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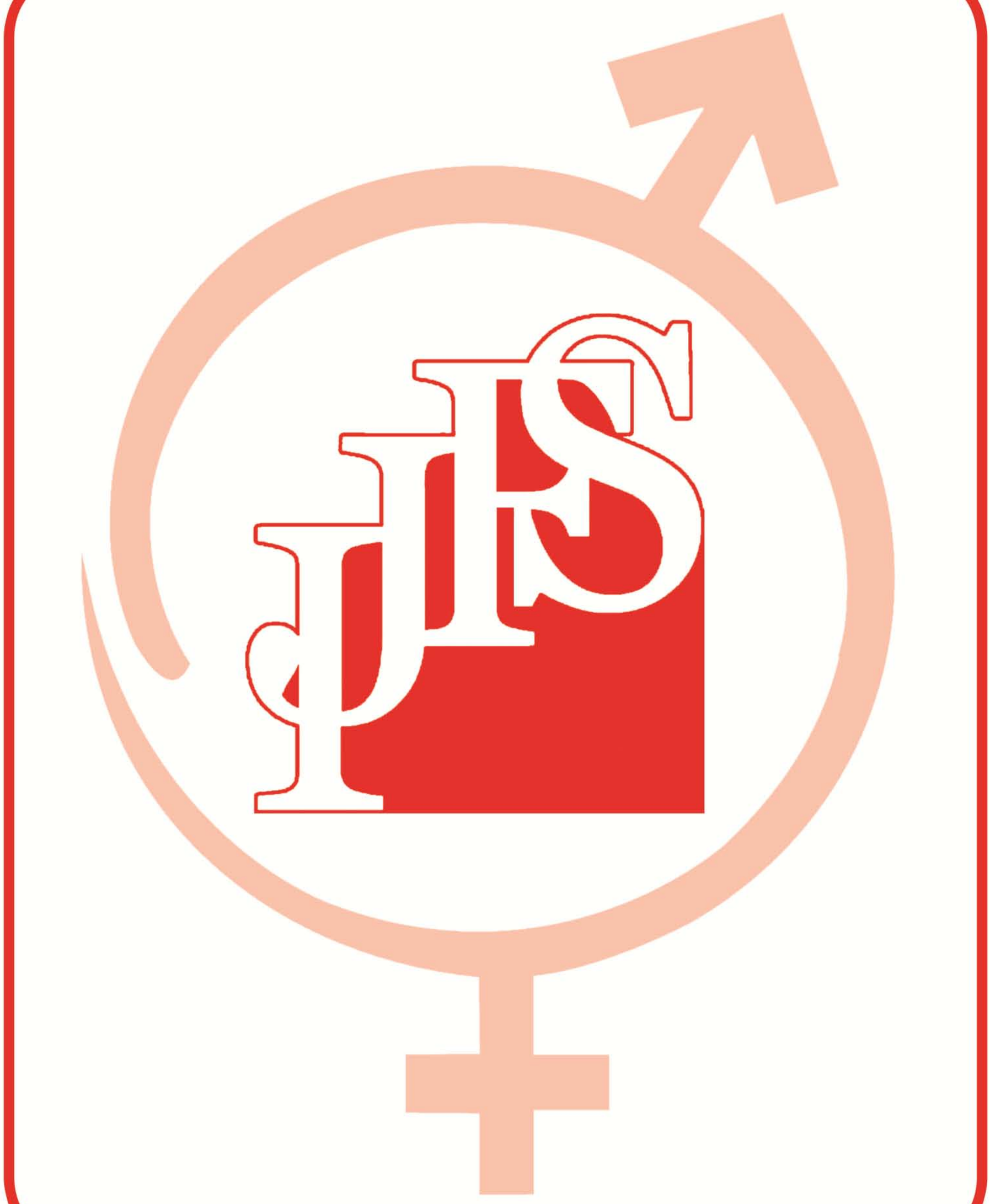

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Contents

Review Article

Reactive Oxygen Species Generation and Use of Antioxidants during *In Vitro* Maturation of Oocytes

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Abstract

In vitro maturation (IVM) is emerging as a popular technology at the forefront of fertility treatment and preservation. However, standard *in vitro* culture (IVC) conditions usually increase reactive oxygen species (ROS), which have been implicated as one of the major causes for reduced embryonic development. It is well-known that higher than physiological levels of ROS trigger granulosa cell apoptosis and thereby reduce the transfer of nutrients and survival factors to oocytes, which leads to apoptosis. ROS are neutralized by an elaborate defense system that consists of enzymatic and non-enzymatic antioxidants. The balance between ROS levels and antioxidants within IVM media are important for maintenance of oocytes that develop to the blastocyst stage. The effects of antioxidant supplementation of IVM media have been studied in various mammalian species. Therefore, this article reviews and summarizes the effects of ROS on oocyte quality and the use of antioxidant supplementations for IVM, in addition to its effects on maturation rates and further embryo development.

Keywords: Oxidative Stress, Reactive Oxygen Species, Antioxidant, *In Vitro* Maturation

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Introduction

In vitro embryo production (IVEP) allows the production of a high and inexpensive number of embryos to conduct basic research and apply emerging biotechnologies such as cloning and transgenesis. IVEP is a three-step methodology that comprises the following procedures: i. *In vitro* maturation (IVM) of oocytes recovered directly from follicles, ii. *In vitro* fertilization (IVF) or co-incubation of capacitated spermatozoa with *in vitro* matured oocytes, and iii. *In vitro* culture (IVC) of zygotes up to the blastocyst stage. According to reports, IVM is the key factor that determines the proportion of oocytes which develop to the blastocyst stage.

IVM of oocytes is a complex process influenced by the interplay of regulatory factors that include gonadotrophins and a growing list of secreted molecules, the biochemical state of the oocyte, and interactions between the oocyte and cumulus cells (1-5). Therefore, the *in vitro* advancement of an oocyte from the diplotene stage of prophase I [germinal vesicle (GV)] to metaphase II (MII), along with cytoplasmic maturation that encloses a broad set of still ill-defined cellular events are essential for fertilization and early development of the embryo (6-8).

Although substantial progress has been made to improve the efficiency of an IVM protocol, however, there is a lack of consistency in the success rate of conventional *in vitro* matured oocytes compared to *in vivo* matured oocytes. Multiple factors likely contribute to the overall poor quality of *in vitro* matured oocytes. One of the important factors may be oxidative stress (OS). The generation of pro-oxidants such as reactive oxygen species (ROS) is an invariable phenomenon in the culture condition. It is possible that OS also influences oocyte development *in vitro*. On the other hand, ROS are considered signal molecules in oocyte physi-

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ology and their impact on maturation promoting factor (MPF) destabilization has recently been reported (9-11).

Oocyte protection against ROS may play important roles in pre-implantation embryonic development. On the other hand, antioxidants are ROS scavengers, thereby helping to maintain the oocyte's oxidant/antioxidant balance. The effects of antioxidant supplementation to IVM media have been studied in various mammalian species (12-14). Our purpose was to incorporate the role of ROS in oocyte physiology, impact of OS in downfall of oocyte quality (15, 16), and the role of enzymatic as well as non-enzymatic antioxidants in reducing ROS levels and deterioration of oocyte quality under IVC conditions. This review article summarized the effects of ROS, the use of antioxidant supplementations for IVM, and its effects on maturation rates. In this systematic review, we used IVM, OS, ROS, and antioxidant as keywords from scientific databases between 1990 and 2016. After a review of all abstracts, we included strong, reliable research in this report.

Production of reactive oxygen species and generation of oxidative stress

OS is caused by an imbalance between pro-oxidants and antioxidants (17). This ratio could change with increased levels of pro-oxidants, such as ROS, or a decrease in antioxidant defense mechanisms (18-20). ROS represents a wide class of molecules that indicate the collection of free radicals (hydroxyl ion, superoxide, etc.), non-radicals (ozone, single oxygen, lipid peroxides, hydrogen peroxide) and oxygen derivatives (21). They are highly reactive and unstable. Hence, ROS can react with nucleic acids, lipids, proteins, and carbohydrates to acquire an electron and become stable. These reactions induce a cascade of subsequent chain reactions that eventually result in cell damage (22-24). ROS can diffuse and pass through cell membranes and alter most types of cellular molecules (nucleic acids, proteins, and lipids), leading to mitochondrial alterations (25), meiotic arrest in the oocytes (26), embryonic block, and cell death (27). On the other hand, OS occurs when increased ROS levels which disrupt cellular redox circuits, result in disturbances of redox-regulated cellular processes and/or oxidatively damage cellular macromolecules (28).

