamethasone suppression</td>
<td>221.20 μg/dL</td>
<td>24–537 μg/dL</td>
</tr>
</tbody>
</table>

FSH; Follicle-stimulating hormone, LH; Luteinizing hormone, PRL; Prolactin; PRGE; Progesterone, E2; Estradiol, T; Testosterone, hCG; Human chorionic gonadotropin and DHEA-S; Dehydroepiandrosterone sulfate.

### Table 3: Levels of other adrenal secretions

<table>
<thead>
<tr>
<th>Time</th>
<th>Level</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALD</td>
<td>103.70 ng/dL</td>
<td>3.81–31.33 ng/dL</td>
</tr>
<tr>
<td>17-KS</td>
<td>46.4 mg/24 hours</td>
<td>10–25 mg/24 hours</td>
</tr>
<tr>
<td>17-OH</td>
<td>25.2 mg/24 hours</td>
<td>6–22 mg/24 hours</td>
</tr>
<tr>
<td>VMA</td>
<td>9.6 mg/24 hours</td>
<td>1.4–8 mg/24 hours</td>
</tr>
</tbody>
</table>

ALD; Aldosterone; 17-KS; 17-ketone steroids; 17-OH; 17-hydroxyl steroids and VMA; Vanilla almond acid.
We performed a genetic analysis of the patient, as well. DNA sequencing was performed according to the method of Zhang et al. (8).

Computed tomography (CT) and magnetic resonance imaging (MRI) were performed using an Aquilion16-slice spiral CT (Toshiba, Japan) and an ultra-high field 3.0T scans (Siemens, Germany), respectively, to examine the patient’s adrenal glands and testicles. Hematoxylin-eosin (H&E) staining and immunohistochemistry were performed to study the pathology of the TARTs.

The urine test results showed a decreased specific gravity (1.003), but liver and kidney function, routine blood test results, and the levels of tumor marker proteins were normal. DNA sequencing revealed a homozygous mutation (I2 g) on intron 2 of the patient’s CYP21A2 gene. Multislice CT (non-contrast-enhanced and contrast-enhanced) revealed a mass with a soft tissue density on the right side of both adrenal glands; its maximum size was 4.5×3.7 cm. The mass was irregularly shaped with a heterogeneous density, and exhibited a "fast-in and fast-out" pattern. MRI (non-contrast-enhanced and contrast-enhanced) showed that both testes were irregularly enlarged, especially the left gonad. The mass within each testis had heterogeneous signal intensity and was surrounded by liquid signals. Several partitions were seen within the lesions. The testes themselves showed obvious and heterogeneous enhancement. Pathologically, the testes showed a polygonal or circular eosinophilic cytoplasm within the testicular tissue. Interstitial cells were in a cord-like arrangement, and lipid pigment was observed within the cytoplasm without Reinke crystals. Pathological investigation of the testicular tissue indicated that spermatogenic cells in the seminiferous tubules were considerably diminished or even absent. Sertoli cell hyperplasia was observed, but not typical spermatogenesis. Together, these imaging and clinical results suggested an initial diagnosis of adrenogenital syndrome, but the possibility of Leydig cell tumors was excluded (Fig.1).

On the basis of all the aforementioned results, the patient was diagnosed to have bilateral TARTs associated with CAH. Consequently, hydrocortisone was first administered for three months (20 mg, orally, twice daily) for treatment of the disease. However, his clinical symptoms did not improve. We then started the patient on dexamethasone replacement therapy (daily, oral dose of 0.75 mg). After six months of therapy, the patient’s skin lightened, and ultrasonography revealed gradual narrowing of his testicular nodules; the levels of sex hormones, ACTH, CO, and DHEA-S also normalized. After two years of treatment, his symptoms further alleviated, after which his oral dexamethasone dosage was reduced (0.5 mg, daily). Recent CT and MRI reexaminations showed shrinkage of the hyperplastic nodules on the adrenal glands and the testicular tumors. MRI showed that the soft tissue-density mass remained in the intramural, collateral branch of the right adrenal gland, but it had reduced in size. The mass in the left adrenal gland had disappeared, and bilateral adrenocortical hyperplasia had recurred. The effectiveness of the treatment was proven by the reduced size of the masses in both testes (Fig.1).

