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Contents

Review Article

Varicocele-Induced Infertility in Animal Models

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Abstract

Varicocele is characterized by abnormal tortuosity and dilation of the veins of the pampiniform plexus within the spermatic cord. Although several reports show the mechanisms by which the varicocele exerts its infertility impact, the exact pathophysiology for varicocele-induced inflammation and its relationship with testicular endocrine disruption remain largely unknown. This review article will update previous findings by discussing the pathophysiology of long term-induced varicocele in rats. Testicular endocrine disruption in experimentally-induced varicocele, new findings related to biochemical alterations in germinal epithelium, and sperm cells apoptosis are highlighted. Recent observations show that varicocele down-regulates first and second maturation divisions, results in Leydig and Sertoli cell inflammation, and increases immune cell infiltration in the testes of the rat as an animal model. Ultimately, previous findings of our laboratory have revealed that varicocele decreased sperm motility, viability and severe DNA damage. Damage in sperm significantly lowers the animal's fertility potential. Varicocele not only exerts its pathologic impact by lowering the testicular antioxidant capacity but it also down-regulates first and second maturation divisions by exerting biochemical alterations such as reducing the intracytoplasmic carbohydrate ratio in germinal epithelium.

Keywords: Infertility, Inflammation, Oxidative Stress, Varicocele, *In Vitro* Fertilization

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Introduction

According to clinical reports, varicocele is observed in 10-20% of the male population, 35-40% of males with primary infertility problems, and up to 80% of men with secondary infertility (1, 2). Annually, 20000 to 40000 infertile men undergo surgery for varicocele (3). Despite numerous studies that emphasize the relation between varicocele and infertility, there are many unsolved questions that remain about the pathophysiology of this impairment. Several studies have shown that varicocele actually causes approximately 35-40% of testicular dysfunction such as damaged seminiferous tubules, a remarkable decrease in Leydig cell distribution and severe testosterone decline in humans (4) and animals, which result in abnormal spermatogenesis and tubules with increased cellular apoptosis (5-7). Increased sperm damage occurs approximately twice (70-85%) as much as seen in testicles and presents as significant decreases in sperm count, motility, viability and remarkable elevations in sperm abnormalities (5, 8, 9). One can hypothesis that the varicocele-induced damages are progressive and a simple pathological analysis of the testicles does not clarify the depth of varicocele-dependent derangements. Thus, the present review focuses on the latest finding of various aspects of varicocele in relation to male infertility in humans. These findings will be compared to the results from our laboratory using rat models with induced varicocele in terms of germinal and sperm cell apoptosis, antioxidant status, inflammation, endocrine function, biochemical changes in carbohydrates, and lipid foci accumulation in the germinal epithelium (6-8). Finally, the varicocele-induced impact on *in vitro* fertilizing potential of rats will be clarified.

Current understanding about varicocele pathophysiology

Varicocele develops from retrograde blood flow through the internal spermatic and cremasteric veins into the pampiniform plexus. According to previous observations the venous retrograde blood flow is attributed to the absence of and/or incomplete valves

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(10, 11). In particular, the reversed blood may lead to severe damage to testicles, partly by two mechanisms: significant resistance to blood flow as measured by the resistive index of capsular branches in varicocele patients (8, 12) and increased scrotal temperature which at least can promote heat-dependent apoptosis (13). Other findings have illustrated that following retrograde blood circulation, the multiple pathophysiologic derangements such as damaged endocrine system, biochemical changes and oxidative stress (6, 14, 15) enhance varicocele-induced impairments. In this regard studies on animal models aim to clarify the pathways where varicocele provokes biochemical changes. Left varicocele induction is used in various studies on animal models in order to induce blood flow into pampiniform plexus (3, 5). We have reduced the left renal vein diameter to less than one mm by ligating the junction of the adrenal and spermatic veins. Then, the anastomotic branch between the left testicular vein and the left common iliac vein was ligated (6, 7).

Apoptosis in spermatogenesis cell lineage

Spermatogenesis is a proliferative process in which millions of spermatozoa are produced daily. Apoptosis occurs in both pathologic and physiologic conditions as a unique pathway in order to control normal spermatozoa development. In physiologic conditions, apoptosis depends on the capacity of Sertoli cells and mainly occurs in order to eliminate defective germ cells. Thus, it can be considered a critical mechanism to estimate infertility in men (16, 17).

There are many pathways that result in apoptosis in the germinal epithelium; these processes seem to be synchronized in three levels - cellular membrane (18), cytoplasmic (19) and nuclear (20). Apoptosis can affect all three classes of cellular lineages, the spermatogonia, spermatocytes and spermatids (16). At the cell membrane level there are specific membrane receptors which mediate death signals of the tumor necrosis factor receptor family, known as the Fas and Fas ligand (17, 21). Apoptotic cells are recognized by Sertoli cells through binding their membrane receptor to phosphatidylserine, which appears on the surface of the apoptotic germ cells. In this situation the death germ cells are then rapidly phagocytized by Sertoli cells (22, 23).

At the cytoplasmic level there are signal transduction pathways that involve cysteine proteases called caspases (23). At the nuclear level there are specific apoptosis regulatory genes such as *P-53* and *Bcl-2* (24-26). The *P-53* tumor suppressor gene responds to DNA damage and temporarily arrests the cell division cycle at the G_1 phase. As a result, *P-53* provokes the DNA recovery process (17). However, if the DNA damage is irreparable, *P-53* will stimulate cellular apoptosis via the Fas receptor complex (22). As previously mentioned, varicocele promotes its pathological impact via induction of apoptosis through nuclear and cell membrane levels. We have recently found that in long-time varicocele-induced rats the intracytoplasmic carbohydrates and lipids indirectly participate in germinal cell apoptosis (7).

Biochemical changes in germinal epithelium and relation with apoptosis at the cytoplasmic level

Although there are several reports for nuclear and membrane level apoptosis in varicocele, herein we discuss apoptosis at the cytoplasmic level. In the case that Fas binds to its ligand on Sertoli cells, the generated union forms a complex on the inner surface of the cell which is known as the death-inducing signaling complex that involves procaspase-8. By this mechanism the physiologic elimination of abnormal germinal cells occurs by apoptosis, which is in line with the Sertoli cells physiologic capacity to control normal spermatogenesis (24). Independent to these findings, the glucose transporters I, II, III, IV and VIII mainly mediate transportation of glucose in the spermatogenesis cell lineage (26-28). Since spermatogenesis develops by remarkable utilization of carbohydrates as a main source of energy, any disruption in glucose and/or hexose carbohydrate transport and/or metabolism can promote apoptosis and cellular degeneration in these series of cells (7, 29, 30). Our most recent findings have shown that varicocele-induced rats had significantly decreased carbohydrate content in the spermatogenesis cells versus control animals (7). The cells with faint cytoplasmic carbohydrate content had increased lipid accumulation, at the same time Sertoli cells exhibited a high intracytoplasmic lipid content. In order to explain how varicocele causes enhancement of lipid accumulation in these mentioned cells, one should note, that the lipid supplement in Sertoli cells differs depending on various conditions (Fig.1A-D). For instance, when the Sertoli cells begin phagocytosis of residual bodies or damaged cells, the ratio of lipids increases in the cytoplasm of these cells (7, 27, 31, 32). In addition, the varicocele-induced derangements force the cells to switch principal energy from glucose to lipids. The newly selected source of energy will not be able to support cell demands for mitosis (7, 27, 33). Therefore, the cells with defective metabolisms that result from insufficient principal energy sources undergo apoptosis. Ultimately, the involved Sertoli cells begin phagocytosis of the apoptotic cells (Fig.2).

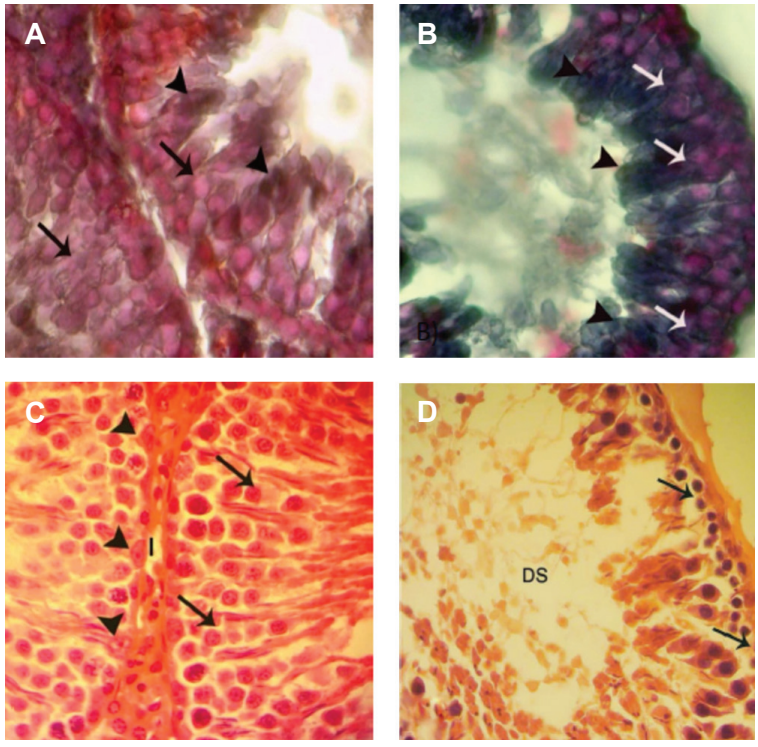

Fig.1: Cross-section from the testes. **A.** Control group: note spermatogenesis cell lineage with negative Sudan-Black B stained cytoplasm (arrows). The area of spermatogenesis appears with dense reaction sites (head arrows). A comparison of varicocele-induced testis. **B**. The control group indicates that in non-varicocele-induced testis the spermatogenesis cell line shows faint lipid stained cytoplasm (arrows) and the spermiogenesis area (head arrows) has a dense stained pattern. Varicocele-induced testis is presented with darkly stained cells in all cell lineages (arrows and head arrows). **C**. Control group: note interstitial connective tissue (I) without edema, Sertoli cells with dense stained cytoplasm (head arrows), spermatogenesis cell lineage with powerful reaction for PAS staining which indicates high cytoplasmic carbohydrate supplement. **D**. varicocele-induced testis: note faint reaction for PAS. The spermatogonial cells have a negative reaction to PAS staining (head arrows). A, B: Sudan-black B staining; C, D: PAS staining, (×600).

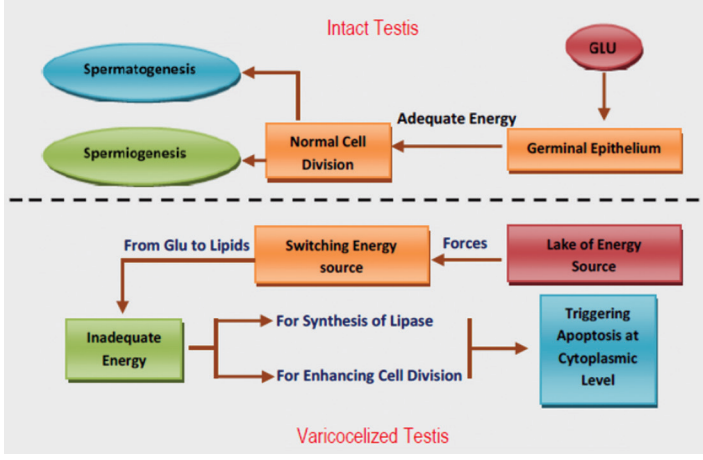

Fig.2: Energy dependent pathways in intact and varicocelized testes; Under normal conditions, glucose (GLU) is transferred the germinal cells via different GLU transporters, which supply enough energy for vital activities of cells such as germinal cell division. In contrast, lack of appropriate sources of energy forces the cells to switch energy sources from GLU to lipids. In order to use lipids the cells need adequate energy to synthesize essential enzymes such as lipase. In varicocele-induced animals synthesis of the lipase enzyme is down-regulated over time. Thus, the cells miss their ability to use the lipids as a secondary source of energy, which leads to remarkable reduction in cell division and continuation of vital functions. The induced impairments trigger cytoplasmic level apoptosis independent of the Fas pathway.

First and second maturation divisions arrest in varicoceles

There are physiologic correlations (supportive and nutritional) between Sertoli cells and spermatocyte type I cells in the male reproductive system (34, 35). Spermatocyte type I cells are considered to be the precursor cells of spermatogenesis (34-36). Any detrimental effect on these cells can disrupt the first maturation division which in turn results in severe reduction in the population of cells that undergo second maturation division. Recently we have shown that long-term-induced varicocele in rats resulted in a significant reduction in the maturation division ratio. Accordingly, 58% of the tubules manifested with arrested second maturation division and approximately 40-45% of the tubules were revealed with stopped first maturation division after 8 months. The primary outcome was that the varicocele impacted not only by down-regulation of the first maturation division but it also decreased the second maturation division dependent/independent to the first division of precursor cells (36).

Possible mechanisms for maturation division arrest

Inflammation

Interleukins (ILs) play an essential role in normal testicular tissue. Physiologic levels of ILs regulate functions of Sertoli and Leydig cells (mainly steroidogenesis) and participate in spermatogenesis (36-38). IL-1β generates severe oxidative stress in different tissues and its compensatory up-regulation in varicoceles testes is well known. Over expression of IL-1β in varicoceles can result in a remarkable increase of reactive oxygen species (ROS) levels which can cause an inflammatory response detrimental to testicular tissue (37). Previously, we have shown that varicocele increased ROS stress in a time-dependent manner. Animals in the eight month–induced varicocele group had remarkable increases in malondialdehyde (MDA) levels accompanied with severely reduced thiol molecule levels in testicles (6). Remarkably increased poly- and mononuclear immune cell infiltration in connective tissue was observed in long-term varicoceles in a rat model. Beside these findings, our observations showed that Sertoli cells in varicocele-induced rats exhibited up-regulated intracytoplasmic alkaline phosphatase levels which suggested that Sertoli cells were directly influenced by inflammation (7, 36). Any inflammatory detrimental effects on Sertoli cells would be able to influence spermatogenesis, particularly at the first maturation division (39, 40). These findings suggested that varicocele-induced inflammation negatively impacted Sertoli cell physiologic function by two mechanisms of extensive ROS stress (via the ILs pathway) and directly by influencing Sertoli cells (alkaline phosphatase positive Sertoli cells). Therefore, damaged Sertoli cells lost their physiologic correlation with the cells that participated in first and second maturation divisions.

Endocrine system dysfunction

A constant level of testosterone at normal concentrations has been clarified as an essential substance to initiate and promote spermatogenesis (41, 42). Serum levels of follicular stimulating hormone (FSH) and luteinizing hormone (LH or ICSH) are extremely important to promote Sertoli and Leydig cell endocrine function. As FSH increases, Sertoli cell synthesis of an androgen binding protein is needed to maintain the high concentration of testosterone (41, 43, 44). LH stimulates the Leydig cells to synthesize essential testosterone. Our experiments on long-term varicoceles in rats have shown a significant reduction in Leydig cell steroidogenesis. Accordingly, the serum level of testosterone remarkably decreased in a time-dependent manner, particularly eight months after varicocele induction, compared to control animals. The histopathological observations for long-term varicoceles testes in rats showed that Leydig cell distribution reduced after eight months. These cells were shown to exhibit vacuolated cytoplasms (7, 36). Although there are contradictory reports about the gonadotropin levels in varicoceles, long-term varicocele-induced rats had decreased serum levels of FSH and LH (7). This disorder might be attributed to the decreased feedback response of Leydig and Sertoli cells to upper axis secreting hormones over time. Thus, it may be concluded that varicocele-induced dysfunction in the endocrine system affects spermatogenesis at both first and second maturation divisions by two mechanisms: a) directly influencing testicular endocrine cells as a-1; reducing Leydig cell distribution and steroid activity accomplished with a-2; and Sertoli cell dysfunction (which it-

self largely depends on testosterone levels) as well as b) affecting hypophysis-gonadal axis feedbacks (Fig.3).

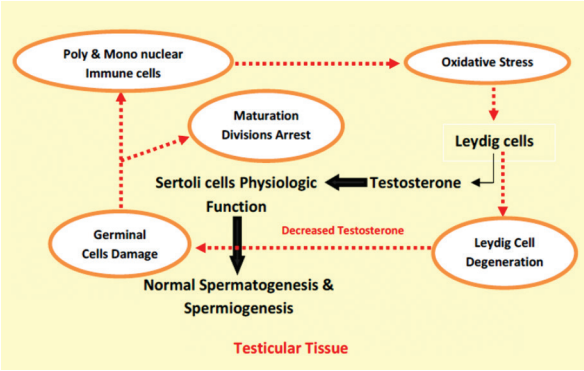

Fig.3: On intact testicular tissue the Leydig cell-produced testosterone promotes Sertoli cell physiologic function, which results in normal spermatogenesis and spermiogenesis processes. On varicocele-exposed testes, the varicocele -induced inflammation impacts the Sertoli cell physiologic role by inhibiting the testosterone synthesis from Leydig cells. The last impairment promotes oxidant generation by two different pathways, including enhancing immune cell infiltration and elevating germinal cell derangement.

Male infertility and oxidative stress

The correlation between oxidative stress and male infertility has been extensively studied (45, 46). ROS include hydrogen peroxide and unstable free radicals with unpaired electrons in their outer orbits. It has been clarified that the mitochondria and plasma membranes of morphologically abnormal and damaged spermatozoa produce ROS through the nicotinamide adenine dinucleotide phosphate-dependent and nicotinamide adenine dinucleotide-dependent oxidoreductase systems, respectively (38). Considering that the duration of the complete spermatogenesis cycle in rats is 45 days (39), normal levels of ROS play an essential role in physiological spermatogenesis, viability, capacitation and sperm motility (38, 47, 48). Excessive ROS generation and/or decreased total antioxidant capacity (TAC) of the testicular tissue result in remarkable increases in ROS levels, which damages the spermatogenesis processes (6, 38, 49). We have shown varicocele reduced testicular antioxidant capacity after eight months in rats by measuring TAC, total thiol molecules (TTM) and MDA levels. The MDA level can be defined broadly as a biomarker for ROS-induced lipid peroxidation. These findings have agreed with previous reports. For example, in some adolescent patients with varicocele, an increased level of MDA indicated extensive lipid peroxidation (50, 51).

Reactive oxygen species and germinal cell degeneration in varicocele

ROS elevation and/or TAC reduction in the testicular microenvironment of patients with varicocele have been reported (6, 46, 47, 51). The level of 8-Hydroxydeoxyguanosine (8-OHdG), a marker of oxidative stress, and the incidence of 4977bp deletion called "common deletion" ($mtDNA^{4977}$) in mitochondria are increased in varicocele patients. These impairments have been shown to be reversed in patients subjected to varicocelectomy (52, 53). In addition, the end products of lipid peroxidation, such as aldehydes, are alkylating agents that damage cellular DNA and form adducts with proteins that initiate apoptosis (38). Most recently we have shown that the testes samples obtained from six- and eight-month varicocele-induced rats had decreased TAC and TTM levels associated with severe germinal epithelium degeneration (6). A remarkable increase in damaged precursor cells (spermatogonia and spermatocytes), apoptotic spermatozoa and high infiltration of immune cells in testicular tissue, led to a change in the balance between ROS generation and testicular anti-oxidative status. Therefore, animals in the eight-month varicoceles group had the highest level of oxidative stress. Possibly, not only the direct varicocele-induced damages led to severe ROS generation but also the generated ROS in turn enhanced damage by impairing cells via genomic, mitochondrial and lipid peroxidation-dependent pathways.

Correlation between increased venous pressure-induced oxidative stress and nitric oxide (NO) in varicocele

Locally produced NO is known to be involved in the regulation of testicular vasculature. In testicular tissue, NO is synthesized from L-arginine by the catalytic activity of two main isoforms of NO synthase-endothelial and inducible NO synthase (NOS). Leydig cells and vasculature of the testes are responsible for the expression of endothelial NOS forms. In varicocele testes, the expression of inducible NOS is enhanced to maintain testicular

arterial blood flow as a protective mechanism to elevate blood circulation, which may be detrimental to spermatogenesis. In some adolescent patients with varicocele, increased MDA levels occur together with elevated NO levels, which indicate excessive lipid peroxidation (36). Reaction between produced NO with superoxide anions results in peroxynitrite and peroxynitrous acid generation, which act as powerful oxidants (36, 49, 50). Therefore, it can be concluded that previously produced severe oxidative stress in varicoceles reverses the protective role of NOS into degenerative impact by yielding peroxynitrite and peroxynitrous acid. The generated agents promote oxidative stress-dependent disorders such as DNA damage in varicocelized testes. On the other hand, several studies have shown that NOS plays an important role in provoking heat-dependent apoptosis. Accordingly, the "knock out" of NOS in mice results in improved spermatogenesis, elevated sperm output as well as resistance to heat-induced apoptosis (49, 50). NOS exerts it pathological impact not only by enhancing oxidative stress-dependent disorders, In addition, the produced NOS in varicocele can increase heat-induced apoptosis in testicular tissue.

Reactive oxygen species and sperm cell physiology in varicocele

Low amounts of ROS can regulate normal sperm function. Exposure of human spermatozoa against low levels of ROS enhances the sperms' ability to bind the zona pellucida of the oocyte, whereas the presence of antioxidants reverses this situation (54). Although incubation of sperm with low amounts of oxidants such as H_2O_2 can promote sperm capacitation and hyperactivation, the abnormal increased ROS levels pathologically impact sperm. Every ejaculation in humans and even in rodents contains potential sources of ROS, which induce oxidative damage to sperm. The extent of damage largely depends on the amount, duration of exposure, and nature of the oxidants. For example the lipid peroxidation of sperm plasma membrane, immobility and DNA disintegrity enhance depending on the duration of exposure to ROS and extracellular factors such as ions (54, 55). In order to evaluate the effect of long-term induced varicocele on sperm parameters, the epididymis of the animals were dissected out and the caudal section of the epididymal tissue minced in Ham's F10 culture medium. The sperm were incubated at 37˚C under 5% CO_2. Vital staining of these sperm showed decreased viability by the time after varicocele induction, which paralleled MDA elevation. The sperm motility and DNA integrity decreased in long-term varicocele-induced rats. Accordingly, the Comet assay for DNA fragmentation showed the highest level of DNA damage in eight-month varicoceles rats (6). Figure 4-A and B show sperm DNA damage. The link between ROS and reduced motility in sperm may be explained by a cascade of events that result in a decrease in axonemal protein phosphorylation as well as oxidant diffusion across the cellular membrane to inhibit the activity of enzymes such as glycerol-3-phosphate dehydrogenase (GPDH). Therefore, the antioxidant defense of sperm decreases, which in turn results in peroxidation of membrane phospholipids (Fig.5) in addition to a severe reduction in sperm motility (6, 55-57).

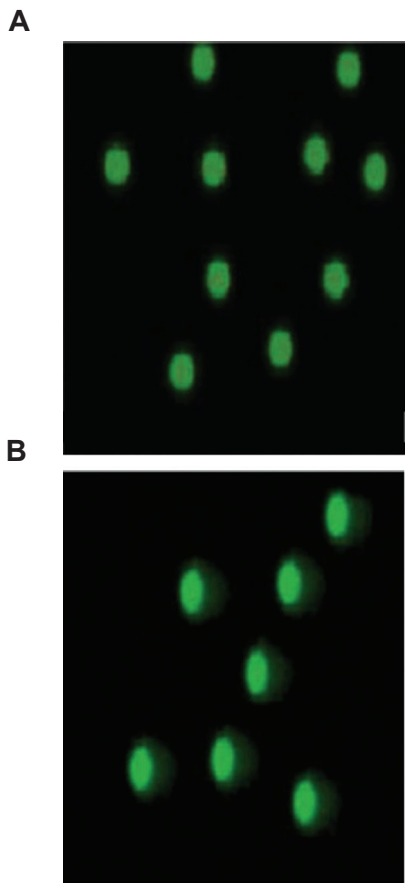

Fig.4: Epi-fluorescent architecture of rat sperm by the Comet assay. **A**. Sperm from the control group; the green spots without any tails are normal sperm. **B**. Sperm collected from the left testes of varicocelized rats with intensive DNA fragmentation. The spots with tails indicate DNA fragmentation. Comet assay (×1000).

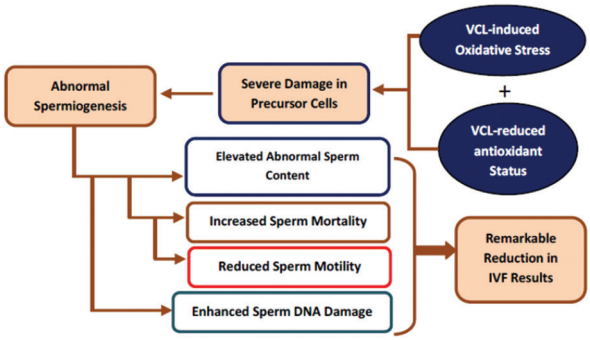

Fig.5: The varicocele (VCL), both by increasing oxidative stress and down-regulating antioxidant capacity, enhance germinal epithelium degeneration which results in abnormal spermiogenesis. Damaged spermiogenesis-induced DNA de-condensation associated with plasma membrane peroxidation via increased reactive oxygen species (ROS) enhances sperm DNA disintegrity and mortality. Oxidative stress-induced damages in sperm axonemal proteins lower sperm motility. Taken together, the sperm fertilizing potential reduces over time, which leads to a low *in vitro* fertilization (IVF) outcome in varicocele patients.

Reactive oxygen species-induced damages in sperm and *in vitro* fertilizing ability

Reports indicate that any disorder which leads to a failure in epididymal sperm maturation processes causes disorders to sperm fertilizing ability (58-60). Developments of spermatozoal ability to expose forward motility, such as undergoing capacitation and penetration in the zona pellucida of the oocyte, are examples of several important properties required by the spermatozoa during epididymal sperm passage (61). In order to analyze the effect of long-term induced varicocele on *in vitro* fertilization (IVF) outcome of animals, samples that contained spermatozoa were prepared from sperm suspensions as mentioned earlier. Then, 0.1 ml from superficial sperm of suspensions was added to 150 µl of tissue culture medium (TCM) that contained the oocytes delivered from superovulated normal rats. A drop of medium with 2 oocytes was allocated with a 10 µl sperm suspension (total: 80000 sperm). For each animal, 20 oocytes were divided into 10 drops. Observations showed that the animals in six- and eight-month varicocele-induced groups had the lowest IVF outcomes. In this regard our previous studies showed that the plasma membrane unsaturated fatty acid of sperm have undergone severe damage following varicocele induction (6). These unsaturated fatty acids are essential to give fluidity to the plasma membrane in order to participate in membrane fusion events associated with fertilization. When the associated double bonds with unsaturated fatty acids are deformed, membrane fluidity decreases, leading to a consequent loss of sperm function. Of note, the lowest results for IVF outcome have been shown after eight months. Previous observations showed that embryo development negatively correlated with the level of DNA fragmentation in the germ line (62). Studies showed that DNA-damaged sperms could not fertilize the oocyte (62, 63). According to our findings, sperm from varicoceles rats caused some of the fertilized oocytes to discontinue division in the two-cell embryo phase whereas others were not fertilized at all. Thus, it might be suggested that the loss of fertilizing potential and remarkable reduction in embryonic cell division could be attributed to severe reduction in plasma membrane fluidity as well sperm DNA damage (Fig.5).

Conclusion

Varicocele associated infertility depends on a cascade of evidence that includes germinal cell apoptosis at the cytoplasmic, nuclear and membrane levels both in human and animal models. Damages that follow experimentally induced varicocele are enhanced. They not only depend on the testicular antioxidant status but also the reduced testicular endocrine function promotes these disorders by stimulating immune cell-dependent inflammation. Finally, development of varicocele-reduced semen quality is time-dependent and negatively impacts sperm fertilizing potential in animal models.

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Original Article

A Comparative Study of Serum and Follicular Fluid Leptin Concentrations among Explained Infertile, Unexplained Infertile and Fertile Women

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Abstract

Background: The relationship between metabolism and reproduction has been always considered as an important topic in female endocrinology. It seems that leptin is one of the involved factors in infertility. Leptin, in addition to regulating body weight plays an important role in regulation of endocrine, reproductive and immune systems. The aim of this stduy is to compare serum and follicular fluid leptin concentrations in order to find the role of leptin level in infertility.

Materials and Methods: This case-control study was performed from September 2010 to March 2013. A total of 90 women referred to the Infertility Center of Afzalipour Hospital, Kerman, Iran, and divided into three equal groups (n=30/per group) of explained infertile (including 4 subgroups), unexplained infertile and normal fertile (control group). The three groups were matched in regard to demographic features [age: 20-40 years and body mass index (BMI): 20-25]. In order to determine leptin level, blood sample and follicular fluid were taken one hour prior and at the time of follicular puncture, respectively. Serum and follicular fluid leptin levels were measured using enzyme-linked immune sorbent assay (ELISA). Data were analyzed using descriptive-analytic tests, like Mann-Whitney and Kruskal Wallis tests, through Statistical Package for the Social Sciences (SPSS) version 16.

Results: In explained infertile and fertile groups, as opposed to unexplained infertile group, mean leptin level was lower in follicular fluid than in serum. Mean follicular fluid leptin concentration in women with unexplained infertility was higher compared to the other two groups. Women with unexplained infertility had lower level of serum leptin in comparison to the other two groups. Follicular fluid leptin level in all subgroups of explained infertile group was lower as compared to unexplained and fertile women.

Conclusion: The results suggested that high leptin level of follicular fluid is one of the main factors involved in infertility.

Keywords: Infertility, Leptin, Follicular Fluid, Serum

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Introduction

Infertility has been always addressed as one of the important, serious and costly health issues in different societies (1, 2). According to the previous studies performed in different countries, about 10-15% of couples suffering from infertility regard this disability as the worst experience in their life (3-5). This problem might cause marital conflicts, social injuries, divorce and other psycho-social problems (2, 6). At present, in Iran, about 3 mil-

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lion couples suffer from infertility. In 7% of infertile couple population, the cause of infertility is unknown.

The relationship between metabolism and reproduction has been always considered as controversial issues in the field of female endocrinology. Insulin, amino acids and low molecular weight IGF-binding protein-1 (IGFBP-1) have been introduced as effective signals in alterations of body fat and body mass index (BMI), but these alterations have been recently attributed to leptin level (7). Leptin, in addition to regulating body weight, plays an important role in regulating the functions of endocrine, reproductive and immune systems through suppressing food intake and increasing energy consumption. Deficiency of leptin or its receptors, in addition to causing obesity, leads to disturbing reproductive cycle, hormonal imbalance, as well as disorders of immune system, hematopoietic system and bone metabolism (8). These observations have indicated the important role of leptin in several physiologic processes and the relationship between abnormal leptin levels and many disorders (9).

Leptin affects menstrual cycles, directly and indirectly. It have been reported that leptin directly affects ovaries and hypothalamic-pituitary axis. Furthermore, its effect on follicle stimulating hormone (FSH)-dependent estradiol production in animals and its role in preventing starvation-induced delay in ovulation in mice show indirect effect of leptin on the luteinizing hormone (LH) concentration (10).

Leptin is an adipocyte hormone acting as a link between adipose tissue and reproductive system. It is also considered as a type 1 cytokine, due to its role in cell growth and maturation (11). Recent studies have reported that leptin is produced by both granulosa and cumulus cells of ovarian follicles (10, 11).

Leptin is used in the treatment of hypoleptinemia due to energy deprivation state, leptin deficiency and obesity-related hyperleptinemia. Due to leptin resistance in some obese individuals, leptin treatmentis used in patients with complete or relative leptin deficiency including patients with hypothalamic amenorrhea and lipoatrophy, but coadministration of this hormone with leptin sensitizers has been resulted in better outcomes in the treatment of obesity (8).

Since it has been proved that there is a definite relationship between infertility and menstrual irregularities in women with abnormal obese (OB) gene expression and peritoneal fluid is also known as an active biologic environment that is essential for regulation of ovarian function, ovulation, zygote implantation, and follicle collection (9, 11), any change in the concentration of substances in this environment is likely to affect ovarian function. Furthermore it can be postulated that the origin of some substances like leptin might be from follicular fluid.

The aim of this study was to compare serum and follicular fluid leptin concentrations among explained infertile women, unexplained infertile women and fertile women in order to find the role of leptin level in infertility. In the present study, without applying any invasive method more than the treatment process, three groups were compared in regard to serum and follicular fluid leptin concentration using blood sample and follicular fluid of assisted reproductive technology (ART) candidates.

Materials and Methods

In this case-control study, 90 women using convenient sampling method with regard to the inclusion criteria referred to the Infertility Center of Afzalipour Hospital, Kerman, Iran. After ensuring, all personal information remained confidential and there was no intervention with their treatment process. This study approved by Ethical Committee of Kerman University of Medical Sciences and done from September 2010 to March 2013 and all participants provided an informed consent.

Inclusion criteria were as follow (the presence of all 7 criteria was necessary): i. Age between 20 to 40 years, ii. BMI between 20 to 25 kg/m^2, iii. Normal levels of FSH, LH (on days 2 and 3 of menstrual cycle), prolactin, testosterone and progesterone (between days of 19 and 21of menstrual cycle), iv. Normal semen fluid analysis and pelvic ultrasonography, v. Absence of any underlying complex disorders like diabetes, obesity, cardiovascular disease, any type of metabolic diseases and malignancies, vi. Negative result of autoantibody test and vii. No use of anti-inflammatory medicines. It should be mentioned that only infertile participants underwent laparoscopy and hys-

terosalpingography (HSG). First the information regarding demographic and clinical characteristics were collected and infertility investigations, including ovulation state, cervical and uterine factors, and patency of fallopian tubes, were then performed in couples referred for *in vitro* fertilization (IVF). All partners had normal sperm analysis. If infertility was due to male factor, the case was excluded from our study because they had no history of pregnancy and were not considered as unexplained infertility.

Subjects were divided into three groups of explained infertile group, unexplained infertile group and fertile group. The explained infertile group (n=30) contained women with one or more infertility factors. This group was divided into the four subgroups including cervical, endometrial, tubal and peritoneal factors based on medical history and findings of physical exam, pelvic ultrasonography, HSG or laparoscopy. The unexplained infertile group (n=30) contained women with unknown causes of infertility. The normal fertile group (n=30), as control group, contained normal fertile women referred for oocyte donation who had regular menstrual cycles with normal fertility factor.

In all groups, the long agonist protocol for controlled ovarian hyperstimulation (COH) was used. Briefly, COH was performed by administration of human menopausal gonadotrophin (HMG, Ferring, Germany) after pituitary suppression with buserelin (superfact, Aboureyhan, Iran), starting in the midluteal phase of the preceding cycle. The dosages of gonadotropins were individualized according to serum estradiol (E_2) levels and transvaginally ultrasonic measurements of the follicles. When at least three follicles reached to diameter of 16-18 mm, ovulation was induced by the administration of 10,000 IU human chorionic gonadotropin (hCG) 36 hours before puncture. Average number of follicles of each case was 12 (9-16).

In each patient before oocyte aspiration, a peripheral blood sample was taken from antecubital vein one hour prior to puncture. Blood samples were transferred into sterile tubes. Then under general anesthesia (same protocol), a surgeon performed the ultrasound-guided transvaginal oocyte aspiration using a 16-17 gouge long needle. The follicular fluid samples were carefully collected from the first aspirated follicle of each ovary, and the follicular fluid samples without visible blood contamination were used in this study. The oocyte retrieval was continued for all IVF candidates. Blood and follicular fluid samples were immediately centrifuged at 3000 rpm for 10 minutes and the supernatants were stored at -70˚C for further analysis.

To measure the serum and follicular fluid leptin concentrations, enzyme-linked immunosorbent assay (ELISA) kit (Labor Diagnostica Nord GmbH, Germany) was used. All measurements were carried out in duplicate. The intra- and inter- assay coefficients of variation were less than 3.7 and 6.8%, respectively, with the standard range of 0.5-100 ng/ml.

Statistical analysis

To present descriptive statistics, mean ±standard deviation (SD) was used. In order to compare follicular fluid and serum leptin concentrations among three groups, Kruskal Wallis test was used. To compare follicular fluid and serum leptin concentrations within groups, Mann Whitney test was used. All statistical analyses were performed throughthe Statistical Package for the Social Sciences (SPSS, SPSS Inc., Chicago, IL, USA) version 16 and P value less than 0.05 was considered as statistically significant.

Results

In the present study, 90 women including 30 explained infertile women (group 1), 30 unexplained infertile women (group 2) and 30 fertile women (group 3) were studied. Demographic and clinical data regarding subsequent IVF of these groups are shown in table 1. The age and BMI displayed no significant differences among three groups. Progesterone, testosterone, FSH and LH levels were not significantly different ($P>0.05$ for all). The number of transplanted embryos demonstrated no significant difference. The percentage of fertility rate and good quality embryos in unexplained infertile group were lower in comparison to explained and control groups, but differences were not significant ($P>0.05$).

In explained infertile group, mean follicular fluid leptin level (19.92 ± 17.87) was lower than mean serum leptin level (33.13 ± 17.31), but the difference was not significant ($P=0.11$). In unexplained infertile group, mean follicular fluid leptin level (48.9 ± 20.20) was significantly ($P<0.001$) higher than mean serum leptin level (27.83 ± 25.29).

In fertile group, mean follicular fluid leptin level (25.07 ± 22.36) was lower than mean serum leptin level (31.27 ± 11.02), but the difference was not significant (P=0.19,Table 2). Mean follicular fluid leptin levels showed a significant difference within the groups (P<0.001). Among the groups, mean follicular fluid leptin level was higher in unexplained infertile women (group 2), fertile women (group 3) and explained infertile (group 1), respectively. In regard to mean serum leptin, explained infertile women had higher mean serum leptin level (33.13 ± 17.31) compared to unexplained infertile women (27.83 ± 25.29) and fertile women (31.27 ± 11.02), but the difference was not significant (P=0.070, Fig.1).

Follicular fluid and serum leptin levels in the each subgroup of explained infertile women were determined and the results were compared with unexplained infertile and fertile woman. Follicular leptin level in all explained infertility subgroups was significantly lower in comparison to unexplained and fertile groups (P<0.001), but serum leptin level in the explained infertile subgroups showed no significant difference as compared with unexplained infertile and fertile groups.

Table 1: Demographic data and clinical characteristics

	Median (range)			
	Unexplained infertile women (n=30)	**Explained infertile women (n=30)**	**Fertile women (n=30)**	**Significance**
Age (Y)	30 (20-38)	31 (25-40)	29 (20-38)	>0.05
BMI (kg/m^2)	24.84 (20.08-24.34)	23.38 (21.01-24.58)	22.26 (20.17-24.71)	>0.05
No. of follicles >14 mm (after COH)	12 (7-14)	13 (9-15)	12 (7-15)	>0.05
No. of oocytes	8 (7-10)	10 (6-13)	9 (7-14)	>0.05
Fertility rate %	76.90	78.80	79.75	>0.05
Good quality embryo (%)	68.20	73.92	75.60	>0.05

No; Number, BMI; Body mass index and COH; Controlled ovarian hyperstimulation.

Table 2: Leptin concentration in serum and follicular fluid of three studied groups

	Leptin level		
Group	**Follicular fluid**	**Serum**	**P value***
Explained infertile	19.92 ± 17.87[&]	33.13 ± 17.31[¥]	0.110
Unexplained infertile	48.90 ± 20.20[#]	27.83 ± 25.29	<0.001
Fertile	25.07 ± 22.36	31.27 ± 11.02	0.19
P value**	<0.001	0.07	

*; Mann-Whitney test, **; Kruskal-Wallis test, [&]; There is a significant difference compared to unexplained infertile group, [¥]; Data are shown as mean ± standard deviation and [#]; The unit of measurement is ng/ml. P<0.05 is considered statistically significant.

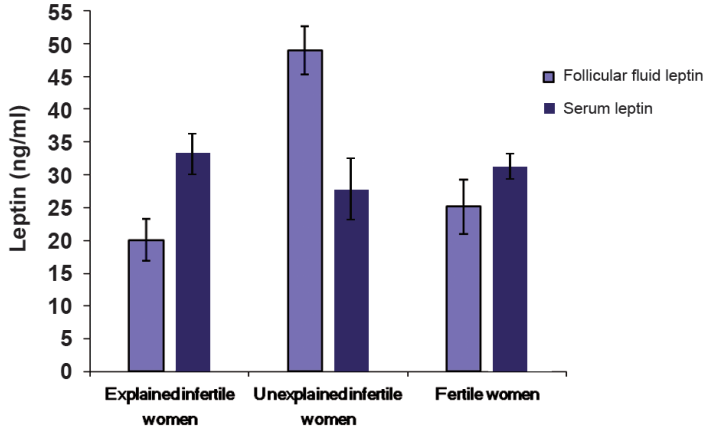

Fig.1: Comparison of leptin concentration in follicular fluid and serum of three studied groups (n=30/per group). Data are presented as mean ± SD.

Discussion

In the current investigation, follicular fluid leptin level in unexplained infertility group was more than that in explained infertility and normal fertile groups.

Infertility has been always addressed as one of the important, serious and costly health issues in different societies (1, 2). For this, various studies have already been performed to discover the causes of infertility, among them high leptin level has been considered as one of the important and effective factors in several studies (12).

Leptin is the product of the OB gene (13). It is involved in body weight control via inhibition of food intake and enhancement of energy expenditure. It is also recognized as an important hormone regulating ovarian function, so is closely related to infertility (14). Malfunction of the leptin system may impair human reproduction through altering hypothalamic and/or pituitary function, affecting ovarian function through direct action on the ovarian follicle and other mechanisms including induction of insulin resistance, hyperandrogenism, and elevated leptin levels (15, 16). Leptin levels are pulsatile and follow a circadian rhythm, with highest levels between midnight and early morning and lowest levels in early- to mid-afternoon, while after only 2 or 3 days of fasting, leptin levels drop to 40 or 10% of baseline, respectively (8).

Leptin has a wide range of functions from acting as an anti-obesity factor to an effective factor in reproduction, hematopoiesis, angiogenesis and T lymphocytes system (17).

Some investigators have suggested that leptin might exert a double role in regulation of reproduction. They showed that when leptin level is lower than normal, it can exert a negative effect on endocrine system, regulating reproduction, while when leptin level is higher than normal, it negatively affects normal function of ovary and fetus development (18, 19).

The present study suggested that high level of follicular fluid leptin has a negative effect on reproduction in unexplained infertility. Enhanced leptin level may inhibit aromatase activities and prevent the transformation of androgen to estrogen, leading to the elevation of serum androgen and interferers with ovarian follicle growth and ovulation by suppressing estrogen production (20).

A recent study revealed that serum leptin level is significantly higher in unexplained infertile women compared to the fertile group and suggested that leptin as cytokine-like or hormone affects pathophysiology of infertility, but due to study limitation, leptin levels in serum and peritoneal fluid were not compared (19).

In a study with Takeuchi and Tsutsumi (21), serum leptin level in unexplained infertility group was higher compared to polycystic ovary syndrome (PCOS) group, but difference was not sig-

nificant, whereas in a study with Demir et al. (22), comparison of serum leptin levels in unexplained infertile woman and fertile woman demonstrated significant higher serum leptin levels in unexplained infertile woman. However, in the present study, unexplained infertile group had lower serum leptin level in comparison to the explained infertile and fertile groups, but the difference was not significant. It seems that further studies are required to clarify this point.

Gogacz et al. (10) have reported a significant increase in peritoneal fluid leptin level in endometriosis and unexplained infertility. They had no control group for comparison and suggested similar studies containing a control group. They also suggested peritoneal fluid leptin might be originated from follicular fluid. To consider the mentioned study and association between the increased level of peritoneal leptin with endometriosis and unexplained infertility, it can be proposed that leptin stimulates toxic factors in peritoneal fluid and also decreases the quality of oocyte in endometriosis. It should be noted that the cause of unexplained infertility needs to be identified with new advances in infertility treatment and leptin is likely to be considered as one of the involved factors.

Another study represented that peritoneal fluid leptin level was significantly higher in unexplained infertile group compared to patients with PCOS. In the mentioned study, serum leptin level was also higher in unexplained infertile group but not significantly (22).

The results of our study showed significant higher follicular fluid leptin level in unexplained infertile women compared to the explained infertile and fertile groups, but serum leptin level in this group was insignificantly lower than the explained infertile and fertile groups. Fertility rate and number of good quality embryos in unexplained infertile group were lower in comparison to explained and control group, but there were no significant differences. It seems that further studies are required to establish effect of high follicular leptin level on infertility. It seems that systemic effects of leptin in blood flow differ from its local effects in follicular fluid as it was observed in the present study that unexplained infertile group had higher follicular fluid leptin level and lower serum leptin level in comparison to the other two groups. Whether these effects act independently or not is a point deserving attention.