Oxidative stress and *in vitro* maturation

Under physiological conditions, the oocytes are major sources of ROS because they use oxygen to produce energy through mitochondrial oxidative phosphorylation. Their ROS production is increased during IVM when compared to *in vitro* maturation (13, 29). Increased levels of ROS beyond the physiological range that may lead to OS can result in deterioration in oocyte quality and thereby affect reproductive outcomes (30). A better understanding of the OS status and its regulation during IVM is needed. However, one must also consider whether and how OS may influence the process of IVM. This section focuses on reports that refer to mechanistic roles for OS in oocyte maturation, especially with respect to key features of nuclear and cytoplasmic events within the oocyte.

Reactive oxygen species and nuclear and cytoplasmic maturation

Increased levels of ROS associated with induce cell cycle arrest in human oocytes as well as in mouse embryos (31). A multitude of key factors regulate the generation of ROS in the media and include various cellular metabolic reactions, oxygen concentration, light, oocyte handling, and general physicochemical parameters that may have a negative impact on oocyte physiology by inducing apoptosis (Fig.1). One of the major constituent that may alter developmental responses in the oocyte is relevant to OS since light is known to result in an imbalance of pro- and antioxidants in somatic cells and embryos. Similarly, a relationship has been shown in a mouse model between a type of light commonly used in the laboratory with increased ROS concentrations and compromised embryonic and fetal development (32). Oxygen tension is another important difference between the *in vivo* and *in vitro* environments for the oocyte culture. Toxic effects of atmospheric oxygen concentration under standard culture conditions and the beneficial effects of lower O_2 concentrations (5-7%) on developmental competence of oocytes *in vitro* have been reported in mice (33, 34), hamsters (35, 36), rats (37), sheep and cattle (38-40), and humans (41-43).

The conditions of an IVC generate ROS, which could exert some beneficial effects if the ROS levels remain under physiological levels (44). The

tonic generation of ROS triggers meiotic resumption from diplotene as well as the MII arrest stage in several mammalian species (44, 45). It has been reported that levels of ROS beyond the physiological range could induce destabilization of maturation MPF, reduce survival factors, and trigger mitochondria-mediated apoptosis of oocytes (15, 46). The biphasic role of ROS must be sufficiently discussed in order to update OS and its impact on oocyte quality (15). The beneficial role of ROS comes from the observations that non-enzymatic antioxidants, such as ascorbic acid and 3-tert-butyl-4-hydroxyanisole (BHA), inhibit spontaneous meiotic resumption from diplotene arrest (47). These results suggest a beneficial threshold level for ROS.

Antioxidants

Antioxidants scavenge ROS, which helps maintain the cell oxidant/antioxidant balance. On the other hand, antioxidants are the compounds which either suppress the formation of ROS or oppose their actions. There are two types of antioxidants: enzymatic and non-enzymatic (Table 1).

Table 1: List of studies that show the effects of antioxidant supplements that improve *in vitro* maturation

Antioxidant	Experimental model
Enzymatic antioxidants	
Superoxide dismutase (SOD)	Mouse
Thioredoxin	Porcine
Catalase (CAT)	Bovine
Sericin	Bovine
Non-enzymatic antioxidants	
Glutathione (GSH)	Hamster, pig, ovine, Bovine and equine
Cysteamine	Canine, mice, goats, porcine
Vitamin C (Ascorbic acid)	Mouse
Vitamin E and trolox	-

Enzymatic antioxidants neutralize excess ROS and prevent it from damaging the cellular structure. Enzymatic antioxidants are composed of superoxide dismutase (SOD), catalase (CAT), various peroxidases and peroxiredoxins (PRDXs), including glutathione peroxidases (GPXs), which can convert peroxides to water and alcohol (48). SOD enzymes catalyze the dismutation of superoxide anion (O_2^-) into O_2 and H_2O_2 while CAT converts H_2O_2 to O_2 and H_2O. The enzyme SOD exists as three isoenzymes (49): SOD1, SOD2, and SOD3. SOD1 contains Cu and zinc (Zn) as metal co-factors in the cytosol. SOD2 is a mitochondrial isoform that contains manganese (Mn), whereas SOD3 encodes the extracellular form. Nutrients such as Se, Cu, and Zn are required for the activities of some antioxidant enzymes, although they have no antioxidant actions. Non-enzymatic antioxidants are composed of glutathione (GSH), vitamin C, taurine, hypotaurine, vitamin E, Zn, selenium (Se), beta carotene, and carotene (47). GSH is a tripeptide thiol compound with many important functions in intracellular physiology and metabolism. One of the most important roles of GSH is to maintain the redox state in cells which protects them against harmful effects effects caused by oxidative injuries. The protective action of GSH against ROS is facilitated by the interactions with its associated enzymes, such as GPx and GSH reductase (Fig.2).

Vitamin C (ascorbic acid) is a known redox catalyst that can reduce and neutralize ROS (50). Based on its chemical structure, ascorbic acid is an electron donor and therefore a reducing agent. It has two different biochemical roles-antioxidant and enzymatic cofactor. Ascorbic acid is maintained through reactions with GSH and can be catalyzed by protein disulfide isomerase and glutaredoxins. Cysteamine is a low-molecular weight amino acid that contains thiol (51). The addition of cysteamine not only enhances the GSH content in MII oocytes but also protects the membrane lipids and proteins due to indirect radical scavenging properties (52). The concentrations of many amino acids, including taurine and hypotaurine are non-enzymatic antioxidants that help maintain the redox status in oocytes (53).

Vitamin E (α-tocopherol) is a lipid soluble vitamin with antioxidant activity. It consists of eight tocopherols and tocotrienols. Vitamin E may directly destroy free radicals such as peroxyl and alkoxyl (ROO•) generated during ferrous ascorbate-induced lipid peroxidation (LPO), thus it is suggested as a major chain breaking antioxidant (54). Hyaluronan, melatonin, tea and sericin are known to act as indispensable antioxidants in IVEP. They can block the release of pro-oxidant factors released as a result of OS (12, 55, 56).

Hyaluronan, an essential component of the extracellular matrix and non-sulfated glycosaminoglycan may play an important role in meiotic resumption of oocytes (57). The hormone melatonin (N-acetyl-5-metoxy tryptamine) is an antioxidant that, unlike GSH and vitamins C and E, is produced by mammals. In contrast to other antioxidants, however, melatonin cannot undergo redox cycling. Once oxidized, it is unable to return to its reduced state because of the formation of stable end-products after the reaction (14). As an antioxidant, green tea has been shown to improve IVM and embryo development of sheep COCs to the blastocyst stage in IVM medium (58). Sericin a water-soluble globular protein (protein hydrolysate) is derived from the silkworm Bombyx mori. This protein represents a family of proteins whose molecular mass ranges from 10 to 310 kDa (59). Dash et al. (60) have reported that sericin might provide a protective effect on fibroblasts by promoting endogenous antioxidant enzymes *in vitro*.

Antioxidant supplements and improving *in vitro* maturation

The addition of enzymatic antioxidants such as SOD, CAT, and thioredoxin are effective for pre-embryo development as scavengers of ROS and serving embryos a low OS condition in mice (61, 62), porcine (63), and bovines (64). Sericin, an antioxidant protein, improves embryo development (60, 65) and is a critical supplement for oocyte maturation (12, 56).

A series of non-enzymatic antioxidants protect oocytes against ROS damage during oocyte maturation. GSH is one of the naturally synthesized antioxidants that protect cells from ROS toxicity and regulate the intracellular redox balance (66). The intracellular level of GSH increases during oocyte maturation in hamsters (67), pigs (68), ovine (69), bovines (70), and equines (71). Recent reports have shown that addition of low molecular weight thiol compounds, such as cysteamine and b-mercaptoethanol to IVM media improved the cytoplasmic maturation of oocytes and embryo development by increasing GSH synthesis (66, 72, 73).

Cysteamine supplementation during IVM reportedly improved nuclear maturation rates in canines (74), mice (75), goats (76), and porcine (77). Although, other studies in goats (78), pigs (79), horses (13), buffalos (80), and cattle (81) did not show any increase in nuclear maturation rates. Addition of cysteamine to the IVM medium improved embryo development to the blastocyst stage in mammalian oocytes (82).