Fig.1: Multislice computed tomography, magnetic resonance imaging, and pathological examination of testicular adrenal rest tumors and testes. A. Mass with soft tissue density is noted on the right side of both adrenal glands; its maximum size is 4.5×3.7 cm. B. The adrenal masses are reduced in size following treatment. C. Both testes are enlarged, especially that on the left side. D. An obvious reduction in size of the masses on both testes is evident after treatment. E. A polygonal or circular eosinophilic cytoplasm is evident within the testicular tissue (H&E stain) and F. The spermatogenic cells in the seminiferous tubules are considerably diminished in size or absent (H&E stain).
Discussion

CAH comprises a group of autosomal recessive genetic disorders resulting from mutations in the genes involved in CO synthesis in the adrenal glands. Non-classic (delayed-type) CAH involves mild 21-OHD, resulting in mild clinical manifestations of hyperandrogenism. In boys, these symptoms usually manifest as an abnormally large penis, advanced puberty or slightly accelerated growth, shorter stature, dark skin, spermatogenic dysfunction, or even infertility. The boundary between simple virilizing and non-classic CAH is difficult to define because the level of 17α-hydroxyprogesterone (17-OHP) varies across a continuum between mild and severe cases. Increased levels of 17-OHP are specific indicators of 21-OHDs. Based on clinical manifestations and the level of 17-OHP, a relatively accurate diagnosis of classic CAH can usually be made. When serum 17-OHP levels are approximately at normal levels and do not provide sufficient diagnostic information, an "ACTH stimulation test" is necessary. In our case, a whole body check of our patient revealed signs of hyperandrogenism, including skin pigmentation, testis/adrenal hyperplasia, and precocious puberty. After determining ACTH and CO levels, we administered a “low-dose dexamethasone suppression test” and found the patient’s level of ACTH could be effectively restrained. Moreover, his levels of ACTH, urinary 17-KS, and DHEA-S, and serum PRGE (which can convert into 17-OHP) were all increased, but his CO level was reduced. These results excluded a hypothalamic pathogeny, because of which we considered that the patient’s symptoms were caused by an adrenal disease, such as CAH. Unfortunately, our hospital lacks the facility for detecting 17-OHP levels. Therefore, an ACTH stimulation test could not be conducted. In general, however, the patient’s clinical signs and metabolic features indicating simple virilizing CAH were relatively obvious, and a definite diagnosis was made after all of the available results, including DNA sequencing, we considered.

Pathologically, hyperplasia of the adrenal glands and accumulation of precursor material can lead to excessive synthesis of androgen; elevated levels of adrenal androgen may thereby inhibit gonadotropin levels. Therefore, determination of LH and FSH levels can indirectly provide an evaluation of a male CAH patient’s gonadal function. Compared to patients with low gonadotropin levels due to thyroid hypofunction, T levels of CAH patients are usually normal or only slightly reduced because of the elevated adrenal androgen levels (9). In our patient, the decreased levels of FSH and LH might have been associated with his prepubescence or his suggested pseudo-precocious puberty. Furthermore, the patient’s testosterone level was within the normal range, eliminating a low gonadotropin level caused by hypothyroidism.

In some cases, pseudo-precocious puberty may activate the hypothalamus-pituitary-gonadal axis, causing central precocious puberty. Large doses of androgens may also cause premature epiphyseal closure in patients. Our patient’s radiographic examination indicated that his bone age was more than three years older than his chronological age, suggesting the etiology of his short stature. In addition, pigmentation in his gums and external genitalia may have been due to weakened feedback inhibition involving ACTH and stimulating hormones that regulate melanin secretion.

The gene responsible for the production of 21-OH, in our patient, was composed of an inactive \textit{CYP21A1} (pseudogene) and an active \textit{CYP21A2} (true gene). In humans, about 1/60 individuals carry a \textit{CYP21A2} mutation, allowing genotyping to contribute to a precise diagnosis of CAH (10). DNA sequencing can clearly detect both heterozygous alleles in patients and may provide valuable guidance for genetic counseling and prenatal diagnoses. In our case, DNA sequencing revealed a homozygous mutation on intron 2 of the \textit{CYP21A2} gene, leading to 21-OHD and adrenal insufficiencies. Such an intron 2 splice usually results in salt wasting CAH; however, the mutation may also be found in approximately 10% of cases of simple virilizing CAH, but almost never in patients with non-classic CAH.

Due to insufficient endocrine regulation, the compensatory secretion of pituitary ACTH may reach a sufficiently high level to cause hyperplasia of testicular adrenal-derived cells. The presence of abnormal, residual adrenal cells may lead to TARTs, but are not associated with malignant tumors. The immunohistochemical results also excluded a Leydig cell tumor diagnosis.