This relationship presents differently in persons with different BMI; for example, in patients with anorexia and low BMI and in obese individuals with high BMI, leptin shows contradictory effects. It can be hypothesized that a specified concentration of leptin is required for female productivity and both high and low levels of leptin can affect fertility. This effect is seen for both systemic leptin and follicular and peritoneal leptin (18).

In another study, systemic and central effects of leptin on gonadotropins have been less accepted and leptin effect on ovary has been considered more. There are more leptin receptors on ovary than in central nervous system (CNS) (19).

Conclusion

The obtained results showed that high leptin level in follicular fluid affect unexplained infertility. Therefore, our finding suggested that high leptin level of follicular fluid is one of the main factors involved in infertility. The fact that whether leptin acts independently or as an associated factor requires more studies.

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Original Article

Cut-Off Levels of Anti-Mullerian Hormone for The Prediction of Ovarian Response, *In Vitro* Fertilization Outcome and Ovarian Hyperstimulation Syndrome

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Abstract

Background: Evaluation of anti-mullerian hormone (AMH) cut-off levels in assisted reproductive technology (ART) as predictive factor for individualization of stimulation protocols and to avoid ovarian hyperstimulation syndrome (OHSS).

Materials and Methods: In a retrospective study, 177 infertile patients were assessed for AMH in serum and follicular fluid (FF) on the day of follicular puncture (FP), between 2012 and 2013 in Kiel, Germany. AMH levels and pregnancy rates were compared between low, moderate and high responders and cut-off levels of low and high responders. AMH cut-off levels in pathological cases were evaluated in analysis 1 (OHSS) and in analysis 2 [polycystic ovarian syndrome, (PCOS)] and compared in analysis 3 to normal endocrinological parameters.

Results: AMH levels in FF were higher than in serum (P<0.001). AMH levels in serum and FF increased from low through moderate to high responders (P<0.001). Pregnancy rates were 14.7, 23.3 and 44.9% (P=0.009), respectively. AMH cut-off level for poor responders was 0.61 ng/ml in serum with a pregnancy rate of 13.8 and 37.1% for below and above of this level, respectively. For FF, it was 1.43 ng/ml. AMH levels in analysis 1 and 2 were significantly higher than in analysis 3 (P=0.001). AMH cut-off level for OHSS was 1.5 ng/ml in serum with OHSS rates of 80.8 and 19.2 % for above and below of the level, respectively. For FF, it was 2.7 ng/ml. PCOS patients had an AMH cut-off level of 3.9 ng/ml in serum and 6.8 ng/ml in FF, resulting in a PCOS rate of 100% above this level.

Conclusion: AMH levels can help to assess ovarian response potential and guide ovarian stimulation while avoiding OHSS.

Keywords: Anti Mullerian Hormone, Ovarian Hyperstimulation Syndrome, Cut-Off Levels, Pregnancy Rate

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Introduction

Anti-mullerian hormone (AMH) in the female ovary is produced by granulosa cells of pre-antral and antral follicles (1). The main physiological role of AMH in the ovary is limited to the inhibition of the early stages of follicular development (2-6).

According to current literature, AMH appears to be a promising and reliable marker of the number of small antral follicles, with essentially constant levels across the cycle and a superior intercycle reproducibility compared with that of follicle stimulating hormone (FSH) and early antral follicle count (7-13). Hence, it has the potential to determine the plan of ovarian stimulation in an assisted reproductive technology (ART) cycle. As AMH levels steadily decline with age from adulthood toward menopause, AMH is a promising parameter for early detection of reduced ovarian reserve as well as ovarian dysfunction (12, 14-17). Patients with ovarian hyperstimulation syndrome (OHSS) have high serum levels of AMH prior to controlled ovarian stimulation (COS). For these patients, the COS protocol can be individualized to suit their requirements (6, 18, 19). The specific risk factors for OHSS include young age, low body mass index (BMI) and signs of polycystic ovarian syndrome (PCOS) (20-22). PCOS affects 5-20% of women of reproductive age and is the primary cause of anovulatory infertility, with an increased number of antral follicles and a resulting rise of AMH (11, 23, 24).

Serum AMH levels in women with PCOS are higher than in ovulatory women. However, urgently needed cut-off levels of AMH in patients with endocrinological risk factors, such as PCOS and the hormonally induced overreaction of the ovarian response as in OHSS, are still missing to support the clinical decision (11, 25-28). In this retrospective study, we attempted to assess and compare the mean and cut-off levels of AMH in serum and follicular fluid (FF) on the day of oocyte retrieval in response to ovarian stimulation with recombinant follicle stimulating hormone (rFSH) and their relation to pregnancy rates. Furthermore, we planned to identify the AMH mean and cut-off levels in serum and FF in patients with endocrinological risk factors and pathological factors, such as OHSS or PCOS, and compare these to the values of patients without these risk factors.

Materials and Methods

Patients

For this retrospective study, we collected serum and follicular fluid for the first or second treatment cycle of 177 patients undergoing *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (between 2012 and 2013) in Kiel. Serum and FF were collected on the day of follicular puncture (FP). The age of the patients ranged from 20 to 42 years, median 33 years, and the size of the leading follicle on the day of follicular puncture measured between 19 and 24 mm. The FF for AMH analysis was only aspirated from the first dominant follicle. All 177 patients presented with tubal or male factor infertility.

The patients were analysed repeatedly and pathological cases were evaluated in analysis 1 (OHSS) and analysis 2 (PCOS) and compared to normal endocrinological parameters (analysis 3). All patients treated (n=177) revealed in their initial endocrine check, on days 3-5 of the cycle, an AMH value >0.5 ng/ml and a FSH value <8 IU/ml. For analysis 2 and 3, we specially collected the cases between 2012 and 2013.

AMH levels of all patients were analysed for:

• Correlation between serum and FF with respect to AMH levels and correlation among serum AMH, serum estradiol (E_2), the number of follicles, injected dose of rFSH and age of patients.

• Evaluation of mean AMH and cut-off levels in serum and FF in response to ovarian stimulation with rFSH in low (n=41), moderate (n=66) and high (n=70) responders and pregnancy rates.

In analysis 1: AMH cut-off levels were evaluated for patients with hormonally induced overreaction of the ovary, such as OHSS (n=26). In analysis 2: AMH levels were evaluated for patients with the endocrinological risk factor, PCOS (n=30) and in versus analysis 3: patients with normal endocrinological parameters (n=121).

Analysis 1 consisted of patients with peak serum levels of E_2>3000 pg/ml on the day of ovulation induction and with signs or symptoms consistent with OHSS, such as ultrasonographic evidence of ascites and increased ovarian size of 8-12 cm, abdominal bloating and pain, or considerable weight

gain (18, 29).

Analysis 2 consisted of patients with signs of PCOS who were diagnosed by the Rotterdam criteria with two of the following three manifestations: irregular or absent ovulation, elevated levels of androgenic hormones and/or enlarged ovaries containing at least 12 follicles each (30).

Ovarian stimulation

rFSH (Gonal F, Merck, Serono, Munich, Germany) after down-regulation with gonadotropin-releasing hormone agonist (GnRH-a) (Synarela, Pharmacia, Erlangen, Germany) was used in the long protocol. The FSH doses were adapted according to the following criteria: age of patient, number of antral follicles, AMH, basal FSH and patient's diagnosis. Monitoring of follicle development by real-time ultrasound scans and serum E_2 levels was performed from day 6 of stimulation every two to three days until the day of human chorionic gonadotropin (hCG) application. Ideally, once the leading follicle measured >16 mm in diameter and the 17 β - E_2 level had adequately increased ideally to around 3000 pg/ml in serum, 6500 IU of hCG were administered subcutaneously. The number of follicles was determined on the day of ovulation induction (n=10.2 ± 6.4). Progesterone (Pr) levels were measured parallel to E_2 and luteinizing hormone (LH). Follicles were aspirated 36 hours after administration of hCG. After embryo transfer (ET), the patients were treated with Pr vaginally (Utrogest, 600 mg daily, Dr. Kade/Besins, Berlin, Germany) for luteal support until confirmation of pregnancy by beta-hCG (β-hCG) determination, 14 days after ET.

In response to ovarian stimulation, the patients were sub-grouped as low, moderate and high responders, according to a scoring system based on the total injected dose of rFSH [± standard deviation (SD)] up to the day of hCG injection, the increase in E_2 levels, the age of patients and the number of follicles (low ≤7, moderate=8-14 and high ≥15) on the day of ovulation induction (31-33). Only the clinically continuing pregnancy rates per ET were evaluated.

Biochemical analyses

Anti-mullerian hormone assay in serum

Blood and FF were taken from all patients undergoing IVF or ICSI and ET on the day of follicular puncture, processed by being centrifuged for 10 minutes at 350×g and 5˚C, shock-frozen and kept at −80˚C. After pick-up of the oocytes, the FF samples underwent the same procedures as the blood. AMH levels in serum and FF were measured in duplicate by a solid-phase enzyme-linked immunosorbent assay (ELISA) using an AMH kit (AMH Gen II Assay, Immunotech, Beckman Coulter Company, Kiel, Germany). This assay uses the quantitative sandwich enzyme immunoassay technique. The AMH precision from manufacture was CV=3.2-12.3% for intra-assay and CV=5.8-14.2% for inter-assay. Only those cases in which both FF and serum could be collected simultaneously on the day of oocyte retrieval were included in this study.

Estradiol assay in serum

E_2 levels were measured by a solid phase, competitive chemiluminescent enzyme immunoassay with the Immulite 2000 auto system (DPC-Biermann; Siemens, Bad Nauheim, Germany) within the range of 0–2000 pg/ml for E_2 (sensitivity 15 pg/ml).

Statistical evaluation

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, SPSS Inc., Chicago, IL, USA) version 20. Based on the Kolmogorov-Smirnov test, normal distribution for most of the parameters could not be assumed. Therefore, in descriptive statistics median values and interquartile ranges (IQR) were given additionally to means and SDs. Nonparametric test procedures were used for statistical evaluation of the study data.

We performed a Kruskal-Wallis test to analyse differences in AMH levels between patients with low, moderate and high response to ovarian stimulation. In the case of any significance, pairwise comparisons between different groups were performed with the U test in an exploratory intention. The differences in pregnancy rates between low, moderate and high response patients were analysed according to a chi-squared test. Association between serum and FF with respect to AMH and between AMH and E_2 in

serum was measured and tested by Spearman rank correlation coefficients (r_s).

A P value of P<0.05 was considered to be statistically significant throughout the study. To evaluate an AMH cut-off value in serum and in FF related to patients with (low/moderate and high) response for ovarian stimulation as well as to patient's risk for OHSS or PCOS, a receiver operating characteristic (ROC) analysis was performed to achieve minimal false positive and false negative results. The U test was also used to evaluate the hypotheses of differentiation [area under the curve (AUC) >0.5, i.e. low responder vs. moderate and high responder].

Ethical considerations

An informed consent was obtained from all patients in the university IVF program. Under the stipulations of the Universitaetsklinikum Schleswig-Holstein (UKSH), Kiel Institutional Review Board (IRB), an approval had not to be obtained for a retrospective observational study.

Results

AMH levels in serum and FF on the day of oocyte retrieval

The median AMH level in FF, 2.2 ng/ml (1.32-3.6) on the day of oocyte retrieval, was significantly higher than that in serum, 1.14 ng/ml (0.52-2.17, P<0.001). On the basis of non-normal distributed values of AMH levels in serum and FF, we found a positive correlation (Spearmann-Rho r_s= 0.88, P<0.001, Fig.1). Additionally, we observed a significant positive correlation between some characteristic clinical parameters and AMH in serum or in FF on the day of FP as follows: E_2 (r_s=0.43), number of follicles (r_s-=-0.71), number of total retrieved oocytes and oocytes in MII (r_s=0.34), number of fertilized oocytes (r_s=0.32) and a significant inversed correlation with regard to age (r_s=-0.55), total injected dose (r_s=-0.63) and BMI (r_s=-0.21). The median AMH level, 1.76 ng/ml (1.0-3.73) in serum and 2.9 ng/ml (1.77- 6.75) in FF, of patients who became pregnant was significantly higher than in those who did not become pregnant, 1.0 ng/ml (0.4-1.65) in serum and 1.8 ng/ml (1.0-2.9) in FF (P<0.004 and P<0.01, respectively).

Evaluation of AMH levels in response to ovarian stimulation and pregnancy rate

The AMH levels in serum and in FF of patients with ET and their response to ovarian stimulation with gonadotropins are summarised in table 1 and figure 2A, B.

AMH levels in serum and in FF increased significantly from patients with low response through moderate and reached a maximum in patients with high response. Similar to AMH, E_2, number of oocytes and pregnancy rate increased from low to high responders. In contrast, age and the injected total rFSH-dose showed a significant decrease.

The differences in AMH, total injected dose, E_2 concentrations, number of oocytes and pregnancy rate in serum and FF among the three groups were statistically significant (P<0.001). Paired comparisons of AMH levels and all other clinical parameters in serum and in FF were significant in all pairs (Table 1). The total pregnancy rate was 31.6% related to ET.

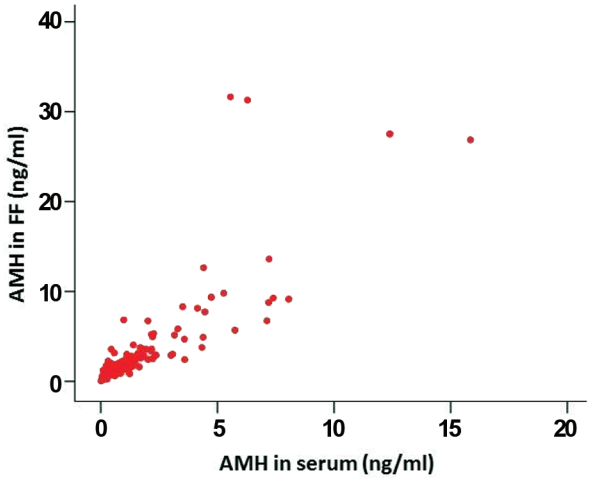

Fig.1: Correlation between AMH levels (n=177) in serum and in FF on the day of oocyte retrieval (r_s=0.88, P<0.001).

Table 1: 177 patients sub-grouped according to their response to ovarian stimulation with rFSH as low, moderate and high responders related to AMH values and pregnancy rates

Response	Low	Moderate	High	P value	*P
	(n=41)	(n=66)	(n=70)		
Variables	Mean ± SD	Mean ± SD	Mean ± SD		
	Median (IQR)	Median (IQR)	Median (IQR)		
Age (Y)	38 ± 4.1	35.7 ± 4.2	33.6 ± 4.4	0.001	0.07 [b],0.001 [c]
	39 (35-41)	37 (33-39)	33 (30-37)		0.011 [d]
Total mean level of rFSH (iu/ml)	4901 ± 1452	3204 ± 1311	2460 ± 977	<0.001	<0.00 [b, c]
	5400 (3525-6000)	2887 (2250-4200)	2250 (1762-3362)		0.005 [d]
E_2 (pg/ml)	527 ± 305	1021 ± 451	1285 ± 470	<0.001	<0.001 [b, c]
	590 (203-732)	954 (774-1269)	1297 (994-1594)		0.002 [d]
Number of oocytes	3.4 ± 1.5	7.5 ± 2.9	12.3 ± 5.5	<0.001	<0.001 [b, c, d]
	3 (2.5-4)	8 (5-10)	12 (8-16)		
AMH in serum (ng/ml)	0.54 ± 0.46	1.1 ± 1.0	3.03 ± 2.6	<0.001	0.001 [b]
	0.3 (0.13-0.54)	0.84 (0.42-1.32)	1.7 (1.3-4.36)		<0.001 [c, d]
AMH in FF (ng/ml)	1.3 ± 1.12	2.01 ± 1.18	5.94 ± 4.3	<0.001	0.001 [b]
	0.87 (0.45-1.41)	1.75 (1.24-2.56)	2.9 (2.07-6.78)		<0.001 [c, d]
Pregnancy rate	14.7%	23.3%	44.9%	0.009[a]	

rFSH; Recombinant follicle stimulating hormone, E_2; Estradiol, FF; Follicular fluid, AMH; Anti-mullerian hormone, IQR; Interquartile range, P; Kruskal-Wallis test, *P; Pair wise comparisons between sub-groups, [a]; Chi-square test, [b]; Low/moderate, [c]; Low/high and [d]; Moderate/high. Values are mean ± SD unless otherwise noted.

Evaluation of AMH cut-off levels in serum and FF in response to ovarian stimulation

a. Low responder

Figure 2 (c+d) shows the typical receiver ROC AUC for AMH, indicating low responders versus moderate and high responders (n=136) in serum (c) and in FF (d) on the day of FP.

We found an AMH cut-off level (based on best sensitivity and best specificity) in serum of 0.61 ng/ml and a pregnancy rate of 13.8 (n=6/44) below and 37.6% (n=50/133) above this cut-off level (Table 2). AMH cut-off level in FF was 1.43 ng/ml and revealed a pregnancy rate of 12.5 (n=5/40) below and 38.3% (n=51/133) above this cut-off level.

b. High responder

AMH cut-off level (based on best sensitivity and best specificity) in serum was 1.03 ng/ml for high responders versus low and moderate responders, and resulted in a pregnancy rate of 21.1 (n=16/76) below and 38.6% (n=39/101) above this cut-off level (Table 2).

The AMH cut-off level in FF was 2.23 ng/ml with a pregnancy rate of 21.5 (n=17/79) below and 39.8% (n=39/98) above this cut-off level.

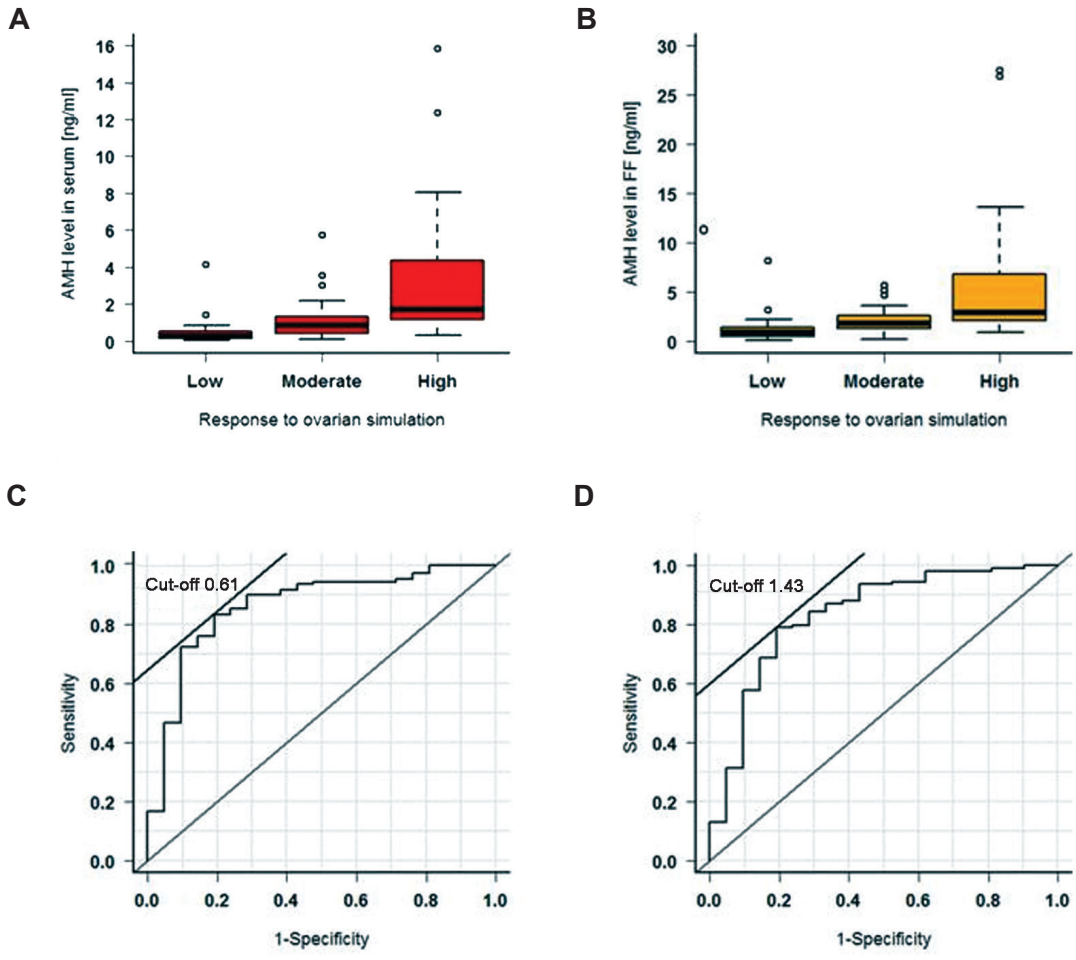

Fig.2: Relationship between anti-mullerian hormone (AMH, n=177) levels in serum **(A)**, in follicular fluid (FF) **(B)** and low, moderate and high response to ovarian stimulation (**C-D**). Receiver operating characteristic (ROC) area for low responder patients versus moderate and high responders in serum **(C)** and in FF **(D)** on the day of FP (Follicular Puncture).
The differences between low, moderate, and high responders were significant (P<0.001). See table 1 for paired comparisons according to the Mann-Whitney U test.

Table 2: Calculated cut-off levels of AMH predicting low and high responders in serum and in FF, sensitivity (true positive rate), specificity (true negative rate) and pregnancy rate

AMH	ROC-AUC	P value	Sensitivity %	Specificity %	Cut-off ng/ml	Pregnancy rate % ≤ cut-off	Pregnancy rate % > cut-off
Serum [a]	0.86	<0.001	83.5	81	0.61	13.8	37.6
FF [a]	0.84	<0.001	78.9	81	1.43	12.5	38.3
Serum [b]	0.83	<0.001	87.7	71.6	1.03	21.1	38.6
FF [b]	0.80	<0.001	73.9	75.4	2.23	21.5	39.8

AMH; Anti-mullerian hormone, FF; Follicular fluid, ROC-AUC; Receiver operating characteristic-area under curve, [a]; Low versus both moderate and high and [b]; High versus both low and moderate.

Comparisons of AMH levels between patients with OHSS, PCOS and patients with normal endocrinological parameters

Patients with OHSS (analysis 1) or PCOS (analysis 2) revealed a significantly lower median level of total injected dose of rFSH and a higher count of follicles, suggesting higher level of E_2 (on the day of hCG injection) than patients with normal endocrinological parameters (analysis 3) (Table 3). A significantly higher number of oocytes was obtained on the day of FP only from patients with OHSS. As seen in table 3 and figure 3a-b, patients with normal endocrinological parameters revealed the lowest levels of AMH and patients with an endocrinological risk of PCOS showed the highest mean levels of AMH for serum and for FF.

According to the Mann-Whitney test, paired comparisons of AMH levels in serum and in FF between sub-groups were significant: OHSS/normal (P=0.009) and PCOS/normal (P<0.001).

Table 3: Comparison between patients with normal endocrinological parameters and patients with OHSS or PCOS

	Normal (n=121) Mean ± SD Median (IQR)	**OHSS (n=26) Mean ± SD Median (IQR)**	**PCOS (n=30) Mean ± SD Median (IQR)**	**P value**	***P**
Total injected dose (IU/ml)	3394 ± 1480	2477 ± 725	2084 ± 1194	<0.001	0.018 [a]
	3000 (2325-4500)	2100 (1912-2850)	1575 (1200-3350)		0.002 [b]
Number of follicles	14 ± 6.4	23.5 ± 6.5	27.3 ± 9.5	<0.001	<0.001 [a]
	13.5 (8-19)	24 (18-28)	26 (23-35)		<0.001 [b]
Number of oocytes	8.1 ± 4.5	15.9 ± 6.2	8.6 ± 5.8	<0.001	<0.001 [a]
	8 (4.2-11)	14 (10-21)	8 (3-12)		NS [b]
E_2 (pg/ml)	1844 ± 536	2907 ± 1430	2759 ± 1143	<0.01	<0.001 [a]
	1795 (1275-2165)	3063(1911-3371)	2561 (1860-3094)		<0.001 [b]
Mean AMH Serum (ng/ml)	1.2 ± 1.1	2.52 ± 2.1	7.2 ± 3.5	0.001	<0.001 [a]
	0.85 (0.4-1.4)	1.64 (1.38-3.1)	6.3 (4.4-7.4)		<0.001 [b]
Mean AMH FF (ng/ml)	2.1 ± 1.4	4.4 ± 3.1	14.5 ± 8.5	0.014	0.009 [a]
	1.7 (1.05-2.8)	2.52 (2.27-3.1)	9.35 (8.3-26.8)		<0.001 [b]

OHSS; Ovarian hyperstimulation syndrome, PCOS; Polycystic ovarian syndrome, E_2; Estradiol, AMH; Anti-mullerian hormone, FF; Follicular fluid, IQR; Interquartile range, [a]; OHSS/normal and [b]; PCOS/normal. Total injected dose of rFSH up to the day of hCG injection, E_2 level on the day of hCG injection. The differences between these 3 groups were analysed by the Kruskal-Wallis test (P) and paired comparisons (*P) by the Mann-Whitney U test.

Evaluation of AMH cut-off levels in serum and in FF in patients with OHSS or PCOS

We found an AMH cut-off level (based on best sensitivity and best specificity) in serum of 1.5 ng/ml, and in FF of 2.7 ng/ml (Table 4, Fig.3C, D), between patients with OHSS and those without, occasioning an OHSS rate of 19.2 (5/26) below and 80.8% (21/26) above these levels. Additionally, we found that 90% of patients with OHSS had an AMH level below 4 ng/ml in serum.

The AMH cut-off level in serum for PCOS in comparison to normal patients was 3.9 ng/ml, with a high sensitivity and specificity, resulting in a PCOS rate of 100% above this level.

In FF, the cut-off level was 6.8 ng/ml, also resulting in a PCOS rate of 100% above this level.

Table 4: Calculated cut-off level of AMH predicting OHSS and PCOS in serum or in FF, specificity (true negative rate), sensitivity (true positive rate)

		ROC-AUC	P value	Sensitivity (%)	Specificity (%)	Cut-off level (ng/ml)	Risk of ≤ cut-off level %	Risk of > cut-off level %
OHSS (n=26)	Serum	0.79	<0.001	79	73	1.5	21	79
	FF	0.73	0.004	79	66	2.7	21	79
PCOS (n=30)	Serum	0.98	<0.001	93	97	3.9	0	100
	FF	0.97	<0.001	93	98	6.8	0	100

OHSS; Ovarian hyperstimulation syndrome, PCOS; Polycystic ovarian syndrome, AMH; Anti-mullerian hormone, FF; Follicular fluid and ROC-AUC; Receiver operating characteristic-area under curve.

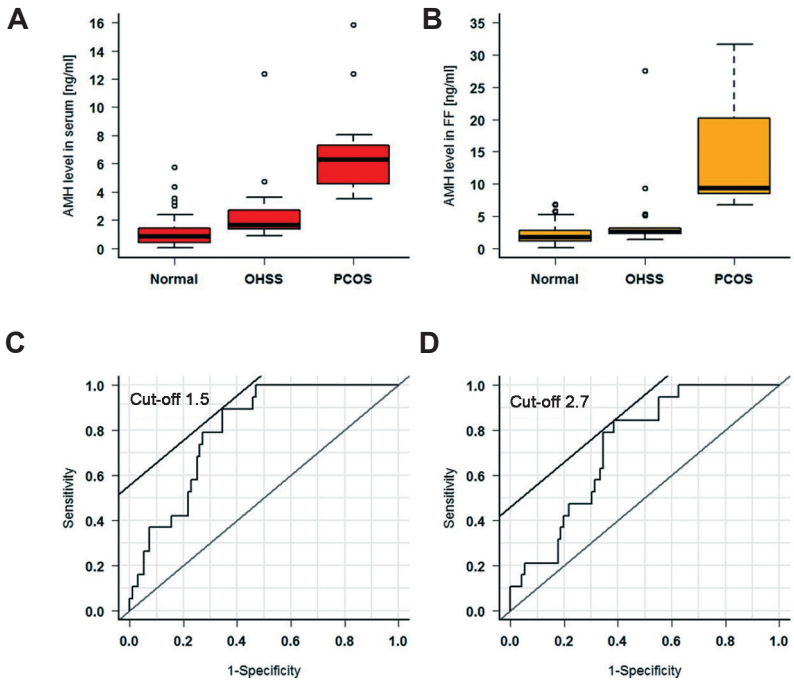

Fig.3: Comparison of anti-mullerian hormone (AMH) levels in serum **(A)**, in follicular fluid (FF) **(B)** between patients with normal endocrinological parameters and patients with ovarian hyperstimulation syndrome (OHSS) or polycystic ovarian syndrome (PCOS) **(C-D)**. Typical receiver operating characteristic (ROC) for AMH level in serum **(C)** and in FF **(D)** for OHSS patients versus normal patients.

Discussion

In this study, we analysed for the first time simultaneously the AMH levels in serum and in FF on the day of oocyte retrieval and compared them between patients with OHSS and PCOS with nomal endocrinological parameters.

Our results showed that AMH concentrations in FF are significantly higher than in serum, as found in the study of Takahashi et al. (34). This implies an intrafollicular production and a potential autocrine or paracrine role of AMH within the follicular environment. AMH is expressed only in the ovarian granulosa cells of primary follicles and plays an important role in ovarian function, especially in follicle differentiation, development and selection (1, 5, 10, 34). The mean AMH levels in serum and in FF of patients who became pregnant were significantly higher than in those who did not become pregnant. Some studies have also reported that AMH can predict pregnancy (7, 13, 34, 35). AMH levels in serum and in FF on the day of FP increased significantly with the decreasing age of patient and with an increasing follicle count on the day of hCG injection. Similar results in serum on day three of the cycle have shown that the loss of follicles with increasing female age is variable and that the chronological age of the ovary does not always reflect its biological and reproductive age (14, 36).

With regard to the response to ovarian stimulation with rFSH, AMH levels increased in serum and in FF significantly from low through moderate to high responders with a respective pregnancy rate of 14.7, 23.3 and 44.9 %. Thus, AMH levels in serum and in FF may reflect successful stimulation and ample follicle maturation.

The characterisation of AMH as a sensitive marker for poor ovarian reserve (13, 18, 37-39) was further evaluated by our AMH cut-off levels for low and high responders with a high sensitivity and specificity. Our evaluated cut-off levels for AMH are in the range proposed by the European Society of Human Reproduction and Embryology (ESHRE) consensus meeting (cut-off level from 0.5-1.1 ng/ml) (33). With these cut-off levels and a very high accurancy for AMH (AUC=0.86 in serum), there were distinct significant differences in the pregnancy rate between low responders (13.8 below versus 37.1% above) and high responders (21.7 below versus 39.8% above). Broer et al. (40) also found similar levels of AUC=0.78 and for AMH, AUC predicting poor response. In accordance with these cut-off levels, patients can be counselled regarding the expected outcome of ovarian response, number of follicles and the anticipated cost of ovarian stimulation drugs, thereby reducing the emotional and financial burden of cycle cancellation. Fleming et al. (35) also reported that AMH is one of the best accepted markers of ovarian reserve and a strong marker for response to stimulation.

We found a significant inverse correlation between AMH level and BMI, which is in good agreement with other authors (41, 42). OHSS represents one of the most serious complications subsequent to COS and can be life-threatening (43). The mean AMH levels in serum and in FF were significantly higher in patients with OHSS and PCOS than in normal patients.

Other authors predict a basal AMH cut-off level of around 3.36 ng/ml for OHSS with a high sensitivity and specificity (22, 43, 44), indicating high rates of OHSS above this level. Our evaluated AMH cut-off level for OHSS patients of 1.5 ng/ml in serum (patients with PCOS excluded) corresponds well with the evaluated level of 1.6 ng/ml found by Ebner et al. (45). This level revealed a high sensitivity, specificity and high accuracy of AUC, resulting in significantly distinct differences in the OHSS rate of 19.2 below versus 80.8% above these levels. About 90% of patients with OHSS had a serum AMH below 4 ng/ml, suggesting that clinicians have become cautious in stimulating patients with an AMH above 4 ng/ml. It has been reported that AMH levels in serum decline gradually during controlled ovarian hyperstimulation (COH), whereas other hormones, such as E2, inhibin A, inhibin B and Pr, increase (46, 47). It has been suggested that this reflects the reduction in the number of small antral follicles parallel to the increase in the number of larger ones. This could also indicate that basal AMH levels and AMH levels on the day of FP differ considerably and should be investigated further.

The assessed AMH cut-off level for PCOS patients in serum and in FF showed a high sensitivity and specificity resulting in all patients, showing an AMH value above this level.

Supporting our results, other authors have also

reported a relatively high specificity of 92% but a low sensitivity of 67% of AMH as a diagnostic marker for PCOS (6, 11). On this basis, it has been proposed that in situations where accurate ultrasound data are not available, AMH could be used in addition to the follicle count as a diagnostic criterion for PCOS (11, 48).

Conclusion

AMH levels are a sensitive parameter for the prediction of response to ovarian stimulation with gonadotropins. The levels can be used as a tool for pre-stimulation patient counselling regarding the expected ovarian response (poor, moderate and high) and outcome (pregnancy rate, OHSS and cycle cancelation). Additionally, it can be used as a marker for PCOS. Its application for guiding appropriate stimulation protocols can be used to avoid OHSS.

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Original Article

Pregnancy Outcome after Office Microhysteroscopy in Women with Unexplained Infertility

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Abstract

Background: Hysteroscopy offers diagnostic accuracy and the ability to treat uterine pathology. The current study aimed to review the findings and feasibility of the proposed office-based diagnostic and operative microhysteroscopy in previously diagnosed women with unexplained infertility and to evaluate the post-microhysteroscopic pregnancy outcome in a-year follow-up period.

Materials and Methods: This prospective controlled study was conducted between 2006 and 2013. Two hundreds women with unexplained infertility were randomized into two groups: A. study group including 100 women recruited for office micohysteroscopic session and B. control group including 100 without the proposed microhysteroscopic intervention. A malleable fiberoptic 2-mm, 0 and 30 degrees angled hysteroscopy along with an operative channel for grasping forceps, scissors, or coaxial bipolar electrode were used for both diagnostic and operative indications. The findings, complications, and patient tolerance were recorded. A-year follow-up of pregnancy outcome for both groups was also performed.

Results: Seventy cases (70%) of patients had a normal uterine cavity. Twenty women (20%) had endometrial polyps. Other pathology included submucous myomas in 3 cases (3%), intrauterine adhesions in 3 cases (3%), polypoid endometrium in 3 cases (3%), and bicornuate uterus in one case (1%). The pathological findings were treated in all patients without complication. Also a-year follow-up of the total developing cumulative pregnancy rate (CPR) was evaluated in groups A and B (control). Group A revealed the total CPR of 28.5%, among which 25% in women without pathology, 40% in women with endometrial polyps, 23% in women with adhesions, 33% in women with polypoid endometrium, and 21% in those with bicornuate uterus. However, A-year follow-up of spontaneous pregnancy outcome in group B showed a total CPR of 15%.

Conclusion: Women tolerance, safety, and feasibility of simultaneous operative correction make the proposed office microhysteroscopy an ideal and routine procedure in order to diagnose and to treat missed intrauterine abnormalities, especially in cases with unexplained infertility, with additional improvement of the pregnancy outcome after the procedure.

Keywords: Abnormal Uterine Bleeding, Polyps, Fibroids, Cost-Effectiveness

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Introduction

Hysteroscopy is still considered the gold standard procedure for uterine cavity exploration. Hysteroscopy is only recommended by the World Health Organization (WHO) when clinical or complementary exams [ultrasound or hysterosalpingogram (HSG)] suggest intrauterine abnormality or after *in vitro* fertilization (IVF). However, many gynecologists feel that hysteroscopy is a more accurate tool because of the high false-positive and false-negative rates of intrauterine abnormality with HSG. Therefore, many specialists have used hysteroscopy as their first-line of routine exam for infertility patients regardless of guidelines (1-5).

Recently, Hystero-Salpingo-Contrast-Sonography (HyCoSy), saline infusion sonography (SIS) and gel infusion sonography (GIS) are inexpensive and non-invasive techniques, while they have been shown to be excellent diagnostic tools to detect subtle intrauterine abnormalities, but they are still so many missed diagnosis. Office hysteroscopy has been increasingly recommended as a routine procedure in the infertility work-up. It has become easy to perform in an outpatient setting without anesthesia. Moreover, it offers direct visualization and enables specialists to diagnose and to treat intrauterine pathology during the same office session (6-10).

Although hysteroscopy is generally accepted as the gold standard in diagnosis and treatment of uterine cavity pathology, many gynecologist are reluctant to perform hysteroscopy as an initial test without a high degree of suspicion for pathology due to the need for anesthesia in an operating room setting. Therefore, the advent of smaller diameter instruments makes office-based operative hysteroscopy as an ideal first-line procedure and can efficiently treat infertile patients with uterine abnormalities in the same setting, thus facilitating a rapid transition from diagnosis to treatment and subsequent pregnancy (11, 12).

The objective of this study was to review office-based diagnostic and operative microhysteroscopic findings and treatment in women with unexplained infertility to evaluate whether microhysteroscopy should be recommended to these patients who had the diagnosis of missed uterine abnormalities and to evaluate the impact of this proposed office procedure on subsequent pregnancy outcome for those women.

Materials and Methods

Two hundreds infertile women, previously diagnosed as unexplained infertility, were recruited for the study between 2006 and 2013. The participants were randomized using a computer software into two groups: A. study group including 100 infertile women who were shortlisted for the studied office microhysteroscopic procedure and B. control group including 100 women with unexplained infertility who were followed up without the proposed office microhysteroscopic intervention. The demographic characters of the women are shown in table 1. The institutional ethical board approval was obtained for women in both groups recruited in Arafa Hospital (a private hospital) in Fayoum city. Each couple signed an appropriate informed consent for the procedure.

Table 1: Demographic characters of the women included in the study

Parameter	Cases (n=100)	Control (n=100)
Age (Y)	25 ± 5	26 ± 3
Menarche age (Y)	12.5 ± 2.5	11.1 ± 3
Regular cycles	89 ± 4	90 ± 3
Weight (kg)	60 ± 5	57 ± 4
Height (m)	1.57 ± 2.3	1.61 ± 1.6
BMI (kg/m^2)	24 ± 3.6	23 ± 1.7
Type of infertility		
Primary	70	75
Secondary	30	25
Duration of infertility	2 ± 2.1	2.1 ± 1.3
Previous ART:		
IUI	40 cycles	38 cycles
ICSI	12 cycles	11 cycles

BMI; Body mass index, ART; Assisted reproductive techniques, IUI; Intra-uterine Insemination and ICSI; Intra-cytoplasmic sperm injection.

All office microhysteroscopies were performed using a malleable 0-degree diagnostic and 30-degrees operative 2-mm fiberoptic microhysteroscope (Circon, Germany) with an operative channel for the use of grasping forceps, scissors, or coaxial bipolar electrode. Instruments were placed through the built in operative channel when needed for treatment of pathology after the diagnostic portion had been completed. Typically, less than 1 liter of normal saline was used as the distention media for procedures, except with myomectomies which occasionally required larger volumes.

Operative procedures including hysteroscopic resection of endometrial polyps and submucous myomas, excision of intrauterine septum and postoperative management plan for bicornuate uterus were performed, where another conventional operative session for bicornuate uterus was arranged by another team. For those longer cases, fluid balance was monitored by ancillary staff throughout the procedure. Diagnostic findings, operative outcomes, complications, and patient tolerance during the procedure were noted.

The coaxial bipolar electrode surgical system (Versapoint, Gynecare, NJ) was used for myomectomies. Power settings were from 60 W (desiccation) to 130 W (cutting). Office microhysteroscopies were performed during the early postmenstrual period. Patients received oral premedication with midazolam (Sigma, Egypt), intramuscular analgesia with diclofenac (Epico, Egypt), and a paracervical uterine block with 1% lidocaine (Kahira, Egypt). Five patients requested conscious sedation with intravenous fentanyl (Cid, Egypt) and midazolam in place of the above regimen. All women were discharged immediately after the procedure, except those who were discharged after 2 hours due to prolonged operative indications.

All patients had a transvaginal ultrasound scanning performed in the office prior to the procedure to screen for uterine pathology, including uterine anomalies and intramural or subserosal myomas, as well as to assess uterine position. Those patients with an anteverted uterus had a full bladder at the time of microhysteroscopy to facilitate placement of the microhysteroscope.

Women tolerance during the whole procedure, pain perception scoring, the need for intraoperative conscious sedation, an extra postoperative analgesia, and the duration of the postoperative and lapse period before discharge were recorded to be analyzed (3).

For a 12-month follow-up period, pregnancy outcome were evaluated after the office microhysteroscopic procedure in A and B groups, for spontaneous pregnancy without any intervention, while each pregnancy developed after the microhysteroscopic procedure was correlated to each uterine abnormality diagnosed and treated during the microhysteroscopic procedure. Early pregnancy complications were evaluated for both groups, and some of the successful ongoing pregnancies were recorded as well.

Statistical analysis

Chi-square test and students' t test were used to analyze different sub-groups. Univariate and multivariate logistic regression were applied in order to identify factors that could predict the presence of unsuspected uterine cavity abnormalities. A $P<0.05$ was considered statistically significant. All statistical analyses were performed in SPSS version 15.1 (SPSS Inc., IL, USA).

Results

Table 1 shows the different demographic characteristics of the women included, indicating there are no significant differences between the case and control subjects. Table 2 lists the findings, both normal and pathologic cases, of the 100 office microhysteroscopies performed. Figure 1 shows the intrauterine abnormal findings in relation to women age category. All procedures were performed without complications. Treatment of adhesions and removal of polyps and submucous myomas were undertaken and completed in all patients. Division of septi was performed in patients with a known single fundus confirmed by laparoscopy at a prior time. No procedures were aborted secondary to patient intolerance.

Abnormalities included the followings: i. Atypical polypoid adenomyoma of endometrium in 3 cases (3%), ii. Intrauterin adhesion (IUA) synechiae in 3 cases (3% of all microhysteroscopies), iii. A case with uterus bicornis (1% of all microhysteroscopies), v. Submucous myoma in 3 cases (3% of all microhysteroscopies), vi. Deformed uterine cavity resulting from intramural myoma in 6 cases

(6% of all microhysteroscopies), vii. Endometrial polyps in 20 cases (20% of all microhysteroscopies) and viii. unique in 10 cases (10% of all microhysteroscopies). Their location was either corporeal (14 cases) or cornual (6 cases). Table 3 shows the degree of patient compliance of the women included for the studied office microhysteroscopic procedure without general anesthesia. Most women accepted the procedure with a good degree of compliance, and none of the procedures was aborted due to the patient non-compliance.

The microhysteroscopic images were quite similar to those following the conventional 5-mm hysteroscopy, and might be better. Normal microhysteroscopic image appears as a regular cavity with reddish glistening endometrial lining with both ostial openings seen as black spots at 2 and 10 o'clock of the uterine cavity. Endometrial polyps and polypoid endometrium could be easily diagnosed, although mostly available together. Intrauterine adhesions could also diagnosed with a change of the reddish glistening soft endometrium to become rough non-glistening with areas of whitish myomas. Submucosal myomas could be diagnosed with the raised endometrial lining.

Table 2 shows the cumulative pregnancy rate during the postoperative one-year follow-up, following the office microhysteroscopic procedure, appears to be 25% in women without pathology (spontaneous pregnancies without interventions), 40% for endometrial polyps, 35% for adhesions, 33% for polypoid endometrium, and 22% for bicornuate uterus. The average total ongoing pregnancy rate is 25% after office microhysteroscopic procedure in group A versus 15% in group B. In group A, the best pregnancy rate belonged to after treated endometrial polyps and worst rate belong to the abnormal uterine configuration in uterus bicornis (0%). The total miscarriage rate is not significantly different in developing pregnancies after the different corrected abnormalities, managed after the office microhysteroscopic procedure. None of the office microhysteroscopic procedure was aborted.

Table 2: Office microhysteroscopic findings of 100 women with unexplained infertility and the reproductive outcomes after the procedure in group A compared to the related values in group B

Findings	Cases n (%)	CPR n (%)	OPR n (%)
Normal finding	70 (70 %)	35 (25%)	28 (20%)
Endometrial polyps	20 (20%)	16 (40%)	12 (30%)
Submucous fibroids	3 (3%)	2 (34%)	1 (23%)
Intrauterine adhesions	3 (3%)	1 (23%)	1 (22%)
Polypoid endometrium	3 (3%)	2 (33%)	1 (23%)
Bicornuate uterus	1 (1%)	1 (21%)	0 (0 %)
Total number in group A	100	57 (28.5%)	43 (21.5%)
Total number in group B	100	15 (15%)	10 (10%)

CPR; Cumulative pregnancy rate and OPR; Ongoing pregnancy rate.