Ascorbate is concentrated in granulosa cells, theca cells, luteal cells, and oocytes (28). Choi et al. (83) reported a beneficial role for vitamin C in protecting spindle structures of MII mouse oocytes and chromosomal alignment against an oxidant (hydrogen peroxide)-induced damage. It is suggested that the effect of vitamin C is associated mainly with its capability to promote ooplasmic maturation during IVM. The beneficial role of ROS comes from the observations that non-enzymatic antioxidants, such as ascorbic acid, inhibit spontaneous meiotic resumption from diplotene arrest. We have presented a number of these observations. Tatemoto et al. (84), Kere et al. (85), and Córdova el al. (86) found that the addition of vitamin C to the oocyte maturation medium exerted no effect on the maturation rates of oocytes. Similarly, antioxidants such as vitamin E and trolox had no effect on oocyte maturation, but other antioxidants such as propyl gallate and 2,4,5-trihydroxybutrophenone inhibited the spontaneous resumption of meiosis (87). Together, these studies emphasized the beneficial roles of ROS during IVM at certain concentrations (low level).

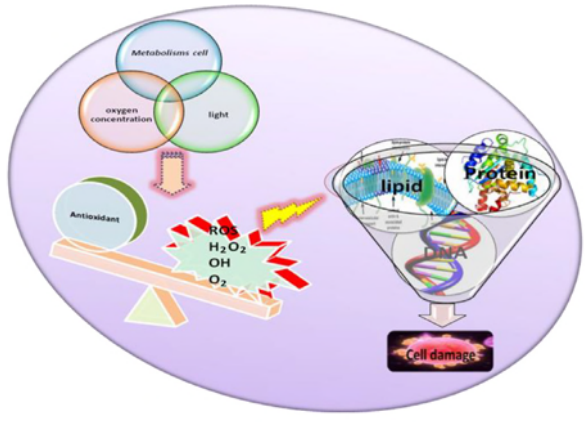

Fig.1: The possible factors that induce generation of reactive oxygen species (ROS) in the oocyte. The imbalance between ROS and antioxidants, the impact of high levels of ROS, and the resulting oxidative stress (OS) on meiotic arrest and apoptosis in oocytes.

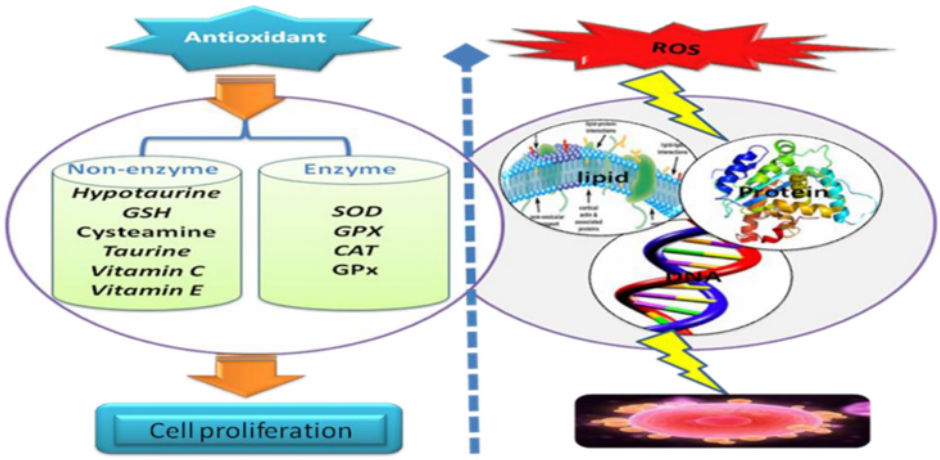

Fig.2: The presence of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidases (GPx), and catalase (CAT) as well as non-enzymatic antioxidants, such as vitamin E and C (ascorbic acid), glutathione (GSH), uric acid, and albumin in the oocytes. Excess amounts of reactive oxygen species (ROS) may be involved in oxidative stress (OS) of oocytes and granulosa cells.

Conclusion

It is well-known that high levels of ROS beyond the physiological range could induce MPF destabilization, reduce survival factors, and trigger apoptosis in oocytes of several mammalian species. Antioxidants are the main defense factors against OS induced by ROS. Many reports suggest that antioxidant supplementation of IVM media improves cytoplasmic maturation by alleviating OS during oocyte maturation via increasing GSH storage, and contributes to further protect the embryo against oxidative aggressions during its early developmental stages. On the other hand, supplementation by antioxidants during IVC improves oocyte quality by reducing ROS levels and apoptotic factors. However, some of the non-antioxidants such as ascorbic acid and 2, 4, 5-trihydroxybutrophenone do not improve oocyte maturation; rather, they inhibit spontaneous resumption of meiosis. Improvements to culture conditions are complex challenges that depend not only on the choice of an antioxidant but also on its concentration, the medium and its components, the species, and the dynamic changes of the specific requirements of the oocyte according to its developmental stage. Future efforts should be placed on understanding the involvement of ROS in oocyte apoptosis and for guiding antioxidant-based strategies to selectively control ROS-induced damage without compromising the physiological functions of these species.

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Pictorial Review

Hysterosalpingography in The Assessment of Congenital Cervical Anomalies

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Abstract

Cervical abnormalities may be congenital or acquired. Congenital anomalies of the cervix are rarely isolated, and more commonly accompany other uterine anomalies. Various imaging tools have been used in the assessment of Müllerian duct anomalies (MDAs). Currently, magnetic resonance imaging (MRI) is the modality of choice for definitive diagnosis and classification of these MDAs. Hysterosalpingography is a basic tool for evaluation of infertility and allows us to detect a spectrum of anatomical malformations of the utero-cervix in the setting of MDAs. It provides good outlines of the uterine cavity and fallopian tubes, as well as the cervical canal and isthmus. However, hysterosalpingograms (HSG) cannot be performed in patients with isolated congenital maldevelopment (agenesis/disgenesis) of the cervix. This part of pictorial review illustrates the various radiographic appearances of congenital malformations of the utero-cervix with a brief overview of the embryologic features. Accurate diagnosis of such cases is considered essential for optimal treatment and categorization of each anomaly.

Keywords: Hysterosalpingography, Congenital, Cervix, Uterine, Anomalies

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Introduction

Female genital malformations include various forms of developmental and morphological malformations of the vagina, cervix, uterus, adnexa and associated malformations (VCUAM) (1). Genital malformations have an incidence of up to ~7% in the general population (2).

Congenital anomalies of the cervix are rarely isolated, and are more commonly associated with other uterine and vaginal anomalies. Cervical anomalies may manifest as agenesis, dysgenesis, obstruction, abnormal length, inadequate width, and hypertrophy (3); however, complete or partial duplication of the cervix with a normal uterus and an unusual Müllerian anomaly have been reported (4, 5). Cervical atresia usually presents with primary amenorrhea and cyclic abdominal pain. Depending on the type and degree of maldevelopment of the uterine cervix, a woman's reproductive potential may be adversely affected.

Several imaging tools have been used in the assessment of Müllerian duct anomalies (MDAs). Although magnetic resonance imaging (MRI) is considered the gold standard for definitive diagnosis and classification of genito-urinary anomalies, especially for complex cases, hysterosalpingography is still an important tool in the early evaluation of infertility. Contrast medium which is slowly injected into the uterus through the cervical canal, provides good outlines of the uterine cavity and fallopian tubes, as well as the cervical canal and isthmus (6).

We retrospectively reviewed 38574 hysterosalpingograms (HSGs) performed over a 29-year period (January 1985-December 2013) by one author (G.Sh.). The indications for HSG included infertility, abnormal uterine bleeding, and symptoms

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related to uterine fibroids. This review illustrates the various radiographic appearances of congenital malformations of the utero-cervix with a brief overview of the embryologic features.

Embryology

The female genital tract develops from a pair of Müllerian ducts between 6 and 12 weeks of gestation. The process involves three main stages: i. Development of both Müllerian ducts that form the fallopian tubes, uterus, and cervix and upper two thirds of the vagina, whereas failure of this stage results in agenesis/hypoplasia or a unicornuate uterus, ii. Fusion of the lower Müllerian ducts leads to formation of the uterus and cervix while defects in this phase result in a bicornuate uterus, and iii. Canalization and septal resorption of the central septum which results in a single uterine cavity and cervix, whereas failure of this stage leads to a septate or arcuate uterus. Mesonephric or Wolffian ducts play a role as inductors for adequate development, fusion, and resorption of the walls of Müllerian ducts (7). There is controversy over formation of the vagina. Recent studies have stated that the mesonephric ducts together with the Müllerian tubercle form the vagina (7, 8).