Claahsen-van der Grinten et al. (9) reported that ultrasonography provides the best detection and
follow-up method for TARTs, especially for non-palpable tumors. In imaging studies, the diameter of a TART is usually <2 cm, and it is surrounded by a hypoechoic area. The tumor may clog the seminiferous tubules, affecting testicular function, or even lead to infertility (1, 11). MRIs of the testicular tumors in the present case showed irregular nodules in both testes and a nonhomogeneous signal in the TARTs. Diaphragms were also present in the TARTs, surrounded by hypoechoic signals. Thus, we recommend ultrasonography as a regular follow-up method in such patients in order to avoid iatrogenic radiation and to minimize patient discomfort. However, when the patient’s condition changes, an MRI should be performed, as it contributes greatly to the disease diagnosis (12, 13).

Benvenga et al. (14) found LH receptors in TARTs and speculated that increased LH levels during puberty were an additional stimulus for the pathogenesis of TARTs. This might explain the increased incidence of TARTs in CAH patients during puberty or postpuberty. In clinical practice, testicular nodules are easily mistaken for Leydig cell tumors, because of which orchiectomy may be performed (15). Electron microscopic examinations have shown that TARTs are histologically similar to Leydig cell tumors, and that they involve the same steroid-secreting cells. However, TARTs do not contain the Reinke crystals observed in Leydig cell tumors (11). Up to 80% of CAH patients may exhibit TARTs in both testes, but only 3% of patients with interstitial cell tumors exhibit a TART in the same testis (9). Therefore, when CAH is accompanied by nodules in both testes, a TART diagnosis should be considered first.

Histological analysis indicated that the identification of the differences between atypical proliferation of steroid-hormone-secreting cells and rapid mitosis is an effective method for distinguishing benign TART from malignant testicular interstitial cell tumors. In addition, real-time-polymerase chain reaction (real time-PCR) analysis of Leydig cell tumors results in unique gene products (14). In the present case, immunohistochemistry showed that the mass was vimentin (+), indicating that both testicular masses were derived from the mesenchyme. Additionally, the absence of Reinke crystals excluded the possibility of malignancy.

Adrenal hyperplasia accompanied by TART can highly increase the probability of low fertility in men. Although TART has no malignant features, the tumor near the testicular mediastinum may oppress seminiferous tubules and eventually decrease fertility (7). Reduced diameters of the seminiferous tubules, fibrosis, and hyalinosis may be observed in the testis, and the number of spermatogenic cells may decrease considerably. TART also has a paracrine role. Steroidal toxic substances produced by tumor cells may damage Leydig and germ cells (16), and thus indirectly disrupt reproductive hormone levels. In addition, excessive glucocorticoid replacement therapy may also affect the quality of semen and thus affect fertility. The inhibin B level in TART patients is most likely to affect spermatogenic function in infertile men (17, 18), but appropriate medical treatment may improve semen quality.

In the present case, spermatogenic cells of the patient reduced significantly owing to support cell proliferation. Glucocorticoid replacement therapy compensated for the lack of CO2, while the secretion of ACTH was inhibited. Hydrocortisone is usually the drug of first choice in this treatment, although it has relatively weak efficacy; it has few side effects. We first administered hydrocortisone treatment to our patient for three months, but noted little improvement. Thus, we switched to dexamethasone, a relatively strong drug. After adequate drug treatment, the levels of the adrenal precursors and DHEA-S, which can cause hyper-androgenism, decreased, and gonadal hormone levels returned to normal. The growth and maturation of the testicular seminiferous tubules were restored, and testicular nodules either decreased in size or disappeared. Unfortunately, sperms were not detected in the semen of our patient during the three-year follow-up.

In some patients with hormone-insensitive CAH, accompanied by TARTs, the clinical symptoms may not improve after the administration of conservative hormone replacement therapy. In these cases, because of improvements in the general condition of the patients, as well as in their quality of life, fertility maintenance, and prevention of glucocorticoid and mineralocorticoid side effects (18), testicular tumor excision should be considered. Tiryaki et al. (19) reported 2 cases of hormone-sensitive adenomatous hyperplasia in which nodular enucleation was performed, and good long-term results were obtained; the nodules and
metastasis did not recur. Walker's testis-sparing surgery has also been found to be useful as the testes retained a satisfactory postoperative vascular flow, and tumor recurrence was not observed (20). However, enucleation of multiple nodules may be accompanied with an increased risk of testicular atrophy. Thus, for fertile TART patients, we recommend preoperative sperm-banking.