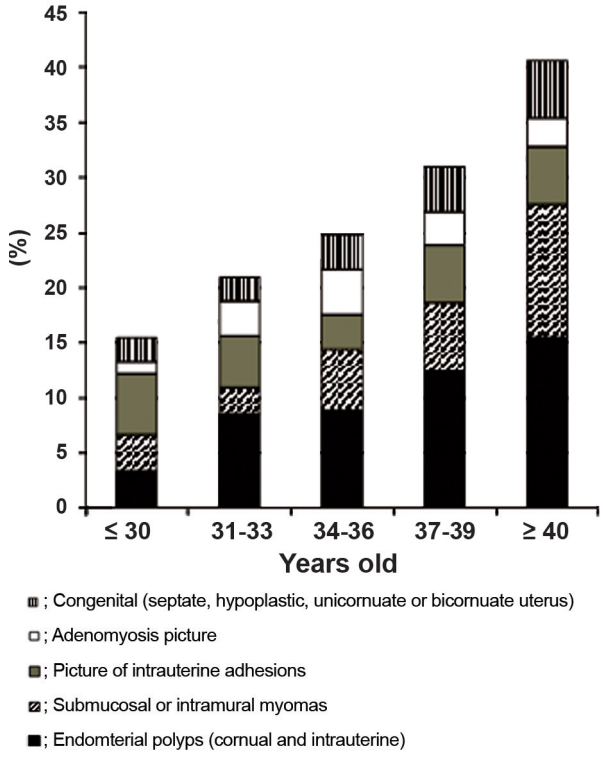

Fig.1: Microhysteroscopic abnormalities in relation to women age.

Table 3: Patients compliance during and after the office microhysteroscopy

Patient compliance	n (%)
No or minor discomfort	90 (90%)
Discomfort	5 (5%)
Major discomfort	3 (3%)
Difficult examination	2 (2%)
Total	100

Discussion

The basic infertility work-up has included a HSG to evaluate the uterine cavity and tubal patency. However, HSG does not allow for simultaneous correction of uterine pathology. Moreover HSG may miss 35% of uterine abnormalities. The high false-negative rate, the low-positive predictive value, and the inability to treat abnormal findings concurrently with the diagnosis have limited the use of HSG to assess the endometrial cavity (12-15).

Sonohysterography (SHG) has been proposed as a better diagnostic test of the uterine cavity. However, it also suffers from a sensitivity and specificity inferior to that of hysteroscopy in most studies. Additionally, it does not allow for correction of presumed pathology. Perhaps because hysteroscopy has traditionally required general anesthesia in an operating room setting, physicians do not consider hysteroscopy as a first-line test. Additionally, distention media are typically composed of low osmolality and electrolyte-free for operative work, and thus require careful surveillance of fluid status to minimize complications of hyponatremia and fluid overload. These requirements have made many practitioners reluctant to perform operative hysteroscopy (16-18).

Patient tolerance of hysteroscopes 2-5-mm allows for their use in an office setting where anesthesia is not required. Additionally, office hysteroscopy is no more costly than HSG at many institutions. Moreover using newly advanced microhysteroscope favors it over other tools. The professional fees for performing and reading a hysterosalpingogram (HSG) are 30% higher than the cost of an office hysteroscopy. Although SHG may offer a cost reduction, for many patients in whom pathology is found or suspected, a hysteroscopy is then indicated adding expense, delay, and inconvenience (18-22).

It has been reported that up to 20-50% of infertile patients have uterine abnormalities (30% in this study), including myomas, polyps, intrauterine adhesions, and uterine malformations. This is in agreement with our study that found 30% of patients undergoing office micohysteroscopy had uterine pathology. The high incidence of endometrial polyps in some patients may be related to prior therapy with gonadotropins due to higher levels of estrogen. Because pathology is present in 20 to 50% of infertile patients, as mentioned previously, practitioners should be more inclined to recommend hysteroscopy as part of the infertility work-up in conjunction with the routine laparoscopy and dye test, due to its simultaneous operative management (23-26).

Outpatient hysteroscopy has been shown to be easily performed with excellent surgical results in previous studies. Nagele et al. (26) and Vercellini et al. (27) found comparable success rates of 98% for performing the procedure. In this study, it was

quite possible to perform all diagnostic and operative procedures in the office setting. Grasping forceps allow for removal of polyps with the ability to retain a clean specimen for pathologic confirmation. Scissors can be introduced for adhesions and septi. Bipolar electrode allows for removing submucous myomas. Using the cutting mode is primarily used for preservation of the delicate endometrium, minimizing the risk of postoperative adhesions, whereas the desiccation mode is applied when specific blood vessels are encountered.

Using saline as uterine distention medium helps to minimize medium-related complications. Hyponatremia and cerebral edema are of a concern when using hypotonic, electrolyte-free media, such as glycine or sorbitol. However, fluid overload, pulmonary edema, and congestive heart failure are likely to occur when an excessive volume of saline is used, especially if patients have underlying medical conditions predisposing them to fluid-related complications.

Air embolism is also considered as a potential complication. This could be minimized by avoiding to place the patients in an exaggerated Trendelenburg positioning, excessive fluid pressure overflow, prolonged operative times, dilating the cervix without instruments sealing air entry, and purging the tubing of air. Post-procedure complications like endometritis could be reduced by pre- and post-treatment with prophylactic antibiotics (16-18), and by avoiding operating on patients with known active vaginal infections (22-28). Patient tolerance of the electrosurgical equipment was excellent, confirming what El Toukhy et al. (29), Lorusso et al. (30) found in their studies on outpatient hysteroscopy.

Office-based operative hysteroscopy has also been found to be extremely safe. In this study, no complications occurred, and no patients needed extended monitoring or laboratory studies for fluid overload. Typical complications associated with hysteroscopy may be procedure-related, media-related, or post-procedure-related. Procedure-related complications, such as uterine perforation; cervical laceration; and damage to tissues including bowel, bladder, and vagina, could be almost minimized using the proposed office malleable 2-mm fiberoptic microhysteroscope, which did not need any cervical dilatation, passing smoothly within the undilated cervix. Moreover, the images produced were nearly similar or almost better than those after using the conventional 5-mm lens system hysteroscope, leading to minimal degree of patient's discomfort.

An increase in pregnancy rates after performing office microhysteroscopic procedure might be attributed to the removal of endometrial polyps, polypoid endometrium, submucous myomas, or intrauterine synechiae at the time of microhysteroscopy that resulted in improving implantation in this population at risk. However, those pregnancies developed after microhysteroscopic confirmation of absence of any intrauterine pathology, the irrigation of the cavity with saline may have a beneficial effect on implantation and pregnancy rates in those women, as suggested in previous studies (20, 25, 30). The explanation of the highest pregnancy rate after excisions of polyps and myomas is logic, but the least pregnancy rate that was observed with uterus bicollis or acutely arcuate uterus might be due to the abnormal uterine cavity configuration. Suspected associated non-mechanical factors with diagnosed adenmyosis may explain the relatively lower pregnancy outcome developed after the procedure.

The higher ongoing pregnancy rate after the managed polyps, polypoid endomtrium, submucous myoma, and those after exclusion of any pathology might confirm the causality of those abnormalities as the main etiology for embryo implanatation, either mechanically or biochemical; however, after confirmation of the integrity of the endometrium and uterine wall, it is suggested to keep pregnancy safe beyond 20 weeks gestation. Regardless of whether these adjunctive benefits are confirmed by further study, office-based operative microhysteroscopy is definitely hold a great value as the gold standard of diagnostic procedures for uterine cavity abnormalities with the ease, safety, and efficiency of simultaneous therapeutic correction of abnormalities.

The spontaneous pregnancy outcome during the follow-up of group B was within the reported incidence before, although it was significantly lower than those following the microhysteroscopic procedure in group A. Taking into account, using any of the assisted reproductive techniques (ART) might increase this lower pregnancy outcome, but both groups were followed up without using any of those techniques, as that might interfere with

the final pregnancy outcome. Still this spontaneous pregnancy outcome in group B was developed with no surgical intervention; no use of any type of anesthesia, conscious sedation, or analgesia; and no application of office procedure that was used for group A.

So our findings showed that in infertile population where office microhysteroscopy is performed routinely prior to the confirmation of un-explained cases of delayed conception, a significant percentage of patients are found to have uterine pathology, which had been missed to be diagnosed by the routine fertility work-up performed before. Endometrial polyps were found most frequently, with smaller numbers of myomas, adhesions, and septi. These abnormalities may impair the success of future treatment cycles, so removal of the pathology was advised. Patient tolerance and the feasibility of operative management, simultaneous with diagnosis, would make the proposed office-based operative microhysteroscopy in conjunction with/or after the routine laparoscopy as an ideal first-line procedure with minimal risk to the patient.

Conclusion

Scheduling the office microhysteroscopy as one of the routine steps in the fertility work-up program has become mandatory before the final diagnosis of unexplained infertility. This technique is considered not only an ideal gold test to diagnose many intrauterine abnormalities that are undiagnosed with other routine tools, but also the significant improvement in the pregnancy outcome following the microhysteroscopic procedure, supports the previously mentioned recommendation. In addition, it is recommended to conduct future research works to support this recommendation.

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Original Article

Association of Glomerular Filtration Rate with Inflammation in Polycystic Ovary Syndrome

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Abstract

Background: We aimed to estimate the glomerular filtration rate (GFR) in women with polycystic ovary syndrome (PCOS) and to determine the relationship between GFR with C-reactive protein (CRP) and uric acid.

Materials and Methods: In this cross-sectional study, one-hundred and forty PCOS women and 60 healthy subjects were evaluated. The study was carried out at Endocrinology Outpatient Clinic, Erzurum Training and Research Hospital, Erzurum, Turkey, from December 2010 to January 2011. GFRs were estimated by Modification of Diet in Renal Disease (MDRD) formula. CRP, urinary albumin excretion (UAE) and uric acid levels were also measured.

Results: GFRs were significantly higher in PCOS group than control (135.24 ± 25.62 vs. 114.92 ± 24.07 ml/min per 1.73 m^2). CRP levels were significantly higher in PCOS patients (4.4 ± 3.4 vs. 2.12 ± 1.5 mg/l). The PCOS group had significantly higher serum uric acid levels (4.36 ± 1.3 mg/dl vs. 3.2 ± 0.73 mg/dl). There was also significantly higher proteinuria level in PCOS patients.

Conclusion: Even though PCOS patients had higher GFR, serum uric acid and UAE values than control patients, the renal function was within normal limits. Increased GFR in PCOS women positively correlates with elevated serum CRP and uric acid.

Keywords: CRP, Glomerular Filtration Rate, Polycystic Ovary Syndrome, Uric Acid

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Introduction

Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder affecting 5-10% of women of reproductive age (1). It is characterized by oligo/amenorrhea, hyperandrogenism and polycystic ovaries (2, 3). The insulin resistance, dyslipidemia, glucose intolerance, hypertension and obesity are metabolic disorders accompanying with this syndrome (4-6). It has been assumed that PCOS is also a proinflammatory state. Recent studies have demonstrated that glucose is responsible for inflammatory response in mononuclear cells of women with PCOS independent of body mass index (BMI) (7, 8). There is also an association between inflammation at the molecular level and insulin resistance in this disorder (8, 9). Elevations of a number of circulating proatherogenic inflammatory mediators have been independently reported in PCOS (10, 11). Meta-analysis of the 31 articles reported that circulating C-reactive protein (CRP) was 96% higher in women with PCOS compared to healthy con-

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trols (12). The relationship between CRP with atherothrombotic cardiovascular disease and renal function abnormalities has been reported in a number of studies (13).

Serum uric acid was associated positively with interleukin 6 (IL-6), CRP and tumor necrosis factor- alpha (TNF-α) and negatively with IL-1 beta (IL-1β). These results suggest that uric acid contributes to systemic inflammation in humans and is in line with experimental data showing that uric acid triggers sterile inflammation (14). It is also known that hyperuricemia is an independent risk factor for renal dysfunction in the normal population (15).

Urinary albumin excretion (UAE) is also a marker of atherogenesis and predicts early endothelial damage (13). Factors predisposing for endothelial injury, including hyperinsulinemia, insulin resistance, dyslipidemia and chronic low-grade inflammation, which often accompany with PCOS (16). Several studies have shown that microalbuminuria is an indicator for increased permeability to macromolecules of peripheral vascular beds. UAE may predict renal function abnormalities (17).

The aim of this study was to investigate renal function by the way of GFR measurement (MDRD formula) in PCOS patients. We tried to find any relationship between glomerular filtration rate (GFR) with CRP and uric acid as inflammatory markers. Also UAE was evaluated for renal function in PCOS patients.

Materials and Methods

Study population

The study was carried out at Endocrinology Outpatient Clinic, Erzurum Training and Research Hospital, Erzurum, Turkey, from December 2010 to January 2011. One-hundred and forty patients with PCOS and 60 healthy subjects were enrolled in this cross-sectional study. We included healthy women as controls with normal menstrual cycles, with no evidence of hyperandrogenism, and with normal ovarian morphology on pelvic ultrasonography. Ferriman-Gallwey scores of all control patients were under 8 (18). PCOS was defined as the presence of two of the following three features after the exclusion of other etiologies (3): i. oligo-or anovulation (fewer than six menstrual periods in the preceding year), ii. hyperandrogenism and/or biochemical signs of hyperandrogenism and/or iii. polycystic ovaries.

All of the participants are nonsmokers and with body mass index (BMI) lower than 25. The exclusion criteria in control and PCOS groups were as follows: patients with any type of renal disease, diabetes mellitus, cardiovascular events, endocrine disease, pregnancy, or antihypertensive drug use including use of oral contraceptives, antidiabetics, glucocorticoids, and anti androgenic agents within the last 3 months. Leukocyte count was less than 10,000/μL in all cases. Patients with older than 40 and younger than 16 years old were excluded from the study.

Assessments

BMI was calculated as weight (kg)/height $(m)^2$. Systolic (SBP) and diastolic blood pressure (DBP) were measured twice in the right arm in a relaxed sitting position. Two measurements were taken 15 minutes apart and the average of two was used. Blood samples were collected during early follicular phase of menstrual cycle after at least 12 hours fasting. Levels of glucose, insulin, serum urea (not blood urea nitrogen), creatinine, hormone profile [follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E_2), and thyroid-stimulating hormone (TSH)], total and free testosterone (Total-T and Free-T), dehydroepiandrosterone sulfate (DHEAS), 17 OH-progesterone (17OH-P), prolactin (PRL), and serum lipids [total cholesterol (Total-C), high-density cholesterol (HDL-C), lowdensity cholesterol (LDL-C), and triglycerides (TG)] were determined. Plasma glucose was determined with the glucose hexokinase method (Cobas Integra 400 Plus, Roche Diagnostics, Mannheim, Germany). Hormone profile was measured with electrochemiluminescence assays (Elecsys 2010 Hitachi, Roche Diagnostics, Germany). Lipid profile was measured with enzymatic colorimetric assays (Roche Diagnostics, Mannheim, Germany).

Plasma concentrations of insulin were measured by chemiluminescent immunoassay (Immulite One, BioDPC, Los Angeles, CA, USA). Insulin resistance was measured with homeostasis model assessment for insulin resistance

(HOMA-IR) (19).

UAE was determined in 24-hour urine samples (Roche/Hitachi 912 Autoanalyzer, Roche Diagnostics, Germany). A UAE of 30-300 mg/24-hour was considered as microalbuminuria, whereas the value >300 mg/24-hour was considered as proteinuria. GFR was estimated from serum creatinine using the MDRD formula (20) as follows:

$GFR\ (ml/min/1.73m^2) = 175 \times (Serum\ Creatinine)^{-1.154} \times (age)^{-0.203} \times 0.742$

Serum uric acid levels were measured by uricase method using an Abbott Aeroset autoanalyzer (Abbott Laboratories, Abbott Park, IL, USA) with a 0.01 mmol/l limit of detection and mean coefficients of variations <2%.

Serum CRP levels were measured using a nephelometric assay (Boehringer, Mannheim, Germany). Complete blood and polymorphonuclear leukocyte counts (%) were measured with a Coulter MaxM analyzer (Philadelphia, PA, USA).

Study ethics

The study was conducted according to the revised guidelines for clinical studies described by the World Medical Association's Declaration of Helsinki (http://www.wma.net). The study protocol was approved by the Ethical Committee of Erzurum Training and Research Hospital. A written informed consent was obtained from all participants.

Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS; SPSS Inc., Chicago, IL, USA) version 13.0, while the differences within- or between-group were analyzed by Student's paired and unpaired t tests. Results were expressed as mean ± standard deviation (SD). Pearson's correlation was used to calculate correlations. A multiple regression analysis was performed to determine the independent association between potential predictor variables and GFR as the dependent variable. A P value ≤0.05 was considered statistically significant.

Results

The demographic variables and biochemical features of PCOS and controls women are shown in tables 1 and 2. A hundred and forty PCOS patients (median age: 24.6 ± 5.5 year) and 60 healthy subjects (median age: 25.2 ± 4.38 year, P=0.687) were included in the study. There were no significant differences between groups with respect to age, height, weight, waist circumference, BMI, serum total cholesterol, LDL, HDL, TG, TSH, FSH and E_2 levels (P>0.05). PCOS group had significantly higher LH values (P=0.02). Both groups were normotensive regarding SBP and DBP (P=0.43, P=0.8, respectively). Serum insulin levels and HOMA-IR were significantly higher in PCOS patients (P=0.02, P=0.007, respectively), while fasting plasma glucose level was not statistically different between two groups (P=0.07). C-reactive protein was significantly higher in PCOS patients (4.4 ± 3.4 vs. 2.12 ± 1.5 mg/l, P=0.01).

The PCOS group had significantly higher serum uric acid (4.36 ± 1.3 vs. 3.2 ± 0.7 mg/dl, P=0.002) beside the fact that statistically similar urea and creatinine levels for each group were reported (P=0.72, P=0.09, respectively). GFR was significantly higher in PCOS group than controls (135.2 ± 25.6 vs. 114.9 ± 24.1 ml/min per 1.73 m^2, P=0.001).

Multiple regression analysis was performed with GFR as a dependent variable. Some parameters such as glucose, BMI, and HOMA-IR were used as an independent variables. Since obesity and diabetes mellitus can cause hyperfiltration (21, 22), GFR was significantly higher in PCOS group in multiple regression analysis including BMI, HOMA-IR, glucose, age, waist circumference, CRP and insulin.

To assess the correlation with GFR, a Pearson's correlation analysis was performed on each variable. GFR was positively correlated with uric acid (Correlation of determination=0.065, P=0.01) and CRP (Correlation of determination=0.23, P=0.000).

In PCOS group, UAE ranged from 3 to 105 mg/ml with a median of 13 mg/ml, whereas in control groups, UAE ranged from 2 to 43.8 mg/ml with a median of 7 mg/ml. There was no patient with macroscopic proteinuria in both groups. Mean UAE was statistically higher in PCOS group than controls (P=0.021). Eleven percent of control groups and 28% of PCOS groups had proteinuria. This difference was statistically significant (P=0.02).

Table 1: The demographic variables and biochemical parameters of patients

Parameters	PCOS (n=140)	Control (n=60)	P values
Age (Y)	24.6 ± 5.4	25.2 ± 4.38	0.170
Height (m)	1.63 ± 0.07	1.62 ± 0.05	0.68
Weight (kg)	59.6 ± 17.1	55.2 ± 10.4	0.1
BMI (kg/m^2)	24.6 ± 6.4	22.9 ± 4.3	0.42
Waist circumference (cm)	78.7 ± 15.3	76.7 ± 8.3	0.56
Urea (mg/dL)	23.4 ± 9.6	24.3 ± 7.2	0.72
Crea (mg/dL)	0.83 ± 0.1	0.6 ± 0.12	0.09
GIucose (mg/dl)	89.9 ± 19.7	85.6 ± 6.6	0.07
Insulin (mU/L)	10.4 ± 6.5	7.22 ± 4.33	0.02
HOMA-IR	1.79 ± 1.66	0.85 ± 0.87	0.007
GFR (ml/min per 1.73 m^2)	135.2 ± 25.6	114.9 ± 24.1	0.001

PCOS; Polycystic ovary syndrome, BMI; Body mass index, Crea; Creatinine, HOMA-IR; Homeostasis model assessment-insuline resistance and GFR; Glomerular filtration rate.

Table 2: The biochemical parameters of patients

	PCOS (n=140)	Control (n=60)	P values
Total-C (mg/dL)	166.7 ± 37.3	156.5 ± 23	0.46
LDL-C (mg/dL)	94.2 ± 30	87.3 ± 24.2	0.3
TG (mg/dL)	93.9 ± 52.6	78.8 ± 48.5	0.08
HDL-C (mg/dL)	54.2 ± 17.5	52.6 ± 14.6	0.26
UAE (mg/ ml)	13 ± 6.1	7 ± 3	0.021
Uric acid (mg/dL)	4.36 ± 1.3	3.2 ± 0.7	0.002
CRP (mg/L)	4.4 ± 3.4	2.12 ± 1.5	0.01
TSH (mIU/L)	2.79 ± 1.5	2.91 ± 2.6	0.29
FT3 (pg/dL)	3.1 ± 1.3	3.6 ± 2.3	0.82
FT4 (ng/dL)	3.7 ± 1.8	3.2 ± 1.9	0.73
FSH (mIU/mL)	5.5 ± 1.9	6.5 ± 1.4	0.06
LH (mIU/mL)	8.8 ± 3.5	6.4 ± 4.8	0.02
E_2 (pg/mL)	75.6 ± 33	80.8 ± 40.5	0.3
Progesteron (ng/mL)	3.2 ± 2.4	0.73 ± 0.4	0.33
PRL (ng/mL)	10.3 ± 3.5	13.3 ± 3.7	0.2
DHEAS (mcg/dL)	241 ± 96	192 ± 83	0.012
Total-T (ng/dL)	37.6 ± 31	21.7 ± 21	0.001
17 OH-P (ng/mL)	1.2 ± 0.4	1.02 ± 0.04	0.83
Free –T (pg/mL)	2.7 ± 0.9	1.7 ± 0.4	0.69
SBP (mmHg)	121.7 ± 13	114.1 ± 11	0.43
DBP (mmHg)	80.8 ± 10.5	74.4 ± 9	0.8

PCOS; Polycystic ovary syndrome, Total-C; Total cholesterol, LDL-C; Low-density cholesterol, TG; Triglycerides, HDL-C; High-density cholesterol, UAE; Urinary albumin excretion, CRP; C- reactive protein, FT3; Free triiodothyronine, FT4; Free thyroxine, FSH; Follicle stimulating hormone, LH; Luteinizing hormone, E_2; Estradiol, PRL; Prolactine, TSH; Thyroid-stimulating hormone, DHEAS; Dehydroepiandrosterone, Total-T; Total testosterone, 17 OH-P; 17-Hydroxiprogesterone, Free-T; Free testosterone, SBP; Systolic blood pressure and DBP; Diastolic blood pressure.

Discussion

To our knowledge, this is the first study for the demonstration of significantly higher GFR in PCOS women as compared with the healthy subjects. However, GFR values of PCOS patients were within normal limits. Hyperfiltration is typically defined by a GFR between 125 to 140 ml/min per 1.73 m^2, or greater than 2 standard deviations above the mean GFR, in healthy individuals (23, 24). According to the National Kidney Foundation (NKF), normal range is defined between 90 and 120 ml/min per 1.73 m^2 (25). No commonly agreement upon definition of glomerular hyperfiltration exists. Even though in our study, overt hyperfiltration was not found in PCOS patients, they had significantly higher GFR values than controls. Yanes et al. (26) reported increased GFR in a rat model of PCOS. In humans, hyperfiltration is observed in diabetes mellitus patients, and also seen in patients with pre-diabetic conditions, such as the metabolic syndrome (21). Similarly the individuals with obesity exhibit a significant increase in GFR (22). In our study, GFR was also significantly higher in PCOS group in multiple regression analysis including BMI, HOMA-IR, glucose, and insuline. Lakhani et al. (27) has shown that there was no difference in GFR between women with PCOS and controls (102.2 vs. 114.4 ml/min per 1.73 m^2). However, 15 PCOS patients were included in their study.

There might be vascular and tubular factors contributing to the pathogenesis of hyperfiltration (21). Hyperfiltration is also associated with lower arterial stiffness and endothelial dysfunction, suggesting that hyperfiltration represents a distinct physiologic state of generalized vascular dysfunction. It has, therefore, been suggested that the hyperfiltration state reflects generalized microvascular and macrovascular functional changes (27, 28). In this study, we found relatively higher GFR in PCOS patients.

In the present study, GFR showed a significantly positive correlation with CRP and uric acide. Inflammatory state may be responsible for increased GFR process, which is the result of vascular, tubular and endothelial changes.

CRP is a circulating marker of the proinflammatory state in PCOS as evidenced by the 2-fold elevation in circulating CRP compared to controls (12). Similarly in our study, C-reactive protein was significantly higher in PCOS patients. A meta-analysis of the most comparable studies indicates that elevated circulating CRP in PCOS suggests the chronic low-grade inflammation present in the disorder. They also found that elevated circulating CRP in PCOS is independent of obesity since this finding persisted after excluding all the studies with mismatches in frequency of obesity or BMI between groups from the meta-analysis (12). Although Stuveling et al. (13) showed that elevated CRP was positively associated with diminished filtration, based on our findings, the chronic inflammation in PCOS patients may be responsible for increased GFR levels. On the other hand, in their studies, highest CRP quartile groups were positively associated with hyperfiltration. This association is important because increased GFR is associated with declining renal function (29, 30).

In this study, the PCOS group had significantly higher uric acid, but showed statistically similar urea and creatinine levels with control group. Additionally, increased GFR was positively correlated with uric acid in this paper. It is paradoxical because increased GFR is associated with increased clearance of uric acid from blood that leads to low plasma levels of uric acid. These results may be associated with PCOS itself. Increased uric acid levels in PCOS women were demonstrated in several studies (16).

In the studies, renal dysfunction was correlated with elevated serum uric acid (31-33). Price et al. (34) reported that uric acid is transported into endothelial cells via urate transporter-1, and it then induces oxidative stress. In addition, it has been reported that hyperuricemia increases juxtaglomerular renin expression and decreases macula densa neuronal nitric oxide synthase expression (35). Thus, uric acid may cause renal injury by interacting synergistically with the renin-angiotensin system beside oxidative stress (36). Uric acid has also been shown to directly stimulate the production of inflammatory mediators, such as CRP, in vascular cells (37). In the present study, elevated uric acid levels probably contributed to hyperfiltration like CRP as an inflammatory marker.

In our study, there were significantly higher UAE levels in PCOS group. In one study it appears that excessive UAE may be even more common in PCOS than in subjects with overt diabe-

tes and/or hypertension (38). Urinary excretion of albumin reflects renal function and is directly related to endothelial function or endothelial leakiness. Albumin leakage into the urine is a reflection of widespread vascular dysfunction and increased intraglomerular pressure (39, 40). The National Health and Nutrition Examination Survey (NHANES; 1999-2000) reported the microalbuminuria prevalence as 8.8% in a subpopulation with no risk factors (41). In our study, 11% of control group and 28% of PCOS group showed proteinuria. Ganie et al. (42) have shown that about 24.6% of women with PCOS showed presence of microalbuminuria in the first void spot urine sample.

This study has some limitations. Only CRP and uric acid were used. Mean values of serial measurements of CRP, high-sensitive CRP and other inflammatory markers probably improved study results. Even though relatively higher uric acid, CRP and GFR levels were found in PCOS group, all of these were within normal limits. This may be related with low-grade chronic inflammation (12). Further studies are needed to assess current outcome.

Conclusion

UAE level and increased GFR are important because they are associated with declining renal function (27). Early inflammatory process may predispose the kidney to glomerular hyperfiltration-related renal function loss. Even though PCOS patients had higher GFR, serum uric acid and UAE levels than control group, they had renal function within normal limits. Further studies may be helpful for understanding PCOS long-term effect on renal function.

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Original Article

Effect of Surgical Removal of Endometriomas on Cyclic and Non-cyclic Pelvic Pain

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Abstract

Background: Endometriosis is a complex disease with a spectrum of pain symptoms from mild dysmenorrhea to debilitating pelvic pain. There is no concrete evidence in the literature whether endometriotic cyst per se, causes pain spectrum related to the disease. The aim of the present study was to evaluate the effect of surgical removal of endometriomas on pain symptoms.

Materials and Methods: In this prospective, observational, before-after study, which was conducted between March 2012 and January 2013 in Training and Research Hospital,Adana, Turkey, a total of 23 patients including 16 sexually active and 7 virgin symptomatic women were questioned for non-cyclic pelvic pain (NCPP), intensity of the NCPP, presence of cyclic dysmenorrhea, and dyspareunia before and after the endometrioma operation. Participants who were sonographically diagnosed and later pathologically confirmed as having endometrioma without sign and symptoms of deep infiltrative endometriosis (DIE) were also questioned for pain symptoms before and after the laparoscopic removal of cyst wall. Patients with intraabdominal adhesions, history of pelvic inflammatory disease, and pathological diagnosis other than endometrioma were excluded. No ancillary procedures were applied for pain management, but if pain was present, pelvic peritoneal endometriotic lesions were ablated beside the removal of ovarian endometriotic cysts.

Results: Out of 23 cases with endometrioma, 91 and 78% reported to have NCPP and dysmenorrhea, respectively, before the operation, while 60 and 48%, respectively, after the operation (McNemar's test, P=0.016 for both figures). Among the sexually active cases, 31% (5/16) had dyspareunia before the operation and only 1 case reported the pain relief after the operation (McNemar's test, P=1). Intensity of NCPP were reported to be none (8.7%), moderate (21.7%), severe (56.5%) and unbearable (13%) before the operation and decreased to none (43.5%), mild (43.5%), moderate (4.3%) and severe (8.7%) after the operation (Wilcoxon signed-rank test, P<0.001).

Conclusion: In symptomatic cases with ovarian endometrioma, without sign and symptoms of DIE, laparoscopic removal of the cysts with/without ablation of the peritoneal endometriotic lesions yields relief of NCPP and cyclic dysmenorrhea, but not dyspareunia.

Keywords: Endometrioma, Pelvic Pain, Endometriosis, Ablation

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Introduction

Endometriosis is the most common gynecological pathology causing cyclic or non-cyclic pelvic pain (NCPP) accounting for 12-32% of women of reproductive age and for 45-70% in adolescents (1). Endometriosis is defined as the presence of endometrial glands and stroma outside of the endometrial cavity. Pelvic pain has long been recognized as a critical concomitant of the endometriosis syndrome. Up to 75% of symptomatic endometriosis causes cyclic pelvic pain with menstruation (2), though it is often associated with several different pain symptoms including noncyclical, nonmenstrual pelvic pain (3, 4). Indeed, in Sampson's treatise (5), 12 of the 17 symptomatic cases he reported presented for surgery due to intolerable pain. As long as 90 years, the relationship between the extent of adhesion and severity of pain has not been well recognized. This lack of correlation continues to confound modern era gynecologists in large part (6) because mediation of painful stimuli are inadequately understood.

Microscopic studies have documented nerve fibres in endometriotic peritoneal lesions (7-9), deep infiltrating endometriosis (10, 11) and ovarian endometriomas (12). Berkley et al. (13) described the growth of efferent sympathetic and afferent sensory nerves into the ectopic implants of endometriosis in women and in a rat model of disease. The studies about neurogenesis in endometriosis caught the attention of gynecologists, physiologists and neuroscientists to evaluate the causes and develop new methods with transdisciplinary effort to ameliorate pain associated with endometriosis. On the other hand, surgery has long been an important part of the management of endometriosis. In 2011, Stratton and Berkley (14), described current approaches to surgical treatment of endometriosis based on "oncological principle" to remove all visible lesions and restore normal anatomy. Endometriomas are not amenable to medical treatment and need to be removed surgically even if symptoms improve with medical treatment (15), while the preferred therapeutic approach for women with symptomatic endometriomas was surgery to relieve the patient's pain (16). Regardless of the stage of endometriosis, randomized controlled trials comparing the effect of surgery to conservative management have shown that surgery and excision of endometriosis results in symptomatic improvement (17, 18). The purpose of the present study was to discuss the benefits of surgical treatment for different types of pain associated with endometriomas.

Materials and Methods

In a prospective, observational, before-after study, was conducted between March 2012 and January 2013 in Adana Numune Training and Research Hospital, Adana, Turkey. Twenty three cases including 16 sexually active and 7 virgin women (mean age: 31.9, range: 20-43) who were sonographically diagnosed and later pathologically confirmed as having unilateral endometrioma (3-8 cm in diameter) without sign and symptoms of deep infiltrative endometriosis (DIE), as dyschezia, hematuria, rectal bleeding, constipation, diarrhea and bloating, formed our study group. The patients whose rectal/rectovaginal examination, imaging studies [ultrasound and magnetic resonance imaging (MRI)], and intraoperative findings suggested DIE as well as the patients who had intra-abdominal adhesions, history of pelvic inflammatory disease, and pathological diagnosis other than endometrioma were all excluded. Women completed a preoperative questionnaire that collected demographic characteristics and data on presenting problem as full menstrual history, medical and surgical history and characteristics of pain symptoms. Pain was assessed using a verbal scale which has good correlation with visual analogue scale and higher compliance in clinical settings (19). The verbal scale offered descriptors such as "no pain, moderate pain, severe pain, and unbearable pain". Women were questioned for pain symptoms preoperatively and 3-6 months after the laparoscopic removal of endometrioma.

All patients were operated using stripping method by the same operator. No ancillary procedures as presacral neurectomy, uterosacral interruptions of sensory nerves and uterine suspension were applied for pain management, but if pain was present, pelvic peritoneal endometriotic lesions were ablated beside the removal of ovarian endometriotic cysts. The diagnosis of endometrioma was confirmed by histological examination of specimens removed at surgery.

Statistical analysis

Pain relief was analyzed by the McNemar's test for pre- and post-operative symptoms. Analyses of pain scores were performed using the Wilcoxon

signed-rank test for paired non parametric data. Analyses was undertaken Statistics Package for the Social Sciences (SPSS, SPSS Inc. Chicago, IL, USA) version 15. A P value of <0.05 was accepted as statistically significant. Qualitative data are expressed in percentage (%) and quantitative data are expressed as the means ± standard deviation (SD).

The study protocol was elaborated according to the revised Declaration of Helsinki and was approved by the Local Research and Ethics Committee of Adana Numune Training and Research Hospital in Adana, Turkey. All subjects were provided a written informed consent.

Results

Average age of women at the time of surgery was 31.9 (range 20-43). Out of 23 subjects, 43.5% (10/23) were nulliparous and 56.5% were parous. Average endometrioma cyst diameter were 43.7 ± 21.7 mm. Twenty two women had no previous abdominal procedure; only one woman had a laparoscopic endometrioma ablation before (Table 1).

Table 1: The demographic characteristics of the patients

Mean age (range), Y	31.9 (20-43)
Parity, n (%)	
Nulliparity	10 (43.5%)
Multiparity	13 (56.5%)
Sexual behaviour, n (%)	
Sexually active	16 (69.5%)
Virgin	7 (30.4%)
Previous abdominal procedure, (%)	1 (4.34%)
Average endometrioma cyst diameter (mm), mean ± SD	43.7 ± 21.7
Postoperative medical therapy, n (%)	0 (0%)

mm; Millimeter.

Out of 23 cases with unilateral endometrioma, 91% (21/23) reported to have NCPP before the operation, but this ratio decreased to 60% after the operation (McNemar's test P=0.016). The frequency of dysmenorrhea was also felt by 30% after the operation (78 to 48%, McNemar's test P=0.016, Fig.1).

Among the sexually active cases, 31% (5/16) had dyspareunia before the operation and only 1 case reported pain relief after the operation (McNemar's test P=1, Fig.2).

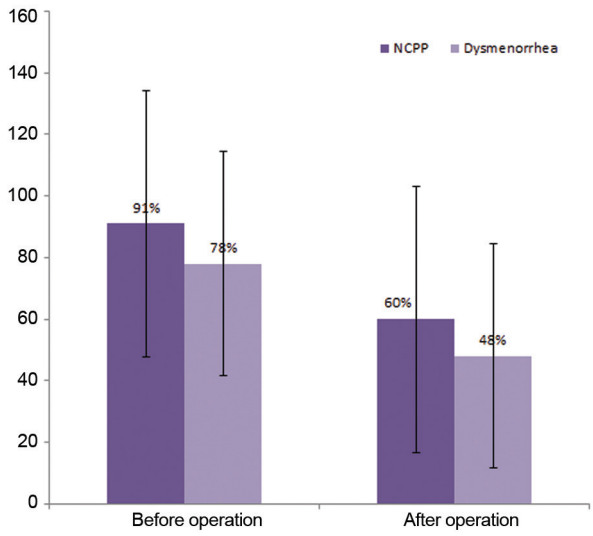

Fig.1: Percentages and %95 confidence intervals (CI) for non-cyclic pelvic pain (NCPP) and dysmenorrhea before and after the operation.

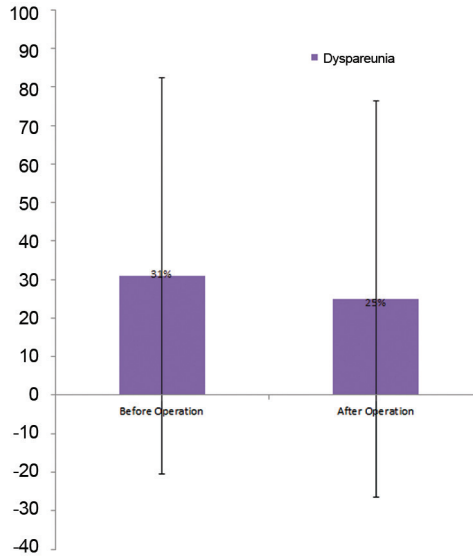

Fig.2: Percentages and %95 confidence intervals (CI) for dyspareunia before and after the operation.

Intensity of NCPP were reported to be none (8.7%), moderate (21.7%), severe (56.5%) and unbearable (13%) before the operation and decreased to none (43.5%), mild (43.5%), moderate (4.3%) and severe (8.7%), after the operation (Wilcoxon signed-rank test P<0.001, Fig.3).

Nine of 23 patients had mild lesions on peritoneal surfaces and were ablated by bipolar cautery. None of the patients were scheduled for long term pain management.

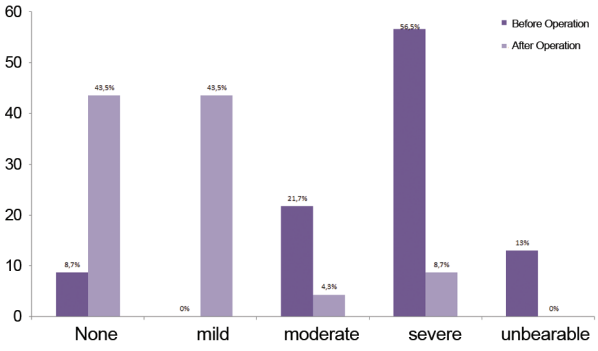

Fig.3: Pain grades for non-cyclic pelvic pain (NCPP) before and after the operation.

Discussion

Women with endometriosis either may have diverse and nonspecific symptoms or may be asymptomatic. The prevalance of endometriosis in asymptomatic women in general population are not known, but pain is the most common symptom associated with endometriosis, diagnosed by visulization of pelvic organs via laparoscopy. Approximately three quarters of symptomatic patients experience nonmenstrual pelvic pain and/or dysmenorrhea (20). In the present study, all participants had different types of pain as follows: 91% had NCPP, 78% had dysmenorrhea, and 21.7% had dyspareunia.

According to the current guideline by European Society of Human Reproduction and Embryology (ESHRE 2013) (21), asymptomatic endometriosis that is incidentally diagnosed should not be operated. Both surgical and medical treatments show improvements in pain scores of symptomatic cases. However, there is no published trials directly comparing one againist the other; therefore, we must rely on other evidence to weigh up the pros and cons of each approach. Unlike medical treatments, surgery can diagnose and remove all macroscopic disease at the same procedure in the majority of cases. In the case of symptomatic endometrioma, suggested and preffered therapeutic approach is surgery. Medical therapy is unlikely to result in complete regression of endometriomas larger than 1 cm and precludes a definitive histologic diagnosis (22, 23).

There have been very few studies in the current literature evaluating the effect of removal of endometrioma on pain symptoms. The efficacy of surgical management of endometriosis was demonstrated by a randomized trial, comparing the outcome of women after therapeutic laparoscopy with the outcome of women who underwent diagnostic laparoscopy alone. Laparoscopic excision of implants led to symptomatic improvement in 80% of patients at six months compared to 32% of controls undergoing diagnostic laparoscopy (3). Ideally if the surgery is performed for diagnosis, consent has to be obtained for surgical resection/ablation of endometriosis at the same time (20).

According to a review by Jadoul et al. (24) in which they analyzed the arguments in favour of and against of surgical treatments of endometriosis and showed that more than 50% of the patients reported pain relief. Also the operation technique used for endometrioma removal affects the pain relief. Several techniques have been described to treat endometriomas. In most of these techniques, the procedure consists of opening and draining the cyst followed by either excision (stripping technique), fulguration, or vaporisation of the cystic wall (ablative technique) (25-28). Drainage is alone not recommended because of the high recurrence rate (29). Hart's Cochrane systematic review found that excisional surgery provides better improvement in pain scores and decreases chance of recurrence compared with ablation (30). In our study the stripping technique was used, while the incidence and severity of NCPP and dysmenorrhea were significantly improved after the operation, as similiar to these studies. Only dyspareunia symptom was remained following the surgery. Ovary is one of the most frequent location for endometriosis, leading to the extensive pelvic and intestinal disease. Caution must be paid not to underdiagnose or undertreat these women (31). Although we

tried to exclude the DIE preoperatively by asking symptoms and performing rectovaginal/rectal and imaging examinations and our operative findings also excluded DIE, we still thought that the presence of endometriotic invisible lesions caused dyspareunia. Milingos et al. (32) found that symptoms of deep dyspareunia was correlated with the presence of dense pelvic adhesions and related to advanced endometriosis. The stripping and ablating of endometriotic lesions seemed to be not enough to improve dyspareunia. On the other hand, the nature of endometriotic pain was reported to be memorized in the brain that might not be resolved by excision of endometrioma. The possible explanation of remaining dyspareunia after the endometrioma excision could be the painful intercourse memorized by the certain brain area (14).

There were some limitations in our study. Due to small number of participants, we were unable to categorized the subjects according to the size of endometriomas, although no larger study was conducted on this subject yet.

Conclusion

One hundred and fifty years after endometriosis was first described, we are still debating both its etiology and management. Although main questions remain unanswered, solid evidence shows that laparoscopic surgery appears to be the most logical approach to treatment. As a result of our study, we want to emphasize that in cases of symptomatic endometriosis, without sign and symptoms of DIE, laparoscopic removal of the cysts with/without ablation of the peritoneal endometriotic lesions may relive NCPP and cyclic dysmenorrhea, but not dyspareunia.

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Original Article

Assessment of Correlation between *Androgen Receptor* CAG Repeat Length and Infertility in Infertile Men Living in Khuzestan, Iran

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Abstract

Background: The *androgen receptor (AR)* gene contains a polymorphic trinucleotide repeat that encodes a polyglutamine tract in its N-terminal transactivation domain (N-TAD). We aimed to find a correlation between the length of this polymorphic tract and azoospermia or oligozoospermia in infertile men living in Khuzestan, Iran.

Materials and Methods: In this case-control study during two years till 2010, we searched for microdeletions in the Y chromosome in 84 infertile male patients with normal karyotype who lived in Khuzestan Province, Southwest of Iran. All cases (n=12) of azoospermia or oligozoospermia resulting from Y chromosome microdeletions were excluded from our study. The number of CAG repeats in exon 1 of the *AR* gene was determined in 72 patients with azoospermia or oligozoospermia and in 72 fertile controls, using the polymerase chain reaction (PCR) and polyacrylamide gel electrophoresis.

Results: Microdeletions were detected in 14.3% (n=12) patients suffering severe oligozoospermia. The mean CAG repeat length was 18.99 ± 0.35 (range, 11-26) and 19.96 ± 0.54 (range, 12-25) in infertile males and controls, respectively. Also in the infertile group, the most common allele was 19 (26.38%), while in controls, it was 25 (22.22%).