By week 20, the process of development is completed. Development of both Müllerian ducts and the urinary tract occurs from a common ridge of the mesoderm; hence, anomalies of the urinary tract are commonly observed in females with genital malformation.

Classification system

Accurate classification of a female genital tract malformation is necessary to prevent inadequate surgery and achieve optimal treatment. Until now, several classification systems for female genital tract anomalies have been proposed: the American Fertility Society Classification System (currently American Society for Reproductive Medicine, AFS/ASRM) (9); the embryological-clinical classification system of genito-urinary malformations proposed by Acién and Acién (8, 10), the vagina, cervix, uterus, adnexae and associated Malformations system based on the tumor node metastasis (TNM) principle in oncology (1), and the new European Society of Human Reproduction and Embryology (ESHRE) and European Society for Gynaecological Endoscopy (ESGE) classification systems (11). Most of these classification systems seem to be associated with limitations, especially in the diagnosis of unusual and complex malformations (12).

The AFS classification is based mainly on the description of uterine changes and most widely used as the main classification system for its simplicity, friendliness and clinical usefulness. However, this system is associated with limitations in efficient categorization of female genital anomalies. The accompanying malformation (such as duplex vagina), unusual or/and complex malformations especially those in group I (hypoplasia/agenesis) not fit and are described completely in the AFS classification and so often fails to correctly identified and treated (12). In addition, some other cases of utero-vaginal anomalies have been classified in nine subtypes of septate and bicornuate communicating uteri schemes, as suggested by Toaff et al. (13).

The use of embryological-clinical classification of genitor-urinary malformations seems to unify the current embryological and pathogenic concepts and appear to be the most clinically useful (10). Based on the AFS classification scheme, the cervix is classified as follows: IB (under segmental Müllerian duct agenesis or hypoplasia), III (complete non-fusion of MDAs), IV (incomplete fusion of the superior segments of the uterovaginal canal), V (partial or complete non-resorption of the uterovaginal septum), and VII [MDAs related to diethylstilbestrol (DES) exposure in utero].

The accurate diagnosis of such cases is considered essential for optimal treatment and in support of the embryologic concept.

Cervical agenesis/dysgenesis

Agenesis/dysgenesis of the cervix is rare and usually occurs in association with complete or partial vaginal agenesis (2). It is difficult to diagnose cervical agenesis. Clinical examination has limited diagnostic value and in most cases hysterosalpingography is impossible.

Utero-cervical anomalies

Uterus didelphys

Uterus didelphys results from complete fail-

ure of Müllerian duct fusion and accounts for approximately 5% of MDAs (2). On hysterosalpingography, two symmetric separate cavities and two cervical canals are present; a double vagina is often present (Fig.1). Didelphys uterus is usually asymptomatic, while cases with unilateral vaginal obstruction may manifest with hematometrocolpos and dysmenorrhea at menarche.

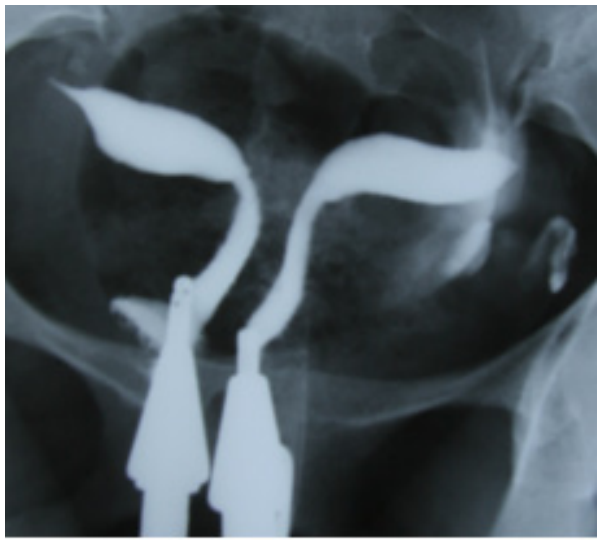


Fig.1: Didelphys uterus in a 31 year-old-woman with 3 years of primary infertility. Hysterosalpingogram demonstrates two symmetrical separate cavities, two cervical canals and presence of double vagina.

Bicornuate uterus

The bicornuate uterus represents approximately 25% of MDAs and results from incomplete fusion of the Müllerian ducts at the level of the uterine fundus (2). The two separate uterine cavities are fused caudally and communicate in the lower segment, mostly at the uterine isthmus; a single cervix and vagina are present. Hysterosalpingography demonstrates separate fusiform uterine horns, often with an intercornual angle of >105º (14). There are various degrees of separation between the two horns, as follows: a complete bicornuate uterus, in which the failure to fuse extends the length of the uterine body inferiorly to the internal os; and lesser degrees of a bicornuate uterus, in which the partial interfering cleft is variable in length, extending from the fundus to the cervix (Fig.2).

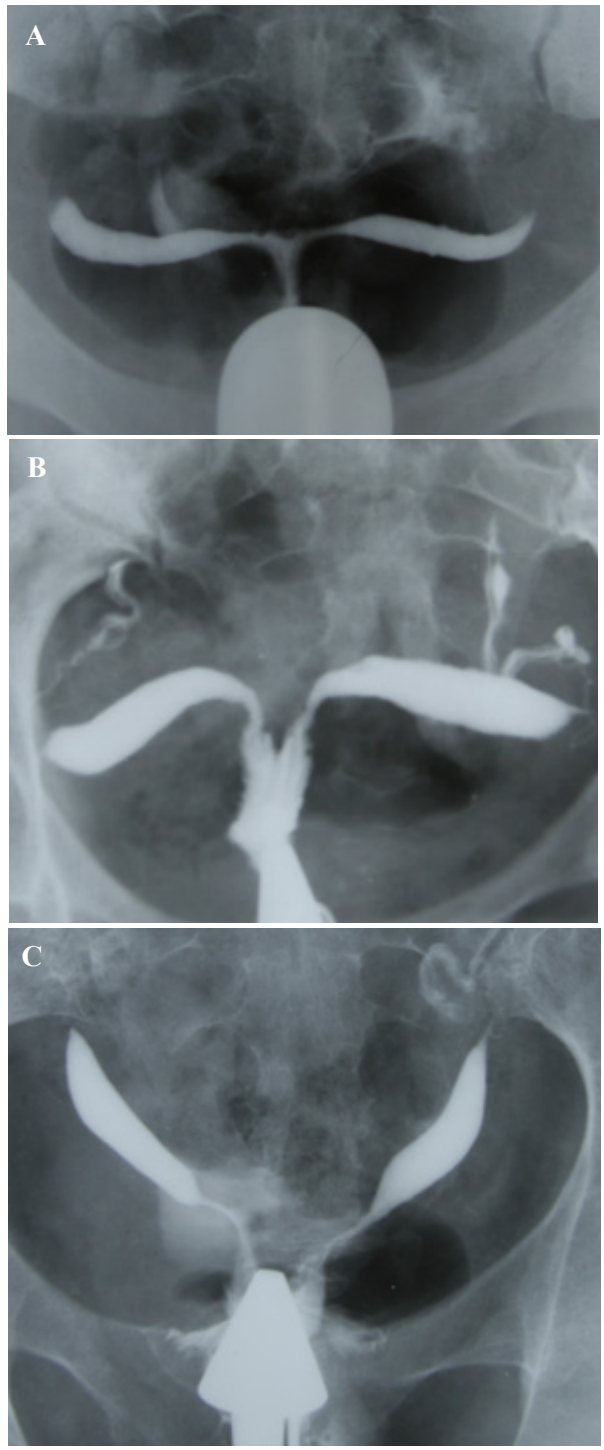
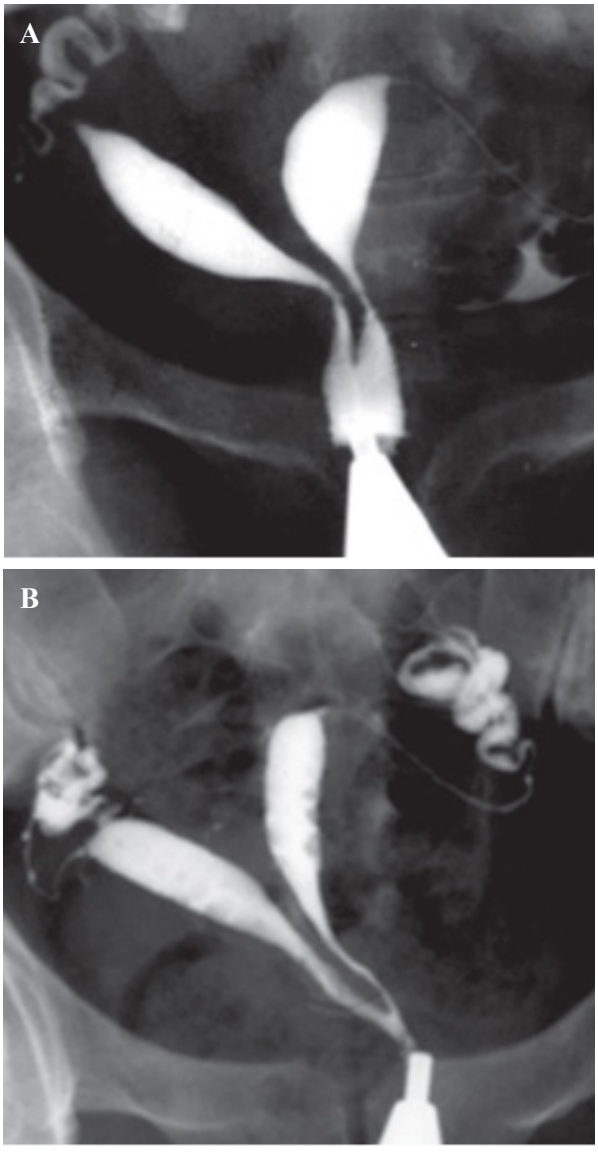