Conclusion

We described a case of simple virilizing CAH with TARTs in a 15-year-old boy. Physicians should consider CAH, especially in patients with bilateral TARTs, especially since TARTs are rarely the presenting symptoms of CAH. The occurrence of TARTs may be related to the continuous production of ACTH in patients with delayed treatment for CAH. If a malignant tumor is ruled out, however, it is necessary to carefully determine the need for surgical removal of the tumor. Satisfactory curative effects may be obtained with appropriate glucocorticoid replacement therapy. Upon encountering such a case, early diagnosis and timely treatment are required to avoid adverse consequences caused by misdiagnoses and also to improve the patient’s quality of life, as much as possible.

Acknowledgements

We appreciate X.M.M from Department of Radiology, Maternal and Child Health Hospital, Xiamen City, China, for his help in image processing. We declare that we do not have any conflicts of interest.

References

Case Report

Molecular Dissection Using Array Comparative Genomic Hybridization and Clinical Evaluation of An Infertile Male Carrier of An Unbalanced Y;21 Translocation: A Case Report and Review of The Literature

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Abstract

Chromosomal defects are relatively frequent in infertile men however, translocations between the Y chromosome and autosomes are rare and less than 40 cases of Y-autosome translocation have been reported. In particular, only three individuals has been described with a Y;21 translocation, up to now. We report on an additional case of an infertile man in whom a Y;21 translocation was associated with the deletion of a large part of the Y chromosome long arm. Applying various techniques, including conventional cytogenetic procedures, fluorescence in situ hybridisation (FISH) analysis and array comparative genomic hybridization (array-CGH) studies, we identified a derivative chromosome originating from a fragment of the short arm of the chromosome Y translocated on the short arm of the 21 chromosome. The Y chromosome structural rearrangement resulted in the intactness of the entire short arm, including the sex-determining region Y (SRY) and the short stature homeobox (SHOX) loci, and the loss of a large part of the long arm of the Y chromosome, including azoospermia factor-a (AZFa), AZFb, AZFc and Yq heterochromatin regions. This is the first case in which a (Yp;21p) translocation has been ascertained using an array-CGH approach, thus reporting details of such a rearrangement at higher resolution.

Keywords: Translocation, Azoospermia, Hypogonadism, Y Chromosome


Introduction

Chromosome anomalies contribute frequently to reproductive failure in men, accounting for approximately 7% of all infertility cases and 10-15% of azoospermia (1-3). Sex chromosomal abnormalities, in particular, may be considered relatively common, as the Klinefelter syndrome occurs in approximately 0.1-0.2% in newborn males, and Y chromosome microdeletions in the azoospermia factor (AZF) regions have been detected in approximately 8% of nonobstructive azoospermic and 5% of severely oligozoospermic men (4, 5). Some discrepancies between different investigations, probably due to ascertainment biases and/or real differences in populations studied, do not alter the assumption that variations of the Y chromosome, in particular microdeletions, impact on spermatogenesis. More rarely, rearrangements of the Y chromosome may take the form of translocations in which variable Y-chromosome-derived material translocates to different recipient chromosomes. When unbalanced, such a rearrange-
ment can be responsible for a large spectrum of different clinical phenotypes, depending on the translocation breakpoints on Y chromosome and autosomes and/or on the chromosomal regions possibly deleted (6-10). The resulting phenotypes may range from Turner syndrome to infertile males with azoospermia or, at least, with severe oligozoospermia as cardinal feature (11, 12). Occasionally, a Y-autosome translocation may be characterized for an apparent discrepancy between the karyotype and the expressed sexual phenotype. In fact, an apparently 45,X karyotype may be associated with maleness, due to the translocation of the testis-determining gene [sex-determining region Y (SRY)] onto an autosome. Less than 40 cases of Y-autosome translocation have been reported in literature still now; all of them are different from each other as regard to the autosome and/or the involved regions of the Y chromosome (13-20). However, in all the previous reports, the characterization of the critical regions involved in such a chromosomal imbalance was carried out by conventional karyotyping or fluorescence in situ hybridisation (FISH) studies. Here, we describe for the first time the results of a detailed molecular dissection using array comparative genomic hybridization (array-CGH) for the characterization of the chromosomal regions involved in a Y;21 translocation occurred in an infertile man. Applying this molecular cytogenetics method, we were able to accurately map the specific genomic regions involved in the translocation breakpoints, offering refined molecular data in such a condition.