Conclusion: Y chromosome microdeletions could be one of the main reasons of male infertility living in Khuzestan Province, while there was no correlation between CAG length in *AR* gene with azoospermia or oligozoospermia in infertile men living in Khuzestan, Iran.

Keywords: Male Infertility, Androgen Receptor, CAG Repeats, Y Chromosome

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Introduction

It seems that male factor is the reason of infertility in approximately half of infertile cases (1). The most common defects in infertile men are severe oligozoospermia or azoospermia, and these patients frequently undergo assisted reproductive technology (ART), such as intra-cytoplasmic sperm injection (ICSI), which may results in the transmission of these defects to next male generation. Different factors including environmental and genetic factors may cause alternation in sperm production, while several genetic studies have been recently conducted in this regard. The role of androgen as the main male hormone in determination of male sexual differentiation and male secondary sexual characteristics is well known. Also the initiation

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and maintenance of spermatogenesis is due to well action of this hormone. It is clear that androgen act on target cells with the help of androgen receptor (AR). Locus of the *AR* gene is on X chromosome at position Xq11-12. The *AR* gene with 8 exons can produce AR with three following domains: i. Exon 1 encoding transactivation domain, ii. Exon 2 and 3 encoding DNA-binding domain, and iii. Exon 4-8 encoding C-terminal ligand-binding domain. Exon one has two polymorphic sequences known as CAG and GGN that contain three nucleotide repeats. The CAG repeats encode polyglutamine residues with different length in transactivation domains of the receptors. The CAG repeats are unstable and the number of their repeats may change during meiotic division. Many mutations in *AR* gene cause various degree of androgen resistance (2-4).

A negative correlation between CAG repeats number and AR transcriptional activity has demonstrated *in vitro* system. Some clinical studies have shown association of longer CAG repeat with oligozoospermia and azoospermia (2-7). In contrast, others have not reported this correlation (8-12). According to these notions, we studied CAG repeats length in *AR* gene in oligozoospermic and azoospermic men living in Khuzestan Province, Southwest of Iran.

Materials and Methods

Individuals

In this case-control study during two years till 2010 in Khuzestan Province, Iran, in conformity with the ethical committee, 84 azoospermic or oligozoospermic men were selected according to World Health Organization (WHO) criteria (13) with sperm concentration less than 15 million/ml as in patient samples. Also, 72 men with normal semen analysis who had at least one child were studied as control samples. Patient samples were collected from Shafa Genetic Lab and the IVF Center of Imam Khomeini Hospital, Ahvaz, Khuzestan Province, Iran, while control samples were obtained from Khuzestan Blood Transfusion Organization, Ahvaz, Khuzestan Province, Iran. Microscopic semen analysis method was used for patient assessment. We selected only patients with idiopathic azoospermia and severe oligozoospermia; therefore, twelve individuals were excluded due to different cases of azoospermia or oligozoospermia resulting from endocrine causes or Y chromosome microdeletions.

An informed consent form and agreement for human specimen analysis were taken from all participants. Peripheral blood samples were stored at -70˚C with ethylenediaminetetraacetic acid (EDTA, Merck, USA) as anticoagulant in order to extract DNA.

Molecular analysis

Genomic DNA was extracted using Diatom DNA Prep extraction kit (Gene Fanavaran Co., Iran), according to the manufacturer's instructions. Then 100 µg purified DNA were diluted and stored at 4˚C before analysis. Next using the polymerase chain reaction (PCR, BioRad, USA) for detection of following six sequence-tagged sites (STS): the sY84 and sY86 within the azoospermia factor a (AZFa) region, the sY127 and sY134 in the AZFb area, and the sY254 and sY255 located within the AZFc site on the long arm of the chromosome Y (Table 1). The applied amplification system, recommended by the European Academy of Andrology (EAA), allowed us to detect 90% of the microdeletions in the AZF region (14, 15). The multiplex PCR product was run by electrophoresis on a 3% agarose (Gene Fanavaran Co., Iran) gel impregnated with ethidium bromide at 5 µg/mL concentration and visualized under UV light.

CAG repeats in exon 1 of the *AR* gene were amplified with these following primers: forward primer including 5′-GCT GTG AAG GTT GCT GTT CCT CAT-3′ and reverse primer including 5′-TCC AGA ATC TGT TCC AGA GCG TGC-3′. Only patients without Yq microdeletions were then analyzed (n=72). We used 25 µl PCR solution (Gene Fanavaran Co., Iran) containing 5 µl PCR buffer, 3 µl of DNA, 0.25 µl of each dNTP, 0.75 µl $MgCl_2$, 0.5 U Taq DNA polymerase, and 1 µl of each forward and reverse primer. PCR was done under these conditions: an initial denaturation step at 94˚C for 3 minutes, 40 cycles of denaturation at 94˚C for 15 seconds, annealing at 49˚C for 1 minute and extension at 72˚C for 1 minute. Final step of extension was 10 minutes at 72˚C. To confirm amplification, PCR products were electrophoresed through a 1.5% agarose gel. Then PCR products, were separated on a 8%-polyacrylamide gel with 1X tris-borate-EDTA (TBE, Merck, USA) at 150 V for 8 hours, impregnated with ethidium bromide

at 5 µg/ml and visualized under UV light. After comparing all polyacrylamide gels, alleles with the same band and the same size (CAG repeats) were categorized into one group. One allele from each group was selected and sequenced, so the size of the PCR band was determined.

Statistical analysis

Results are reported as mean ± SD in case and control groups. Statistical analysis was carried out using t test. The data were considered significant when $P<0.05$.

Results

In this case-control study, 72 fertile and 84 infertile males were analyzed. Forty-eight patients showed severe oligospermia (sperm concentration ≤15 million/ml), and 36 were non-obstructive azoospermic males. Summary of clinical data of fertile and infertile groups are shown in table 2. The azoospermic patients aged between 23 and 47 years, with mean age of 31 years. The oligozoospermia patients' age ranged from 22 to 38 years, with mean age of 32 years. The control group aged between 21 and 67 years, with mean age of 38.79 ± 10.24 years.

Table 1: Sequence of primers used to amplify specific regions to assess Y chromosome microdeletions

STS	Region	Sequence 5′ to 3′	bp
SRY	Yp11.3	F 5′-GAA TAT TCC CGC TCT CCG GA-3′	472
		R 5′ -GCT GGT GCT CCA TTC TTG AG-3′	
Y84	AZFa	5′ -AGA AGG GTC TGA AAG CAG GT-3′	325
		5′-GCC TAC TAC CTG GAG GCT TC-3	
Y86	AZFa	5′- GTG ACA CAC AGA CTA TGC TTC-3′	320
		5′- ACA CAC AGA GGG ACA ACC CT-3′	
Y127	AZFb	F 5′-GGC TCA CCA ACG AAA AGA AA -3	274
		R 5′-CTG CAG GCA GTA ATA AGG GA -3′	
Y134	AZFb	F 5′-GTC TGC CTC ACC ATA AAA CG-3′	301
		R 5′-ACC ACT GCC AAA ACT TTC AA-3′	
Y254	AZFc	F 5′-GGG TGT TAC CAG AAG GCA AA-3′	370
		R 5′-GAA CCG TAT CTA CCA AAG CAG C-3′	
Y255	AZFc	F 5′-GTT ACA GGA TTC GGC GTG AT-3′	126
		R 5′-CTC GTC ATG TGC AGC CAC-3′	

AZF; Azoospermia factor and STS; Sequence-tagged sites.

Table 2: Summary of clinical data of fertile and infertile groups

Classification (n)	Age (Y)	Sperm concentration (million/ml)	Bitesticular volume (ml)	Morphology (%)	Motility (%)
Control (72)	21-67 (38.79 ± 10.24)	20-100 (53.19 ± 17.83)	34-70 (49 ± 9.29)	40.08 ± 7.53	60.94 ± 9.89
Total infertile (72)	24-65 (38.62 ± 7.51)	0-4 (0.53 ± 0.62)	2-66 (28.97 ± 14.30)	13.03 ± 9.07	21.56 ± 14.47
Azoospermia (36)	23-47 (31.25 ± 8.05)	0	6-51(24.13 ± 12.73)	0	0
Sever oligospermia (48)	22-38 (32.98 ± 5.21)	0.01-4 (1.09 ± 1.17)	2-60 (25.67 ± 13.61)	11.25 ± 4.78	11.75 ± 4.25

Our results revealed that deletions were found in 12 out of 84 (14.3%) infertile men. Therefore, 1.2% of the infertile men had microdeletions in the AZFa region, 11.9% in the AZFb regions, and 1.2% in the AZFc regions (Table 3). It means that among 48 severe oligozoospermic patients, one had deletions only in the AZFa region, 10 had microdeletions in the AZFb region, and one had in the AZFc region. All patients and controls were shown amplification of the *sex-determining region (SRY)* gene. In control males, no microdeletions were identified (Figs.1, 2).

Then patients with microdeletions were excluded from the CAG repeat analysis (Table 3). After gels analysis (Fig.3), we were able to identify 6 and 12 different alleles in the infertile and control groups, respectively. Due to the facts that males have one X chromosome and individuals belonging to a population have the same allele, we were able to specify the frequency of distribution of the alleles. The distribution of the allele frequencies in both groups is depicted in table 4 and figure 4.

Table 3: Frequency of AZF Y chromosome microdeletions in infertile men

Patients	Azoospermia number (%)	Oligospermia number (%)	Total number (%)
Deletions	36 (42.86%)	48 (57.14%)	84 (100%)
AZFa	0	1 (2.1%)	1 (1.2%)
AZFb	0	10 (20.8%)	10 (11.9%)
AZFc	0	1 (2.1%)	1 (1.2%)
Total number (%)	0	12 (25%)	12 (14.3%)

AZF; Azoospermia factor.

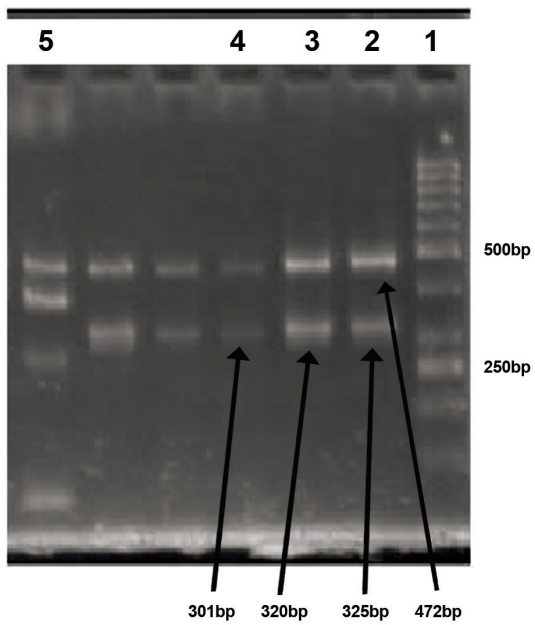

Fig.1: Amplified sequence-tagged sites (STS) in an infertile man with no deletion. line 1; 50 bp marker (two sharp bands are 250 bp and 500 bp markers), line 2; The heavy band is SRY (472bp) and the other is Y84 STS (325bp), line 3; The heavy band is SRY (472bp) and the other is Y86 STS (320bp), line 4; The heavy band is SRY (472bp) and the other is Y134 STS (301bp) and line 5; The heavy band is SRY (472bp) and the others are Y254 (370bp), Y127 (274bp) and Y255 (126bp) STSs.

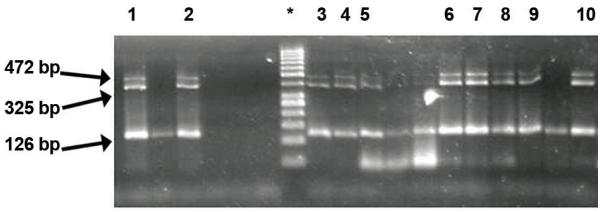

Fig.2: Analysis of polymerase chain reaction (PCR) products using Y chromosome with STS markers on 3% agarose gel indicates 10 infertile men (1-10) with microdeletion in Y127(274 bp). line*; 50 bp marker (two sharp bands are 250 bp and 500 bp markers) and line 1-10; The heavy band is SRY (472bp) and the others are Y84 STS (325bp) and Y255 STS (126bp).

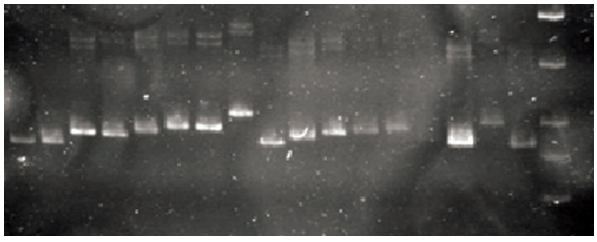

Fig.3: Analysis of polymerase chain reaction (PCR) products using *androgen receptor (AR)* gene on 8% polyacrylamide gel indicates difference between alleles size. First line from right is 10 bp DNA marker that its bands (from down to up) are 10, 20, 30, 40 and 50 bp, while other lines are different CAG alleles.

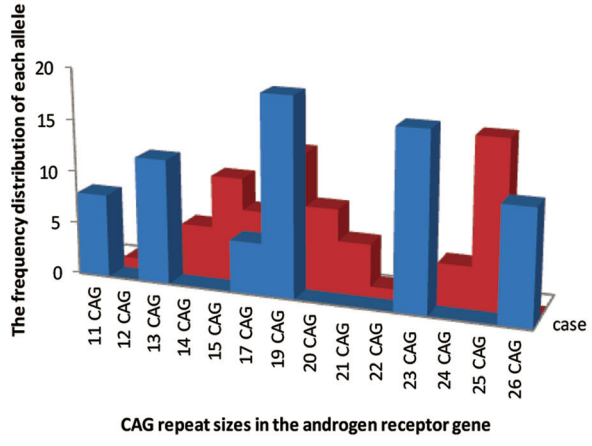

Fig.4: Distribution of CAG repeats sizes in the *androgen receptor (AR)* gene of infertile men (blue line) and fertile controls (red line).

Table 4: Distribution of CAG repeat sizes in the *androgen receptor (AR)* gene of infertile (case) and fertile (control) men

Group	Allele (the number of CAG)	Number	Frequency
Case	11	8	11.1%
	13	12	16.7%
	17	5	7%
	19	19	26.4%
	23	17	23.6%
	26	11	15.3%
Control	12	1	1.4%
	13	1	1.4%
	14	5	7%
	15	10	13.9%
	17	7	9.7%
	19	13	18.1%
	20	8	11.1%
	21	5	7%
	22	1	1.4%
	23	1	1.4%
	24	4	5.5%
	25	16	22.2%

According to the frequencies, the most and the least common alleles were seen in both groups. In the infertile group, the allele with 19 repeats of CAG was the most common allele (26.38%), while in fertile group, it was the allele with 25 repeats (22.22%). The least allele in case group has 17 repeats, while in the control group, the least alleles were seen with 12, 13, 22 and 23 repeats. The mean CAG repeat length is 18.99 ± 0.35 (range, 11-26) and 19.96 ± 0.54 (range, 12-25) in infertile males and controls, respectively. There is no association between CAG repeat length and azoospermia or oligozoospermia in infertile men living in Khuzestan in this study (Table 5).

Table 5: Correlation between severity of impairment of spermatogenesis and CAG length

Group	CAG, mean ± SD	P value	Sperm count	n	CAG, mean ± SD	P value
Infertile	18.99 ± 0.35	NS	Azoospermia	36	19.80 ± 0.75	NS
			Sever oligospermia	36	18.16 ± 0.63	NS
Fertile	19.96 ± 0.54	NS	Normal	72	19.96 ± 0.54	NS

NS; Non significant.

Discussion

As mentioned, male factor is the reason of infertility in approximately 50% of infertile cases, while the causes of more than half of these cases are poorly understood. It is clear that androgen as the main male hormone has critical roles in male sexual differentiation and regulation of spermatogenesis. It is also noted that androgen can act on target cells through its receptors.

Our study investigated the association between the number of CAG repeats of exon 1 in androgen receptor gene and sperm counts in 72 azoospermic or oligozoospermic men and 72 control men from Khuzestan, Iran. The infertile men with idiopathic azoospermia and severe oligozoospermia as the case group were selected after screening for other reason of infertility such as Yq microdeletion. Among 84 infertile men, 12 individuals (14.3%) with severe oligozoospermia were found to have microdeletions. The frequency of microdeletions was 25% in the severe oligozoospermic group and zero in the azoospermic group. Our findings showed that 1.2% of the infertile men had microdeletions in the AZFa region, 11.9% in the AZFb regions, and 1.2% in the AZFc regions. In other words, among 48 severe oligozoospermic patients, one had deletions only in the AZFa region, 10 had microdeletions in the AZFb region, and one had microdeletions in the AZFc regions. Therefore, 83.3% of deletions were located at the AZFb region.

Although several studies have investigated the proposed association between CAG repeat length in *AR* gene and infertility, these reports have yielded conflicting results. Different studies from the United States, Singapore (2), France (3), Japan (6) and Spain (7) have reported an association between higher CAG repeats and low sperm count, while other studies from Germany (8, 10), India (9), Nigeria (16), Mexico (17), Chile (18) and Egypt (19) have not demonstrated a significant correlation between them. One meta-analysis (20) using 33 published studies understood correlation between CAG repeat number in *AR* gene and infertility in men. The observed variations in the results from previous studies may originate from several factors: i. Ethnically diverse populations which can change some environmental and genetic factors in them, ii. The studied infertile men may represent a heterogeneous group with respect to the causes of infertility and may be under the effect of different genetic mutations or even epigenetic phenomena, and iii. Different inclusion criteria in each study. The infertile populations in different studies may be included various categories of infertility such as patients with varicocele or infection in genital tract and also different semen parameters, for example azoospermia or oligozoospermia (10, 21, 22). Most importantly, the control groups in many of these previous studies were not well-matched in terms of ethnicity and age. The control groups in these studies often included not only individuals with proven fertility, but also individuals with normal sperm count but not proven fertility and/or individuals from unselected populations (23-25).

At first our results showed differences between the cases and controls in the length of CAG repeats. In the case group, it was only found 6 alleles ranging from 11 to 26 repeats of CAG. Diversity between the numbers of CAG repeats in controls is more than the cases, indicating that there are 12 different alleles in fertile individuals ranging between 12 and 25 repeats, while four of the alleles are common in both groups that are alleles with 13, 17, 19 and 23 repeats. Despite that, both groups have differences in the frequencies of the alleles. In group of infertile men, alleles with 13 and 23 repeats have high frequencies, but the numbers of these alleles in control group are very low.

Also in fertile men, we did not find alleles with repeat number of 11 and 26, but these alleles showed high frequencies in infertile men. It can be mention that in case group, the allele with 19 CAG repeats is the most common, but in control group, the most common allele has 26 repeats.

Despite the facts we found no differences in the mean number of CAG repeats between infertile men (18.99 ± 0.35, range, 11-26) and controls (19.96 ± 0.54, range, 12-25). The infertile group was further subdivided according to sperm counts, and no differences were found in any subgroup when compared to controls. These results are in agreement with studies in which no association was found. Thus, it might be assumed, at least in our studied population, the AR could act without being associated with any pathologic phenotype.

Our study presented the range of alleles in an ethnically and geographically restricted population of Iranian men with normal fertility. There was no significant correlation between CAG repeat length and risk of male infertility in our ethnically restricted experimental population compared with the matched control population. In other word, polymorphism detected in the CAG-rich region of the *AR* gene may not be a useful genetic indication of male factor infertility.

Conclusion

With this high percentage of deletion in Yq chromosome in oligospermic men in this study, we can say that Y microdeletions is likely to be one of the main reasons of male infertility in this region, and the most frequent type of microdeletion is located at the AZFb region. Also, in this study, we did not find a correlation between CAG repeats in *AR* gene and sperm count, showing that there was no significant correlation between CAG repeat length and the risk of male infertility in this part of Iran. Therefore, polymorphism detected in the polyglutamine-rich region of the AR could not be a useful genetic indication of male infertility.

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Original Article

Expression Levels of *PPARγ* and *CYP-19* in Polycystic Ovarian Syndrome Primary Granulosa Cells: Influence of ω-3 Fatty Acid

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Abstract

Background: The omega-3 fatty acid (ω-3 fatty acid) such as eicosapentaenoic acid (EPA) is currently used in the clinic as a nutritional supplement in the treatment of polycystic ovarian syndrome (PCOS). The present study was designed to investigate the effect of EPA on the expression levels of peroxisome proliferator-activated receptor gamma (*PPARγ*) and cytochrome P450 aromatase (encoded by the *CYP-19*) in primary cultured granulosa cells (GC) from patients undergoing *in vitro* fertilization (IVF), and also to compare these effects with those in GC of PCOS patients.

Materials and Methods: In this experimental study, human GC were isolated, primary cultured *in vitro*, exposed to a range of concentrations of the EPA and investigated with respect to gene expression levels of *PPARγ* and *CYP-19* using real time-polymerase chain reaction (PCR). The participants (n=30) were the patients admitted to the IVF Center in February-March 2013 at Alzahra Hospital, Tabriz, Iran, who were divided into two groups as PCOS (n=15) and non-PCOS (n=15) women (controls).

Results: All doses of the EPA significantly induced *PPARγ* mRNA gene expression level as compared to the control recombinant follicle stimulating hormone (rFSH) alone condition. High doses of EPA in the presence of rFSH produced a stimulatory effect on expression level of *PPARγ* (2.15-fold, P=0.001) and a suppressive effect (0.56-fold, P=0.01) on the expression level of *CYP-19*, only in the PCOS GC.

Conclusion: EPA and FSH signaling pathway affect differentially on the gene expression levels of *PPARγ* and *CYP-19* in PCOS GC. Altered FSH-induced *PPARγ* activity in PCOS GC may modulate the *CYP-19* gene expression in response to EPA, and possibly modulates the subsequent steroidogenesis of these cells.

Keywords: Eicosapentaenoic Acid, PPAR Gamma, Aromatase, Granulosa Cells, Polycystic Ovary Syndrome

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Introduction

Polycystic ovarian syndrome (PCOS) is the most commonly occurring cause of female infertility (1). In PCOS, there is an imbalance of female sex hormones, which may lead to ovarian cysts and irregular or absent menstrual cycle. The abnormality has been mainly attributed to the suppression of the follicle stimulating hormone (FSH) secretion by an excess androgen produced from the ovary. Accelerated early follicular growth leads to attenuated FSH responsiveness and the premature luteinisation of granulosa cells (GC). In turn, the development of the dominant follicle is disrupted which causes cystic follicular arrest (2).

The cytochrome P450 aromatase, encoded by the *CYP-19* gene, in ovarian GC that converts testosterone to estradiol is induced by FSH during early follicle development. The timely expression of *CYP-19* in GC plays a critical role in follicle development. In the *CYP-19* knockout mice, antrum formation is arrested at a stage before ovulation and no corpora lutea are formed (3). The follicular arrest of PCOS has been characterized by the lack of *in vivo* FSH-induced *CYP-19* activity in GC (4).

The expressions of *CYP-19* is coordinately regulated and efficiently inhibited by thiazolidinediones (TZDs) in human GC obtained from *in vitro* fertilization (IVF) (5, 6). TZDs are known as agonists of the gamma isoform of the peroxisome proliferator-activated receptor (*PPARγ*), a family of nuclear receptors regulating the expression of genes involved in lipid metabolism, insulin sensitivity, and cellular differentiation. *PPARγ* expression has been found in the GC (7). The *PPARγ* may regulate the steroidogenesis, thereby contributes to the regulation of ovarian function (8). Previous studies have reported that retinoid X receptor (RXR) response elements are present in the *CYP-19*; however, no exact region that responds independently to *PPARγ* has yet been identified (9).

There is a strong indication that omega-3 fatty acids (ω-3 fatty acids) have protective action against PCOS (10). In particular, eicosapentaenoic acid (EPA), a long-chain ω-3 fatty acid (PUFA), is a natural high-affinity ligand for *PPARγ*. Despite the increasing clinical use, the mechanisms by which EPA exerts its effects is yet relatively unknown. The aim of the present study was to investigate the effects of EPA on gene expression levels of *PPARγ* and *CYP-19* in cultured GC from patients undergoing IVF, and also to compare these effects with those in GC of PCOS patients.

Materials and Methods

This experimental study was approved by the Ethics Committee of Tabriz University of Medical Sciences. All patients gave a written informed consent and their confidentiality and anonymity were protected.

Primary cell culture

Sampling was done by a simple consecutive method covering all patients (n=30) who were admitted to the IVF Center in February-March 2013 at Alzahra Hospital, Tabriz, East Azerbaijan Province, Iran. PCOS were defined as the presence of 12 or more follicles measuring 2-9 mm with clinical (a Ferriman–Gallwey score >7) and/or biochemical hyperandrogenism (total testosterone >3 nmol/l) (11). The participants (n=30) were divided into two groups as PCOS (n=15) and non-PCOS (n=15) women (controls).

Inclusion criteria were no alcohol consumption and no smoking habit. Uterus abnormalities, endometriosis, anovulation, positive history of endocrine disease and inflammatory disorders such as thyroid and adrenal disorders, hormonal treatment, and history of recurrent infections were considered as exclusion criteria in this study. Control group (n=15) included individuals with age- (27.62 ± 4.14 years) and body mass index (BMI)- (25.11 ± 2.57 kg/m^2) matched with no evidence of hyperandrogenemia or menstrual irregularities. All patients underwent a standard infertility evaluation, including hormonal testing and assessment of the uterus and fallopian tubes by means of hysterosalpingography. Patients underwent a long gonadotropin-releasing hormone (GnRH) agonist (decapeptyl, Debio Pharm, Geneva, Switzerland)/ FSH-long down regulation protocol as described previously by us (12). GC was isolated from aspirated follicular fluid by hyaluronidase digestion, followed by Percoll gradient centrifugation (13).

Three sets of experiments with both PCOS and control groups were performed. GC was pooled because the number of cells from follicles was insufficient to perform individualized culture. In the experiments, each group composed of GC pooled from 5 women. In total, GC were isolated and pooled from 15 PCOS and 15 control women of reproductive age. The GC were counted with a homocytometer, and approximately 1×10^6 cells were plated in a 12-well culture plate containing dulbecco's modified eagle medium/ nutrient mixture/F-12 (DMEM/F12, Cellgro, USA)

medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin, for 24 hours. Cells were maintained at 37˚C in 5% CO_2 in a humidified incubator. EPA (Sigma, St. Louis, MO) was conjugated with bovine serum albumin (BSA) fatty acid-free (Sigma, St. Louis, MO) before treatment (14). GC, after serum starvation overnight, were treated with indicated concentrations of EPA (25-100 µM), both either with or without pretreatment with recombinant (r)FSH (100 ng/mL).

Real-time polymerase chain reaction analysis

Total RNA was isolated using RNX-Plus according to the instructions of the manufacturer. RNA pellets were ethanol-precipitated, washed, and resuspended in sterile ribonuclease-free water. Two µg of total RNA were reverse transcribed into cDNA using SuperScript II reverse transcriptase (Life Technologies, Carlsbad, CA, USA). Real-time polymerase chain reaction (PCR) was carried out using the fluorescent dye SYBR-Green and a Bio-Rad CFX real-time PCR system (BioRad Co, CA, USA). The primers used for qPCR were as follows: *PPARγ*, 5′ ATGACAGACCTCAGACAGATTG 3′ (sense) and 5′ AATGTTGGCAGTGGCTCACGTG 3′ (antisense); *CYP-19*, 5′ TCTTGGTGTGGAATTATGAG 3′ (sense) and 5′ TTGAGGACTTGCTGATAATG 3′ (antisense); glyceraldehydes 3-phosphate dehydrogenase *(GAPDH)*, 5 AAGCTCATTTCCTGGTATGACG 3 (sense) and 5′ TCTTCCTCTTGTGCTCTTGCTGG 3′ (antisense).

Samples were assayed in duplicates. The amount of specific PCR products was normalized to the *GAPDH* mRNA content, and quantities were expressed as an x-fold difference relative to a control.

Statistical analysis

Values are presented as mean ± standard deviation (SD) of 3 separate experiments done in duplicate. Data in all groups were normally distributed. Statistically significant differences in mean values between groups were assessed by t tests. Analysis of variance test were used for comparing the group means. Calculation of significance between groups was done according to analysis of variance (ANOVA) with post hoc Tukey's tests for multiple comparisons. Repeated-measures ANOVA was used for measures of response times, and a P value of <0.05 was considered statistically significant.

Results

Figure 1 shows the genes expression levels measured by quantitative PCR method in GC from patients with PCOS and non-PCOS women. Primarily, no significant differences were found in the gene expression levels of *PPARγ* and *CYP-19* between the two groups.

To determine the effect of rFSH stimulation on expression levels of *PPARγ* and *CYP-19*, GC was treated with rFSH. Only *CYP-19* showed a significant increase in mRNA level ($P<0.001$, Fig.2), which was more elevated in PCOS than in non-PCOS (mean 4.0-fold vs. 3.5-fold, respectively, $P=0.03$). In contrast, incubation with EPA alone resulted in comparable upregulation of *PPARγ* expression level (1.49 ± 0.12 vs. 1.52 ± 0.11, $P=0.51$) in GCs from non-PCOS and PCOS patients. However, no such changes were observed for *CYP-19* expression level in EPA-treated cells (Fig.2).

Comparison of control rFSH with the combined rFSH-EPA condition showed a similar response compared to the EPA alone. To optimize the assay, cultured GC from non PCOS women were incubated with the 50 µmol/L EPA and the incubation time ranged from 12 hours to 48 hours. While no significant changes were observed in the expression level of *CYP-19*, the expression level of *PPARγ* increased by 30% ($P=0.02$) after 24 hours. However, later no further changes were observed in the expression levels of both mRNAs (Fig.3).

In the next series of experiments, three doses of EPA (0-100 µM) were tested in the presence of rFSH. Treatment of GCs with 50 and 100 µM doses of the EPA significantly increased *PPARγ* mRNA gene expression level compared to the control rFSH alone condition ($P<0.05$). *PPARγ* displayed a larger fold change in the PCOS group than in the non-PCOS group. The magnitude of this difference between non-PCOS and PCOS was more pronounced at the higher doses of EPA (e.g., 1.42-fold at 25 µmol vs. 2.15-fold at 100 µM, $P=0.008$). Moreover, it was identified that the expression level of *CYP-19* was also influenced by the higher doses of EPA in the PCOS GC as compared to the control. The combination of high doses of EPA in the presence of rFSH produced a strong suppressive effect on the *CYP-19* gene expression level in the PCOS GC (0.56-fold, $P=0.01$, Fig.4).

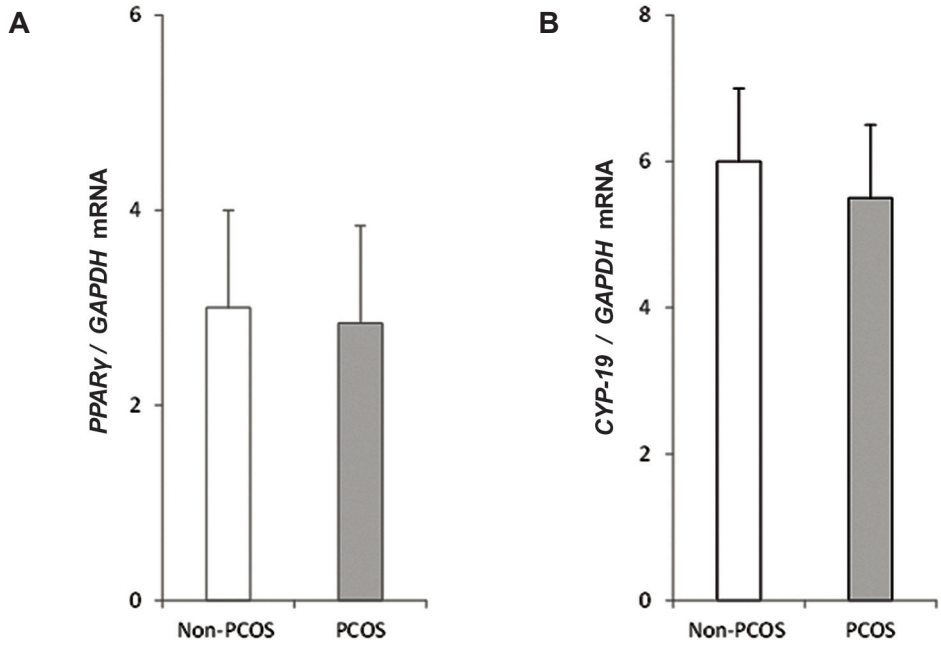

Fig.1: Quantitative analysis of *PPARγ* **(A)** and *CYP-19* **(B)** genes expression levels by real-time PCR in GCs from PCOS and non PCOS-women. Each expression level was normalized to the *GAPDH* levels. The mean ± SD of three independent determinations with cells pooled from 5 women per group per experiment (t test).
PCR; Polymerase chain reaction, GCs; Granulosa cells and PCOS; Polycystic ovarian syndrome.

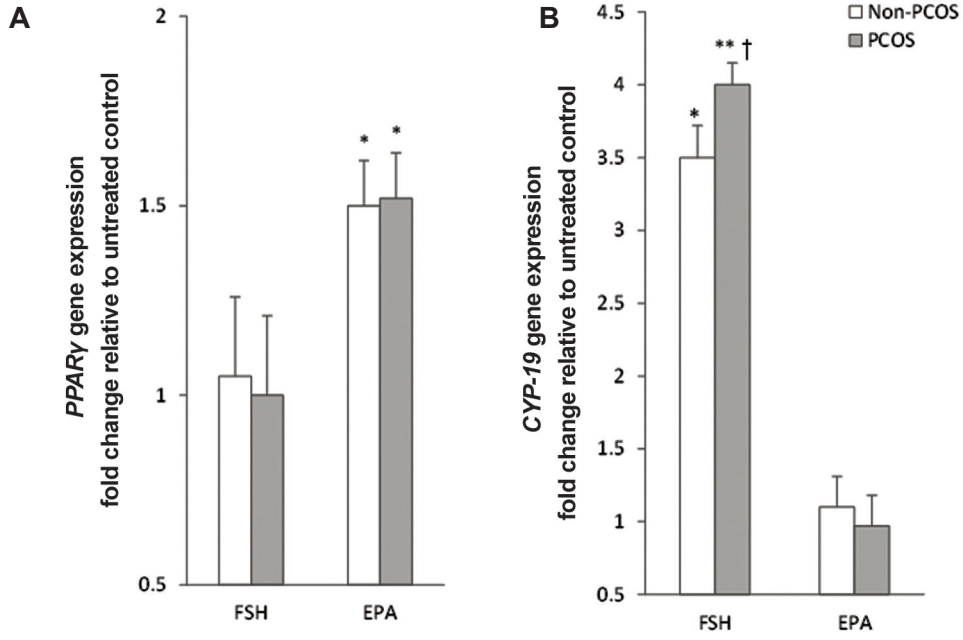

Fig.2: Effect of the follicle stimulating hormone (FSH) and eicosapentaenoic acid (EPA) incubation on mRNA expression levels of *PPARγ* and *CYP-19*. GCs, after serum starvation, were incubated for 24 hours ± 100 ng/mL FSH or 50 µmol/L EPA. Cell lysates were prepared and analyzed by real-time PCR for genes expression levels. Expression levels of *PPARγ* **(A)** and *CYP-19* **(B)** in each lysate were normalized to the amount of *GAPDH* and represented as fold of untreated control. The mean ± SD of three independent experiments with cells pooled from 5 women per group per experiment (t test).
*; P<0.05 and **; P<0.01 vs. untreated control and †; P<0.05 vs. non-PCOS.
PCR; Polymerase chain reaction, GCs; Granulosa cells and PCOS; Polycystic ovarian syndrome.

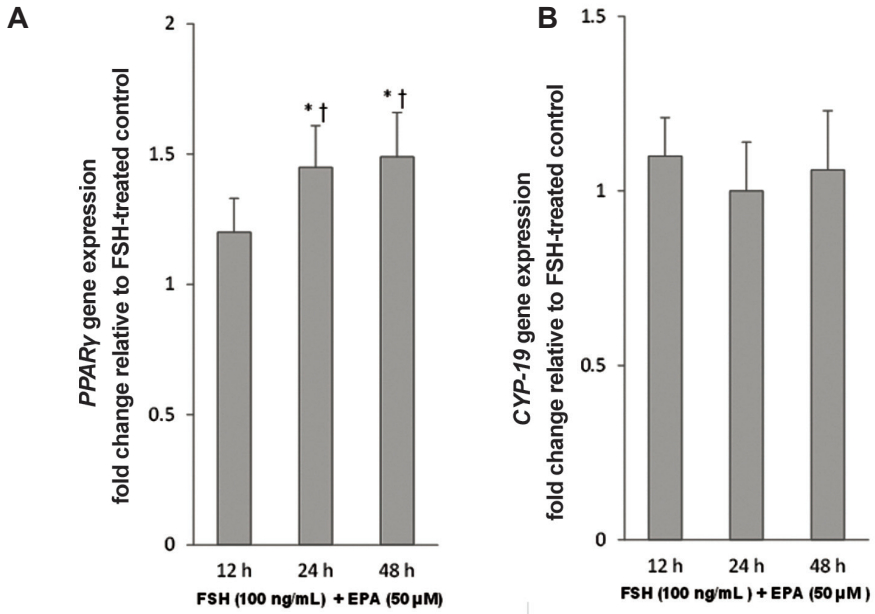

Fig.3: Effect of eicosapentaenoic acid (EPA) incubation time on mRNA expression levels of *PPARγ* and *CYP-19*. GCs, after serum starvation, were incubated in 100 ng/mL follicle stimulating hormone (FSH) alone or in combination with 50 µmol/L EPA for 12 hours, 24 hours and 48 hours. Cell lysates were prepared and analyzed by real-time PCR for genes expression levels. Expression levels of *PPARγ* **(A)** and *CYP-19* **(B)** in each lysate were normalized to the amount of *GAPDH* and represented as fold of FSH-treated control. The mean ± SD of three independent experiments with cells pooled from 5 women per group per experiment (repeated-measures ANOVA. *; P<0.05 and †; P<0.05 vs. FSH-treated control and 12-hour incubation, respectively).
PCR; Polymerase chain reaction, GCs; Granulosa cells and h; Hours.

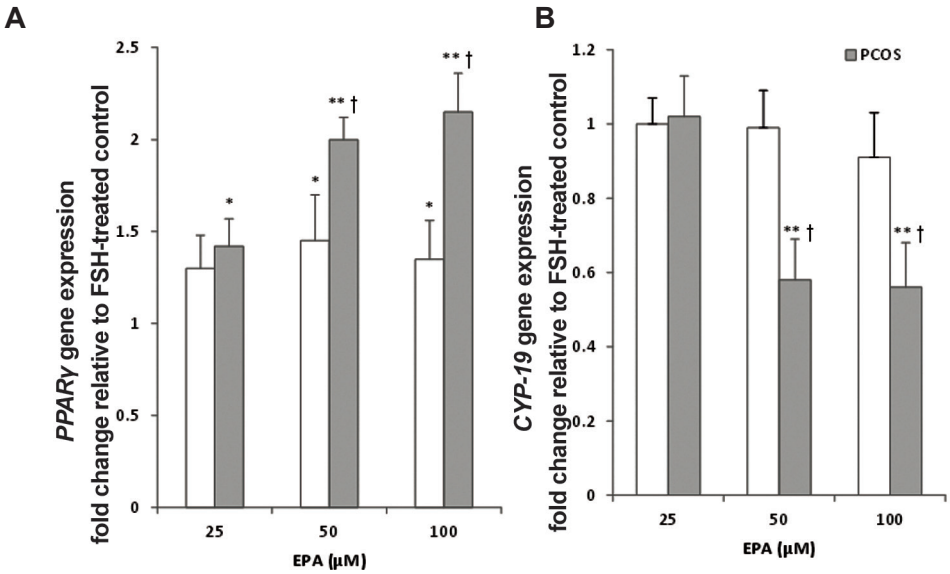

Fig.4: Effect of different doses of eicosapentaenoic acid (EPA) on expression levels of *PPARγ* and *CYP-19* in follicle stimulating hormone (FSH)-stimulated GCs from PCOS and non-PCOS women. GCs, after serum starvation, were incubated in 100 ng/mL FSH alone or in combination with 25 µmol/L, 50 µmol/L or 100 µmol/L EPA for 24 hours. Cell lysates were prepared and analyzed by real-time PCR for genes expression levels. Expression levels of *PPARγ* **(A)** and *CYP-19* **(B)** in each lysate were normalized to the amount of GAPDH and represented as fold of FSH-treated control. The mean ± SD of three independent experiments with cells pooled from 5 women per group per experiment (ANOVA with post hoc Tukey's test, *; P<0.05, **; P<0.01 vs. FSH-treated control and †; P<0.01 vs. non-PCOS).
PCR; Polymerase chain reaction, GCs; Granulosa cells and PCOS; Polycystic ovarian syndrome.

Discussion

PPAR-γ has been shown to be critically important in multiple biological functions such as fertility (12), while EPA and docosahexanoic acid (DHA) are natural, preferentially-binding ligands for this receptor. It has been shown that EPA and DHA down-regulate activation of NF-κB through increasing both PPARγ mRNA levels and protein activity in different types of cells. These effects may be one of the underlying mechanisms for the anti-inflammatory effect of the ω-3 PUFA (15, 16). To the contrary, although no change in *PPARγ* mRNA expression level has been reported previously in certain types of cells after exposure to EPA (17). Our results demonstrated that there were mRNA expression levels of *PPARγ* and *CYP-19* in pre-ovulatory human GC, and that *PPARγ* was increased by EPA. This suggests that EPA may elicit important biological responses in GC via activation of *PPARγ*.

PPARγ is a key transcription factor involved in follicular differentiation (18) and ovarian GC tumor (19). It has been shown that a decrease in expression level of *PPARγ* in response to luteinizing hormone (LH) is important for ovulation and/or luteinization. GC differentiation into the corpus luteum in response to the LH surge is accompanied by reduced *CYP-19* activity. It has been reported that the expression level of mRNA for *PPARγ* in follicles is inversely related to the expression level of mRNA for *CYP-19* (20). Overexpression of *PPARγ* in the KGN ovarian granulosa-like tumor cell line reduced FSH-stimulated *CYP-19* mRNAs (21). These observations suggest that *PPARγ* has an inhibitory effect on the *CYP-19* activity as well as on ovulation and/or luteinization. The complete disruption of FSH-induced estradiol production by synthetic PPAR-γ agonists in cultured human ovarian cells has been attributed to *CYP-19* (5). It has been shown that *PPARγ* agonists suppress the *CYP-19* mRNA expression level in human GC, in a dose-dependent manner, probably via nuclear receptor system *PPARγ*: RXR heterodimer (22). However, the data reported in the literature about the effects of TZDs on *CYP-19* activity in the ovary are controversial. Either no effect (23) or suppressive effects (22) have been shown, which could partly be attributed to a variety of *PPARγ* independent signaling events (24). Furthermore, no specific data is available regarding the effect of either the synthetic or natural *PPARγ* agonists on the expression and activity of GC aromatase in PCOS.

As shown herein and reported previously, FSH induces the expression level of *CYP-19* (25, 26). In contrast, levels of mRNA for *PPARγ* were not affected by treatment with rFSH, in agreement with the observations made previously in rats (27). Co-treatment with EPA and rFSH resulted in enhanced *PPARγ* expression level both in control and PCOS GC. However, altered levels of gene expression in PCOS granulosa in response to the combined drug condition was not similar to that observed in control granulosa. In cultured GC obtained from patients with PCOS, EPA induced a more pronounced effect with rFSH treatment on the mRNA expression level of *PPARγ*. Furthermore, EPA treatment of PCOS GC remarkably down regulated *CYP-19* gene, as compared with non-PCOS patients. Coffler et al. have shown that women with PCOS exhibited dose-dependent GC hyperresponsiveness to FSH and increased production of estradiol (28, 29). The above results implied a possibility that the apparent suppressive effect of EPA on hypersensitivity of PCOS GC to rFSH may be due to a negative regulation of the rFSH signaling by activated *PPARγ*. Accordingly, *CYP-19* down-regulation via induction of *PPARγ* has recently been noted in GC from subjects undergoing IVF (21).

The deregulated synthesis of estradiol (E_2) by PCOS GC has been associated with the arrest of early antral follicle development (30). The GC from PCO antral follicles produce normal or increased E_2 amounts *in vitro* (31), even though follicles in women with PCOS contain low levels of *CYP-19* mRNA (32). This would suggest an *in vivo* blockade of estrogen production by follicular environment in PCOS. This is in accordance with our findings of no statistically significant difference in the expression of *CYP-19* in primary culture between GC from patients with PCOS and those from control non-PCOS.