Fig.2: Bicornuate uterus with various degrees of duplication of the cervix in different patients (AFS class IV). **A.** Bicornuate uterus, consisting of two symmetric uterine cavities with communication at the uterine isthmus, and also intercorneal angle greater than 105º. Single cervix and vagina are present, **B.** Intervening cleft extends to the endocervical canal, and **C.** Extension to the level of external os.

Septate uterus

A septate uterus results from partial or complete failure of resorption of the uterovaginal septum after fusion of the paramesonephric ducts, which occurs in approximately 35% of MDAs (2). Hysterosalpingography of a septate uterus represents varying degrees of the midline septum, extending from the fundus to the cervix and upper vagina, and yielding a V-shaped configuration often with an angle <75º between the two uterine horns (Fig.3). In 25% of cases, complete extension of the septum to the upper vagina is present (Fig.3C) (14).



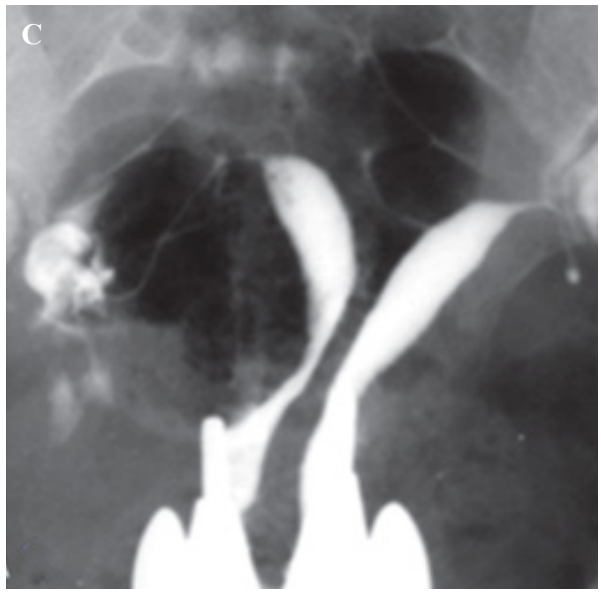


Fig.3: Hysterosalpingography of septate uterus demonstrates a variable degrees of cervical septation in different patients (AFS class V) (12). **A.** Extension of the midline septum to the lower internal os, **B.** Septate uterus with extension of septum to the level of external os and production of two cervical canal with one opening, and **C.** Complete septate uterus with two separate cervical canal (pseudodidelphys).

Diethylstilbestrol-exposed uterus

Exposure to DES in utero results in multiple, benign abnormalities of the genital tract and clear cell adenocarcinoma of the vagina. DES has been associated with T-shaped and irregular configurations of the endometrial cavity, constrictive bands, structural cervical changes, and cervical anomalies that include hypoplasia, cervical ridges, and cervical collars (15).

Hysterosalpingography is an excellent screening tool to diagnose DES-related uterine anomalies. The radiographic appearance includes an irregular, narrowed endocervical canal with a shortened upper uterine segment and small, typically irregular cavity that yields a T-shaped uterine configuration (Fig.4).

Communicating uterus

In 1984, Toaff et al. (13) proposed a classification scheme for nine subtypes of septate and bicornuate uteri that identified the presence of a communication between two separate uterocervical cavities. All types of communicating uteri have an isthmic communication, except for type 9, which has a low cervical communication.

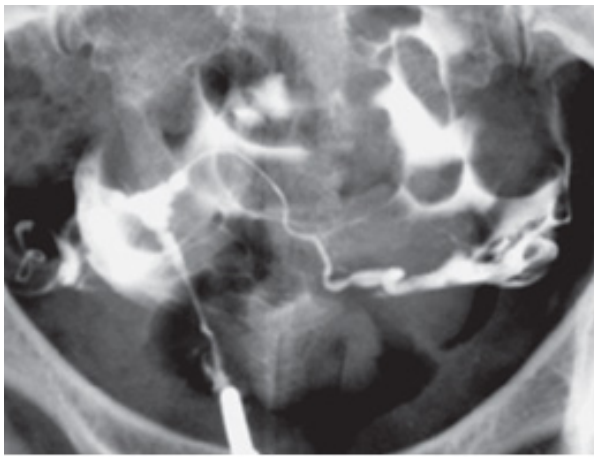
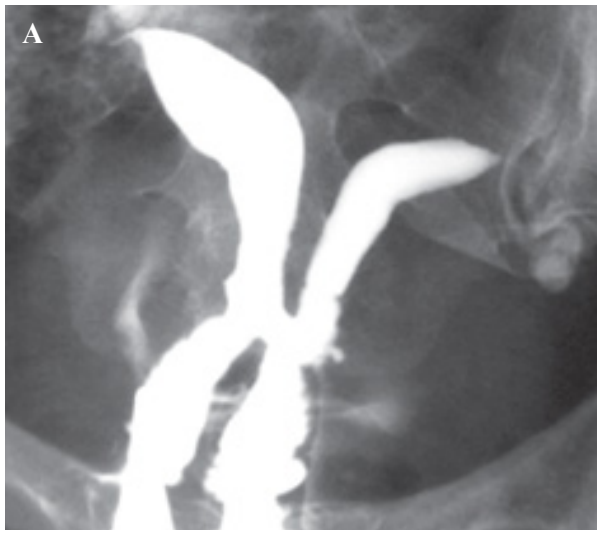
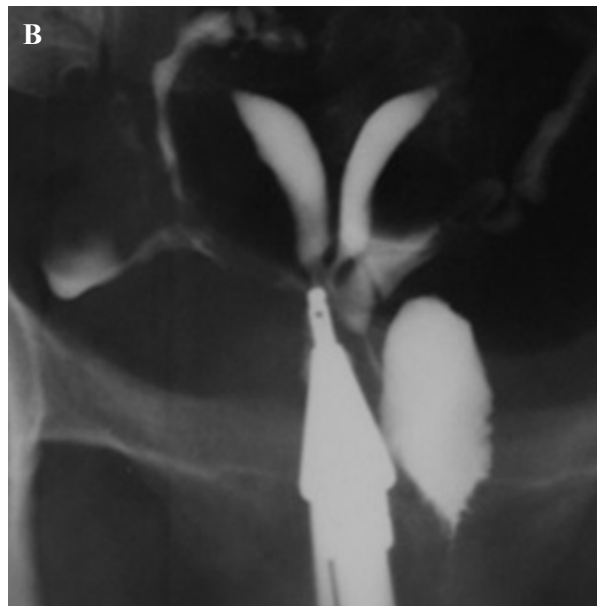
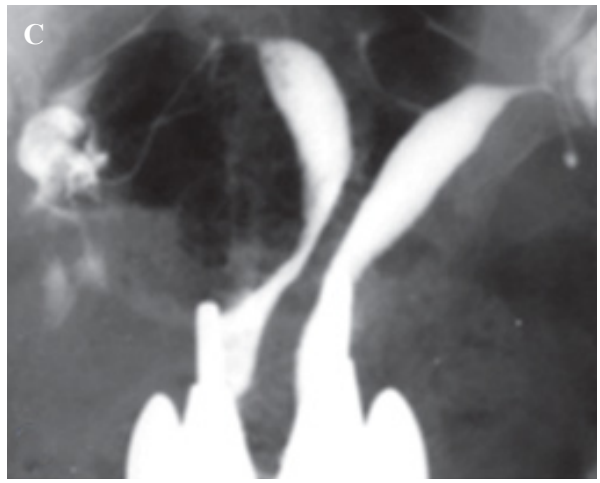