Case report

Clinical findings

An apparently healthy man, 34-year-old, attended our services after a two years history of infertility. An informed consent was obtained from the patient after the aims and procedures of the investigation were fully explained. He appeared as a well-developed male, 170 cm tall, weighing 68 kg. Physical examinations revealed normal male habitus except for a high-pitched voice and testis slightly smaller in size: at scrotal echography, the right testis was evaluated about 15 ml in volume and the left approximately 12 ml. Repeated semen analysis revealed azoospermia. Endocrinological examinations showed hypergonadotrophic hypogonadism, with elevated follicle stimulating hormone (FSH, 24.6 mU/ml), moderately low total serum testosterone level (2.74 ng/ml), very low free testosterone (3.74 pg/ml), and normal levels of sex-hormone-binding globulin (34.8 nmol/l) and luteinizing hormone (LH, 4.2 mU/ml). The patient, who received pre- and post-test genetic counselling, refused a testicular biopsy for diagnostic purposes and the possibly recovery of germ cells.

Cytogenetic analysis

High-resolution chromosome analysis [Quinecine (QFQ) banding] was performed from blood lymphocyte cultures according to standard cytogenetic procedures. FISH was carried out according to manufacturer’s instructions, using centromeric probes for chromosomes Y (DYZ3, Yp11.1-q11.1; Kreatech Diagnostics) and 13/21 (D13Z1/D21Z1, 13p11.1-q11.1 and 21p11.1-q11.1; Kreatech Diagnostics), subtelomere-specific probes for both arms of the sex chromosomes (DXYS130, Xp/Yp telomeres; DXYS224, Xq/Yq telomeres; Kreatech Diagnostics) and probes for the whole chromosome Y and 21 (Kreatech Diagnostics). Lymphocyte preparations from male with normal karyotype were used as controls for the FISH assays.

Molecular genetic analysis

Array-CGH was performed using the Human Genome CGH 4X180K Microarray Kit (Agilent Technologies, USA), according to the manufacturer’s protocols. The Agilent Feature Extraction software has been used to perform image analysis. In order to correct systematic spatial and intensity biases, the results were normalized using the lowess function. Normalised log2ratio values were calculated and breakpoint identification was performed applying the Shifting Level Model (SLM) segmentation algorithm (21). The probabilistic classification of each segmented region into biologically motivated status (loss, neutral or gain) was performed by FastCall algorithm (22). A rearrangement was defined by the deviation of at least three consecutive probes, with a practical average resolution of about 100 kb.

In our patient, the Y chromosome was not detectable at a first conventional chromosome analysis, revealing the appearance of a 45,X0 karyotype. As
the result was inconsistent with the phenotype of the patient and some additional material detected on the terminal part of 21p chromosome, might be suspected of Y-derived material, we applied FISH analysis. By this approach, we detected a derivative chromosome originating from a fragment of the short arm of the chromosome Y translocated on the short arm of the 21 chromosome (Fig.1). By applying dual-color FISH using alpha-satellite probes respectively for Y (Yp11.1-q11.1) and 21 (21p11.1-q11.1) chromosomes, we identified two centromeres at the derivative chromosome, originated from centromeres of both Y and 21 chromosomes.

As a whole, the derived chromosome could be described by FISH as [45,X,der(21)t(Y;21)(q11;p11).ish der(21)wcpY+,wcp21+,DXYS130+,DYZ3+, D13Z1/D21Z1+,DXYS224-]). To further characterize the rearrangement, array-CGH analysis was carried out and showed a Yq deletion of about 45 Mb ± 0.2 Mb, spanning from 13,992 kb to 59,031 kb, respectively the first and the last probes on the array platform. According to the University of California Santa Cruz (UCSC) Genome Browser, GRCh37/hg19 deletion breakpoints mapped in Yq11.2-q12 (arr Yq11.21-q12(13.992.304-59.031.421)x1 (Fig.2).