Unlike the response to combination of rFSH and EPA, the gene expression of *PPARγ* in response to EPA alone was not different between control and PCOS GC. On the other hand, rFSH alone exerted no apparent effect on *PPARγ* gene expression level in the both control and PCOS GC. Based on these results, the higher EPA-induced *PPARγ* expression level in PCOS than in control GC may be somewhat explained by concomitant hypersensitivity of

PCOS cells to FSH. FSH activates several signaling mechanisms through its surface G protein-coupled receptor (GPCR) such as the MEK and PI3K pathways, which are potentially involved in the regulation of *PPARγ*-mediated signaling in GCs (33).

Several clinical evidences support the preventive and therapeutic effects of ω-3 fatty acids in menopausal problems (10). Recently, ω-3 fatty acids supplementation has been related to the improvement in insulin sensitivity (34), and less androgenic and atherogenic lipid profiles (35) in women with PCOS. The results of the present study confirmed the potential effect of ω-3 fatty acids on the ovulatory function of PCOS. It is suggested that the modulatory effect of ω-3 fatty acids on the GC steroidogenesis could also play an important role in the oocyte maturation and subsequent ovulation.

Although previous research has shown beneficial effect of *PPARγ* agonists in PCOS, this is the first study to examine the combined effect of EPA and rFSH on the gene expression levels of *PPARγ* and *CYP-19* in human GC. The small sample size, pooled estimate and lack of assessment of *CYP-19* activity may be seen as limitations of this study. However, the regulatory effects were simultaneously analyzed by studying the expression level in control and PCOS GC, which made it possible to identify similarities and differences. Since the preliminary findings of the present study were derived from cultured GC, it remained to confirm the *in vivo* effect of EPA and to further assess the possible mechanism of action of EPA in the treatment of PCOS.

Conclusion

Our study showed that EPA and FSH signaling pathway affect differentially on the gene expression levels of *PPARγ* and *CYP-19* in PCOS GC. We speculated that altered FSH-induced *PPARγ* activity in PCOS GC may modulate the *CYP-19* gene expression level in response to EPA, and subsequently modulates the steroidogenesis of these cells.

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Original Article

Association of Two Polymorphisms in *H2B.W* Gene with Azoospermia and Severe Oligozoospermia in An Iranian Population

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Abstract

Background: During spermatogenesis, the H2B family, member W *(H2B.W)* gene, encodes a testis specific histone that is co-localized with telomeric sequences and has the potential role to mediate the sperm-specific chromatin remodeling. Previously *H2B.W* genetic variants were reported to be involved in susceptibility to spermatogenesis impairment. In the present study, two single nucleotide polymorphisms (SNPs) in 5´UTR and exon 1 of *H2B.W* gene were examined to investigate possible association of these polymorphisms with male infertility in Iranian population.

Materials and Methods: This case control study was conducted in Royan institute during four-year period (2010–2013). Genetic alteration of two SNPs loci, −9C>T and 368A>G, in *H2B.W* gene were indicated in 92 infertile men who were divided into two main groups including azoospermia (n=46) and sever oligozoospermia (n=46), while there was 60 fertile men as control group. Azoosperima was also divided into three sub-groups including sertoli cell only syndrome (SCOS, n=21), complete maturation arrest (CMA, n=17) and hypo spermatogenesis (n=8) according to testicular biopsy. For analysis, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was applied.

Results: The frequency of allele −9T was significantly higher in CMA group than in patients with SCOS (P<0.05). The haplotype TA (corresponding to simultaneous occurrence of −9T and 368A) compared with haplotype CA (corresponding to simultaneous occurrence of −9C and 368A) in patients suffering from CMA significantly increased, compared with patients had SCOS (P<0.05). However, statistical studies indicated that in general, the distribution frequencies of −9C>T and 368A>G had no significant difference between the infertile groups and control (P=0.859 and P=0.812, respectively).

Conclusion: This investigation showed that SNP −9C>T might be contribute to CMA in azoospermic patients and SNP 368A>G had no correlation with male infertility in Iranian population.

Keywords: Histone, Male Infertility, Polymorphism

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Introduction

One of the most common causes of male infertility is impaired spermatogenesis. It is an intricate, temporal process whereby adult stem cells either self-renew or generate daughter cells that are transformed into a specialized testicular spermatozoon (1-3).

Dramatic chromatin remodeling and chromosomes rearrangement can occur during spermatogenesis. These structural alterations are involved in the normal formation of sperm pronuclei that subsequently ensure the successful fertilization.

Telomeric sequences play an important role in the reorganization and integration of sperm chromosomes (4, 5). They also conduct proper arrangement and separation of chromosomes during cell division, mitosis and meiosis (6, 7). Probably migration of telomeric chromatin to the cell membrane during spermatogenesis establishes unique architecture in the human sperm nucleus that are important in early chromatin remodeling at fertilization and early stages of fetal development (8-14).

To fulfill these roles, some features clearly distinguish telomeres of somatic cells with sperm. For example, unlike other mammals, 10-15% of the histones remain in human sperm (15-17). It is assumed that the remaining histones in human sperm tag specific genes for early expression in embryo (18); however, no evidence of nucleosomal ladder has been observed yet (19, 20).

In addition, numbers of testis-specific histone variants preferentially accumulate in telomeres (21-23). Even though this issue is not conserved, specific histone variants can organize particular regions of the genome, like telomeres, within the globally protamine-packaged sperm chromatin (24). Moreover, telomere-binding protein complex in human spermatozoa is different from somatic cells and contains telomeric histones like the H2B family, member W (H2B.W) (8, 25).

The H2B family, member W *(H2B.W)* gene, is one of the testis specific histone variant genes located at Xq22.2. *H2B.W* consists of three exons and two introns, expressed in particular stage of spermatogenesis (spermiogenesis). H2B.W is also present in mature sperm (21).

H2B.W causes chromatin fibers to resist against compaction (26). This special structure of chromatin may explain the dynamic rearrangement of telomeres at late stages of spermatogenesis, especially telomere extension occurring within elongating spermatids (27). This rearrangement can be a decisive factor to determine the position of telomeres in specific regions of mature sperm nucleus (28). These evidences suggest that H2B.W may also be a epigenetic marker necessary to identify and to cause the transmission of the telomeric chromatins through generations (29). Therefore, it is important to study the characterization of human telomere structure by H2B.W involvement to understand the mechanisms of fertilization (26).

According to recent studies, copy number variations of *H2B.W* locus with other genes [plectin *(PLEC)*, tetraspanin 7 *(TSPAN7)* and p21 protein (Cdc42/Rac)-activated kinase 3 *(PAK3)*] were found not only in men with sertoli cell only syndrome (SCOS), but also in women suffering from premature ovarian failure (POF) and XY gonadal dysgenesis. These 5 genes may imply a common genetic origin of lack of spermatogonia in the male and loss of oogonia in the female, leading to SCOS, XY gonadal dysgenesis and POF, respectively (30).

In addition, several studies indicated that genetic polymorphisms may also increase susceptibility to some forms of male infertility; for example, two recent allelic association studies on −9C>T (rs7885967) and 368A>G (rs553509) polymorphisms in *H2B.W* gene among different populations suggested that genetic variations of this gene could influence the susceptibility to spermatogenesis impairment (31, 32).

In this study, two single nucleotide polymorphisms (SNPs), -9C> T and 368A> G of *H2B.W* gene that may interfere in spermatogenesis were investigated in 152 Iranian men with known fertility status (92 infertile men with azoospermia and severe oligozoospermia and 60 fertile men with normal spermatogenesis) using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) technique.

Materials and Methods

Participants

In this case controlled study conducted in Royan institute, ninety-two infertile men, aged from 25 to 46 years, presenting azoospermia and severe oligozoospermia were enrolled. Comprehensive characterizations of all patients including at least two semen analyses, physical examination, chromosome analysis and molecular tests were performed. Azoospermic group (n=46) were divided into three subgroups according to their testicular biopsy including

patients with SCOS (n=21), complete maturation arrest (CMA, n=17) and hypo spermatogenesis (n=8). Severe oligozoospermic group (n=46) were defined with sperm count less than 5 million cells/mL.

Patients with history of cystic fibrosis, trauma, malignancies, varicocele, diabetes mellitus, hypertension, and chemotherapy were not included. Patients with Klinefelter syndrome, azoospermia factor (AZF) genes micro deletions or any identifiable cause of male infertility, including congenital bilateral absence of vas deference (CBAVD), were also excluded from the study groups by review of their records. Controls included healthy, fertile men, with at least one child within 3 years by spontaneous pregnancy and no history of miscarriage. The mean age of control group was 24 to 46 years. All donors gave an informed consent form before participation. The nationality of all groups was Iranian. All samples were collected during four-year period (2010-2013). This study was approved by the Ethical Committee of Royan Reproductive and Biomedicine Research Center.

DNA preparation

The genomic DNA was extracted from the peripheral blood samples of each patient using salting-out method, according to the protocol (33).

Choice of SNPs

Two SNPs in *H2B.W* gene that reportedly impact the impaired spermatogenesis were chosen for genotyping analysis including SNP −9C>T (rs553509) located in 5′ un-translated region (5′UTR) and SNP 368A>G (rs7885967) with a missense mutation in exon 1 (32), which was in contrast to National Center for Biotechnology Information (NCBI) that refers to 368G>A, according to diverse allele distribution of SNPs in different populations. The sequence of normal *H2B.W* gene (NC_000023) was obtained from the NCBI website: http://www.ncbi.nlm.nih.gov (Fig.1).

Polymerase chain reaction

Amplification of a fragment containing each of these SNPs was carried out by PCR according to the protocol of Ying et al. (32). PCR amplifications were performed in a final volume of 25 µl containing about 100 ng of extracted DNA, 200 µmol/L dNTPs, 10 pmol of each primer, 2.5 µl 10X PCR buffer, 1.5 mmol/l $MgCl_2$ and 1.5 U Taq polymerase (CinnaGen, Tehran, Iran). PCR reaction consisted of an initial denaturing step at 95˚C for 5 minutes followed by denaturation at 95˚C for 45 seconds; annealing at 54˚C (−9C>T) and 60˚C (368A>G), respectively, for 45 seconds; extension at 72˚C for 35 seconds for 30 cycles; and a final extra extension at 72˚C for 10 minutes. Specific primer pairs used in these reactions are shown in table 1.

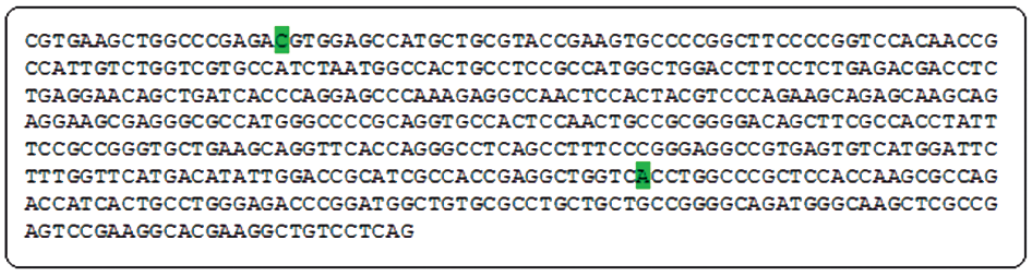

Fig.1: The sequence of 5′UTR and exon 1 of *H2B.W* gene. The position of the SNPs −9C>T and 368A>G are highlighted in green. SNP; Single nucleotide polymorphisms and UTR; Un-translated region.

Table 1: Sequences of oligonucleotide primers used for genotyping analysis of *H2B.W*

	Forward primer	Reverse primer
5'UTR	5′-CATCCAATCAGACGTGAAGCTGGCCCGTGA-3′	5′-TGCTTCTGGGACGTAGTGGA-3′
Exon 1	5′-GTCTGGTCGTGCCATCTAAT-3′	5′-TACCTGAGGACAGCCTTCGT-3′

UTR; Un-translated region.

Restriction enzyme treatment

For the next step, amplified fragments were digested overnight with position specific restriction enzymes. Restriction enzyme Tsp451 was used for genotyping analysis of −9C>T and Eco911 for 368A>G loci according to the manufacturer's protocols (Fermentas, Vilnius, Lithuania).

Electrophoretic separation

Electrophoretic separation were done by 3% agarose gel which indicated 212 bp band for allele T and two bands including 182 bp and 30 bp for allele C of −9C>T locus. Also visualization of 368A>G locus suggested two bands (320 bp and 126 bp) for Allele A and one band (446 bp) for allele G. The representative results of allele analysis for 368A>G and −9C>T loci in *H2B.W* gene by electrophoresis were shown in figures 2 and 3, respectively.

Subsequently genotype alterations of some samples were confirmed performing direct DNA sequencing (Pishgam Biotech, Tehran, Iran). The reaction was carried out by Sanger method using ABI 3730xl capillary DNA sequencer (Fig.4).

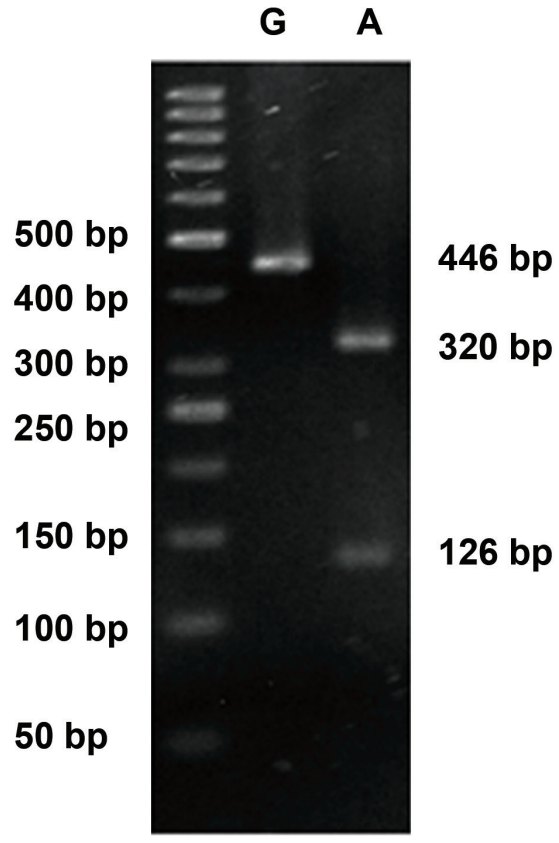

Fig.3: Restriction enzyme digestion of single nucleotide polymorphisms (SNP) 368A>G of PCR product. The marker is a 50bp ladder.

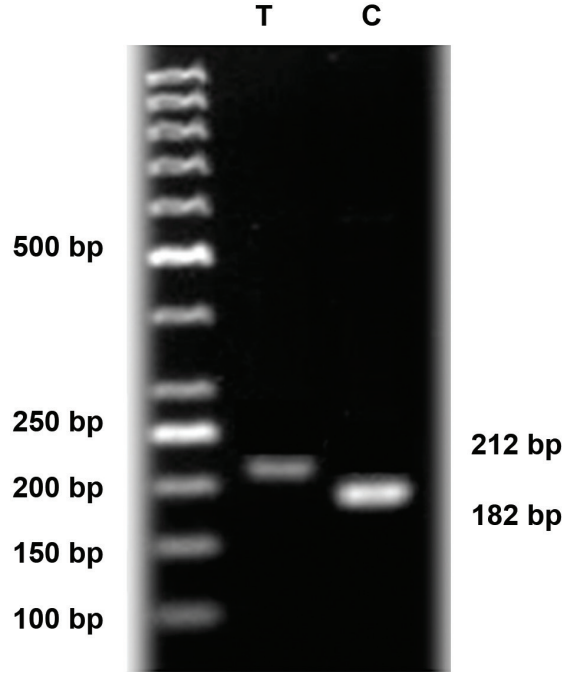

Fig.2: Restriction enzyme digestion of single nucleotide polymorphisms (SNP) −9C>T of polymerase chain reaction (PCR) product (30 bp band for −9C allele not shown in figure). The marker is a 50bp ladder.

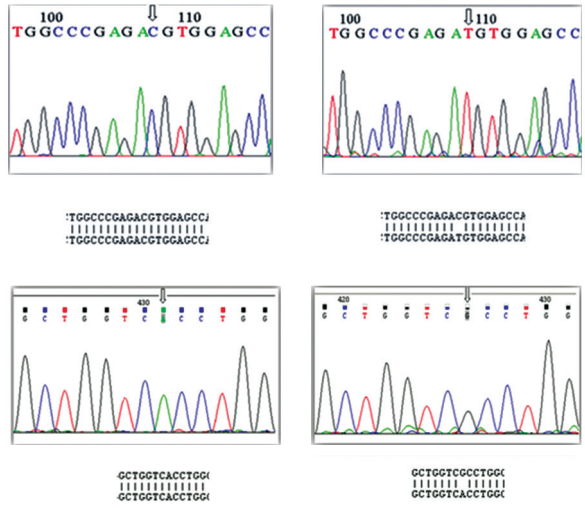

Fig. 4: Direct sequencing of the polymerase chain reaction (PCR) products of 5'UTR and exon 1 of *H2B.W* gene. Arrow marks the sequences of −9C>T and 368A>G polymorphisms.
UTR; Un-translated region.

Statistical analysis

All the statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, SPSS Inc., Chicago, IL, USA). In this study, the genotype frequencies of control and patient groups were compared using the chi-square test. The P value lower than 0.05 was set as statistically significant. A logistic regression analysis was performed to calculate the odds ratio (OR) and 95% confidence interval (95% CI) for measuring the relation of alleles and haplotypes of two SNPs with male infertility.

Results

This study investigated genetic alterations of two SNPs loci, −9C>T and 368A>G, in *H2B.W* gene in 92 infertile patients with azoospermia and severe oligozoospermia and 60 fertile men using a PCR-based RFLP analysis. The distribution frequencies of the two SNPs loci in azoospermic group or severe oligozoospermic subgroups and controls are listed in table 2. As shown in table 2, the frequencies of allele −9T of SNP −9C>T and allele 368G of SNP 368A>G in total patients, azoospermic and severe oligozoospermic patients, had no significant difference in contrast with controls (P>0.05).

In subgroups of azoospermia patients, the frequency of allele −9T in patients suffering from CMA was significantly higher compared with patients suffering from SCOS (P=0.015). The distribution frequencies of the two SNPs loci in azoospermic subgroups are listed in table 3. Also the allele frequency distributions between azoospermic subgroups and controls are shown in table 4.

Four kinds of haplotypes of the two SNPs (CA, TA, CG, and TG) were observed in both infertile patients and controls. Accordingly the haplotype TA compared with haplotype CA significantly increased in patients suffering from CMA, compared with men had SCOS (P=0.029). Tables 5-7 show the results of haplotypes observations.

Table 2: The distributions of allele frequencies of SNPs −9C>T and 368A>G in *H2B.W* gene in infertile patients with azoospermia or sever oligozoospermia and fertile men

Locus		Fertile men	Infertile patients			P value[a]			OR (95% CI)[a]		
	Allele	Total (n=60)	Total (n=92)	Azoospermia (n=46)	Severe oligozoospermia (n=46)	1	2	3	1	2	3
−9C>T	C	58.3% (n=35)	59.8% (n=55)	65.2% (n=30)	54.3% (n=25)	0.859	0.471	0.682	0.942 (0.486-1.824)	0.747 (0.337-1.653)	1.17 (0.542-2.550)
	T	41.7% (n=25)	40.2% (n=37)	34.7% (n=16)	45.7% (n=21)						
368A>G	A	63.3% (n=38)	65.2% (n=60)	65.2% (n=30)	65.2% (n=30)	0.812	0.841	0.841			
	G	36.7% (n=22)	34.8% (n=32)	34.7% (n=16)	34.8% (n=16)				0.921 (0.468-1.815)	0.921 (0.413-2.055)	0.921 (0.413-2.055)

SNP; Single nucleotide polymorphisms, OR; Odd ratio, CI; Confidence interval, [a]; Controls vs. 1; Total infertile patients, 2; Azoospermia, and 3; Severe oligozoospermia. Due to the fact that *H2B.W* is on the X chromosome and that the subjects studied are 46, XY, there are no heterozygous men with both alleles (-9C and -9T; 368G and 368A).

Table 3: The distributions of allele frequencies of SNP −9C>T and 368A>G in *H2B.W* gene in azoospermia according to testicular biopsy

Locus		Azoospermia (n=46)			P value[a]			OR (95% CI)[a]		
	Allele	Men with hypo spermatogenesis (n=8)	CMA (n=17)	SCOS (n=21)	1	2	3	1	2	3
−9C>T	C	75% (n=6)	41.2% (n=7)	81% (n=17)	0.127	0.724	0.015	4.286 (0.661-27.78)	0.706 (0.102-4.891)	0.165 (0.038-0.706)
	T	25% (n=2)	58.8% (n=10)	19.0% (n=4)						
368A>G	A	87.5% (n=7)	70.6% (n=12)	52.4% (n=11)	0.370	0.109	0.257	2.917 (0.281-30.290)	6.634 (0.662-61.199)	2.182 (0.566-8.414)
	G	12.5% (n=1)	29.4% (n=5)	47.6% (n=10)						

SNP; Single nucleotide polymorphisms, CMA; Complete maturation arrest, SCOS; Sertoli cell only syndrome, OR; Odd ratio, CI; Confidence interval, [a]; Men with hypo spermatogenesis vs. 1; CMA, 2; SCOS and 3 SCOS vs. CMA.

Table 4: The distributions of allele frequencies of SNPs −9C>T and 368A>G in *H2B.W* gene in azoospermia according to testicular biopsy and fertile men

Locus		Fertile men	Azoospermia (n=46)			P value[a]			OR (95% CI)[a]		
	Allele	Total (n=60)	Men with hypo spermatogenesis (n=8)	CMA (n=17)	SCOS (n=21)	1	2	3	1	2	3
−9C>T	C	58.3% (n=35)	75% (n=6)	41.2% (n=7)	81% (n=17)	0.374	0.214	0.071	0.467 (0.087-2.505)	2.000 (0.670-5.971)	0.329 (0.099-1.098)
	T	41.7% (n=25)	25% (n=2)	58.8% (n=10)	19.0% (n=4)						
368A>G	A	63.3% (n=38)	87.5% (n=7)	70.6% (n=12)	52.4% (n=11)	0.144	0.364	0.636	0.200 (0.023-1.729)	0.583 (0.182-1.866)	1.273 (0.469-3.454)
	G	36.7% (n=22)	12.5% (n=1)	29.4% (n=5)	47.6% (n=10)						

SNP; Single nucleotide polymorphisms, CMA; Complete maturation arrest, SCOS; Sertoli cell only syndrome, OR; Odd ratio, CI; Confidence interval, [a]; Controls vs. 1; Men with hypo spermatogenesis, 2; CMA and 3; SCOS.

Table 5: The distributions of haplotype frequencies of SNPs −9C>T and 368A>G in *H2B.W* gene in infertile patients with azoospermia or sever oligozoospermia and fertile men

Haplotype	Fertile men	Infertile patients			P value[a]			OR (95% CI)[a]		
	Total (n=60)	Total (n=92)	Azoo-spermia (n=46)	Severe oligozoo spermia (n=46)	1	2	3	1	2	3
CA	33.3% (n=20)	30.4% (n=28)	34.7% (n=16)	26.0% (n=12)	0.693	0.756	0.688	Reference	Reference	Reference
TA	30% (n=18)	34.8% (n=32)	30.4% (n=14)	39.1% (n=18)	0.565	0.954	0.302	1.270 (0.563-2.866)	0.972 (0.373-2.573)	1.667 (0.632-4.392)
CG	26.7% (n=16)	29.3% (n=27)	30.4% (n=14)	28.2% (n=13)	0.664	0.857	0.562	1.205 (0.519-2.802)	1.094 (0.413-2.894)	1.354 (0.487-3.769)
TG	10% (n=6)	5.4% (n=5)	4.3% (n=2)	6.5% (n=3)	0.441	0.321	0.819	0.595 (0.159-2.224)	0.417 (0.074-2.350)	0.883 (0.175-3.965)

SNP; Single nucleotide polymorphisms, OR; Odd ratio, CI; Confidence interval, [a]; Controls vs. 1; Total infertile patients, 2; Azoospermia and 3; Sever oligozoospermia.

Table 6: The distributions of haplotype frequencies of SNPs −9C>T and 368A>G in *H2B.W* gene in azoospermia according to testicular biopsy

Haplotype	Azoospermia (n=46)			P value[a]			OR (95% CI)[a]		
	Men with hypo spermatogenesis (n=8)	CMA (n=17)	SCOS (n=21)	1	2	3	1	2	3
CA	62.5% (n=5)	17.6% (n=3)	38.1% (n=8)	0.246	0.519	0.104	Reference	Reference	Reference
TA	25% (n=2)	52.9% (n=9)	14.3% (n=3)	0.060	0.952	0.029	7.500 (0.921-61.047)	0.937 (0.114-7.728)	0.125 (0.019-0.805)
CG	12.5% (n=1)	23.5% (n=4)	42.9% (n=9)	0.155	0.150	0.851	6.667 0.487-91.331)	5.625 (0.537-58.909)	0.844 (0.143-4.974)
TG	0	5.9% (n=1)	4.8% (n=1)	1.000	1.000	0.532	2.692E9 (0.000- .)	1.010E9 (0.000-.)	0.375 (0.017-8.103)

SNP; Single nucleotide polymorphisms, CMA; Complete maturation arrest, SCOS; Sertoli cell only syndrome, OR; Odd ratio, CI; Confidence interval, [a]; Men with hypo spermatogenesis vs. 1; CMA, 2; SCOS and 3; SCOS vs. CMA.

Table 7: The distributions of haplotype frequencies of SNPs −9C>T and 368A>G in *H2B.W* gene in azoospermia according to testicular biopsy and fertile men

Haplotype	Fertile men	Azoospermia (n=46)			P value[a]			OR (95% CI)[a]		
	Total (n=60)	Men with hypo spermatogenesis (n=8)	CMA (n=17)	SCOS (n=21)	1	2	3	1	2	3
CA	33.3% (n=20)	62.5% (n=5)	17.6% (n=3)	38.1% (n=8)	0.602	0.359	0.358	Reference	Reference	Reference
TA	30% (n=18)	25% (n=2)	52.9% (n=9)	14.3% (n=3)	0.366	0.104	0.244	0.444 (0.077-2.581)	3.333 (0.779-14.26)	0.417 (0.096-1.815)
CG	26.7% (n=16)	12.5% (n=1)	23.5% (n=4)	42.9% (n=9)	0.226	0.540	0.564	0.250 (0.026-2.361)	1.667 (0.325-8.549)	1.406 (0.442-4.473)
TG	10% (n=6)	0	5.9% (n=1)	4.8% (n=1)	0.999	0.933	0.450	0.000 (0.000--)	1.111 (0.097-12.750)	0.417 (0.043-4.034)

SNP; Single nucleotide polymorphisms, CMA; Complete maturation arrest, SCOS; Sertoli cell only syndrome, OR; Odd ratio, CI; Confidence interval, [a]; Controls vs. 1; Men with hypo spermatogenesis, 2; CMA and 3; SCOS vs. CMA.

Discussion

Study mutations in human X-linked genes with a testis-specific pattern in view of male infertility are considered to be remarkable. Firstly this chromosome is enriched for genes expressed in reproduction-related tissues and secondly it is due to its hemizygous exposure in men (34). *H2B.W* is a newfound X-linked gene that its characteristic and association with male infertility have been reported recently.

In this study, the prevalence of two SNPs −9C>T and 368A>G, in *H2B.W* genes, was conducted on a population of Iranian infertile men.

The present study showed that the frequency of −9T at the −9C>T locus was significantly higher in CMA group than in patients with SCOS (Table 3), suggesting that the mutation of allele C to T in *H2B.W* gene might influence mRNA stability or its overall translation rate (35) that leads to arrest the maturation process of spermatids. This could also explain the expression of *H2B.W* gene at late stages of spermatogenesis.

As shown in table 2, in general, no significant differences are found in the frequencies of −9T allele between two groups of controls and patients, proposing that the alteration of allele C to T may be insufficient reasons for infertility in Iranian men. In contrast to previous studies, −9C>T polymorphism is associated with spermatogenic impairment in South Korean and Chinese populations (31, 32) which may be related to the following factors: environmental factors, characteristics of subjects, as well as X chromosome haplogroups in different ethnic populations.

In addition no notable association between SNP 368A>G and the risk of male infertility in Iranian population was found in this investigation. These results are similar to study on South Korean population (31).

Finally, haplotype analysis of patient and control groups was performed in *H2B.W* gene. The results of haplotype analysis showed that the haplotype TA compared with haplotype CA significantly increased in patients suffering from CMA, compared with men had SCOS, suggesting that haplotype TA might arrest maturation process of spermatids during spermatogenesis. In fact patients with TA haplotype seem to be at higher risk of azoospermia caused by CMA of spermatids. Haplotype analysis in the study by Ying et al. also suggested that haplotype CA may be a protection factor from spermatogenesis disorder and haplotype TG may be a risk factor for azoospemia or oligozoospermia (32).

Therefore, it may be suggested that SNPs −9C>T and 368A>G of *H2B.W* genes have no crucial roles in spermatogenic failure in the Iranian population, except those observed in patients suffering from CMA.

To further study, it would be better to investigate the expression level of H2B.W in testis tissue of patients with −9T polymorphism, but unfortunately in our study, their tissues were not available.

Conclusion

The present study showed no significant correlation of SNPs −9C>T and 368A>G in *H2B.W* gene with susceptibility to spermatogenesis impairment in Iranian men, although it could be presumed that allele −9T in 5'UTR of *H2B.W* gene arises the risk of complete maturation arrest in azoospermic patients. Also this study indicated that haplotype CA compared to haplotype TA might be a protective factor for maturation process of spermatids. Further studies in larger size samples are needed to assessment the exact role of *H2B.W* gene in sperm nucleus.

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Original Article

Blastocyst Morphology Holds Clues Concerning The Chromosomal Status of The Embryo

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Abstract

Background: Embryo morphology has been proposed as an alternative marker of chromosomal status. The objective of this retrospective cohort study was to investigate the association between the chromosomal status on day 3 of embryo development and blastocyst morphology.

Materials and Methods: A total of 596 embryos obtained from 106 cycles of intracytoplasmic sperm injection (ICSI) followed by preimplantation genetic aneuploidy screening (PGS) were included in this retrospective study. We evaluated the relationship between blastocyst morphological features and embryonic chromosomal alteration.

Results: Of the 564 embryos with fluorescent in situ hybridization (FISH) results, 200 reached the blastocyst stage on day 5 of development. There was a significantly higher proportion of euploid embryos in those that achieved the blastocyst stage (59.0%) compared to embryos that did not develop to blastocysts (41.2%) on day 5 (P<0.001). Regarding blastocyst morphology, we observed that all embryos that had an abnormal inner cell mass (ICM) were aneuploid. Embryos with morphologically normal ICM had a significantly higher euploidy rate (62.1%, P<0.001). As regards to the trophectoderm (TE) morphology, an increased rate of euploidy was observed in embryos that had normal TE (65.8%) compared to embryos with abnormal TE (37.5%, P<0.001). Finally, we observed a two-fold increase in the euploidy rate in high-quality blastocysts with both high-quality ICM and TE (70.4%) compared to that found in low-quality blastocysts (31.0%, P<0.001).

Conclusion: Chromosomal abnormalities do not impair embryo development as aneuploidy is frequently observed in embryos that reach the blastocyst stage. A high-quality blastocyst does not represent euploidy of chromosomes 13, 14, 15, 16, 18, 21, 22, X and Y. However, aneuploidy is associated with abnormalities in the ICM morphology. Further studies are necessary to confirm whether or not the transfer of blastocysts with low-quality ICM should be avoided.

Keywords: Aneuploidy, Dysmorphism, *In Vitro* Fertilization, Preimplantation Genetic Screening

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Introduction

In order to maximize the success rates of assisted reproductive techniques (ART), a reliable means of identifying the embryo with the best prognosis and the highest potential for implantation is required. Because of the high frequency of aneuploid embryos and the negative outcomes associated with their transfer, the identification and transfer of chromosomally normal embryos is of pivotal importance, thus increasing the likelihood that the embryos are viable, leading to improved implantation and pregnancy rates, and reduced miscarriage rates (1).

Embryo morphology has been proposed as an alternative marker of chromosomal status (1, 2). Some studies suggest a link exists between the distribution and number of nucleoli in the pronuclei and the chromosomal status of the zygote (3, 4). In addition, it has been found that arrested cleavage-stage embryos, as well as embryos that present with abnormal rates of cleavage, exhibited a high frequency of chromosomal abnormalities (5).

Other studies that have searched for a link between aneuploidy and altered embryo morphology (6, 7) suggested that morphology could be a useful indicator of aneuploidy in some embryos and under some conditions. Therefore, the aim of this study was to investigate the association between the chromosomal status of the embryo on day 3 of development and blastocyst morphology.

Materials and Methods

Experimental design, patients and inclusion criteria

Using our centre's computerized database we retrospectively identified 106 cycles, performed from January 2010 to December 2010, which fulfilled the following inclusion criteria: intracytoplasmic sperm injection (ICSI) followed by preimplantation genetic aneuploidy screening (PGS). The indications for chromosome screening were advanced reproductive age (>35 years), history of unsuccessful *in vitro* fertilization (IVF) attempts and/or miscarriages. To minimize the influence of male factor infertility, all cases of sperm concentration less than 1×10^6 M/mL and sperm motility less than 20% were excluded from the study. The relationship between blastocyst morphological features and embryonic chromosomal alteration was evaluated.

Written informed consent was obtained, in which patients agreed to share the outcomes of their own cycles for research purposes, and the study was approved by the local Institutional Review Board.

Controlled ovarian stimulation, oocytes and embryo culture

The stimulation protocol, preparation of oocytes and embryo culture were described elsewhere (8). Full blastocysts onwards, presenting morphologically normal inner cell mass (ICM) and trophectoderm (TE) were defined as high-quality blastocysts. A tightly packed ICM that contained numerous cells was defined as a high quality ICM. Similarly, the TE was classified as high quality by the presence of numerous cells forming a cohesive epithelium (9).

Embryo biopsy

Embryos that reached at least the 5-cell stage on day 3 of development were biopsied by laser zona drilling using a 1.48 μm Infrared Diode Laser (Octax Laser Shot System, MTG, Bruckberg, Germany) and returned to culture. Only one blastomere was removed per embryo. The definition of a successful biopsy was the removal of a cell without lysis, so that the cell could be used for fixation and analysis.

Blastomere fixation and fluorescent in situ hybridization (FISH)

The blastomere of an embryo was fixed on a slide using the HCI/Tween 20 method as previously described (10). A two-round fluorescent in situ hybridization (FISH) procedure was performed which allowed for the detection of chromosomes X, Y, 13, 18 and 21 (Multivision PGT Probe Panel; Vysis, Downers Grove, IL, USA) in the first round and chromosomes 14, 15, 16 and 22 in the second round. The hybridization solution for the second round was prepared by mixing a probe for chromosome 14 (Vysis, Telvysion 14q/D14S1420 probe, Spectrum Orange), 15 (Vysis, Telvysion 15q/D15Z1, Spectrum Aqua), 16 (Vysis, Satellite II DNA/D16Z3 probe, Spectrum Orange) and 22 (Vysis, LSI 22, 22q11.2, Spectrum Green). The results were analyzed us-

ing a fluorescence microscope.

Fluorescent in situ hybridization scoring criteria

At diagnosis, we considered embryos as normal when two sex chromosomes and two chromosomes (13, 14, 15, 16, 18, 21 and 22) were present. They were considered trisomic or monosomic, respectively, if an extra or missing signal was observed. Finally, we defined embryos as haploid, triploid or polyploid if one, three or more copies, respectively, of the set of chromosomes were present. The presence of two or more chromosomal abnormalities within the same blastomere was characterized as multiple abnormalities.

Embryo transfer

Embryo transfer was performed on day 5 of development using a soft catheter. One to three euploid embryos were transferred per patient.

Clinical follow-up

A pregnancy test was performed 12 days after embryo transfer. A positive pregnancy test confirmed biochemical pregnancy. All women with a positive test had a transvaginal ultrasound scan 2 weeks after the positive test, a clinical pregnancy was diagnosed when the fetal heartbeat was detected. Pregnancy rates were calculated per transfer. Miscarriage was defined as pregnancy loss before 20 weeks.

Statistical analysis

We compared the incidence of euploid and aneuploid embryos according to the morphologic characteristics of the embryo on day 5 of development. Qualitative variables were compared using the chi-square or Fisher's exact tests. The influence of chromosomal constitution on the blastocyst morphology was investigated through binary logistic regression, adjusted for maternal age. The results were expressed as odds ratio (OR), confidence intervals (CI) and P values. Results were considered to be significant at $P<0.05$. Statistical analysis was carried out using MINITAB 16 Software.

Results

The general characteristics of the cycles are shown in table 1. The mean ± SD female age was 37.0 ± 4.7 years (range: 25–46 years). Of 106 cycles started, 90 were transferred (84.9%). The implantation rate was 26.7%, pregnancy rate was 28.9% and no miscarriage occurred for any of the patients who became pregnant.

Table 1: General characteristics of the intracytoplasmic sperm injection (ICSI) cycles

Variable	Value
Female age (Y)	37.0 ± 4.7
Male age (Y)	40.8 ± 6.7
FSH (IU)	2448.6 ± 641.6
E_2 (pg/mL)	2220.0 ± 1461.0
Follicles (n)	18.0 ± 11.9
Oocytes (n)	13.2 ± 8.7
MII oocytes (n)	10.4 ± 7.4
MII oocyte rate (%)	78.8
Injected oocytes (n)	10.5 ± 6.8
Fertilization rate (%)	75.6
High-quality embryo rate (%)	70.7
Transferred embryos (n)	1.3
Transferred cycles (%)	90/106 (84.9)
Implantation rate/ transferred embryos (%)	31/117 (26.5)
Pregnancy/transferred cycle (%)	26/90 (28.9)
Miscarriage/pregnancy (%)	0/26 (0.0)

FSH; Follicle-stimulating hormone, E_2: Estradiol and MII; Metaphase II.

Out of 596 embryos successfully biopsied on day 3 of development, 564 had FISH results. An inconclusive diagnosis was obtained in 32 (5.4%) cells due to technical issues that included hybridization failure, signal overlapping yielding false-negative results, and split or diffuse signals. A total of 240 embryos were euploid (42.6%) and 324 were aneuploid (57.4%). The detailed distribution of aneuploidy is shown in table 2.

Table 2: Distribution of aneuploidy in embryos on day 3 of development

Type of abnormality	Affected embryos (%)	Affected chromosomes								
		13	14	15	16	18	21	22	X	Y
Multiple	107/324 (33.0)	56	0	18	0	56	70	0	39	4
Mosaic	2/324 (0.6)	0	0	0	0	0	2	0	0	0
Monosomy	96/324 (29.6)	28	2	4	4	20	16	0	20	2
Trisomy	119/324 (36.7)	36	2	2	18	14	39	4	4	0

Note: Columns 3-11 represent number of embryos with the respective chromosome affected.

Of the 564 embryos with FISH results, 200 reached the blastocyst stage on day 5 of development (35.5%). A total of 118 blastocysts were euploid (59.0%) and 82 were aneuploid (41.0%) on day 3 of development.

There was a significantly higher proportion of euploid embryos in those that achieved the blastocyst stage (59.0%) compared to embryos that did not develop to a blastocyst on day 5 (41.2%, P<0.001).

In terms of blastocyst morphology, we observed that all embryos with abnormal ICM were aneuploid. There was a significantly higher euploidy rate in embryos with a morphologically normal ICM (62.1%, P<0.001). An increased rate of euploidy was observed in embryos that showed normal TE (65.8%) compared embryos with abnormal TE (37.5%, P<0.001). Finally, we observed a 2-fold increase in the euploidy rate in high-quality blastocysts that had both high-quality ICM and TE (70.4%) compared to low-quality blastocysts (31.0%, P<0.001, Fig.1, Table 3).

The results of the logistic regression models demonstrated an increase in the probability of euploidy when: i. embryos reached the blastocyst stage on day 5 of development (OR: 2.09, CI: 1.29–3.39, P=0.002), ii. blastocysts showed normal TE (OR: 3.21, CI: 1.24–8.31, P=0.015) and iii. blastocysts showed both normal TE and ICM (OR: 5.29, CI: 2.07–13.51, P<0.001).

Neither the presence of monosomies (OR: 1.77, CI: 0.87–3.59, P=0.113), nor the presence of trisomies (OR: 2.98, CI: 0.79–11.21, P=0.880) influenced blastocyst formation. However, the presence of multiple abnormalities negatively influenced the odds of development to the blastocyst stage (OR: 0.20, CI: 0.01–0.56, P=0.012). Finally, the percentage of euploid blastocysts did not influence implantation (Slope: 47.65, R^2: 1.7%, P=0.413) or pregnancy (OR: 1.03, CI: 0.98–1.08, P=0.273) rates.

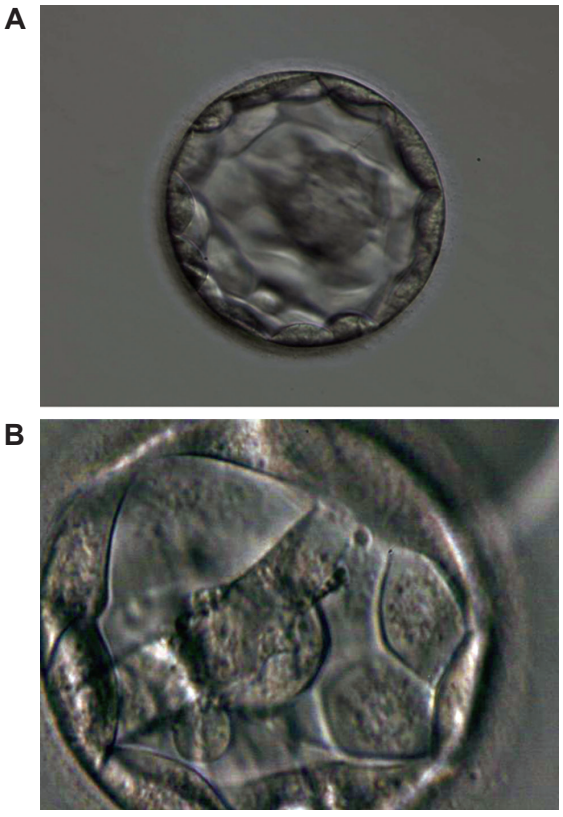

Fig.1: Blastocysts showing high- and low-quality inner cell mass (ICM) and trophectoderm (TE).
A. A high-quality blastocyst showing a normal ICM with many cells that are tightly compacted, and a normal TE with many cells that form a cohesive epithelium lining the blastocoel cavity. **B.** A low-quality blastocyst showing an abnormal ICM that is loosely made up of only a few cells. Large TE cells that stretch over great distances to reach the next cell.

Table 3: Comparison of euploidy and aneuploidy rates according to blastocyst development and morphology

	Dependent variables	
Predictor variables	**Euploidy (%)**	**Aneuploidy (%)**
Embryo development on D5		
Blastocyst	118/200 (59.0)	82/200 (41.0)
Non-blastocyst	150/364 (41.2) [a]	214/364 (58.8) [a]
Blastocyst morphology		
ICM		
Normal	118/190 (62.1)	72/190 (37.9)
Abnormal	0/10 (0.0)[b]	10/10 (100) [b]
TE		
Normal	100/152 (65.8)	52/152 (34.2)
Abnormal	18/48 (37.5) [c]	30/48 (62.5) [c]
ICM+TE		
Normal	100/142 (70.4)	42/142 (29.6)
Abnormal	18/58 (31.0) [d]	40/58 (69.0) [d]

D5; Day five of development, ICM; Inner cell mass, TE; Trophectoderm, [a]; Significantly different from blastocyst group, [b]; Significantly different from normal ICM group, [c]; Significantly different from normal TE group and [d]: Significantly different from normal ICM+TE group.

Discussion

The objective of this study was to investigate the relationship between blastocyst morphology and the chromosome status of embryos on day 3 of development. Our results demonstrated significant differences in the euploidy rate between embryos that achieved blastocyst stage on day 5 compared to embryos that did not. As for blastocyst morphology, we observed significant differences in the euploidy rate between the groups with i. normal and abnormal ICM, ii. normal and abnormal TE and iii. normal and abnormal ICM plus TE. The results of the logistic regression models demonstrated a 2-fold increase in the probability of euploidy when embryos reached the blastocyst stage on day 5 of development, a 3-fold increase in the probability of euploidy when blastocysts showed normal TE, and a 5-fold increase in the probability of euploidy when blastocysts showed both normal TE and ICM.