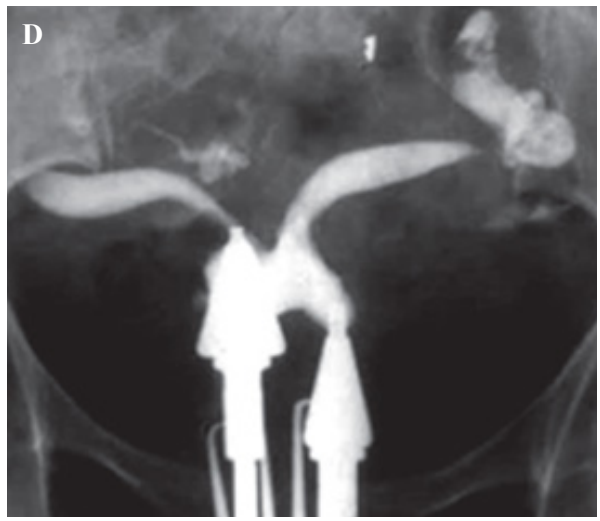
Fig.4: DES-exposure uterus in a 28-year-old infertile patient. Note T- shaped appearance of the endometrial cavity with a long narrowed irregular endocervical canal.

During >29 years of one author's experience (G.SH) in performing HSGs, some rare cases of communicating uteri have been observed and reported (Fig.5). Of these, three represented communications at unusual sites. They were identified as non-isthmic communicating uteri and classified as a new subclass of type 9 (16). The first case was a complete septate uterus with a mid-corporeal communication, in which the septum ended inferiorly several millimeters above the external os (Fig.6). Another case was a bicornuate uterus with two sites of communication, at the mid-cervical and isthmic levels (Fig.7), and a third case with communication that involved the bicornuate uterus with a low cervical communication, left cervico-vaginal atresia, and left renal agenesis (Fig.8).









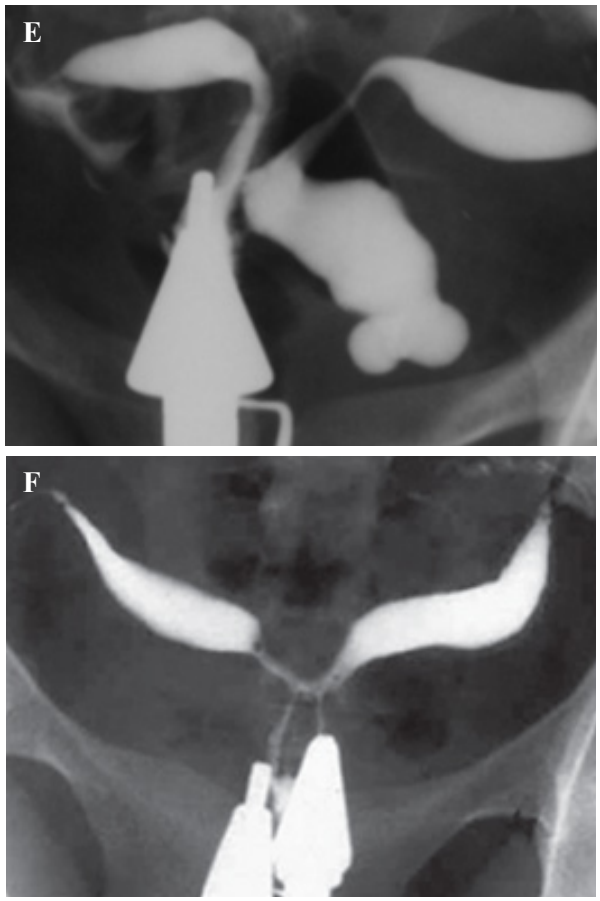


Fig.5: Some cases of communicating uteri with duplication of cervical canal (12). **A.** Uterus communicants septus, cervix duplex, vagina septa (type 1a). Vaginal septum was observed in vaginal examination, **B.** Uterus communicants septus, cervix duplex, vagina septa unilateralis atretica (type 2a), **C.** Uterus communicants septus, cervix septa, vagina septa (type 3a).Vaginal septum was visualized in vaginal examination, **D.** Uterus comunicants bicornis, cervix duplex, vagina septa (type 4a). Vaginal septum was seen in vaginal examination, **E.** Uterus communicants bicornis, cervix duplex, vagina septa unilateralis atretica (type 5a), and **F.** Uterus communicants bicornis, cervix septa, vagina simplex (type 6).

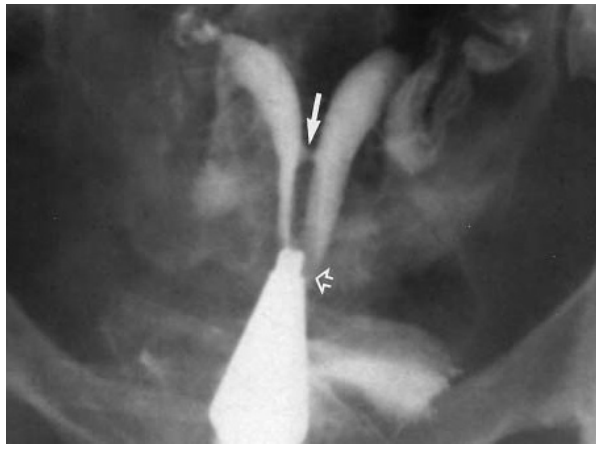


Fig.6: Hysterosalpingography in a 23 year-old-woman with 5 years of primary infertility. The uterus is septate with a midcorporeal communication (straight arrow). The cervix is partially septate and the cervical septum ends a few millimeters above the single external os (open arrow) (16).

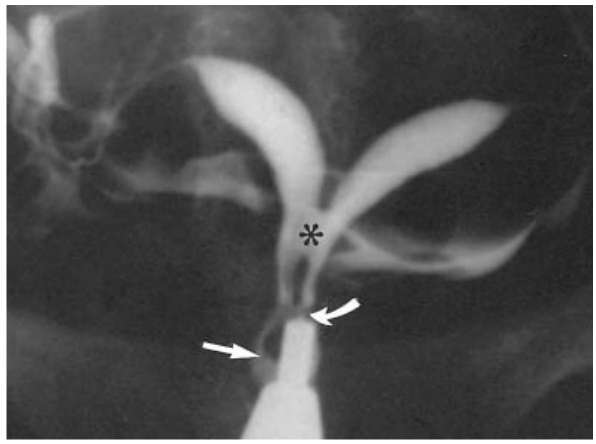


Fig.7: Bicornuate uterus in a 26 year-old-woman with 3 years of primary infertility. Hysterosalpingogram shows the bicornuate uterus with two sites of communication between two uterocervical canal, midcervical (curved arrow) and isthmic (asterisk); septum of the cervix ends several millimeters above the single external os (straight arrow) (16).

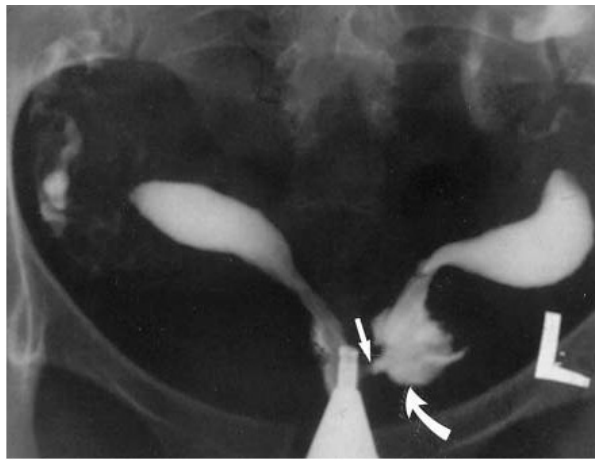


Fig.8: Hysterosalpingography in a 27-year-old woman with primary infertility of 5 years. The uterus is bicornuate with a low cervical communication and left atretic hemicervix. The cannula is located in the right hemicervix. The right external os is visible. Injection of contrast into the right hemicervix was noted to opacify the left hemiuterus and cervix through the low cervical communication (arrow) (16).