Discussion

Chromosomal imbalances can severely affect male fertility. The prevalence of karyotype abnormalities in infertile males are reported about 8-10 fold higher than in general population (23, 24). In particular, reciprocal translocations between autosomes are evaluated to occur in approximately 1% of severely oligo- or azoospermic men, versus 0.1% of general population (25). Moreover, one of the most common genetic contributions to male infertility is represented by Y chromosome microdeletions involving the three AZF regions.
located on the distal Yq11 region. In contrast to such submicroscopic chromosomal imbalances, translocations involving the Y chromosome occur very rarely. To our knowledge, less than 30 cases of material exchange between a Y chromosome and an autosome has been reported in literature, only three of them involving the 21 chromosome (16, 26, 27). More recently, a mosaicism for an unbalanced Y:21 translocation with the loss of chromosome 21 material has been reported (28). In our case, we report on a constitutional translocation between the short arms of the chromosomes Y and 21 [(45, X, t(Yp:21p) (p12;p1.1)], with the loss of Yq material. The breakpoints were detected in the proximal region of the long arm of the Y chromosome (Yq11) and in the distal region of the short arm of chromosome 21 (21p12), resulting in a derived chromosome with both Y and 21 centromeres. More specifically, array-CGH showed a Yq11.21q11.2 deletion of about 45 Mb, encompassing a genomic region that includes about 200 genes and transcripts, the majority of which involved in the events of sperm maturation, specifically expressed in the male germline. The Y chromosome structural rearrangement results in a conserved short arm of the Y chromosome, including the SRY and the short stature homeobox (SHOX) loci, although translocated on the 21 chromosome, and the loss of a large part of the long arm of the Y chromosome, including the AZFa, AZFb, AZFc and Yq heterochromatin regions. In agreement with the three cases previously reported (16, 26, 27), the retention of the SRY gene allowed the complete masculinization of the patient, although azoospermic. Among the secondary sexual traits, only a persistent high-pitched voice resulted as an additional and distinctive phenotypic sign in our patient, supporting the well-known negative relationship between the voice pitch and circulating levels of testosterone in men (29, 30).

Due to the complexity of the information regarding his genetic status, as previously agreed with patient, he was seen in several rounds of genetic consultation after the test, alone and in the company of his partner, to disclose the result and to discuss all the possible implications. As we inform that sperm retrieval from a testicular biopsy is considered ineffective for males with such a Y chromosomal deletion (entire AZF region), a more invasive evaluation was refused and no testicular tissue studies were performed for histological ex-

aminations. Overall, the patient did not show adverse psychological reactions after the disclosure of genetic information: he did not exhibit anxiety and/or depression symptoms and refused a proposed psychological support as considering it unnecessary. These findings are consistent with the notion that the psychological distress associated with the communication about positive genetic test results may be reduced by careful pre- and post-test genetic counseling.

Our study, discussing a new case of a rare Y;21 unbalanced translocation, highlights the usefulness of the high-throughput molecular diagnostic methods for detection of subtle chromosomal rearrangements. To our knowledge, this is the first case in which a (Yp:21p) translocation has been ascertained using an array-CGH approach, thus reporting details of such a rearrangement at higher resolution.

Acknowledgements
The authors declare no conflict of interest.

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International Journal of Fertility and Sterility (Int J Fertil Steril)

Guide for Authors

Aims and Scope: The "International Journal of Fertility & Sterility" is a quarterly English publication of Royan Institute of Iran. The aim of the journal is to disseminate information through publishing the most recent scientific research studies on Fertility and Sterility and other related topics. Int J Fertil Steril has been certified by Ministry of Culture and Islamic Guidance since 2007. It has also been accredited as a scientific and research journal by HBI (Health and Biomedical Information) Journal Accreditation Commission since 2008. This open access journal holds the membership of the Committee on Publication Ethics (COPE).

1. Types of articles

The articles in the field of Fertility and Sterility can be considered for publications in Int J Fertil Steril. These articles are as below:

A. Original articles: are scientific reports of the original research studies. The article consists of English Abstract (structured), Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgements, and References.

B. Review articles: are the articles written by well experienced authors and those who have expertise in the related fields. The corresponding author of the review article must be one of the authors of at least three articles appearing in the references. The review article consists of English Abstract (unstructured), Introduction, Discussion, Conclusion, and References.

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E. Editorial: should be written by either the editor in chief or the editorial board.

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Each article should be accompanied by a cover letter, signed and dated by corresponding author specifying the following statement: The manuscript has been seen and approved by all authors and is not under active consideration for publication. It has neither been accepted for publication, nor published in another journal fully or partially (except in abstract form). I hereby assign the copyright of the enclosed manuscript to Int J Fertil Steril. Corresponding author can also suggest three peer reviewers in the field of their article.

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