Previous studies have investigated the relationship between embryo morphology and aneuploidy. Although preliminary, these studies have shown a weak association between aneuploidy and abnormal embryo morphology. (2, 5-7, 11-13). Alfarawati et al. (2) showed that aneuploidy negatively affected the ICM and TE grades. Morphologically, poor blastocysts had a higher incidence of monosomy and abnormalities that affected several chromosomes. Magli et al. (5) observed that the incidence of chromosomal abnormalities was significantly higher in embryos that divided according to a time frame and a symmetry plan which were different from expected.

The main question is whether morphological analysis can be of assistance in the selection of euploid embryos for transfer. A recent study has shown that the aneuploidy rate observed on day 5 could be reduced from 56 to 48% if only embryos that achieved the top grades were selected for transfer (2). In addition, Munne et al. (6) showed an euploidy incidence of 44% in morphologically normal embryos and 30% in morphologically abnormal embryos. These results were consistent with the findings of the present study which showed that a high incidence of aneuploidy could be found in morphologically normal embryos.

This study showed a link between euploidy and normal blastocyst ICM and TE morphologies. We found increased euploidy rates amongst blastocysts with good ICM and TE morphology and a lower likelihood of euploidy in low-quality blastocysts. In light of these results we could suggest that blastocyst morphology might be a useful indicator of embryo chromosome constitution. This would be an attractive possibility, as chromosome assessment based upon morphology would allow embryo biopsy to be avoided, resulting in an inexpensive test with no impact on the embryo. However, as seen in the present study, it was important to note that over 40% of embryos which reached the blastocyst stage were aneuploid. Moreover, 35% of the blastocysts that presented with morphologically normal TE and approximately 30% of high-quality blastocysts were aneuploid. Therefore, the development to blastocyst and morphological normalcy of the ICM plus TE could not be used to predict euploidy for the chromosomes analyzed in this study. On the other hand, despite the observation that 38% of blastocysts with normal ICM were aneuploid, in this study all embryos that had abnormal ICM were aneuploid. Therefore, an abnormal ICM could predict aneuploidy for the chromosomes analyzed in this study. Nonetheless,

of note, only 10 embryos showed low-quality ICM in the present study.

Our study possesses three drawbacks, as follows:

i. This is a retrospective study that lacks sample size calculation and therefore is subject to bias and underpowered results.

ii. A single blastomere biopsy, which does not rule out the risk of embryo mosaicism, has been performed. Nevertheless, since no conclusive data has demonstrated the superiority of double- over single-blastomere biopsy (14, 15), a single blastomere biopsy is routinely performed in our center.

iii. We assessed a limited number of chromosomes frequently involved in term pregnancies with potentially severe clinical consequences. Therefore it was inevitable that some of the embryos categorized as euploid were in fact abnormal with aneuploidies that affected chromosomes which were not tested.

It has been suggested that blastocyst culture may select against aneuploidy (16); however, certain abnormalities are compatible with development to term. Despite evidence for improved selection with blastocyst culture, our data suggest that extended culture to the blastocyst stage does not definitively select for euploid embryos.

Conclusion

Chromosomal abnormalities do not impair embryo development as aneuploidy is frequently observed in embryos that reach the blastocyst stage. High-quality blastocysts are not representative of euploidy of chromosomes 13, 14, 15, 16, 18, 21, 22, X and Y. However, aneuploidy is associated with abnormalities in the ICM morphology. Further studies are necessary to confirm whether or not we should avoid the transfer of blastocysts with low-quality ICM.

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Original Article

Timing of The First Zygotic Cleavage Affects Post-Vitrification Viability of Murine Embryos Produced *In Vivo*

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Abstract

Background: Timing of the first zygotic cleavage is an accurate predictor of embryo quality. Embryos that cleaved early (EC) have been shown to exhibit higher developmental viability compared to those that cleaved at a later period (LC). However, the viability of EC embryos in comparison to LC embryos after vitrification is unknown. The present study aims to investigate the post-vitrification developmental viability of murine EC versus LC embryos.

Materials and Methods: In this experimental study, female ICR mice (6-8 weeks old) were superovulated and cohabited with fertile males for 24 hours. Afterwards, their oviducts were excised and embryos harvested. Embryos at the 2-cell stage were categorized as EC embryos, while zygotes with two pronuclei were categorized as LC embryos. Embryos were cultured in M16 medium supplemented with 3% bovine serum albumin (BSA) in a humidified 5% CO_2 atmosphere. Control embryos were cultured until the blastocyst stage without vitrification. Experimental embryos at the 2-cell stage were vitrified for one hour using 40% v/v ethylene glycol, 18% w/v Ficoll-70 and 0.5 M sucrose as the cryoprotectant. We recorded the numbers of surviving embryos from the control and experimental groups and their development until the blastocyst stage. Results were analyzed using the chi-square test.

Results: A significantly higher proportion of EC embryos (96.7%) from the control group developed to the blastocyst stage compared with LC embryos (57.5%, $P<0.0001$). Similarly, in the experimental group, a significantly higher percentage of vitrified EC embryos (69.4%) reached the blastocyst stage compared to vitrified LC embryos (27.1%, $P<0.0001$).

Conclusion: Vitrified EC embryos are more vitrification tolerant than LC embryos. Preselection of EC embryos may be used as a tool for selection of embryos that exhibit higher developmental competence after vitrification.

Keywords: Vitrification, Early Cleavage, Mouse Embryos

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Introduction

One of the major problems in assisted reproductive technology (ART) is identification of good quality embryos. This is very important because the number of transferred embryos has to be low (preferably one embryo) in order to reduce the incidence of multiple pregnancies in women.

Multiple transfers can increase the risk of postpartum hemorrhage, pregnancy induced hypertension and anemia, as well as maternal mortality. It is also associated with higher rates of pre-term delivery, low birth weight, neonatal morbidity and infant death (1). To avoid complications associated with multiple pregnancies, transfer of a single embryo is highly recommended. However, the major concern among practitioners is the reduced success rate after transfer of a single embryo. Hence, if a good quality embryo can be identified and used in single embryo transfers, the likelihood of pregnancy will be increased.

Morphological evaluation has been the common method used in assessment of embryo quality (2). This method requires observational skills and may also be subjective, leading to inconsistencies. There can be a bias in the assessment of an embryo between different evaluators and also between different laboratories or clinics.

Timing of the first zygotic cleavage has been used as an alternative predictor for embryo quality in humans (3-5). Embryos that cleave early were proven to develop into good quality embryos with higher developmental viability compared to their late cleaving (LC) counterparts (5-8). Despite the fact that cryopreservation of human embryos is a common method in ART procedures, comparative studies on the viability of early cleaving (EC) and LC embryos after cryopreservation are lacking.

This study was therefore conducted to compare the cryotolerance of EC and LC murine embryos by evaluating their developmental viability after vitrification.

Materials and Methods

Embryo collection

In this experimental study, a total of 26 female ICR mice, aged 6-8 weeks were superovulated by intraperitoneal (i.p.) injections of 5 IU pregnant mare serum gonadotropin (PMSG, Folligon, Intervet International B.V, Holland) followed 48 hours later by an i.p. injection of 5 IU human chorionic gonadotropin (hCG, Chorulon, Intervet International B.V, Holland). Females were subsequently mated with male mice of the same strain at a ratio of 1:1. The morning after mating, females were checked for the presence of a vaginal plug. After 28 hours from hCG administration, oviducts from the plugged female mice were excised and embryos flushed out in M2 medium (Sigma, USA). Embryos were assessed under an inverted microscope (Leica, Germany). One-cell embryos with 2 pronuclei and embryos at the 2-cell stage were considered fertilized. All procedures that involved animals were approved by the Animal Care and Use Committee (ACUC), UiTM (ACUC-7/11).

Timing of the first zygotic cleavage

Embryos were divided into two groups - EC and LC according to the timing of the first zygotic cleavage. Embryos that displayed 2-cells at 28-30 hours post-hCG administration were categorized as EC embryos while zygotes that contained a second polar body and two pronuclei were categorized as LC embryos. Embryos were cultured in 50 µl drops of the M16 culture medium (Sigma, USA) plus 3% bovine serum albumin (BSA, Sigma, USA) overlaid with mineral oil (Sigma, USA) in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2.

Control group embryos were cultured until the blastocyst stage without being subjected to vitrification. Embryo viability was assessed by embryo development in culture until the blastocyst stage. The developing embryos were observed under an inverted microscope every 24 hours. The developmental kinetics for a normal developing murine embryo is as follows: ≥2-cell at 24 hours, ≥4-cell at 48 hours, at least morulae at 72 hours and expanded blastocysts at 96 hours (9). Experimental embryos were vitrified at the 2-cell stage and subjected to culture after warming.

Vitrification

The vitrification method used in this study was

developed by Kasai et al. (10) and described in detail by Shaw and Kasai (11). The cryopreservation solution consisted of M2 medium with 40% v/v ethylene glycol, 18% w/v ficoll 70 and 0.5 M sucrose (EFS40). A Styrofoam box with a lid was used as a cooling container. This box was filled with at least 5 cm liquid nitrogen and precooled for 30 minutes before use. A Styrofoam boat with a thickness of 1 cm was made with grooves for holding straws.

A total of 30 µl of EFS40 solution were aspirated by connecting the straw to a pipett. This was followed by aspirating a total of 10 embryos in 10 µl of M2 and another 30 µl of EFS40 solution into the straw. Then, the straw was sealed at the open end using polyvinyl alcohol (PVA). The straw was then placed in the horizontal position and embryos equilibrated with the cryoprotectant for 1 minute at room temperature. Once ready, the straw was transferred to a Styrofoam boat and left floating on liquid nitrogen vapor for five minutes before being immersed in liquid nitrogen for 1 hour.

Warming

The straw was transferred from the liquid nitrogen and placed for 5 minutes on the Styrofoam boat inside a Styrofoam box that contained liquid nitrogen. Using a pair of forceps, the straws were lifted from the boat and held in air for 10 seconds before being immersed in a 37˚C water bath for 10 seconds. The contents of the straws were expelled into M2 medium that contained 0.5 M sucrose in a culture dish. After 3 minutes, the culture dish was agitated gently to mix the dilution and cryoprotectant solutions. After a 5-minute incubation in 0.5 M sucrose, embryos were transferred to a new petri dish that contained M2 medium.

Assessment of survival rate of vitrified embryos

The post-vitrification survival rate of embryos was assessed by evaluation of their morphology under an inverted microscope followed by development *in vitro* until the blastocyst stage. Embryos with intact blastomeres and zona pellucida after warming were classified as surviving embryos (Fig.1A). Degenerated embryos were discarded (Fig.1B).

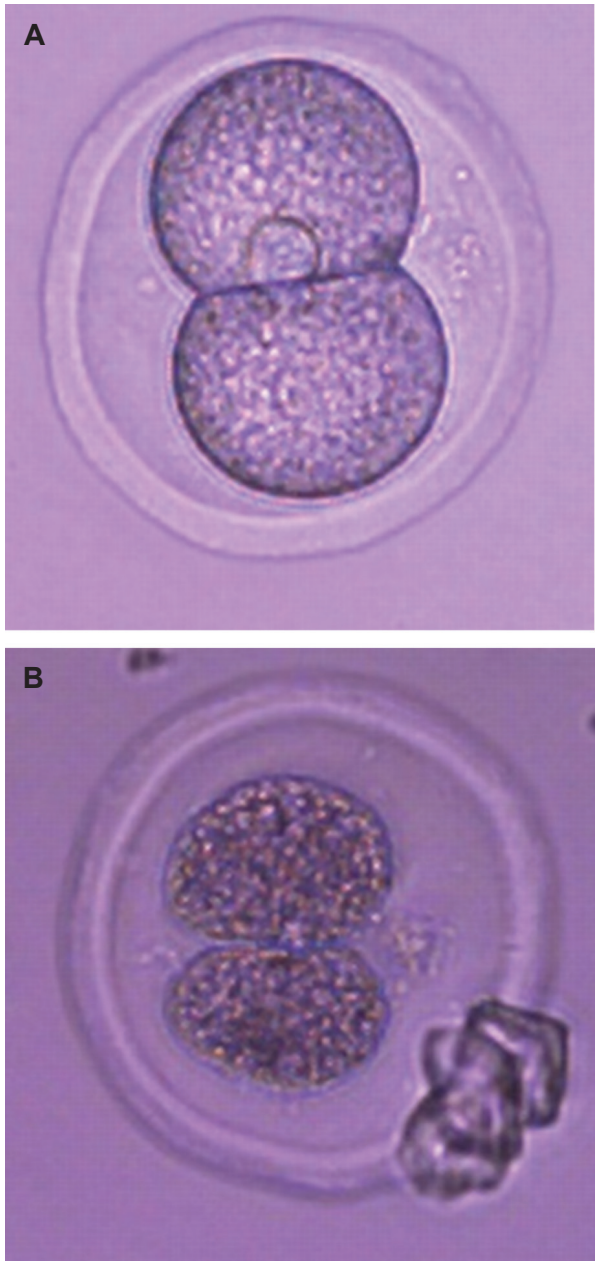

Fig.1: Morphology of murine embryos after vitrification **(A)** intact **(B)** degenerated.

Embryo culture

Thawed embryos that had proper morphology were then transferred into fresh 50 µl droplets of the M16 medium plus 3% BSA, overlaid with mineral oil and cultured in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2.

Embryo viability was assessed by embryo de-

velopment in culture until the blastocyst stage. Embryo development was monitored every 24 hours under an inverted microscope. The developmental kinetics for normal developing murine embryos after vitrification is as follows: ≥4-cells at 24 hours, at least morulae 48 hours and expanded blastocysts at 72 hours.

Statistical analysis

Statistical analysis was performed using the SPSS software for Windows version 19.0.1 (Statistical Package for the Social Sciences, Inc., USA). Embryonic survival rates subsequent to vitrification and thawing, the developmental rates of embryos at different stages, and the blastocyst formation rates were determined and reported as percentages. The difference between the two groups (EC and LC embryos) was analyzed using the chi-square test. A P value of less than 0.05 was considered statistically significant.

Results

Control group (non-vitrified)

There were 234 embryos in the control group (non-vitrified). Out of this number, 60 (25.6%) were EC embryos while the other 174 (74.4%) were LC embryos (Fig.2). The developmental potential of both EC and LC embryos is summarized in table 1.

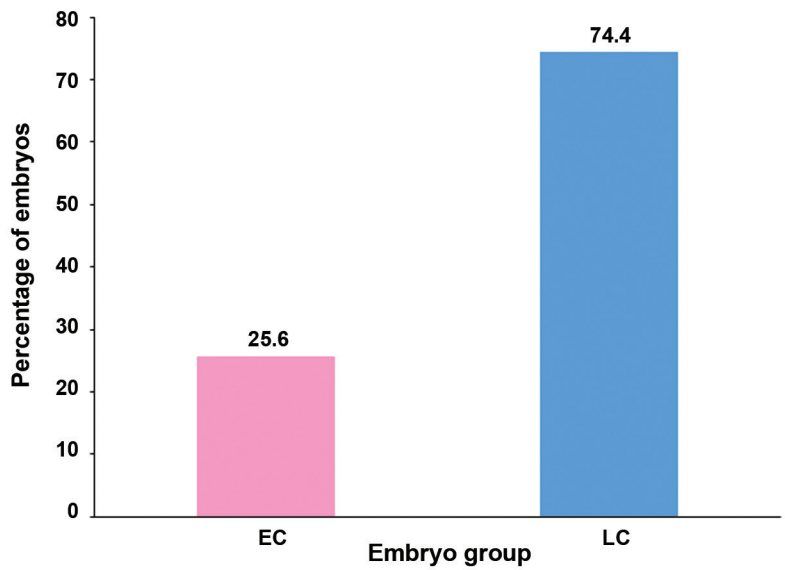

Fig.2: Percentage of early cleaving (EC) and late cleaving (LC) embryos from the control group (non-vitrified).

Table 1: Developmental kinetics of control early cleaving (EC) and late cleaving (LC) embryos following *in vitro* culture for 96 hours

Embryo groups	Number of embryos (%)			
	2-cell stage (24 hivc)	4-cell stage (48 hivc)	Morula stage (72 hivc)	Blastocyst stage (96 hivc)
EC	60 (100)	59 (98.3) **	59 (98.3) **	58 (96.7) **
LC	174 (100)	125 (71.8)	118 (67.8)	106 (60.9)

**; P<0.0001 versus LC embryos and hivc; Hours of *in vitro* culture.

There was a significantly higher percentage of developing EC embryos compared to LC embryos at 48, 72 and 96 hours (P<0.0001). After 48 and 72 hours of culture, 98.3% of EC embryos reached the 4-cell and morula stages. For LC embryos, the percentage was significantly lower than EC embryos after 48 and 72 hours of culture where 71.8% reached the 4-cell stage and 67.8% reached the morula stage (P<0.0001). After 96 hours of culture, the proportion of developing embryos were also significantly higher in EC embryos (96.7%) compared to LC embryos (60.9%, P<0.0001).

The blastocyst rate was significantly higher (96.7%) for EC compared to LC embryos (60.9%, P<0.0001). The remaining EC embryos arrested at the 2-cell (1.7%) and morula (1.7%) stages. In LC embryos, besides the blastocyst stage, developmental arrest occurred at the 2-cell (21.8%), 3-cell (6.3%), 4-cell (4.0%) and morula (6.9%) stages (Fig.3).

Experimental group (vitrified)

A total of 175 embryos were included in the experimental group. Of these, 58 (33.1%) were EC embryos while the other 117 (66.9%) were LC embryos (Fig.4). Evaluation of post-vitrification survival rate showed that vitrified EC embryos demonstrated better post-vitrification survival (62.1%) than vitrified LC embryos (50.4%). However, the difference was not significant (Table 2).

After 24 hours of *in vitro* culture we observed no significant difference between vitrified EC (80.6%) and LC (71.2%) embryos. However, after longer culture (48 and 72 hours) there was a significantly higher percentage of developing embryos in the EC compared to LC embryos (P<0.05). Significantly more EC embryos reached the morula (77.8%) and blastocyst (69.4%) stages compared to LC embryos that reached the morula (42.4%) and blastocyst (27.1%) stages (P<0.0001, Table 2).

The remaining EC embryos arrested at the 2-cell (13.9%), 3-cell (5.6%), 8-cell (2.8%) and morula (8.3%) stages. In LC embryos that did not reach the blastocyst stage, developmental arrest occurred at the 2-cell (18.6%), 3-cell (10.2%), 4-cell (15.2%), 8-cell (13.6%) and morula (15.3%) stages (Fig.5).

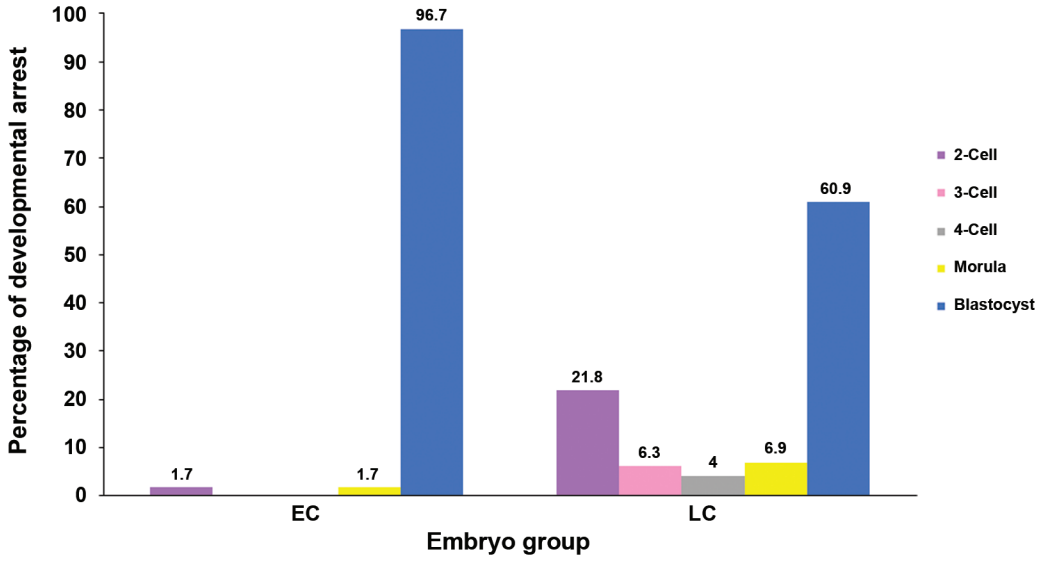

Fig.3: Arrest of early cleaving (EC) versus late cleaving (LC) embryos following *in vitro* culture in M16 medium for 96 hours.

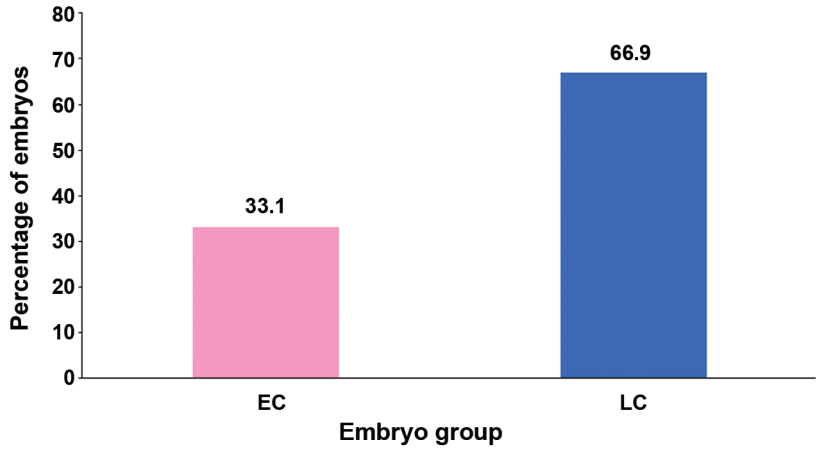

Fig.4: Percentage of early cleaving (EC) and late cleaving (LC) embryos from *in vivo* fertilization (treatment group).

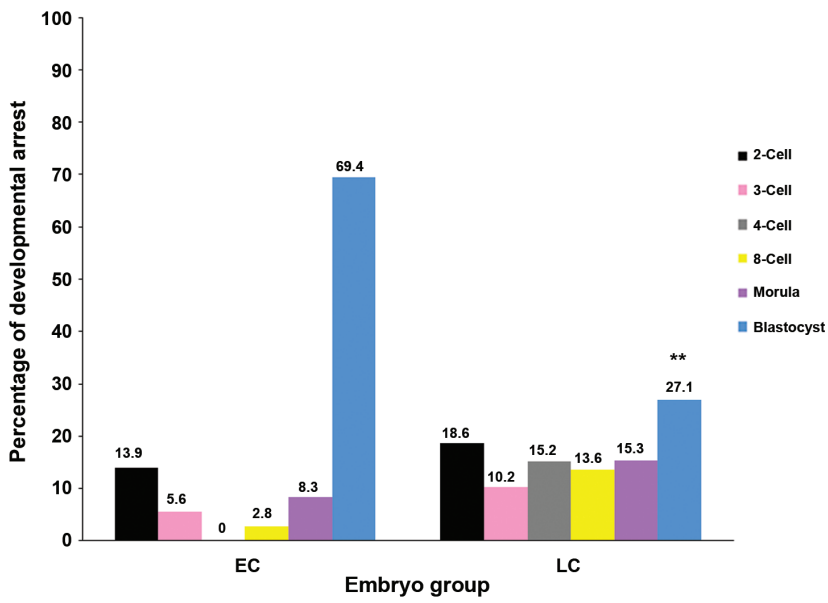

Fig.5: Developmental arrest of early cleaving (EC) and late cleaving (LC) embryos following vitrification and *in vitro* culture in M16 medium for 72 hours. **; P<0.0001 compared to EC embryos

Table 2: Survival and developmental ability of early cleaving (EC) versus late cleaving (LC) murine embryos following vitrification and *in vitro* culture in M16 medium for 72 hours

Embryo groups	Number of embryos (%)				
	Vitrified	Survived	4-cell (24 hours post-vitrification)	Morula (48 hours post-vitrification)	Blastocyst (72 hours post-vitrification)
EC	58	36 (62.1)	29 (80.6)	28 (77.8) *	25 (69.4) **
LC	117	59 (50.4)	42 (71.2)	25 (42.4)	16 (27.1)

*; P<0.05 versus LC embryos and **; P<0.0001 versus LC embryos.

Table 3 presents a comparison of blastocyst formation between control (non-vitrified) and experimental (vitrified) groups. The percentage of blastocyst from vitrified embryos (69.4%) was significantly lower compared with non-vitrified embryos (96.7%) for EC embryos; we observed the same for vitrified (27.1%) compared to non-vitrified (60.9%) embryos among the LC embryos.

Table 3: Comparison of blastocyst formation between control and experimental groups following *in vitro* culture in M16 medium for 96 hours

Embryo group	Blastocyst formation, n (%)	
	EC embryos	LC embryos
Control (non-vitrified)	58/60 (96.7%)**	106/174 (60.9%)**
Experimental (vitrified)	25/36 (69.4%)	16/59 (27.1%)

**; P<0.0001 versus vitrified embryos

Discussion

In order to select the best quality embryo, the embryo scoring system based on morphological assessment has been established for human ART protocols (2). However, few studies have sought to find alternative, non-invasive tools that improve selection of embryos (5, 12, 13). Timing of the first zygotic cleavage is one of the proposed parameters (5, 6, 14). As shown in humans, embryos that have cleaved early more often develop into good quality embryos with higher developmental potential (5, 6).

For human ART protocols, EC embryos are defined as those which have cleaved to the 2-cell stage at 25-27 hours after insemination (hpi) or intracytoplasmic sperm injection (ICSI) (4, 15). This corresponds to the first mitotic division. LC embryos are defined as embryos which have cleaved to the 2-cell stage >27 hpi or ICSI.

In this study, embryos were collected from oviducts of *in vivo*-fertilized mice. The results showed that under the *in vivo* conditions applied to this study, murine EC embryos displayed the 2-cell stage at 28-30 hours post-hCG administration, while LC embryos reached the 2-cell stage ≥30 hours post-hCG administration.

To our knowledge, this is the first report on the timing of the first zygotic cleavage of *in vivo*-derived embryos in a mouse model. The superiority of *in vivo*-derived mouse embryos over *in vitro*-derived embryos is supported by a previous study, which has demonstrated that *in vivo*-derived bovine embryos exhibited a reduced sensitivity to chilling and freezing due to the lower lipid to protein ratio than *in vitro*-produced embryos (16).

As far as the incidence of EC is concerned, a previous study has found that this incidence ranges from 15 to 38% in humans and 32 to 76.8% in cattle (7). Whilst in the present study, we have shown the incidence of early cleavers to be 22.4% for the control group and 33.1% for the experimental group, which suggested that the percentage of early cleavers in mice was within the same range as human IVF-derived embryos.

Concerning the relationship between early cleavage status and embryo quality in the mouse, the results of the present study agreed with other studies (4, 6, 15) of humans which found that EC embryos had a significantly higher developmental potential compared to LC embryos. EC embryos observed in the present study were characterized by a significantly higher developmental rate at 24, 48, 72 and 96 hours post-hCG administration compared to LC embryos. A significantly higher percentage of developing embryos was also maintained in vitrified EC embryos compared to vitrified LC embryos in most stages, except at 48 hours of culture.

The present study revealed that EC embryos (96.7%) significantly reached the blastocyst stage compared to LC embryos (60.9%). In congruence, previous studies on porcine and human embryos also found that EC embryos had higher blastocyst formation compared to LC embryos (8, 14). Similarly, vitrified EC embryos (69.4%) showed a significantly higher percentage of blastocyst formation compared to vitrified LC embryos (27.1%). Greater cryotolerance of post-vitrification EC embryos was indicated by their better morphology, especially the intactness of the zona pellucida and blastomeres (17). This resulted in a higher developmental potential, even after exposure to high concentration of cryoprotectant and high cooling rates during the vitrification procedure.

However, the reasons for better quality and better

developmental viability of EC embryos compared to LC embryos remain unknown. Whether this is related to maternal factors such as the quality of oocytes as speculated by previous studies (4, 6, 15) warrants further investigation. Lechniak et al. (7) has stated that maternal factors in oocytes have more prevalence of an impact on embryo quality than sperm, since the majority of transcripts and other cytoplasmic compounds in a zygote are of maternal origin. However, paternal factors such as the quality of spermatozoa cannot be ruled out as they contribute to the DNA of the embryos (15).

The present study also compared the blastocyst formation between control (non-vitrified) and experimental (vitrified) murine embryos. It was found that blastocyst rate from vitrified embryos was significantly lower compared with that of non-vitrified embryos i.e. 69.4 versus 96.7% for EC embryos; and 27.1 versus 60.9% for LC embryos. This is in accordance with results of a previous study on murine embryos which showed a 22.3% blastocyst rate for vitrified embryos versus 47.1% for non-vitrified embryos (18). The explanation for the decreased blastocyst rate after vitrification remains unclear. However, application of cryoprotectant at high concentrations may increase the osmolarity, which further damage the cells and destabilize cell membranes. Removal of the permeated cryoprotectant from the cell during warming may cause osmotic injuries to cells (19). All factors involved in vitrification may affect the viability of embryos and cumulatively reduce blastocyst rates.

In a study, survival rate of murine embryos following vitrification was reported to be 62% by Miyake et al. (20), whilst Uechi et al. (18) reported 77.4%. However these studies did not compare EC to LC embryos. In the present study, the survival rate of vitrified EC embryos was 62.1%, whereas for LC embryos it was 50.4%. However, there was no significant difference between the survivability of these two groups. Although no significant differences were noted, there was a consistent trend that the survived EC embryos had higher potential to develop into the blastocyst stage, compared to LC embryos.

The present research provides new information on cryotolerance of EC murine embryos. Previous studies on EC mouse embryos have concentrated more on developmental viability, blastocyst rate, pregnancy rate, implantation rate and live birth rate. There was no evidence of the quality and viability of these embryos after vitrification. Although morphological scoring of post-vitrification EC and LC at the 2-cell stage showed no significant difference, our results showed that vitrified EC embryos had higher potential to develop into blastocysts compared to vitrified LC embryos.

Conclusion

The present study has shown that under *in vivo* conditions, EC murine embryos are superior to LC embryos in terms of post vitrification developmental viability. We suggest preselection of EC embryos as vitrification candidates for better cryopreservation outcomes to improve ART procedures.

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Original Article

Beneficial Effects of Nitric Oxide Induced Mild Oxidative Stress on Post-Thawed Bull Semen Quality

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Abstract

Background: Cryopreservation of semen requires optimized conditions to minimize the harmful effects of various stresses. The main approach for protection of sperm against stress is based on the use of antioxidants and cryoprotectants, which are described as defensive methods. Recently, the application of controlled mild stressors has been described for activation of a temporary response in oocyte, embryo and somatic cells. In this study a sub-lethal oxidative stress induced by precise concentrations of nitric oxide (NO) has been evaluated for sperm during cryopreservation.

Materials and Methods: In this experimental study, we used different concentrations of NO [0 μM (NO-0), 0.01 μM (NO-0.01), 0.1 μM (NO-0.1), 1 μM (NO-1), 10 μM (NO-10) and 100 μM (NO-100)] during cryopreservation of bull semen. Their effects on post-thawed sperm quality that included motility and velocity parameters, plasma membrane functionality, acrosome integrity, apoptosis status, mitochondrial activity and lipid peroxidation after freezing-thawing were investigated.

Results: Exposure of sperm before freezing to NO-1 significantly increased total motility (88.4 ± 2.8%), progressive motility (50.4 ± 3.2%) and average path velocity (VAP, 53.8 ± 3.1 μm/s) compared to other extenders. In addition, NO-1 significantly increased plasma membrane functionality (89.3 ± 2.9%) compared to NO-0 (75.3 ± 2.9%), NO-0.01 (78.3 ± 2.9%), NO-0.1 (76.4 ± 2.9%), NO-10 (64 ± 2.9%) and NO-100 (42 ± 2.9%). Sperm exposed to NO-1 produced the highest percentage of viable (85.6 ± 2.3%) and the lowest percentage of apoptotic (10.8 ± 2.4%) spermatozoa compared to the other extenders. Also, NO-100 resulted in a higher percentage of dead spermatozoa (27.1 ± 2.7%) compared to the other extenders. In terms of mitochondrial activity, there was no significant difference among NO-0 (53.4 ± 3.2), NO-0.01 (52.1 ± 3.2), NO-0.1 (50.8 ± 3.2) and NO-1 (53.1 ± 3.2). For acrosome integrity, no significant different was observed in sperm exposed to different concentrations of NO.

Conclusion: Induction of sub-lethal oxidative stress with 1 μM NO would be beneficial for cryopreservation of bull semen.

Keywords: Bull, Cryopreservation, Nitric Oxide, Oxidative Stress, Sperm

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Introduction

The modern cattle industry is interested in improving the ability of cryopreserved semen for oocyte fertilization (1). However, during laboratory manipulation of sperm for the cryopreservation process, various factors such as oxidative, temperature and osmotic stresses lead to reduced sperm fertility (2, 3). This reduction results from damage to the sperms' integrity due to anatomical and biochemical destruction of subcellular organelles

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(4). Therefore, it is necessary to use a strategy that opposes these destructive events (5). The common approach used during the last 20 years has been a defensive one based on the use of reagents that contain antioxidant properties such as catalase, superoxide dismutase and glutathione peroxidase as well as cryoprotectants such as egg yolk or soybean lecithin that protect sperm (4, 6, 7).

In recent years, a reported novel approach has led to improvements in the resistance of oocyte, embryo and sperm cells (8-11). The hypothesis for this approach is the use of a mild sub-lethal stress that will enable cells to improve tolerance to a future stress event such as cryopreservation (12). High Hydrostatic pressure (HHP), osmotic stress and oxidative challenges are the main stressors which have been applied for this purpose (13-15). Sub-lethal HHP has been applied to semen, oocyte, embryo and embryonic stem cells. There was a beneficial effect observed after controlled exposure of these materials to HHP (13). HHP treatment of sperm has been shown to increase the production of special proteins such as the ubiquinol-cytochrome C reductase complex which are thought to play an important role in the fertilization process (16).

Recently, oxidative sub-lethal stress has been reported to induce a temporary resistance to different future stresses. This response is mediated by several physiological pathways, which lead to regulation of programmed cell death (apoptosis) or necrosis (17). Vandaele et al. (15) have reported positive effects of short-term exposure of cumulus oocyte complexes to 50-100 μM H_2O_2. The rate of embryo development parameters significantly improved after *in vitro* fertilization compared to the absence of H_2O_2 concentrations.

Sperm processing for cryopreservation also introduced an additional source of oxidative stress by producing free radicals which attacked the sperm membrane and increased its susceptibility to lipid peroxidation (18). To date, no investigation has been conducted to determine the effects of oxidative stress on sperm before cryopreservation. Therefore, the purpose of this study was an attempt to find the mild range of this oxidative stress induced by nitric oxide (NO) on the ability of a sperm or semen sample to withstand cryopreservation.

Materials and Methods

Chemicals

All chemicals used in this study were purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany). NO was supplied by Sigma Chemical Company (GS-NO, N4148). Approval for the study was given by the Research Ethics Committees of Tehran University and Royan Institute.

Farm management and semen collection

This experimental study was conducted at the Department of Animal Science, University of Tehran. Semen samples were collected from 6 mature Holstein bulls (Zar Gene AI Stud) using an artificial vagina (43˚C) twice weekly for one month. After collection, ejaculates were transferred to a water bath (37˚C) and subsequently evaluated for color, volume, motility, concentration and morphology. Samples used in the study met the following standards: semen concentration of $\geq 1.0\times10^9$ spermatozoa/mL, motility ≥60% and ≤15% abnormal morphology. Ejaculates were pooled to eliminate individual differences. Finally, the pooled semen was split into six equal aliquots for processing according to treatments.

Sperm processing and stress treatment before cryopreservation

Aliquots of ejaculate were diluted at room temperature with Optidyl® (Biovet, France) extender that contained different concentrations of NO [0 μM (NO-0), 0.01 μM (NO-0.01), 0.1 μM (NO-0.1), 1 μM (NO-1), 10 μM (NO-10) and 100 μM (NO-100)]. The semen concentration was set at a final concentration of 100×10^6 spermatozoa/mL. The diluted semen samples in each treatment were gradually cooled and equilibrated at 4˚C for 150 minutes. Semen samples were subsequently aspirated into 0.25 mL French straws (IMV, L'Aigle, France) and sealed with polyvinyl alcohol powder, then cryopreserved in a computerized freezing machine (Digit Cools, IMVs Technologies, L'Aigle Cedex, France) using a previously tested freezing curve (0.1˚C/minute from 4 to -10˚C, 20˚C/minute from -10˚C to -110˚C, 40˚C/minute from -110˚C to -140˚C) for bull semen.

Post-thawing evaluation of sperm parameters

Computerized analysis of sperm motility

We used Semen Class Analysis software (SCA) to evaluate total motility (%), progressive motility (%), average path velocity (VAP, µm/sec), straight linear velocity (VSL, µm/sec), curvilinear velocity (VCL, µm/sec), amplitude of lateral head displacement (ALH, µm), straightness (STR, %), and linearity (LIN, %). For analysis, a 5 µl sample of diluted semen was added onto a pre-warmed chamber slide (20 µm, Leja 4, Leja Products Luzernestraat B.V., Holland).

Plasma membrane functionality

Plasma membrane functionality was determined according to the hypo-osmotic swelling (HOS) test (19). The HOS test relies on membrane resistance to loss of permeability barriers under a stress condition of stretching in a hyper-osmotic medium. We performed HOS by mixing 50 µl of semen with 50 µl of a 100 mOsm/kg hypo-osmotic solution [fructose (9 g/L distilled water), sodium citrate (4.9 g/L distilled water)]. This mixture was incubated at 37˚C for 20 minutes. Then, 200 spermatozoa were randomly assessed to determine the percentage of swollen and non-swollen tails visualized under a phase-contrast microscope (×400 magnification, CKX41, Olympus, Tokyo, Japan).

Phosphatidylserine translocation assay

For apoptosis status, Annexin-V was used to track phosphatidylserine translocation in the sperm plasma membrane. A commercial PS Detection Kit (IQP, Groningen, The Netherlands) was used according to the manufacturer's instructions. After washing spermatozoa with calcium buffer and adjusting the concentration of sperm to 1×10^6, we added 10 µl of Annexin V-FITC to the sperm suspension, which was allowed to incubate for 15 minutes at room temperature. Then, 10 µl of propidium iodide (PI) was added to the sperm suspension and the resultant suspension was analyzed with a FACS Calibur Flow cytometer (Becton Dickinson, San Jose, CA, USA). For each sample, 10000 events were collected and sperm subpopulations classified as follows: i. Live spermatozoa ($Annexin^-/PI^-$), ii. Apoptotic spermatozoa ($Annexin^+/PI^-$) and iii. Dead spermatozoa (PI^+).

Acrosome integrity

Pisum sativum agglutinin (PSA) was used to identify the integrity of the acrosomal region in post-thawed spermatozoa (20). We added 5 µl of the sperm suspension to 100 µl ethanol (purity: 96%). After 15 minutes, 10 µl of the sperm suspension was mixed with 30 µl of PSA on a glass slide. Finally, 200 sperm per slide were counted by a fluorescent microscope (BX51, Olympus) equipped with fluorescence illumination and a FITC filter (excitation at 455-500 nm and emission at 560-570 nm) at ×400 magnification. Sperm heads that fluoresced green were considered to have intact acrosome and those with no fluorescence were recorded as damaged or disrupted acrosome.

Mitochondrial activity

We determined mitochondrial activity by combining fluorescent dyes, Rhodamine 123 (R123, Invitrogen TM, Eugene, OR, USA) and PI. R123 (5 µL) solution was added to 250 µl of diluted semen and incubated for 30 minutes at room temperature in the dark. Then, 5 µl of the PI solution was added to the sample and analyzed with a FACS Calibur Flow cytometer (Becton Dickinson, San Jose, CA, USA). Sperm were analyzed according to their green and red fluorescence stain with R123 and PI. The percentages of live spermatozoa with active functional mitochondria were identified in the $R123^+/PI^-$ quadrant. For each sample we collected 10000 events.

Malondialdehyde production

The amount of malondialdehyde (MDA) in the semen samples, as an index of lipid peroxidation, was measured with the thiobarbituric acid reaction (21). MDA concentration was determined by absorption with a standard curve of MDA equivalent generated by the acid catalyzed hydrolysis of 1, 1, 3, 3-tetramethoxypropane.

Statistical analysis

All data were analyzed using Proc GLM of SAS 9.1 (SAS Institute, version 9.1, 2002, Cary, NC, USA) to determine the effect of different concentrations of NO on post-thawing quality of bull semen. The results were expressed as mean ± SEM. The mean of the treatments were compared using Tukey's tests.

Results

Computerized analysis of sperm motility

Table 1 shows the mean percentage of motility and velocity parameters of the post-thawed sperm exposed to different NO concentrations. NO-1 significantly improved total motility (88.4 ± 2.8) compared to NO-0 (72.5 ± 2.8), NO-0.01 (71.8 ± 2.8), NO-0.1 (79.3 ± 2.8), NO-10 (54.2 ± 2.8), and NO-100 (37.1 ± 2.8). NO-1 also significantly improved progressive motility (50.4 ± 3.2) compared to NO-0 (40.7 ± 3.2), NO-0.01 (41.3 ± 3.2), NO-0.1 (42 ± 3.2), NO-10 (32.6 ± 3.2) and NO-100 (15.7 ± 3.2). NO-1 resulted in significantly higher VAP (53.8 ± 3.1 μm/s) and VSL (40.5 ± 4.2 μm/s) rates compared to the other NO concentrations. There were no significant differences observed between NO-0, NO-0.01, NO-0.1 and NO-1 for VCL and STR. Also, different NO concentrations had no significant effect on the percentage of LIN.

Plasma membrane functionality

Figure 1 shows the alteration in post-thawed sperm plasma membrane functionality and acrosome integrity in different extenders. Plasma membrane functionality showed a similar trend as motility. NO-1 significantly improved plasma membrane functionality (89.3 ± 2.9%) compared to the other extenders. No significant differences were observed among the NO concentrations for the percentage of acrosome integrity.

Phosphatidylserine translocation assay

Results of apoptosis status are shown in figure 2. Sperm exposed to NO-1 produced the highest percentage of viable spermatozoa ($Annexin^-/PI^-$, 85.6 ± 2.3%) and the lowest percentage of apoptotic spermatozoa ($Annexin^+/PI^-$, 10.8 ± 2.4%) compared to other NO concentrations. NO-1 (3.6 ± 2.7%) produced a lower percentage of dead spermatozoa compared to NO-10 (17 ± 2.7%) and NO-100 (27.1 ± 2.7%).

Mitochondrial activity

Figure 3 shows the percentage of post-thawed live spermatozoa with active mitochondria after exposure to different extenders. There were no significant differences between NO-0 (53.4 ± 3.2%), NO-0.01 (52.1 ± 3.2%), NO-0.1 (50.8 ± 3.2%) and NO-1 (53.1 ± 3.2%). However, we observed a significantly lower percentage of live spermatozoa that had active mitochondria in NO-10 (40.6 ± 3.2%) and NO-100 (29 ± 3.2%) compared to the other groups.

Malondialdehyde production

Figure 4 shows the results of MDA concentration as a lipid peroxidation rate. Sperm exposed to NO-1 produced a significantly higher concentration of MDA (1.23 ± 0.24) compared to NO-0 (0.83 ± 0.24), NO-0.01 (0.89 ± 0.24) and NO-0.1 (0.9 ± 0.24). The highest MDA concentrations were observed in NO-10 (1.9 ± 0.24) and NO-100 (1.92 ± 0.24).