Unusual cervical anomaly

Some unclassified cases of normal or septate uteri that communicate with double cervices with or without vaginal septa have been previously described (4, 17-19). We encountered a case of normal uterus with septate cervix and vagina (Fig.9).

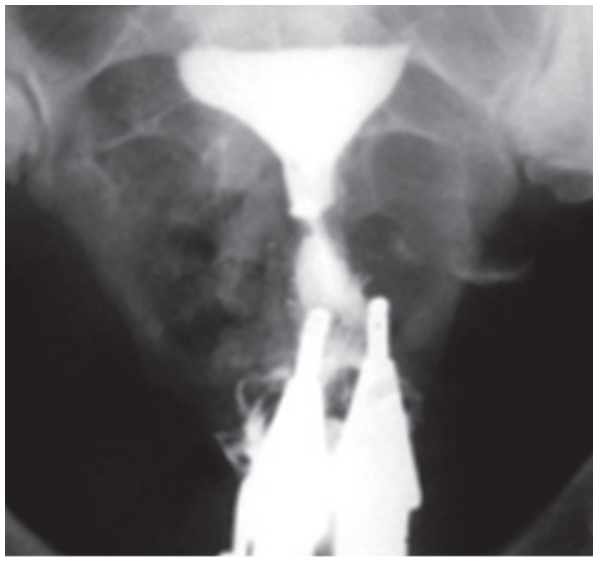


Fig.9: Hysterosalpingogram of a 36 year-old woman shows an normal uterus with septate cervix and vagina.

In the second case, the proximal part of the cervical canal was double, but the distal portion was single and one cervical opening was present (Fig.10).

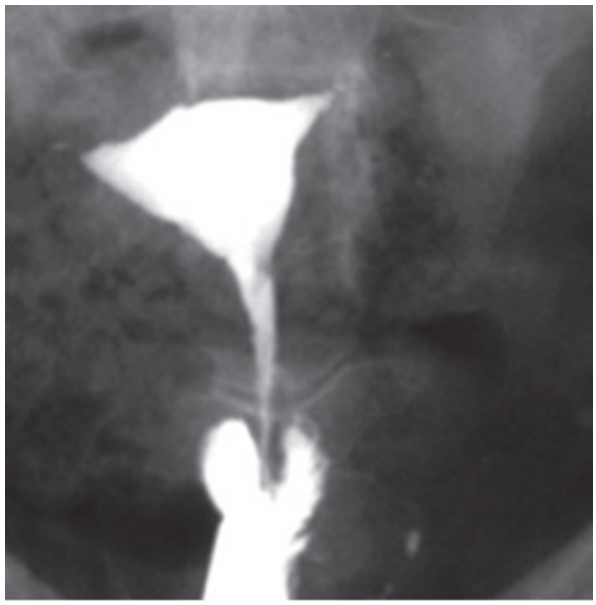


Fig.10: Infertility of a 27 year-old woman investigated. HSG demonstrates invagination of distal part of cervical canal within proximal part of cervix. In this patient proximal part of cervical canal is double but the distal portion is single and the patient has one cervical opening.

The third case was a bicornuate uterus with communication at the isthmic level, a septate cervix, and normal external cervical os and vagina (Fig.11). The patient had no history of any previous vaginal or cervical septum resection.

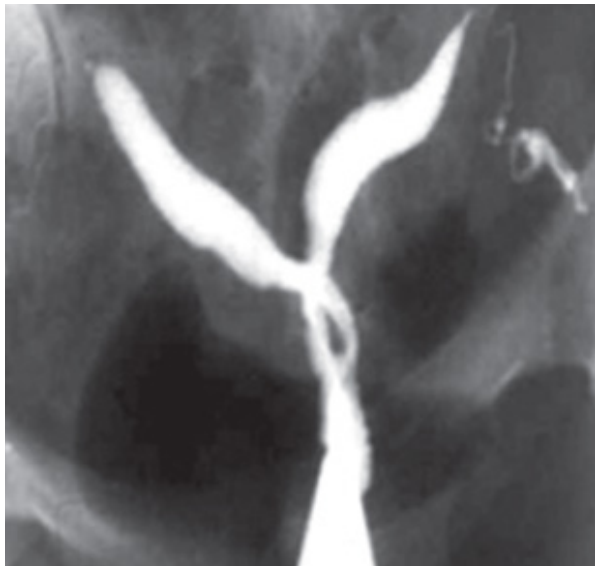


Fig.11: A 29- year-old woman with a history of 5 years of primary infertility. HSG represents a bicornuate uterus with communication at the level of isthmus, a septate cervix, and normal cervical os and vagina. The patient has no history of previous vaginal and cervical septum resection.

In the last case, HSG demonstrated a left blind hemivagina (atretica) with left renal agenesis (Herlyn-Werner- Wunderlich syndrome, Fig.12).

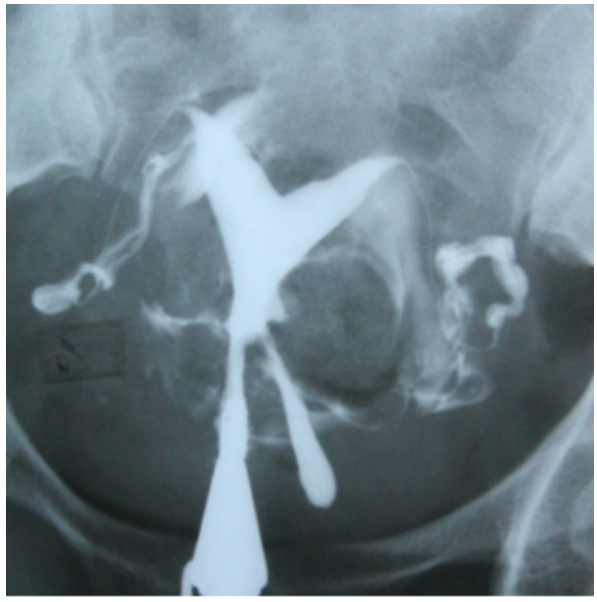


Fig.12: A 24-year-old woman with a history of 3 years infertility. HSG shows a septate uterus with a left blind hemivagina(atretica) and left renal agenesis (Herlyn-Werner- Wunderlich syndrome).

Conclusion

Although congenital anomalies of the utero-cervix in the setting of MDAs are rare, the impact on a woman's reproductive potential can be significant. Anomalies of the cervico-uterus are widely diagnosed by HSG. The diagnostic value of HSG in the detection of anomalies varies, depending on the type of malformation. Accurate diagnosis of these cases, especially the cases with any classification system, is important for optimal treatment and categorization of each anomaly.

Acknowledgements

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Original Article

A Comparison of Outcomes from *In Vitro* Fertilization Cycles Stimulated with Follicle Stimulating Hormone Plus either Recombinant Luteinizing Hormone or Human Menopausal Gonadotropins in Subjects Treated with Long Gonadotropin Releasing Hormone Agonist Protocols

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Abstract

Background: This study compared rates of pregnancy and *in vitro* fertilization (IVF) parameters in subjects stimulated with follicle stimulating hormone (FSH) plus either recombinant human luteinizing hormone (r-LH) or human menopausal gonadotropin (hMG) in a long gonadotropin releasing hormone (GnRH) agonist IVF protocol.

Materials and Methods: This cohort study enrolled patients who underwent IVF stimulation with a long GnRH agonist protocol and received FSH plus r-LH or hMG. Outcomes measured included: FSH and LH doses, number of oocytes and embryos obtained, pregnancy rate per cycle, and clinical pregnancy rate per cycle. Stepwise logistic regression was performed on continuous and categorical variables to control for confounding effects between all variables analyzed.