Table 1: The effect of nitric oxide (NO) induced oxidative stress on motility and velocity parameters of post-thawed bull sperm

Variable	Extenders					
	NO-0	NO-0.01	NO-0.1	NO-1	NO-10	NO-100
Total motility (%)	72.5 ± 2.8 [c]	71.8 ± 2.8 [c]	79.3 ± 2.8 [b]	88.4 ± 2.8 [a]	54.2 ± 2.8 [d]	37.1 ± 2.8 [e]
Progressive motility (%)	40.7 ± 3.2 [b]	41.3 ± 3.2 [b]	42 ± 3.2 [b]	50.4 ± 3.2 [a]	32.6 ± 3.2 [c]	15.7 ± 3.2 [d]
VAP (μm/s)	44 ± 3.1 [b]	46.1 ± 3.1 [b]	45.9 ± 3.1 [b]	53.8 ± 3.1 [a]	24.2 ± 3.1 [c]	22.3 ± 3.1 [d]
VSL (μm/s)	28.1± 4.2 [b]	29.3 ± 4.2 [b]	39.1 ± 4.2 [a]	40.5 ± 4.2 [a]	26.4 ± 4.2 [b]	27.1 ± 4.2 [b]
VCL (μm/s)	60.5 ± 3.8 [a]	63.75 ± 3.8 [a]	64.62 ± 3.8 [a]	65.41 ± 3.8 [a]	62.8 ± 3.8 [a]	34.7 ± 4.8 [b]
STR (%)	70.4 ± 2.9 [a]	68.7 ± 2.9 [a]	71.1 ± 2.9 [a]	72.7 ± 2.9 [a]	53.8 ± 2.9 [b]	54 ± 2.9 [b]
LIN (%)	38.3 ± 1.4	37.6 ± 1.4	37 ± 1.4	39.5 ± 1.4	38.4 ± 1.4	37.2 ± 1.4

Different letters within the same row show significant differences among the groups (P≤0.05).
VAP; Average path velocity, VSL; Straight linear velocity, VCL; Curvilinear velocity, STR; Straightness and LIN; Linearity.

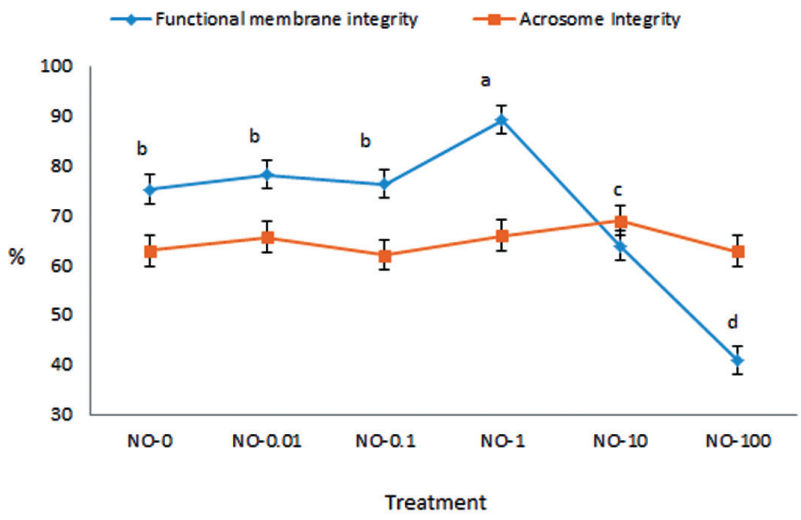

Fig.1: The effect of nitric oxide (NO) induced oxidative stress on plasma membrane functionality and acrosome integrity of post-thawed bull sperm (mean ± SEM).
Different letters within the same line show significant differences among the groups (P≤0.05).

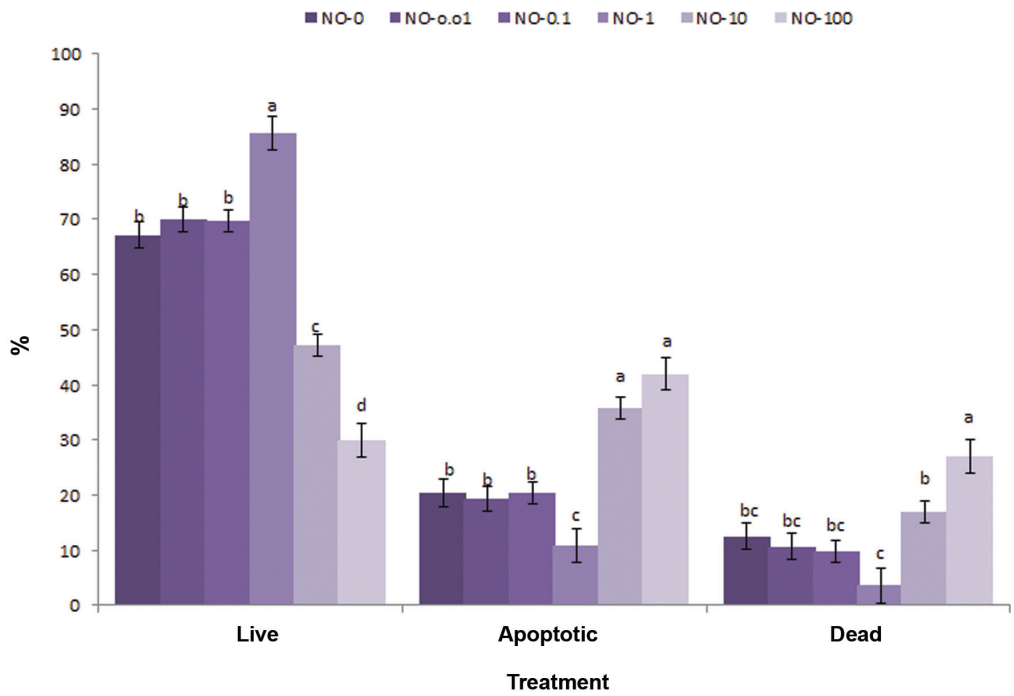

Fig.2: The effect of nitric oxide (NO) induced oxidative stress on the percentage of viable, apoptotic and dead post-thawed bull spermatozoa (mean ± SEM). Viability was assessed by Annexin V and propidium iodide.
Different letters within the same column show significant differences among the groups (P≤0.05).

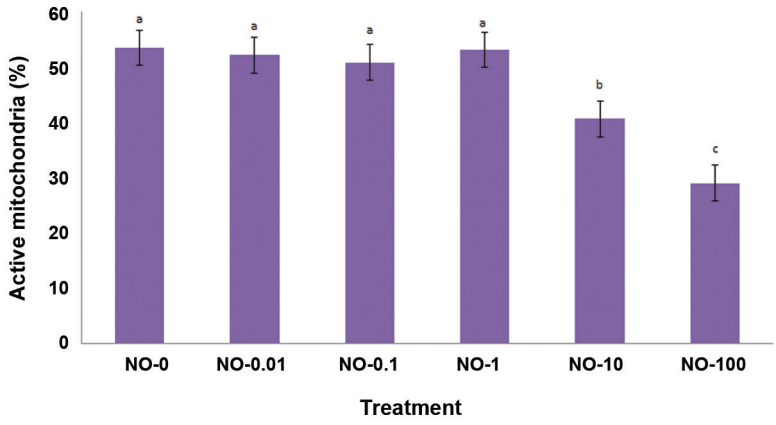

Fig.3. Post-thaw mitochondria potential of bull sperm after oxidative stress by different concentrations of nitric oxide (NO).
Different letters within the same column show significant differences among the groups (P≤0.05). Mitochondrial potential was assessed by R123 and propidium iodide.

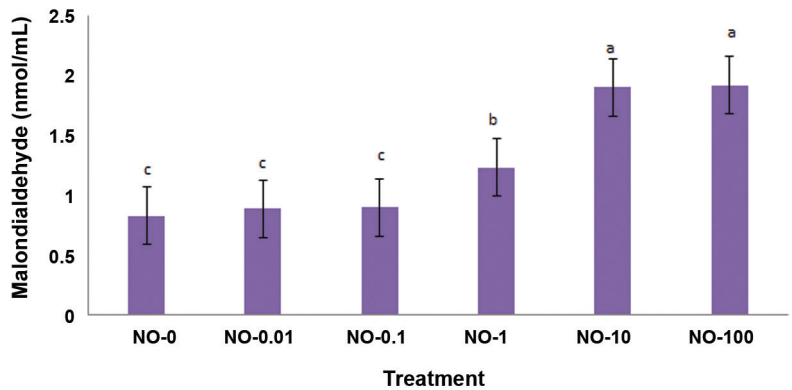

Fig.4: Malondialdehyde (MDA) concentration of frozen-thawed bull sperm after oxidative stress by different concentrations of nitric oxide (NO).
Different letters within the same column show significant differences among the groups (P≤0.05). Lipid peroxidation was assessed by MDA assay.

Discussion

The results of the present study have shown beneficial effects of NO induced sub-lethal oxidative stress for bull sperm during cryopreservation. During the process of freezing-thawing, spermatozoa are exposed to numerous stressful conditions which can cause disruption of cellular organelles and function (22). This experiment is the first study to investigate the controlled offensive approach by oxidative stress for creating a temporary response against future environmental challenges.

Our trial used a design that created a wide range of oxidative stress (0.01-100 µM NO) to determine the best concentration that could induce temporary resistance in the sperm against more serious stress during cryopreservation. Our analysis showed that the effective range was limited to 0.1-1 µM NO which improved motility, plasma membrane functionality, viability and mitochondria activity. A high amount of MDA production as indices of lipid peroxidation was observed in sperm treated with NO concentrations greater than 1 µM of NO. The present results also demonstrated that high oxidative stress to sperm before freezing produced a high amount of MDA which was directly respon-

sible for lower motility, viability, mitochondria activity and plasma membrane functionality in the groups exposed to greater than 1 μM NO. MDA, as an index of lipid peroxidation, increased during cryopreservation due to high activity of reactive oxygen species (ROS) (23).

The fact that sub-lethal stress increased temporary resistance to future stresses has been observed in different types of live cells (24). Conserved proteins in the cells are the main keys that participate in this process, by repair and stabilization of DNA, proteins and the cytoskeleton (25, 26).

Production and phosphorylation of heat shock proteins (HSPs) is another reason for enhancement of resistance after sub-lethal stress (26-28). It has been reported that HSPs directly inhibit the intrinsic and extrinsic pathways of apoptosis in the cell (29). Although we did not measure the amount of HSPs after oxidative stress, however it has been shown to increase HSPs level in both prokaryotes and eukaryotes expose to stress condition. In a recent study, phosphorylation of HSPs 70 and 90 in frozen-thawed sperm significantly increased compared to pre-freezing which assisted sperm to oppose stressful conditions.

However, we observed that exposure to oxidative stress over the limit of tolerance increased apoptosis and necrosis. These findings agreed with Hansen (17) who reported that heat stress greater than tolerance of embryonic stem cells led to higher numbers of apoptotic events. HHP is a one of controlled stress for sperm. It has been shown that HHP stress increased survival and fertility of prolonged storage *in vitro* (9, 30, 31). In a study by Kuo et al. (32), mild stress by HHP did not change the pregnancy rate but increased litter size after insemination with post-thawed semen. Huang et al. (16) stated that stress by HHP particularly increased proteins in sperm which played a key role in fertilization.

Similar to HHP stress, we obtained an improvement in bull semen quality by induction of moderate oxidative stress to the sperm before cryopreservation. Our result agreed with the finding by Vandaele et al. (15) who reported that a low level of oxidative stress by 50-100 μM H_2O_2 for oocytes resulted in a higher blastocyst rate after *in vitro* fertilization. This efficiency might be attributed to increasing levels of antioxidants such as GSH in the oocyte which positively affected development.

However, in other studies, oxidative stress resulted in negative effects on the blastocyst rate by increasing the numbers of apoptosis cells (33-35). This discrepancy might be related to various factors such as manipulation of cells, type and level of stress, and cell type (oocyte, embryo sperm or fibroblast). We have also found that mild stress may reduce the apoptosis rate in sperm exposed to 1 μM NO before freezing which shows a logical relationship with motility. Mitochondria play a primary role in extending the phase of apoptosis as a result of the opening of mitochondrial pores which leads to the subsequent release of pro-apoptotic factors. Synthesis of ATP is under the control of mitochondrial activity and damage to mitochondria lead to non-renewal of ATP, this negatively affects the sperms' ability to move.

Conclusion

This study has shown that a mild level of oxidative stress treatment (1 μM NO) prior to cryopreservation offers an approach to improve the quality of frozen-thawed semen performance such as motility, viability, plasma membrane functionality and mitochondrial activity. Understanding the molecular and cellular mechanism of this phenomenon needs more investigation. A filed study using this approach along with artificial insemination, *in vitro* fertilization, and the application of different procedures for manipulation of sperm are also necessary.

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Original Article

The Effectiveness of Emotionally Focused Therapy on Enhancing Marital Adjustment and Quality of Life among Infertile Couples with Marital Conflicts

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Abstract

Background: The purpose of this study is to investigate the efficacy of emotionally focused therapy (EFT-C) on promoting marital adjustment of infertile couples with marital conflicts by improving quality of life.

Materials and Methods: This is a semi-experimental study with a pre- and post–test design in which 30 infertile couples (60 individuals) were chosen by purposive sampling. Couples were randomly divided into two groups, sample and control, of 15 couples each. Next, couples in the sample population answered questionnaires for marital adjustment, sexual satisfaction and quality of life after which they received 10 sessions of EFT-C.

Results: Pre- and post-tests showed that EFT-C had a significant effect on marital adjustment and quality of life.

Conclusion: According to the results, EFT-C had a significant, positive effect on enhancement of marital adjustment. Life quality of infertile couples significantly increased via application of EFT-C. This approach improved the physical, psychological and social relationships of infertile couples and enhanced their social environment.

Keywords: Emotion, Therapy, Adjustment, Infertile, Quality of Life

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Introduction

Despite the alteration of standpoints on sexual behavior in recent centuries, fertility remains of crucial importance and children play an important role in cementing a marital relationship. Fertility, as one of the major reasons for marriage, actually results from human nature and introduces the concept of eternal life (1, 2). Another concept, sterility, as the opposite of fertility is defined as the inability to bear offspring after a year of regular sexual activity without contraception (1-3). This inability is considered a failure and leads to the feeling of imperfection in sexual identity. Sterility often causes the person to feel a loss of control over one's life, doubting one's manhood/womanhood and generally damages self-confidence and health (4). High-costs of treatment, constant anxiety about treatment outcomes, exhaustion from visiting various clinics, societal repercussions, confronting questions about a childless marriage, potential distress during the treatment and fear for missing the spouse or destruction of the family are among the factors which result in multiple psychological complications. These complications include frustration, personal conflict, disappointment, sharp decline in self-esteem, isolation, identity crisis and loss of marital adjustment (5). Marital adjustment is a process commonly composed of: i. Marital satisfaction, ii.

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Dyadic cohesion, iii. Consensus on matters of importance to marital functioning, iv. Showing affection and warmth toward the spouse and v. Sharing intimacies (6, 7). Couples who are well-adjusted gain great satisfaction out of the marital relationship and think well of the spouse's habits. They enjoy communicating with family and friends, and ask their help with problems. These couples derive immense sexual pleasure as well (8).

Sterility has physical, financial, emotional and psychological impacts on a person (9). Therefore it seriously undermines self-confidence and results in a sexual identity crisis which results in a decline in life quality (10). Life quality has a wide range of definitions. Some believe that it is the ability of an individual to manage life from his/her standpoint, as such, fertility status and its factors can put social and psychological pressures on the person. It lowers sexual pleasure and life satisfaction, meaning a decline in quality (11). Donald considers quality of life as a descriptive term. Quality of life is a perception that an individual holds of their state of health which has a feeling of contentment within, and is accompanied by happiness and joy (12).

Downey and Mckinney (13) declared that women who sought treatment for fertility problems suffered from greater depression, anxiety and stress, along with decreased dyadic cohesion. Most researchers reported increased marital quarrels among infertile couples, which in some cases led to separation (14). Decreasing familial disputes and striving to gratify the partner's desires could alleviate these problems (15).

In this regard, a host of researches have confirmed efficacy of couples therapy for decreasing marital conflicts (13). As emotions play a central role in infertile couples' relationships, the emotionally focused approach is employed as short-term structured counseling of 9-20 sessions. This technique is used because it is a branch of couples therapy and because it takes advantage of emotions to develop the process. This method addresses communication disorders, marital discord, and persuades people to express their emotions and talk over them. From the standpoint of couples therapy, marital distress is mostly caused by negative emotions and attachment injuries (16).

A study on 120 infertile couples in East India suggested that infertile men had problems with their character and social interactions. On the other hand, women displayed symptoms of depression. The study indicated that infertility spoiled gender concept, life quality, marital adjustment, and sexual relationships of the couples (17). Poor quality of marital relationship was followed by a number of social and familial troubles. Sterility along with other differences and problems between couples challenged the mind and social welfare of spouses. In general, a low quality within the marital relationship could be problematic when couples struggled with infertility (18, 19).

As a result, attention to psychological needs of infertile couples is essential for successful infertility treatment. Each partner requires support and empathy while undergoing treatment (20). There are a variety of practices to cope with psychological reactions of infertility, among which is emotionally focused therapy for couples (EFT-C) which merges three approaches of systematic, humanistic (empiricism) and attachment theory. This therapy was founded by Johnson and Greenberg in the early 1980s. Given the major role of emotions in attachment theory, EFT-C emphasizes emotions and employs them to organize interaction patterns (16). Hence, EFT-C concentrates on the emotional relationship of couples as a basis to tackle their problems. EFT-C has a process of 9 steps as follows (21):

Step 1: Evaluation, making contact, and then recognition of tensions between couples from the standpoint of attachment.

Step 2: Identification of the cycle of negative interactions that sustain anxiety and bring about insecure attachment.

Step 3: Discerning the underlying feeling or emotion not yet expressed in couples' interactions that is being concealed.

Step 4: Reframing the problems resulting from the cycle of negative interactions, unmet urges, needs and emotions in order to explore the cycle.

Step 5: Having access to fears and needs of attachment.

Step 6: Promotion of acceptance by the other spouse.

Step 7: Smoothing the way for expression of needs and wants, and restructuring new models of interaction on the basis of perceptions and knowledge obtained from the process.

Step 8: Providing new solutions for old challenges.

Step 9: Strengthening new positions and patterns of behavior (16).

Thus, showing emotions and attachment needs along with sincere fulfillment by the partner constitute the process of EFT-C and are necessary for change (22, 23).

In light of the points previously mentioned, the current study intends to meet the necessity for enhancing marital adjustment of infertile couples - people who suffer from poor life quality and are locked in marital disputes. In addition, the results can serve as a practical map or a manual for counselors, psychotherapists and family therapists to raise their clients' self-esteem and show mismatch in communication methods between individuals.

Materials and Methods

This semi-experimental method with a pre- and post-test design was conducted on a sample group of 15 couples and a control group of the same number. Initially, demographic characteristics of the subjects were collected. Next, they were tested prior to conducting the independent variable (EFT-C). According to Johnson's plan (24), couples in the sample group underwent 10 EFT-C sessions of 120 minutes duration conducted twice per week. By the end of the term, subjects were again tested. To meet ethical standards, a compact course of 4 weeks was offered to volunteers from the control group.

The sample population comprised couples married for 10 years who attended infertility clinics in pursuit of treatment during 2013. According to purposive sampling, 30 couples (60 individuals) were selected in terms of poor marital adjustment and low sexual satisfaction. Couples were then divided into two groups of 15 couples (15 men and 15 women); one as control group and another as sample population.

Infertile couples attended fertility clinics. The data-gathering tools were demographic characteristics and World Health Organization (WHO) quality of life questionnaires.

Data were analyzed using SPSS software (version 18) by application of the methods of mean calculation plus minimum and maximum standard deviation from descriptive statistics and analysis of covariance from inferential statistics (alpha 0.81). In order to use analysis of covariance, first the equality of variances was noted. Hence, the hypothesis was examined by the Levin test. Table 1 points out the treatment protocol used in this study, this protocol is emotionally-focused therapeutic approach, which have been provided to the couple during 10 sessions.

Ethical consideration

In order to observe ethical considerations, a few tutorial sessions were held over four weeks for the control group that did not receive EFT-C.

Research tools

Questionnaire of Demographic Characteristics: This questionnaire comprised parameters of age, gender, education level, occupation, income level, cause of infertility, duration of infertility, duration of marriage, number of surgeries, date of last surgery, history of attending psychological or counseling sessions, history of any chronic physical or psychological disorders.

Spanier's Dayadic Adjustment Scale: This scale (25) consists of 32 questions based on a Likert approach of responding which measures the total score of marital adjustment within a range of 0 to 15. People who score 101 or less, according to Spanier, are supposed to be maladjusted and those with higher scores are considered well-adjusted. In a study by Hassan shahi (26), well-adjusted couples had an average score of 114.7 ± 17.8 whereas the average score of maladjusted couples was 70.7 ± 23.8. Spanier grouped the data into four subscales of marital satisfaction, dyadic consensus, dyadic cohesion, and affectional expression with evaluated validity of 0.94, 0.90, 0.81 and 0.73. The entire scale had a validity of 0.96. Reliability was 0.86 according to Pearson's correlation coefficients between Spanier's scale and the Locke-Wallace Marital Adjustment Scale. Hassan shahi (26) evaluated the validity of Spanier's scale in Iran by calculating the cohesion between the Locke-Wallace Marital Adjustment Scale and Spanier's scale (25).

Table 1: Johnson's Protocol of emotionally focused therapy (EFT-C) for infertile couples

Step	Purpose	Session	To do
1	Identification	1	Collect general information about the couple; introduce the therapist to the partners, investigate grounds and expectations of participation, define the method of EFT-C in addition to concepts of infertility, conflict, marital adjustment, sexual satisfaction, and life quality, ask the couple for their opinion on the method and concepts; identify negative cycles, assess couple's way of dealing with issues, discover attachment blocks as well as personal and interpersonal tensions, evaluate status of marital relationship, sexual satisfaction and quality of life.
			Task: Pay attention to positive and negative emotions, i.e., joy, happiness, anger, hate, sadness, jealousy, anxiety, etc.
		2	Appoint a separate session for each partner to discover significant events and information that is not feasible to discuss in the presence of the other, such as commitment to marriage, extramarital relationship, exporter attachment trauma, assess the fear of revelation.
			Task: Pay attention to your partner's cycle of interaction.
2	Change	3	Ascertain interaction patterns and ease acceptance of the experienced emotion, discern every partner's fears of insecure attachment, help each partner with openness and self-disclosure, continue the therapy.
			Task: Discern pure emotions, thoughts, and sentiment.
		4	Restructure the bond through clarification of key emotional reactions, widen the emotional experience of each spouse to create new ways of interaction, partners should accept new patterns of behavior.
			Task: Express pure emotions and sentiments.
		5	Task: Deepen the relationship by recognizing recently developed needs of attachment; improve personal health and relationship status, express pure emotions and sentiments.
		6	Establish a safe therapeutic alliance, develop new ways of interaction, promote acceptance of the other, discover deep-seated fears and express needs and wants.
		7	Restructure the emotional experiences of the couple, clear the needs and wants of each partner.
			Task: Underline strengths and weakness.
3	Stabilization	8	Support couple in finding new solutions to past problems, change problematic manners of behavior, facilitate steps the couple can take to invest in their responsive and accessible positions, sync the inner feelings and concepts to the relationship, encourage in positive reaction.
			Task: Find new solutions to past problems.
		9	Take advantage of therapeutic achievements within daily life to consolidate intimacy, continue with the therapy and its direction, create secure attachment, discern and support constructive patterns of interaction, help the couple shape a story about their future together.
			Task: Practice the techniques in daily life.
		10	Ease the end of the treatment, Maintain therapeutic changes, draw a comparison between the past and present cycles of interaction, keep an emotional involvement to the deepest status of relationship.

World Health Organization (WHO) Quality of Life-BREF (WHOQOL-BREF): This scale is comprised of 26 questions in areas of physical health (7), psychological health (6), social relationships (3), and environment (8) along with 2 additional questions about quality of life. WHO developed this widely used questionnaire to assess general domains of health. Every question is rated on a Likert scale from 1 to 5. A higher the score assumes better quality of life. The psychometric quality of the questionnaire has received approval in a large number of countries, including Iran (26-28).

According to reports prepared by the scale-makers of WHO from 15 international centers, Cronbach's alpha for the quad subscale and the entire questionnaire ranged between 0.73 and 0.89. Rahimi (29) evaluated reliability of the WHOQOL-BREF and determined it to be 0.88 for the entire scale. Cronbach's alpha of physical health, psychological health, social communication, and quality of life environment were calculated to be 0.88, 0.70, 0.77 and 0.65.

Results

There were 30 participants (15 couples). Of these, there were 30 (31.7%) individuals with diplomas which was the maximum education level and 10 (11.7%) who had secondary school certificates, as the minimum education level. Duration of marriage in subjects was 10 years. The average age of participants was 33.8 ± 5.03 years. Table 2 shows the pre- and post-test scores on marital adjustment and aspects of quality of life in the control and sample groups and table 3 points out the Kolmogorov-Smirnov Test which has used to determine normality of the data.

As the table indicates there was no significant difference between groups in the subscales of marital adjustment (P>0.05). Therefore both groups were the same at the pre-test. According to table 4, it could be inferred that no significant difference existed between groups in the WHOQOL-BREF at the pre-test stage (P>0.05).

Table 2: Average pre-test and post-test scores in control and sample groups

Subscales		Study groups					
		Control		Sample		Total	
		Mean	SD	Mean	SD	Mean	SD
Pre-test	Dyadic satisfaction	22.17	4.32	21.27	4.27	21.72	4.29
	Dyadic cohesion	7.97	2.08	7.37	2.19	7.67	2.14
	Dyadic consensus	24.20	5.93	21.80	6.52	23.00	6.30
	Affectional expression	4.67	1.30	4.40	1.33	4.53	1.31
	Physical health of the couple	18.87	3.10	19.73	2.03	19.30	2.64
	Psychological health of the couple	15.40	2.40	15.27	1.55	15.33	2.01
	Social Relationships	7.10	1.16	6.87	1.36	6.98	1.26
	Social surrounding	21.97	3.34	22.43	2.10	22.20	2.77
Post-test	Dyadic satisfaction	22.57	4.42	41.03	3.59	31.80	10.13
	Dyadic cohesion	7.63	2.06	20.63	2.20	14.13	6.89
	Dyadic consensus	21.33	7.08	54.10	5.13	37.72	17.62
	Affectional expression	4.30	1.34	11.10	0.99	7.70	3.62
	Physical health of the couple	16.90	3.35	31.67	2.26	24.28	7.97
	Psychological health of the couple	14.23	2.18	28.80	1.92	21.52	7.62
	Social relationships of the couple	7.53	2.73	15.13	3.30	11.33	4.87
	Social surroundings of the couple	20.60	3.91	33.57	4.74	27.08	7.83

SD; Standard deviation.

Table 5 shows a significant difference between the control and sample groups (P<0.001). According to the results, equality of the variances of the control and sample groups was approved (P>0.05). The result of the covariance analysis for comparison of average scores is shown in table 5. The degree of change as a result of EFT-C was as follows: marital satisfaction (86%), dyadic cohesion (92%), dyadic consensus (90%), affectional expression (87%), physical and psychological health (93%), social relationships (62%) and social surroundings (80%), which represented a significant improvement attributed to EFT-C.

Table 3: Kolmogorov-Smirnov test to investigate normality of the data

Scales	Subscales	Statistic	Sample size	P value
Marital adjustment	Dyadic satisfaction	1.298	60	0.069
	Dyadic cohesion	0.909	60	0.380
	Dyadic consensus	0.813	60	0.523
	Affectional expression	1.550	60	0.97
Quality of life	Physical health	1.051	60	0.219
	Psychological health	1.091	60	0.185
	Social relationships	1.097	60	0.186
	Environment	1.016	60	0.253

The data was approved as normal for all variables according to the Kolmogorov-Smirnov test (P>0.05).

Table 4: Comparison of the groups in the subscales of marital adjustment and WHOQOL-BREF through the pre-test stage

Subscales	Group	Mean	SD	t *	df **	P value ***
Dyadic satisfaction	Control	22.17	4.32	0.811	58	0.421
	Sample	21.27	4.27			
Dyadic cohesion	Control	7.97	2.08	1.089	58	0.281
	Sample	7.37	2.19			
Dyadic consensus	Control	24.20	5.93	1.491	58	0.141
	Sample	21.80	6.52			
Affectional expression	Control	4.67	1.30	0.787	58	0.434
	Sample	4.40	1.33			
Physical	Control	18.86	3.10	-1.279	58	0.206
	Sample	19.73	2.03			
Psychological	Control	15.40	2.40	0.255	58	0.799
	Sample	15.26	1.55			
Social	Control	7.10	1.15	0.717	58	0.476
	Sample	6.86	1.35			
Environment	Control	21.96	33.3	-0.649	58	0.519
	Sample	22.43	2.09			

WHOQOL-BREF; World Health Organization Quality of Life-BREF, SD; Standard deviation, *; Paired t test, **; Degrees of freedom and ***; Probability of rejecting the null hypothesis.

Table 5: ANCOVA of marital adjustment and WHOQOL-BREF in couples

Aspects	Freedom	Mean square		F Value		P value		Effect size		Statistical power	
	Pretest	Pretest	Group member ship	Pretest	Group member ship	Pretest	Group member ship	Pretest	Group member ship	Pretest	Group member ship
Dyadic satisfaction	1	141.80	5238.84	10.12	373.95	0.002	0.001	0.15	0.86	0.87	1
Dyadic cohesion	1	56.08	61.47	15.38	16.86	0.001	0.001	0.21	0.92	0.97	1
Dyadic consensus	1	417.16	16503.136	13.20	522.54	0.001	0.001	0.188	0.902	0.947	1
Affectional expression	1	42.28	3060.73	5.59	404.70	0.021	0.001	0.089	0.88	0.64	1
Psychological health	1	33.26	3201.08	8.990	865.14	0.004	0.001	0.14	0.94	0.84	1
Social relationships	1	9.083	875.42	0.99	95.62	0.32	0.001	0.017	0.62	0.16	1
Social surroundings	1	116.70	2602.69	10.52	234.60	0.002	0.001	0.16	0.807	0.89	1

ANCOVA; Analysis of convariance and WHOQOL-BREF; World Health Organization Quality of Life-BREF.

Discussion

According to the results, EFT-C had a significant positive effect on marital adjustment. There was improvement in the dyadic satisfaction, dyadic cohesion, dyadic consensus, affectional expression, dimensions of life quality, physical and psychological health, social relationships, and social surroundings subscales. The difference was observed between the control and sample groups as well as between the pre- and post-test results. Findings of this study were consistent with previous studies. Aarts et al. (30) through their research indicated that scores of anxiety, depression, and poor life quality in fertility clinics were related to each other. Consideration of these factors could create positive experiences. They concluded that EFT-C could improve an infertile couple's quality of life and decrease the level of anxiety and depression. This influence has been attributed to the power of emotions over marital relationships. Emotions play a key role in an infertile couple's relationship which deserves attention. It is recommended to apply this approach for 9 to 20 structured sessions, as it is both a branch of couples therapy and focuses on emotions. EFT-C addresses communicative disorders and maladjustment and encourages people to speak about their emotions. From the standpoint of EFT-C, marital distress originates from negative emotions and attachment injuries.

The findings of present study were consistent with results of studies by Soltani et al. (31), Zuccarini et al. (32) and Vizheh et al. (33). According to research by Soltani et al. (31) on the influence of EFT-C on couple intimacy in Shiraz, it was suggested that EFT-C could improve emotional, psychological, sexual, physical, communicative, ethical and mental dimensions of couples. However it had no effect on their spiritual and social dimensions.

The results of present study corresponded with findings of Michelle (34) and Tie and Poulson (35) with respect to dyadic consensus. The results of present study also matched findings of Pinto-Gouveia et al. (36) in terms of mutual affection.

Of note, infertile couples tend to express negative and damaging feelings, remarks, sarcasm and criticism rather than empathy while their spouse is trying to deal with an issue (infertility). According to Morin-Papunen and Koivunen (37), marital satisfaction of infertile couples is significantly lower than fertile couples with regards to mutual affection. Onat and Beji (38), in an investigation into the marital life and relationship of infertile couples, have declared that the stress coming from the inability to conceive negatively influenced the couple's relationship. EFT-C could significantly improve the sexual relationship of the partners.

With regards to physical health of the couples, our findings were consistent with the results of

research by Peterson et al. (39) who stated that counseling could improve physical and psychological health of infertile couples. They asserted that psychological counseling through persuasion of the clients to continue with medical treatments significantly improved both their psychological status and physical health.

Our findings were also compatible with the results of Naamen et al. (40) which revealed that social support and understanding played an important role in psychological health of infertile couples, which motivated the couples to continue with infertility treatment and reduced the call for divorce. Javidi et al. (41) indicated that EFT-C had influential effects on family functioning, inasmuch as this protocol took advantage of a systematic approach which refined the inflexible interaction patterns of couples in distress and strengthened their bonds. Soltani et al. (42) concluded that EFT-C was capable of promoting marital adjustment of infertile couples.

Najafi et al. (43) evaluated studies about questionnaires of life quality among infertile couples. Through screening all studies, they found 10 general and 2 specialized inventories. Although no meta-analysis was found, infertility negatively influenced couples' quality of life. This research indicated that general questionnaires (SF-36, WHO-QoL, and FERTI-QoL) were mostly used for evaluation of infertile couples quality of life.

Ramezanzadeh et al. (44) investigated the emotional adjustment of infertile couples. They concluded that people unable to conceive suffered from psychiatric disorders (particularly stress and depression) which led to emotional maladjustment.

Conclusion

EFT trains couples to give stronger support to each other, corrects their patterns of behavior and raises their accessibility accompanied by responsiveness to the partner's needs in order to achieve an optimal sexual relationship.

The present study showed that EFT-C significantly increased satisfaction, cohesion, consensus and affection expression of the partners. The life quality of infertile couples remarkably grew which was attributed to EFT-C. This method improved the social relationships of infertile couples and improved their physical and psychological health. The findings have opened a window for family therapists to conduct more practical and clear counseling in order to improve their client's self-worth and assist with self-disclosure, as well as revision of wrong communicative patterns which can lead to a decline in marital disputes and increase in adjustment. In addition, the results have shown that EFT-C has a significant effect on enhancement of sexual satisfaction in infertile couples by increasing physical sexual satisfaction as well as emotional sexual satisfaction.

We recommend that similar research be conducted with different populations (in addition to infertile couples) that have diverse levels of education. In addition, follow-ups should be conducted at later months in order to compare the results.

Constraints of the study included the enrollment of couples that had a minimum educational level of a diploma. Hence, to generalize the results of the present study to illiterate individuals, measures of prudence should be taken. The study was performed in one province. Generalization of the findings to other statistical areas should be made with caution.

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Original Article

Effect of Telephone-Based Support on Postpartum Depression: A Randomized Controlled Trial

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Abstract

Background: Postpartum depression (PPD) is one public health issue that affects both maternal and child health. This research studies the effect of health volunteers' telephone-based support on decreasing PPD.

Materials and Methods: This randomized controlled trial evaluated 203 women who had uncomplicated deliveries. The women completed the Edinburg Postnatal Depression Scale (EPDS), 10 to 15 days after childbirth in order to be assessed for pre-trial depression scores. The cut-off point for depression was considered to be a score of >10. We randomly assigned 54 eligible mothers (n=27 per group) with mild and moderate depression to the intervention and control groups. In both groups, mothers received routine postpartum care. The intervention group additionally received telephone support from health volunteers. A questionnaire was used to gather demographic and obstetric information. By the end of the 6th week, mothers completed the EPDS to be reassessed for depression after intervention. Data were analyzed using the chi-square, Fisher's exact, t- and paired t tests.

Results: The mean depression scores before intervention (10 to 15 days after childbirth) in the intervention and control groups did not significantly differ (P=0.682). Depression scores of the intervention and control groups showed a significant difference after 6 weeks (P=0.035). In addition, there was a significant decrease in depression for the intervention and control groups (P=0.045).

Conclusion: Health volunteer telephone-based support effectively decreased PPD and may be beneficial to women with symptoms of mild and moderate PPD (Registration number: IRCT201202159027N1).

Keywords: Postpartum Depression, Postnatal Care, Volunteers, Mother, Women Health

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Introduction

Pregnancy and childbearing are important events in women's lives that greatly impact the physical, mental and social health of the mother. Postpartum blues, depression and anxiety are common realities in today's world, especially in developing countries where many remain underdiagnosed and undertreated due to different reasons (poverty, culture, values and major life events) (1-3). Major depression after childbirth has serious risks such

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as suicide and infanticide (4, 5). It also decreases breastfeeding, induces severe malnutrition and other diseases (6). Postpartum depression (PPD) is a public health issue whose worldwide incidence is approximately 15% or more (7-11). In some cities of Iran, PPD incidence has been reported as 23.7 and 32% (12, 13).

Finding strategies for prevention, early detection and treatment of PPD can have major benefits in the areas of reproductive rights, as well as medical and financial aspects. These strategies can promote public health, especially in societies with limited human and financial resources. Several strategies have been implemented in an attempt to find help-seeking behaviors, that screen, prevent and manage mothers who suffer from this illness (e.g., peer support); provide telephone care management; and establish consultation centers and Crisis Intervention Units (10, 14-18).

Some studies have shown that women preferred "talking therapies" with someone who was non-judgmental rather than pharmacological interventions. Dennis and Chung-Lee (19, 20) studied the effect of telephone-based peer support on prevention of PPD at 12 weeks after childbirth. They found that the group who received postpartum care plus telephone-based peer support reported higher levels of positive relationship qualities than the control group. In their study women preferred to talk with someone than to receive medical interventions. They concluded that peer volunteer support might be a preventive strategy for PPD. Simon et al. (17) compared the benefits of telephone care management with telephone psychotherapy for depression and concluded that telephone psychotherapy was more effective than telephone care management.

In Iran, a limited number of studies have described and explained PPD. Hassan Zahraee et al. (21) studied the supportive role of the midwife in preventing PPD in Isfahan. The results showed that mean depression scores of mothers who received emotional and informational support of a midwife on the 2nd and 10th days postpartum were significantly lower than the control group on the 45th day after childbirth. They concluded that midwife support might be an effective factor in preventing PPD. Sadr et al. (22) studied factors that affected PPD in Tehran and have reported significant relationships between PPD and the husband's education, marital dissatisfaction and lack of social support, unwanted pregnancy, congenital disorders of the newborn, and mental disorders. Additionally, it is possible that genetics may play a role in susceptibility to PPD (2). However, it is important to emphasize that genetic and biologic factors of PPD have yet to be efficiently studied and explained (23).

Telephone-based support can help mothers with access to postpartum information and care as well as receive responses to their questions according to a study by Goodman (24). In Iran, there have not been any studies regarding the effect of health volunteer telephone-based support on PPD. Thus, this research aimed to study the effect of health volunteers' telephone based-support on PPD. The term "health volunteers" were women who interacted with families in primary health care (PHC) and acted as bridges between health centers and the community in Iran.

Materials and Methods

In this randomized controlled trial, 203 postpartum women completed the Edinburg Postnatal Depression Scale (EPDS) during routine postpartum care visits conducted 10 to 15 days after uncomplicated childbirth in hospitals affiliated with Shahid Beheshti University of Medical Sciences. The EPDS was validated in Iran. This scale is a 10-item self-report questionnaire with a possible score range of 0 to 30. Items 1, 2, and 4 are scored 0, 1, 2 or 3 with the top box scored as 0 and the bottom box scored as 3. Items 3 and 5-10 are reverse scored, with the top box scored as 3 and the bottom box scored as 0. The cut-off point for detecting depression was identified as a score of >10 (25). Cases with scores ≥14 were considered to be severely depressed. The aim, benefits and privacy were explained in simple words to participants after which the EPDS was completed by the mothers. If the mother was unable to complete the questionnaire, the interviewer assisted with completion. Thus, 75 cases of mothers who suffered from PPD were detected, 67 cases had moderate depression and the other 8 cases had severe depression.

Inclusion criteria

The criteria for including cases were term pregnancy, live birth and depression score (EPDS score) >10 to <14.

Exclusion criteria

The criteria to exclude cases were: history of mental disorder, episodes of mental disorders during pregnancy or in the last 12 months that necessitated the use of medicines, nonviable fetus, history of PPD, current use of prescribed psychiatric drugs and an EPDS score of ≥14. Mothers with EPDS scores ≥14 and who had suicidal thoughts were referred to a psychiatrist. By considering the exclusion criteria, 21 of 75 depressed mothers were excluded; 54 of the participants were eligible to remain in the trial.

Intervention description and assessment

There were 54 eligible mothers out of 203 postpartum mothers (n=27 per group) who had mild to moderate depression (>10 to <14 EPDS scores). These cases were recruited and randomly assigned into the intervention and control groups. After obtaining informed written consent, demographic and obstetric information of the participants was gathered by a questionnaire as follows: age, parity, number of children, job, education, interpersonal relationship with husband, history of disease, newborn gender, willingness of parents to a specific newborn gender, quality of childbirth, type of childbirth, health status of newborn, and wanted pregnancy.

In both groups, mothers received routine postpartum care as shown in table 1. Routine postpartum care did not have any screening tool for PPD. The intervention group received telephone support provided by eight health volunteers. These health volunteers had been trained in a workshop to enable them to communicate effectively and accurately with mothers and to manage their problems. Volunteers were instructed to refer cases that they could not handle to health centers.

Each trained volunteer called 3 to 4 mothers at intervals of 2 to 3 times per week until 6 weeks after childbirth. During each phone call, after a greeting, they asked the mother about her health status, newborn's condition, complaints, and the mother's relationship with her newborn or husband, and whether there was any problem. The volunteers managed the problem according to their training, and if needed, assistance was given by a principal researcher. During the conduction of research all volunteers were in contact with the main researcher however they kept all data confidential from their fellow volunteers. The control group did not receive any further intervention during the study. At the end of 6 weeks (after intervention), both groups completed the EPDS to reassess depression scores. There were 5 participants from the intervention group and 3 from the control group that were lost to follow up.

Data analysis plan

Data were processed using SPSS version 16. Data with nominal and ordinal scales were compared in two groups using the Chi-square and Fisher's exact tests. Data with ratio scales was tested using the t test. The changes in depression scores between the pre- and post-intervention in each group were tested using the paired t test; the changes of scores between two groups were compared with the t test (26, Table 2). A P value of <0.05 was considered significant.

This study was registered at the Iranian Registry of Clinical Trials (IRCT) website with registration ID: IRCT201202159027N1 and was approved by the Ethics Committee of research at Shahid Behaeshti University of Medical Sciences.

Table 1: Routine postpartum care in Iran

Routine postpartum care (until day 42)	Examples
Medical history	Vaginal bleeding and discharge, urinary complaints, psychological status, etc.
Clinical examinations	Vital signs, breasts, abdomen, eyes, etc.
Education and counseling	Nutrition, breastfeeding, family planning, etc.
Nutritional supplements	Ferrous tablets, multivitamins

Table 2: Baseline characteristics of study group participants

	Intervention (n=22)	Control (n=24)	P value
Age (Y, Mean ± SD)	27.59 ± 4.81	28.37 ± 7.37	0.670
Parity (Mean ± SD)	2.36 ± 1.22	2.12 ± 1.8	0.596
Number of children (Mean ± SD)	2.90 ± 1.15	2.3 ± 1.4	0.121
Job (%)			
Housewife	19 (86.4%)	21 (87.5%)	0.909
Employed	3 (13.6%)	3 (12.5%)	
Educational level (%)			
<Diploma	13 (61.9%)	15 (68.2%)	0.731
≥Diploma	9 (42.8%)	7 (31.8%)	
Relationship with husband (%)			
Good	19 (86.4%)	22 (91.7%)	0.658
Poor	3 (13.6%)	2 (8.3%)	
History of physical disease (%)			
Positive	5 (22.7%)	7 (29.2%)	0.872
Negative	17 (77.3%)	17 (70.8%)	
Newborn gender (%)			
Willingness	12 (54.5%)	15 (62.5%)	0.804
Unwillingness	10 (45.5%)	9 (37.5%)	
Quality of childbirth (%)			
Moderate	8 (36.4%)	7 (29.2%)	0.740
Difficult	10 (45.5%)	13 (54.2%)	
Type of childbirth (%)			
NVD	9 (40.9%)	7 (29.2%)	0.672
Cesarean	13 (59.1%)	16 (66.7%)	
Health status of newborn (%)			
Healthy	14 (63.6%)	18 (75%)	0.606
Unhealthy	8 (36.4%)	6 (25%)	
Wanted pregnancy (%)			
Yes	10 (45.5%)	18 (75%)	0.080
No	12 (54.5%)	6 (25%)	

P<0.05 was considered significant.
NVD; Normal vaginal delivery.

Results

Out of 203 mothers who completed the EPDS, 67 (33%) had mild and moderate depression whereas 8 (4%) had severe depression. The intervention and control groups were the same in baseline characteristics as well as depression scores before intervention. Thus, there were no any significant differences between the intervention and control groups. Contextual variables such as age, parity, number of children, educational level, and relationship with husband were compared in the intervention and control groups as shown in table 2.

Table 3 shows the mean ± SD depression measured by the EPDS scoring scale that included 0, 1, 2 and 3 values as well as changes before and after intervention in the intervention and control groups. Before intervention, the mean ± SD depression score in the intervention group was 12.68 ± 1.35 and in the control group it was 12.83 ± 1.12. There was no significant difference between the groups (P=0.682).

After intervention, the mean depression scores were 7.95 ± 3.45 for the intervention group and 10.33 ± 3.93 for the control group, which significantly differed (P=0.035). Changes in mean depression scores for both the intervention (-4.73 ± 3.83, P≤0.001) and control (-2.5 ± 3.51, P=0.008) groups significantly differed. A comparison of decrease in depression between the intervention (4.7 scores) and control (2.5 scores) groups showed significantly more decrease in the intervention group than the control group (P=0.045).

Table 3: Comparison of mean depression scores and changes in intervention and control groups before and after intervention

	Before (Mean ± SD)	After (Mean ± SD)	Changes (Mean ± SD)	P value
Intervention group	12.68 ± 1.35	7.95 ± 3.45	-4.73 ± 3.83	<0.001
Control group	12.83 ± 1.12	10.33 ± 3.93	-2.5 ± 3.51	0.008
P value	0.682	0.035	0.045	

P<0.05 was considered significant.

Discussion

The incidence of PPD in this study was 36.9% whereas in other Iranian studies this incidence was reported as 23.7 (12) and 32% (13). It should be mentioned that the timing of screening differed among these studies therefore these differences could be time dependent. The PPD incidence in developed countries has been reported as 13% (27). Thus the PPD incidence in Iran appeared to be more than developed countries. Further researches should be done for understanding the influencing factors.

Mean depression scores before intervention in both groups were the same, however after intervention the mean depression scores in the intervention group who received telephone support was significantly lower than the control group. It could be concluded that the decrease of EPDS scores was due to intervention. However, we could not find any study about health volunteer telephone-based support. A similar result was reported by Dennis et al. (16) after peer support. They found that PPD could be decreased after peer support (mother to mother support). Meanwhile, Hassan Zahraee et al. (21) found that midwives' emotional and informational support given on days 2 and 10 after childbirth prevented PPD after 5 weeks. Hantsoo et al. (28) studied the efficacy of treatment with computer-assisted cognitive-behavioral therapy for antepartum depression and suggested this therapy to be used for antepartum depression.

In both groups the depression scores decreased after 6 weeks, but the intervention group showed more decrease than the control group. Mean depression scores of the intervention group decreased in the non-depressed area (decreased under <10 scores), but the control group mean scores remained at the >10 scores (depressed area). In other words, although depression scores in the control group decreased, this was not adequate. A similar result was reported by Dennis et al. (16), they found that in the peer supported group, there were a significantly less proportion of depressed mothers compared to the control group. In contrast, Mohammad-Alizadeh-Charandabi et al. (29) conducted a randomized controlled trial that studied the effect of telephone support on PPD. They found no significant difference regarding frequency of depression as well as mean depression scores between the intervention group that received tele-

phone support by a midwife and the control group. This contradiction might be due to the selection of a different cut-off point for depression. In their trial, the cut-off score was ≥13, whereas in the current study, it was >10, according to findings by Edmondson et al. (25).

The advantage of the findings of the present study when compared with those of other studies and strategies suggested for handling PPD was the decrease in PPD scores which was achieved by engaging inexpert health volunteers and via telephone calls. This was a cost benefit method available in every house. In other words, there was no high-skilled person required, nor the need for any advanced technology or complex tools. In the study by Mohammad-Alizadeh-Charandabi et al. (29), the intervention was performed by trained midwives with no evidence of decreasing PPD reported. In our study, intervention was performed by health volunteers (grassroots community members with a high potential for communication) who worked free of charge and communicated directly with families. This could be used as a model to engage accessible human resources for improving mothers' health in every society. This model, as a simple method, could reduce the burden of disease and prevent the establishment of depression and its severity. Therefore the use of expensive therapies and medications alongside with complications for families and society would be decreased and the national health system could additionally benefit from this approach. Some studies have suggested computer-assisted cognitive-behavioral therapy for antepartum depression (28). Since the computer is not widely used by Iranian women, this cannot be implemented in Iran at the present time.

The present study limitation included support from the family and the husband as an important factor in PPD, which we have not considered in this study. Thus, additional studies with more cases and factorial designs will be necessary to achieve a precise conclusion.

Conclusion

In this study, health volunteer telephone-based support was effective in decreasing PPD and may be beneficial to women with mild and moderate levels of PPD symptoms.

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Ethics, Legal, Social, Counseling Article

An Evaluation of Iranian Judges' Decisions about The Act of Embryo Donation

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Abstract

Embryo donation was one of the infertility treatment methods introduced to the Iranian legal system in 2003 (Act of Embryo Donation) and its by-law passed in 2005 after numerous discussions.

Embryo donation is a new legal issue in Iran. No similar act has been previously legislated in the legal system; however, on the other hand, the importance of the judicial procedure in its execution cannot be ignored since during this treatment process the infertile couples must refer to the court.

In this paper, we analyzed 80 court decisions that concerned permission for embryo donation during 2006-2011. The decisions were made for couples who requested this treatment and referred to Avicenna Fertility Center (Tehran, Iran). In this study, we analyzed the decisions and regulations for the demands, in addition to the medical and legal viewpoints of the judges. The differences among the judges' decisions and in the ways of investigating were discussed.

Keywords: Embryo Donation, Law, Iranian, Assisted Reproductive Technology

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Introduction

Embryo donation is a method of treatment for couples who lack hope in having a child. In this method, the embryo that arises from other couples' gametes is transferred to the woman's uterus for pregnancy and child bearing. The method was introduced to the Iranian legal system in 2003 (Act of Embryo Donation) (1) and its by-law was passed in 2004 following numerous discussions (2).

Sometimes we can find some patients who have come to a clinic 13 times or even more for demanding the treatment (3). In many countries such as Iran, women who cannot have children undergo numerous pressures and threats, along with rejection, family disintegration, cease of financial support and the husband's remarriage (4). Therefore, although the drugs used in assisted reproductive technology (ART) often have side effects, the patient insists on continuing treatment because of pressures from her husband or relatives.

Gamete and embryo donation, as infertility treatments, bring some moral and legal challenges. Therefore, it is necessary to find moral and legal strategies to inform the society. This is particularly true for families of infertile persons because infertility is a benign, curable disease and not a stigma (3).

Some countries have enacted both similar and dissimilar regulations and rules for embryo donation. Most importantly, embryo or gamete donors and recipients should undergo efficient consultation and be aware of the physical, psychological and legal aspects of embryo donation before making any decision (5). Opposition against organ commercialization and inevitable outcomes of ART such as filiations, rights, and interests of children born from this method has been the impetus for codifying a legal regulation in infertility treatment. Therefore, embryo donation in which a third party is involved can be a suitable alter-

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native for patients. Some countries have special legal provisions for ART, particularly those which involve third parties (6-9).

In Iran, family courts apply different methods for ensuring the competence and qualifications of infertile couples. A lack of unity in the judicial procedure may threaten patients' best interests. Moreover, inequality before the law is in opposition with article 20 of the Iranian Constitution.

The present study was not conducted about the Act of Embryo Donation or its scientific, moral and legal effects, and results. On the contrary, the significant focus of the study was the demand for embryo donation and the approach adopted by the courts and Iranian legal system in their decision-making process. In the context of existing literature, no similar studies have been performed.

Materials and Methods

The study was conducted in 80 family courts, which have the authority to permit embryo donation. Their decisions regarding permission for embryo donation during 2006 until the end of 2011 were presented to Avicenna Fertility Center.

Article 8 of the executive by-law regarding embryo donation to infertile couples states that information and documents related to the embryo donation issue are confidential. Moreover, article 648 of the Penal Code of Iran considers the disclosure of medical information and secrets of the patients as a crime. Therefore, for the present study, these decisions have been reviewed by the Clinic Director and all names and numbers were deleted. No data such as claimant's name, judge's name and petition number or court branch were mentioned.

The above-mentioned decisions were discussed with regard to claimant, defendant, court location, and male or female infertility factor. The reasons for these decisions were analyzed by taking into consideration the context of the law and its approach towards embryo donation which was legislated in 2003 and the executive by-law (passed in 2005), which have been called "the Embryo Donation Act" and "the regulation" in this paper.

Results

Judicial procedure for embryo donation

Petition (demand presentation)

According to article 2 of the Act, receiving an embryo should be requested from the court. In the juridical literature, a request or demand is not the same as a petition. In the present study, all 80 mentioned requests were presented to the court in a petition format. Of these, 77 were sent to courts in the centers of provinces and 3 to the courts of small cities.

However, despite the inexplicable nature of this issue, the submission of a petition provides uniform standards and guidelines for the staff.

The participation of both wife and husband in writing and submitting the request

Article 2 prohibits the wife or husband from separately writing and submitting the request. Infertility treatment by embryo donation needs consent from both the wife and husband. A request from one does not assume any commitment or obligation to the other person according to one of the rules of the Civil Code of Iran (10).

Right to have a lawyer

According to the Act, both the claimant and defendant have the right to freely hire a lawyer (11). The only condition in embryo donation that could justify the necessity of submitting the request by the couple to the court is the possibility of the court unawareness of the couple's divorce during the consideration process. However, according to the general context of the law and the term "both wife and husband" where the term "individually" is not mentioned the possibility of submitting the request by a lawyer is undeniable. Different results have been observed in this study. None of the 80 requests presented to the court were submitted by a lawyer. In all requests the plaintiffs undertook this duty personally.

Claimants

According to the courts' decisions considered for the claimant it was determined that different people presented as claimants (Table 1).

The 77 claimants in the provincial courts comprised 9 wives, 29 husbands, 38 couples and 1 infertility clinic. Among 3 claimants in the courts of cities, there was 1 wife and 2 couples. Obviously, judges were not unanimous in determining the claimants.

Table 1: The parties in the Courts

Location	Lawyer		Claimant				Defendant				
	-	+	Wife	Husband	Both	None	Wife	Husband	Both	None	Court
77	77	0	9	29	38	1	30	8	6	6	27
3	3	0	1	0	2	0	0	0	3	0	0

Defendants

Based on courts' decisions, different people were presented as defendants. Among 77 decisions in provincial courts, there were 30 wives, 8 husbands, 6 couples, and 27 Presidents of Family Divisions who were regarded as defendants. In 6 cases, no one was considered to be the defendant. In all 3 cases in the courts of cities, the judge was considered as the defendant. A great disparity existed in determining the defendant.

When the decision was about non-litigious matters, the only requirement was to submit the petition to President of the Family Division or to the prosecuting attorney (12) (Table 1).

Requirements (conditions)

Official ensuring

As previously mentioned, the court is responsible for gathering different types of data about the couples. The adverb "in case of …" insists on the court's duty because the opposite meaning of the above sentence is that "in the case of not obtaining", the court will not issue the permission to receive an embryo.

The most important, ambiguous issues in the judicial process of embryo donation are the conditions mentioned above and the way of their assurance.

In order to determine to what extend the court has authority in evaluating the requirements and identifying alternative methods, the conditions and requirements should be differentiated and separately analyzed.

Infertility and the ability for receiving an embryo

The legal criterion for infertility is an accredited medical certificate. However, there is no accurate definition for the word "accredited". Some issues regarding the impossibility of having a child or the ability of wife to receive an embryo are ambiguous. In this part, we elaborate the issues in detail.

The accredited medical certificate

In the phrase "accredited medical certificate", the word "accredited" is associated with "certificate" rather than the word "medical". As it is perceived by the phrase, classifying certificates into accredited and non-accredited is not the matter and the actual validity of the certificate is not indicated in its name. A medical certificate issued by an authorized person such as a physician seems to be an accredited certificate.

Therefore, we can conclude that a qualified infertility clinic is the same as an accredited infertility clinic. According to article 1 of the Act concerning medical issues legislated in 1954 and its revisions and amendments that every medical institute shall receive a special license from the Ministry of Health, it can be argued that qualified centers are the same as those holding valid licenses. However, by considering the contexts of the article, the certificates issued by other, unmentioned centers or issued by a physician who is not a member of these centers, are not necessarily invalid. Therefore, the conditions for invalidation of these certificates must be clarified.

From 80 studied decisions, 6 had documented medical certificates from infertility clinics and 42 presented forensic medicine certificates issued by the Legal Medicine Organization of Iran (LMO). A total of 31 decisions documented both certificates and 1 decision documented no certificates (Table 2).

Thus, evaluating the requirements by the court should be based on medical certificates. Referring

couples to LMO for confirmation of their condition is not mentioned in the law, even indirectly. This is not a sound decision, which leads to confusion among couples and wastes resources.

Table 2: Accreditation authorities

Accreditation	LMO	Infertility clinic	Both	None
Total=80	42	6	31	1

LMO; Legal medicine organization of Iran.

Of note, the release of information such as a medical certificate number or the agent that has issued the permission is against treatment confidentiality.

The ability of wife to receive an embryo and pregnancy

The ability should be considered from two aspects: the physical ability for embryo transfer and the ability for pregnancy and child birth. The first issue mostly depends on the condition of the wife's uterus whereas the second aspect mostly relates to her body's general health. The Act used the word "ability" in an absolute sense and does not apply to any adverbs.

Therefore, the important question is whether the woman is capable of receiving an embryo based on the above-mentioned criteria or not. The answer depends on our viewpoint about life principles and its philosophy, a child's benefits and expediency, and couple's rights.

Although the conditions mentioned and their associated regulations have been included in the by-law, a tremendous gap exists in the definitions and the requirements of embryo reception.

There is a defensible argument that the wife is able to receive the embryo regardless of medical criteria. Basically, a wife must have the capability to become pregnant. Thus an older woman is excluded from this law even if she can meet the necessary medical requirements. This is closer to the expediency of the child (13).

Proven infertility

As considered in article 1 of the Act, infertility of one or both members of the couple should be clearly proven; though determining incurable infertility is not an easy task.

Infertility is clearly defined in medicine. Infertility refers to the fact that a couple with the aim of having a child is not able to conceive after one year of unprotected sexual intercourse (14). An important point according to the definition of infertility is whether the couple is capable of receiving an embryo or not. In other words, whether the infertility of the couple is sufficient documentation or whether it is necessary for the couple to prove their attempts for treatment and ultimate failure.

As understood from the last part of Article 1, infertile couples should receive medical treatments and according to the article, their infertility must be proven. However it is not mentioned how many times the couples must attempt and whether there is any benefit in obliging them to treat their infertility.

Certainly, in some cases medical treatment can be continued for a long time but it seems that the couple cannot be forced to accept treatment when there is no chance for success with treatment. The side effects of these treatments are indisputable. Therefore, we can claim that the context of the law in terms of this matter is not sufficiently clear.

Of note, the use of ART by postmenopausal women is a matter of controversy. Opponents, by citing the principle of non-malfeasance, believe in high risk of pregnancy of older women. By referring to the principle of beneficence, they believe in the best interests of the child with regards to the high possibility of losing his/her mother at a younger age. However, proponents resort to the principle of justice and strongly advocate the continuation of this right. Younger women are prioritized when an appropriate budget is available (15, 16). There is no explicit article about these types of women in the Act; only article 7 of the executive by-law about embryo donation asks for a certificate that pertains to the ability of the wife for pregnancy and receiving the embryo.

Among 80 reviewed decisions, 70 were related to male infertility and the remaining 10 were related to female infertility. There was no significant difference between provinces and cities. The large difference between the two above-mentioned numbers showed that most cases of infertility which re-

sulted in embryo donation were due to male factor, an important finding which should be investigated thoroughly. Perhaps fertile men could provide an alternative for their infertile wives (by remarriage or polygamy), an opportunity which was unachievable for women. Based on these arguments, further research is needed in this area.

Moral competence

Moral competence shall be determined by the court. In Iranian legal literature, there is no definition of moral competence. In certain cases, instances of incapacity have been noted, such as corruption, drug addiction or previous criminal convictions (article 14 of the Act of Governmental Employment and article 1173 of Civil Code of Iran).

There are two factors to be considered with respect to moral competence. First, the instances of capacity and incapacity and secondly, the way of determining them should be identified. However, these issues have not been included in the Act.

The 80 decisions studied showed that the courts did not mention what should be determined. Mostly they considered the method (how to) of determination.

Among the 80 studied decisions, 14 were cited by local researches, 1 was cited by a police report, 13 were cited by local affidavits and 26 were related to the principle of validity (omnia praesumuntur rite esse acta).

In 24 cases, it was only mentioned that the court determined the general competence of the couple. In 2 cases, there was no mention of the couple's competence (Table 3).

A few judges made use of external assistance for determining competence. However, others believed that they did not need to resort to external help.

Among 77 cases referred to regional courts, in 13 the judges pursued local investigation and in 64, they did not.

Among 3 cases referred to city courts, in 1 local investigation was used as the main method for determining moral competence and in 2, this approach had no place in the judges' decisions.

Some judges have considered accuracy as the main principle for moral competence determination. However, this principle is more applicable in a juridical act and contract, yet not in determining an existing issue. Among 77 cases which had been referred to the provincial courts, in 26 the principle was included in the process of decision making.

None of the 3 cases referred to the city courts had the accuracy principle included.

Among 77 cases referred to the provincial courts, in only 1 case an inquiry by the police helped the judges to determine the couple's moral competence. In 76, the judges did not rely on this method. None of the judges inquired from the police in 3 cases which had been referred to the city courts. In all cases, none of the judges accepted the principle of innocence.

Most judges used certain methods which were against the principles of confidentiality. For example, among 77 cases which had been referred to the provincial courts, in 14 a local affidavit was used. In 63, this method was not used.

Among 3 cases which had been referred to the city courts, in 1 the judge relied on local investigation whereas in the other 2 cases the judges did not adopt this approach.

Among 15 cases in which local research was used, the infertility reason for all cases was male factor.

Table 3: Instances of capacity

Instances of capacity	Local investigation	Police	Principle of validity	Testimony	Mentioned	Not mentioned
80	14	1	26	13	24	2

Among 65 cases in which a local research method was not used, the infertility reason in 55 cases was male factor and for 10 cases, it was female factor.

Iranian nationality of the couple

According to article 976 of the Civil Code of Iran, a native Iranian is someone whose father is Iranian or who holds an Iranian citizenship by one of the mentioned ways in the law. The wife of an Iranian man is considered a native Iranian as long as she is not separated or divorced. Birth and marriage certificates can be considered documents that prove Iranian nationality.

Conclusion

According to the aforementioned points, the enactment and legal issues in embryo donation remain unknown among lawyers and judges. This can be attributed to a lack of an identical judicial process for legal procedures and non-interference by lawyers in judicial dossiers.

The legal issues in embryo donation are mostly studied at the law school level which may be a cause for various judicial processes. For this, it is necessary to consider the following points:

Embryo donation as a treatment method for infertile couples must be carried out exclusively by infertility clinics that have sufficient capabilities. The possibility of carrying out this treatment must be studied separately for each couple. Issuing a certificate based on confirming general physical and mental health of the couple is one of the tasks of treatment centers. This certificate shall be offered to the legal authority (the president of the court) as an attachment with the couple's personal request or by a lawyer.

Obtaining good standing certificate records can result in unity in the judicial procedure.

The court's avoidance from performing local investigations-developing a local affidavit or demanding testimony, researches by local police or publishing the court's decision in more than 1 copy all of which oppose the confidentiality of treatment- are important points that should be taken into consideration. Familial relationships in small cities and towns have conflicts with medical confidentiality. Due to couples' tendency keep silent about the treatment; it is appropriate to allocate special branches of courts in large cities to prevent such conflicts. Educating the staff and judges about the bases of the treatment and its medical, legal, ethical and social aspects would be beneficial.

The court's decision for permitting embryo donation (transfer of donated embryos) should be limited to a definite period of time (e.g., 1 year).

Of note, permission given by the court does not obligate the infertility clinic to perform an embryo donation. Although the clinic should determine the basic capacities and facilities necessary for issuing the certificate to the court, this cannot inhibit these centers from using new methods or additional studies. A physician considers his/her colleagues' opinions about a patient as useful advice and not mandatory orders. Therefore, it is not surprising that the opinion of a physician or a medical team for determining the couple's competence may be considered wrong and unsound by another medical team (even if it leads to the court's permission for receiving a donated embryo).

Couples are recommended to follow their request process from the first step in the infertility clinic to which they will refer after the court's permission, in order to respect their physician's diagnosis and treatment, irrespective of the practices and opinions of other physicians.

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Case Report

Endometrial Adenocarcinoma in A 31-Year Old Woman: A Case Report

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Abstract

Endometrial adenocarcinoma (EC) usually occurs after menopause, whereas in 2-14% of cases, it occurs in young patients (less than 40 years old) who may desire to keep their fertility. It is of importance to evaluate women for EC when they develop polycystic ovarian syndrome and abnormal uterine bleeding. Its treatment includes hysterectomy, bilateral salpingo-oophorectomy and pelvic lymphadenectomy and in some cases, radiation therapy. We report a case of EC in a 31-year-old woman who presented to Royan Institute. She complained about oligomenorrhea with a 10-year history of primary infertility.

Keywords: Endometrial Adenocarcinoma, Polycystic Ovarian Syndrome, Abnormal Uterine Bleeding, Infertility

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Introduction

Endometrial adenocarcinoma (EC) is the most common gynecologic malignancy in the United States, predominantly among postmenopausal women, at the average age of 59 years. The majority of cases are diagnosed when the carcinoma is confined to the uterus, leading to less than 1.5% of cancer deaths (1, 2).

Although 20-25% of EC are diagnosed before the menopause, 2-14% occur among younger women (less than 40), most of whom wish to preserve their fertility (3-6). This complication is more common in developed countries than the developing countries (7).

We report a case of a 31-year-old patient with an endometrial cancer diagnosed at stage II according to the International Federation of Gynecology and Obstetrics (FIGO), 2000 classification of endometrial cancer (8). This case study will provide a useful guide in diagnosis of EC for the sonographer.

The aim of study is to provide opportunity for sonographists to learn the broad spectrum of findings that may be seen at sonohysterography (SHG) in both benign and malignant processes to raise clinician's awareness toward the appropriate diagnosis and treatment.

Case Report

A 31-year-old woman who was nulliparous and overweight [body mass index (BMI)=35.5] with a 10-year history of primary infertility presented to the Imaging Department of Royan Institute in 2012. She had a history of laparoscopy with ovarian cotter, septum and polyp resection by hysteroscopy (HSC) in 2010 at different infertility center. In addition, due to male factor infertility, she underwent ovarian stimulation in IVF cycle and ten embryos were obtained and frozen in 2010. Her chief complaint was oligomenorrhea. Since she was overweight and had abnormal uterine bleeding (AUB), transvaginal sonography (TVS) and SHG were done for patient. TVS showed thickened endometrium with smooth counter (Fig.1), while the result of three dimensional sonohysterography (3DSHG) revealed irregular endometrium and fibrotic bands which involved ½ of uterine cavity in

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fundus and body of the uterus, therefore, this finding proposed intrauterine adhesions (Fig.2A, B). Based on findings at TVS and SHG and consideration of irregular endometrium, she was then considered as a candidate for hysteroscopy operation and dilation and curettage (D&C). The hysteroscopic appearance of the endometrium consisting of multiple polypoid areas, indicated that patient was suspected of having hyperplasia or endometrial cancer, so direct biopsy was done and the specimen was then sent to pathological exam for further examination. The pathology report revealed endometrial adenocarcinoma (stage II). In order to determine the staging of disease, pelvic MRI was done. The result of pelvic MRI just showed endometrial involvement and other pelvic organs were normal, finally after completing oncologic evaluation, conservative management was not considered because she was patient decided to have the surgery to obtain her health promotion, so she underwent hysterectomy with bilateral salpingo-oophorectomy and pelvic lymphadenectomy. Regarding the fact that she had 10 embryos frozen two years ago, she could have a chance of surrogate pregnancy.

This is an interesting case because she was at reproductive age and adenocarcinoma is uncommon in this age group. Also obtained findings from transvaginal ultra sonography (TVUS) and SHG are not specific for adenocarcinoma.

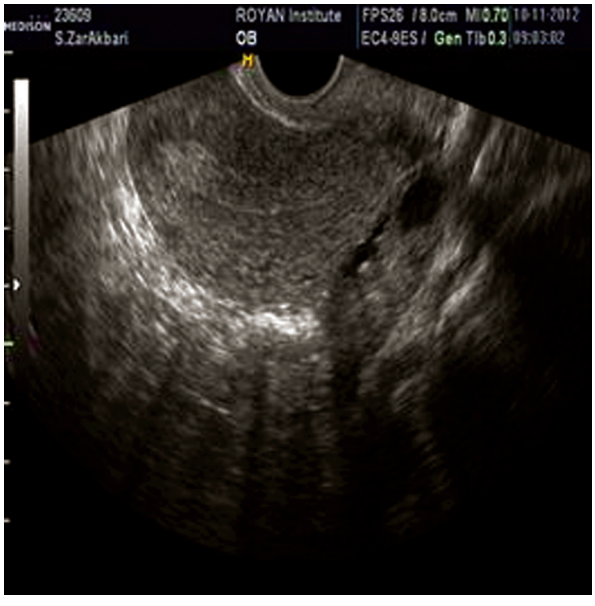
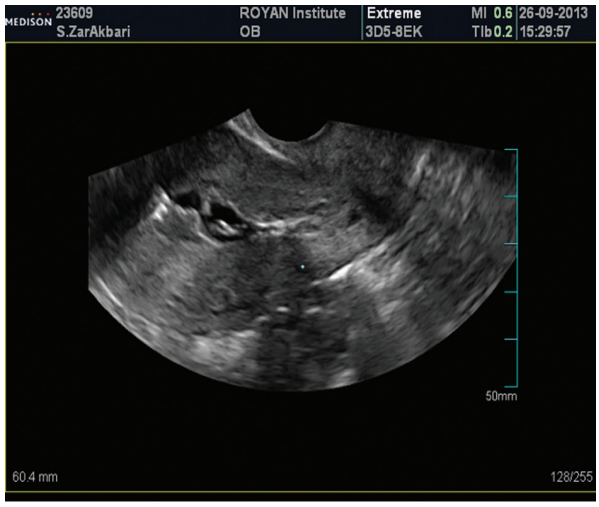


Fig.1: Sagital transvaginal sonogram showing anteverted uterus and endometrial layer of 12 mm.

A



B

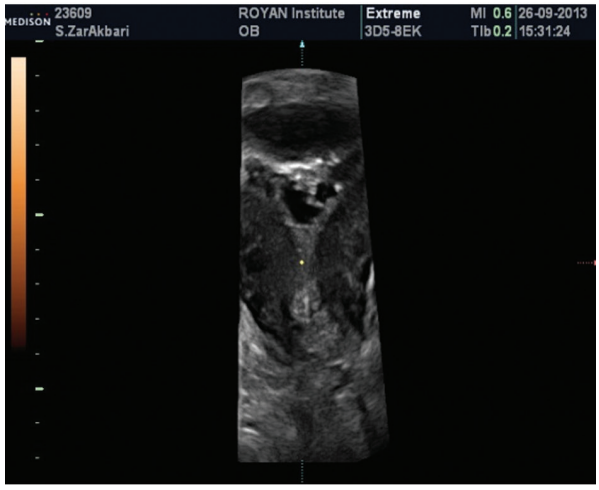


Fig.2: Sagital and coronal saline hysterosonogram showing several echogenic bands with irregular internal border.

Discussion

The sonographic appearance of the normal endometrium highly depends on the age of the patient and the stage of menstrual cycle for woman of reproductive age.

A carcinoma generally originates from the epithelial cells. EC is a type of uterine cancer that involves the endometrium. Adenocarcinoma (adeno=gland) refers to a carcinoma featuring microscopic glandular-related tissue. Prior to the 1980s, EC was broadly characterized as a single disease. However, observations by Lauchlan (9), Hendrickson et al. (10) have led to the descrip-

tion of two distinct types based on histologic and molecular characteristics. Type I EC is commonly referred to endometrioid adenocarcinoma which includes 80-90% of all ECs. Type II EC, nonendometrioid tumors, encompasses the remaining 10-20% of endometrial tumors (11). The etiology and survival of these two subtypes are vastly different.

The risk factors for EC which are related directly or indirectly to estrogen exposure including early menarche, late menopause, nullparity, poly cyctic ovarian syndrome (PCO), diabetes and obesity. Besides, adenomatous polyps, breast cancer and the use of estrogen therapy, are associated with higher incidence of endometrial cancer (6, 12-17). In our case, she had obesity, PCO, and adenomatous polyp, while she was considered nulliparous. History of estrogen use is more frequently seen in young patients. Also hormone-related disorders such as ovarian dysfunction, chronic anovulation, infertility, obesity and PCO detected in these patients (18). In this case, it should be mentioned that she had one stimulation cycle of assisted reproduction treatment (ART) in 2010, and subsequently, had 10 frozen embryos.

Physicians mostly prefer to perform endometrial biopsy if there is abnormal uterine bleeding which is considered as indication of early symptom of EC (18, 19). Although postmenopausal bleeding is a common sign of EC, recent studies have shown that only 4-5% of women with postmenopausal bleeding have endometrial cancer (1, 2).

SHG and TVUS provide a good predictive value for endometrial disease in patients with AUB (20). SHG which is instillation of sterile saline solution by means of a catheter was initially described by Nannini et al. (21). SHG produces better images, whereas TVUS provides more accurate measurement of the endometrial thickness allowing more clear evaluation of the heterogeneity. SHG has greater accuracy in the identification of focal lesions than in the diagnosis of diffuse lesions particularly more associated to malignant lesions. Adding 3D imaging to SHG can help getting optimal results because of allowing real time visualization of the cavity and more accurate assessing than conventional 2D. Other advantages are the ability to observe the coronal plane of the uterus and saving 3D volumes for later study, which leads to reduce the duration of examination and to cause less discomfort for the patients. In premenopausal patients, SHG is preferably performed during the early proliferative phase of the patient's menstrual cycle, when the endometrium is a very thin tissue. Although there is no limitation for normal premenopausal endometrial thickness, the endometrium should be uniform in thickness, homogeneous in echotexture, and not to be displaced by any submucosal, myometrial abnormality (22).

In postmenopausal patients, the normal atrophic endometrium should measure be less than 4 mm in double-layer thickness as seen at TVUS and less than 2.5 mm in single-layer thickness as seen at SHG. In addition, the atrophic endometrium should be smooth and uniform in echotexture and not to be displaced by any submucosal, myometrial abnormalities (19, 23).

The most common appearance of EC at TVUS is nonspecific thickening of the endometrium. Even at SHG, endometrial cancer can be difficult to distinguish from endometrial hyperplasia and polyps. This diagnosis should be suspected when the single layer of the endometrium is thicker than 8 mm, irregular, broad based, or poorly marginated or when the endometrial-myometrial interface is disrupted. One of interest finding in our case is that she had smooth endometrial-myometrial interface. Endometrial thickness measurements often overlap in benign and malignant conditions (24).

At SHG, EC is typically a more diffuse process, while early cases can appear as a polypoid mass (19). An intact subendometrium shows localized disease, whereas extension of heterogeneity and increased echogenicity in the myometrium propose advanced invasive endometrial carcinoma (25). Sonographic findings related to EC in our patient included a thickened, heterogeneous endometrium.

Final diagnosis achieved through HSC finding and pathology. Advantages of HSC in the evaluation of abnormal bleeding or abnormal lesion are notable and the ability to see lesions and to evaluate endometrial cavity is precious. The panoramic HSC, especially with directed biopsy is superior to D&C in patient with abnormal findings (22).

Conclusion

SHG make obvious differentiation between focal and diffuse endometrial lesions, so it becomes reliable test in the imaging evaluation of dysfunc-

tional uterine bleeding and postmenopausal bleeding. It is essential for the radiologist to be familiar with the broad spectrum of findings that may be seen at SHG in both benign and malignant processes in order to direct the clinician toward the appropriate means of diagnostic biopsy or surgery.

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Case Report

Laparoscopic Management of Heterotopic Interstitial Pregnancy with Subsequent Term Delivery

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Abstract

A 35 year-old woman at 7-week gestational age was referred to our hospital. The patient was diagnosed with the heterotopic interstitial pregnancy by transvaginal ultrasonography after receiving *in vitro* fertilization (IVF) and embryo transfer.

Laparoscopic excision and curettage was successfully performed at 8.4-gestational age under general anesthesia and the patient was discharged 2 days after operation without any post-operative complications. The woman had normal antenatal follow-up and delivered a healthy baby at term by cesarean section.

Keywords: Cesarean Delivery, Heterotopic Interstitial Pregnancy, Laparoscopic Surgery

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Introduction

Heterotopic pregnancies, where intrauterine and ectopic gestations co-exist, are very rare with an estimated incidence of 1 in 30,000 pregnancies (1). However, this may be as high as 1% in the setting of *in vitro* fertilization (IVF) where multiple embryos are transferred (2). Recently, there is an increased incidence of abnormal pregnancies such as heterotopic pregnancy because the number of women exposed to risks, such as pelvic inflammatory disease, previous pelvic surgery, tumors, uterine anomalies, and the use of assisted reproductive technologies (ART) increases.

There are limited options for the treatment of heterotopic pregnancy, particularly if the woman desires to continue with her intrauterine pregnancy.

We reported a case of successful laparoscopic surgery for heterotopic interstitial pregnancy and subsequent successful delivery at term.

Case Report

A 35-year-old woman was referred to our department for the further management of a heterotopic interstitial pregnancy. This heterotopic pregnancy was the first episode of pregnancy after the patient received an IVF for primary infertility due to previous surgeries which included right salpingectomy and left tubal pregnancy, respectively. The patient presented with acute abdominal pain, localized to the left lower quadrant area with local tenderness on physical examination. Transvaginal ultrasonography showed an intrauterine pregnancy of about 7 weeks gestation and another gestation of about 4.8×4.5 cm at the left interstitial of the fallopian tube (Fig.1).

Laparoscopic surgery was performed at 8 weeks gestational age. The intra-abdominal pressure was maintained at 13 mmHg with carbon dioxide gas. Once the pneumoperitoneum was achieved, video-laparoscopy (laparoscopic camera provided by Stortz, Germany) was performed using a 10-mm trocar that had been introduced through the umbilicus. Further, three trocars were needed for the operation. A 10-mm trocar for placement of the endoscopic suturing was placed on the left side, a 5-mm trocar on the right side of the lower abdomen, and another 5-mm trocar on the median line

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just above the pubic hairline. The left cornus of the uterus was distended with increased vascularity (Fig.2). The ipsilateral ovary and fallopian tube were grossly normal in appearance.

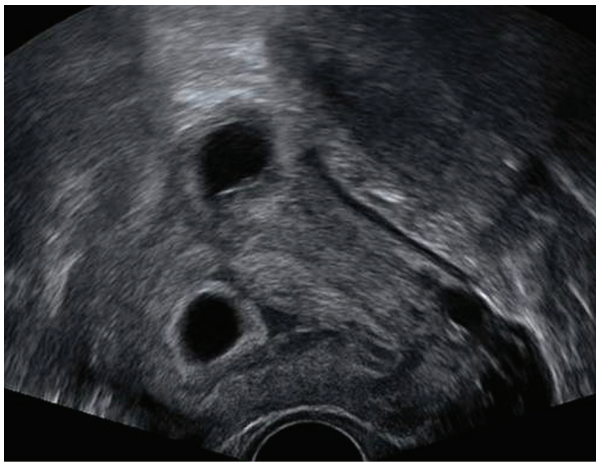

Fig.1: Transvaginal ultrasonography showed the gestational sacs in the two different sites, longitudinal and sagittal views.

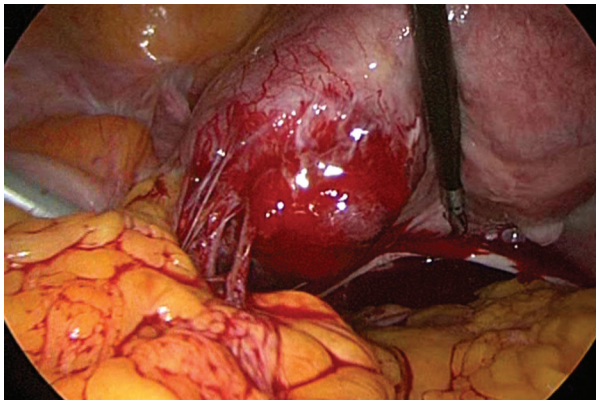

Fig.2: A laparoscopic view showed the mass located in the left cornus of the uterus. The cornus was expanded and had a bulging appearance.

The bulging cornus was transversely incised using a monopolar cutting electrode (The ENDOPATH® Electrosurgery, EPH02, Ethicon inc.) 40 W to expose the ectopic gestation. The mass was removed followed by curettage of the area with a spoon forceps to completely evacuate the ectopic gestation. Laparoscopic suturing was done using 1-0 POLYSORB™ sutures polysorb (Covidien inc.) after controlling the bleeding on the surgical bed of the left cornus. The technique of laparoscopic suturing was a simple interrupted suturing with caution taken to avoid damage to the intrauterine pregnancy (Fig.3).

The time of operation was 40 minutes and the time of anesthesia was 55 minutes. There was no intraoperative complication. A closed drain bag was inserted during the operation. The patient was discharged 2 days after operation without any postoperative complications. During the time from initiation of antenatal care to delivery, the patient stayed healthy and showed no clinical problems. The placenta was located centrally in anterior body of the uterus. Finally, an elective cesarean delivery was performed at 38 weeks gestation and a 3.4 kg healthy female baby was born. Minimal old scars without major deformity or adhesion were found on the cornual operation site.

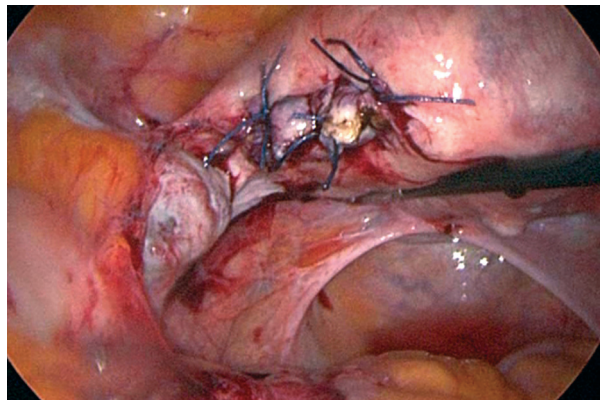

Fig.3: The technique of laparoscopic suturing is a simple interrupted suturing. The ipsilateral ovary was grossly normal in appearance.

Discussion

Heterotopic pregnancy is believed to occur in 1% of all conceptions achieved with IVF, but implantations, specifically in the interstitial, account for only 1% of all ectopic pregnancies (3). There is little agreement regarding the optimal surgical management of heterotopic pregnancy because of the rarity of this type of pregnancy.

To our knowledge, there have been six reports of laparoscopic management of interstitial heterotop-

ic pregnancies (2, 4-8). Two of the six cases with laparoscopic management of intrauterine pregnancy were reported without a good outcome.

In the current case, there were no complications during the laparoscopic operation and no late miscarriage. An elective cesarean delivery was done and a healthy 3.4 kg female baby was born at 38-weeks gestational age.

A selective embryo reduction by direct local injection of potassium chloride or hyperosmolar glucose solution could be considered in women with clinically stable situation. However, the patient in the current case presented with severe abdominal pain and hemoperitoneum with a risk of catastrophic rupture of the interstitial pregnancy; therefore, surgical management was recommended.

The minimal invasive laparoscopic surgery for the interstitial pregnancy was focused on the prevention of intraoperative complications: i. operation time and anesthetic time had to be shortened, ii. the amount of blood loss had to be reduced and iii. during suturing, the tip of the needle must have some distance from the normal gestational sac in the intrauterine cavity.

We did not use pharmacological methods such as vasopressin to control bleeding in order to avoid any potential effect on the circulation to the normal intrauterine. Instead, we tried to shorten the time between the staring time of incision and finishing of suturing to reduce the amount of intraoperative bleeding loss.

Laparoscopic suturing technique is a key factor to reduce the operation time and bleeding. With regard to these points, an expert laparoscopic surgeon can help to carry out a laparoscopic surgical management for a heterotopic pregnancy with less intraoperative bleeding.

Although the increased rates in pregnancy after IVF represent a welcomed trend in the advanced reproductive technologies, the gains have not eliminated the risk of an ectopic pregnancy (9). In fact an iatrogenic transfer of multiple embryos to the uterus after an IVF represents a major risk factor for a heterotopic pregnancy (10).

We suggest that the aim of treatment for heterotopic pregnancy should be the continuation of the normal pregnancy and the careful use of minimal invasive surgical techniques for the ectopic pregnancy. Expert laparoscopic management for a heterotopic pregnancy might be the appropriate treatment modality for fetal and maternal safety. A collaborative approach for larger collection of data on these surgical techniques could help women with heterotopic pregnancy continue their normal intrauterine pregnancy safely.

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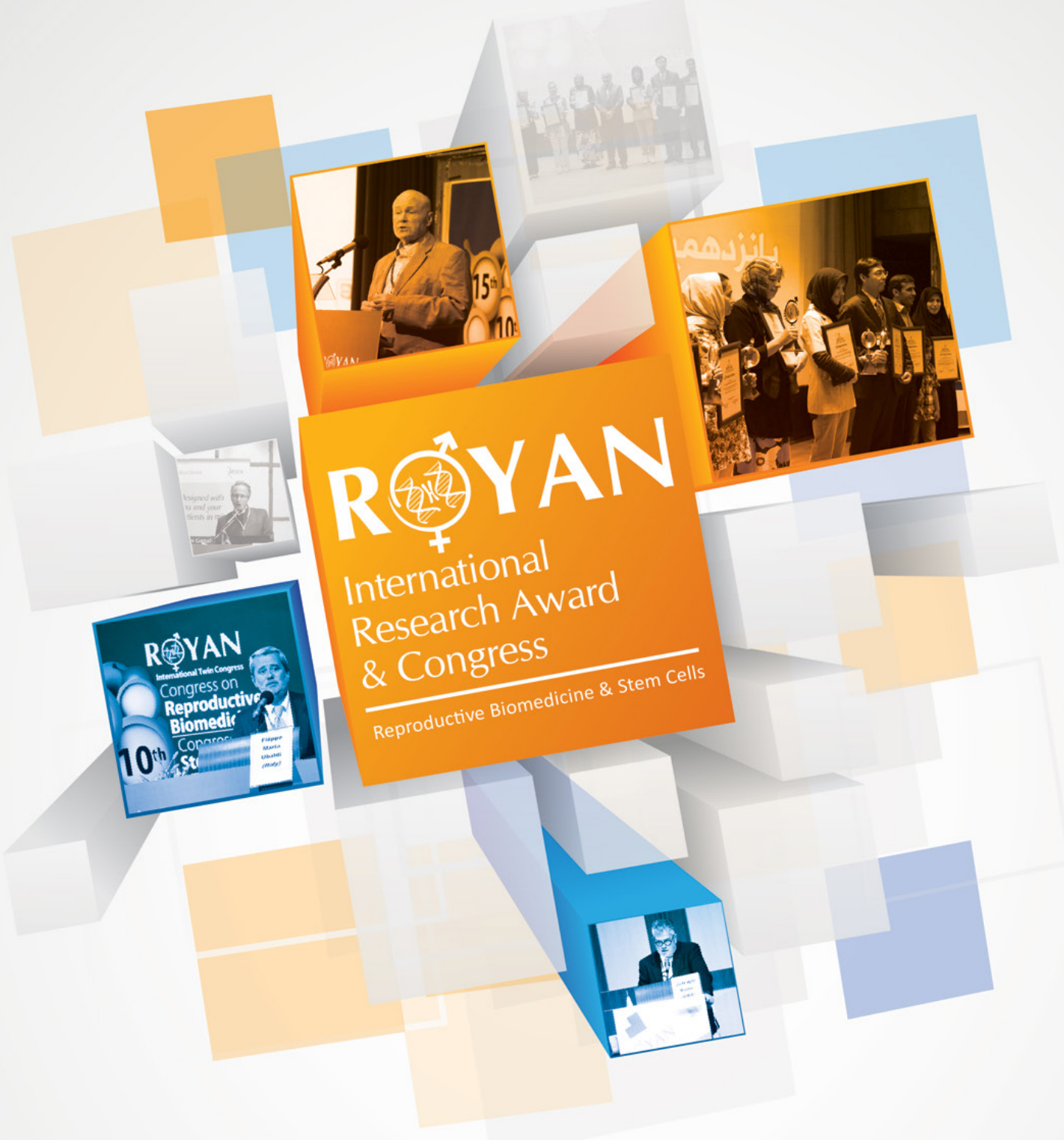

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