Results: There were 122 patients who underwent 122 IVF cycles with long GnRH agonist protocols. Similar baseline parameters existed between groups. Patients that received r-LH required a lower FSH dose (3207 ± 1300 IU) for stimulation compared to the group that received hMG (4213 ± 1576 IU, P=0.0001). The LH dose was also lower in these patients (1332 ± 587 IU) compared to the patients who received hMG (1938 ± 1110 IU, P=0.0001). The number of days of stimulation did not differ between groups (P=1.0). The group that received r-LH also had statistically higher numbers of oocytes (14.4 ± 6.3) and embryos (7.9 ± 4.8) compared to the hMG group with 11.0 ± 5.3 oocytes and 6.0 ± 3.7 embryos. Pregnancy rates per cycle start were higher for patients in the r-LH group (49%) compared to the hMG group (27%, P=0.025). Patients that received r-LH had higher implantation rates (62%) compared to the hMG group (33%, P=0.001). The r-LH group had a higher trend toward clinical pregnancy rates per cycle start (39%) compared to the hMG group (25%, P=0.065).

Conclusion: r-LH may offer benefits compared to hMG when combined with FSH for ovarian stimulation in long GnRH agonist protocols in good responders. Prospective studies should be undertaken to confirm these results.

Keywords: Luteinizing Hormone, *In Vitro* Fertilization, Human Menopausal Gonadotropins, Ovarian Stimulation

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Introduction

Multiple studies and meta-analyses have reported the importance of luteinizing hormone (LH) or LH mediated activity for *in vitro* fertilization (IVF) stimulation cycles (1-4). More precisely, administration of human menopausal gonadotropin (hMG) has led to increased pregnancy, clinical pregnancy, and live birth rates compared to recombinant follicule-stimulating hormone (r-FSH) alone (1, 2, 5). LH is available in two forms, recombinant (r-LH) or in hMG, which contains human chorionic gonadotropin (hCG) that acts as an LH analogue. Few studies have evaluated the role of different types of LH stimulation by comparing r-LH to HMG, which may yield subtle differences. A previous study performed at the McGill Reproductive Center compared subjects who received r-LH and r-FSH to subjects that received hMG alone in women with good or poor ovarian reserve. In subjects with good ovarian reserve, the r-LH group had higher numbers of oocyte and embryos, increased pregnancy rates per cycle, and overall higher clinical pregnancy rates which showed a potential benefit for r-LH stimulation (6). However, no distinction was made between IVF protocols.

The current study compared stimulation parameters, pregnancy and clinical pregnancy rates of patients with normal ovarian reserve parameters treated with a long gonadotropin releasing hormone (GnRH) agonist protocol and received r-LH to those treated with hMG that contained hCG as an LH analogue. Both groups of participants also received daily FSH stimulation.

Materials and Methods

We performed a cohort study from data collected at the McGill Reproductive Center. An analysis of IVF cycles for a two-year period was undertaken to identify all patients treated at our institution that met the inclusion criteria. To be included in the study patients received FSH and either r-LH or HMG but not both forms of LH stimulation. Patients with maximum serum baseline FSH levels under 10 IU/L (drawn menstrual cycle days 2 to 5 inclusively) and baseline follicle counts of 6 follicles or more determined by transvaginal ultrasound (TVUS) as assessed on menstrual cycle days 2 to 5, inclusively, initiated treatment with a long GnRH agonist down-regulation protocol (n=122). A total of 65 women received r-LH whereas 57 received hMG. Cycles were excluded from analysis if the patient had hyperprolactinemia (morning fasting prolactin greater than 26 ng/mL), thyroid abnormalities (TSH below 0.39 or above 4.0 µIU/mL), hypothalamic pituitary dysfunction, and ovarian failure (FSH below 2 IU/L or abover 20 IU/L and estradiol <66 pg/mL). The McGill University Committee for the Protection of Human Research Subjects approved this data collection. All subjects were de-identified in the database. Patients were allotted to their respective treatment regimens by clinic staff to maintain equivalent rates for prescription of different drugs produced by competing pharmaceutical companies. Patients that received hMG (Repronex, Ferring Canada, North York, ON) also received either follitropin beta (64%, Merck Canada, Inc., Pointe-Claire, QC), follitropin alfa (20%, EMD Serono Canada, Mississauga, ON), or purified urofollitropin (16%, Ferring Canada, North York, ON). All patients that received r-LH were treated with follitropin alfa (EMD Serono Canada, Mississauga, ON).

Patients treated with a GnRH agonist long down-regulation protocol initially received stimulation with 112.5 to 225 units of FSH daily at the discretion of their treating physician. The physician selected the dose based on parameters of ovarian reserve noted during the planning of the cycle. After 5 days of FSH stimulation, we reassessed the doses which were titrated up or down depending on serum estradiol levels, as well as the numbers and diameters of follicles noted. Subsequently, patients were monitored at 1 to 3 day intervals with serum estradiol levels and transvaginal ultrasonographic follicle monitoring. Patients were prescribed LH activity such that the ratio of FSH to LH was 3:1 to 2:1 at the discretion of their treating physician. We measured peak serum estradiol levels and either 10000 IU or 5000 IU of hCG were given based on our center's protocol 35 hours prior to egg retrieval. We followed the McGill Reproductive Center protocol for egg retrieval and embryo transfer using either a Cook (Cook Canada, Inc., Stouffville, ON) 17-gauge single lumen needle or a 16-gauge double lumen flushing needle and warmed saline flush. Pressure for aspiration was kept at 145 mmHg by a Cook Vacuum Pump (K-Mar 8200, Cook, Australia).

At 17 to 19 hours after insemination, embryo fertilization was evaluated for the presence of two pronuclei and two polar bodies. The zygotes were transferred to an IVF cleavage medium for further culture (Cook, Australia). The embryos were evaluated on days 2 (41-43 hours after insemination) and 3 (65-67 hours after insemination). Quality of development of the embryos was assessed according to the regularity of blastomeres, the percentage and pattern of anucleate fragments, and dysmorphic characteristics. Good quality embryos on day 2 had at least 2 cells and by day 3, they at least 6 cells with less than 20% anucleate fragments and no apparent morphological anomalies. Embryos were considered low quality if they showed blastomere multi-nucleation, poor cell adhesion, uneven cell division, and cytoplasmic anomalies. We transferred embryos of best quality based on cell number, degree of fragmentation, symmetry of blastomeres, degree of compaction, clarity and texture. Depending on age and physician orders, the transfer was performed on day 2 or 3.

Embryos were transferred under trans-abdominal ultrasound guidance and placed 2.5 to 1.5 cm from the uterine fundus using a Wallace embryo replacement catheter (Smith Medical International Ltd., UK). The number of embryos transferred varied between 2-5 depending on the patient's age, embryo quality, and previous number of unsuccessful IVF cycles. Decisions were based according to recommendations by the American Society for Reproductive Medicine Committee Opinion (7). Progesterone was prescribed for luteal phase support per the center's protocol. We defined pregnancy as a single serum hCG level of ≥20 IU/L measured 16 days after egg retrieval. Clinical pregnancy was defined as an intra-uterine positive fetal heartbeat seen on TVUS at 6-7 weeks of gestational age.

Statistical analysis

Statistical analysis was done using SPSS 11.0 (SPSS Inc., Chicago, IL, USA). Continuous variables were evaluated for normal distribution using the Kolmogorov-Smirnov test. All continuous variables were normally distributed. We performed stepwise logistic regression on continuous and categorical variables to control for multiplicity and confounding effects. Rates for causes of infertility were analyzed by chi-squared tests. Data are presented as means ± SD with statistical significance as a two-sided P≤0.05. Controlled variables included patient age, basal serum FSH level, basal serum estradiol level, antral follicle count, previous pregnancies, previous full term pregnancies, previous miscarriages, previous IVF cycles, total LH and FSH stimulation doses, days of LH and FHS stimulation, as well as the number of oocytes and embryos obtained.

Results

There were no cancelled cycles in any of the patients. Patients were good responders; hence, we did not anticipate any poor response. Ovarian hyperstimulation syndrome was avoided in all cases. No statistical differences in pregnancy rate (P=0.432) or clinical pregnancy rate (P=0.381) existed among the cycles that used follitropin alfa, follitropin beta or urofollitropin, which was combined in all cases with hMG. This result supported the combined analysis of these results. A comparison of patient demographics in subjects treated with the long GnRH agonist protocol is presented in Table 1. The two groups did not differ in any of the baseline characteristics studied, including basal serum FSH level, basal serum estradiol level, and baseline follicle count. The number of embryos transferred did not differ between r-LH (2.2 ± 0.6) versus hMG (2.3 ± 0.6, P=0.36).

Table 1: Patient demographics (mean ± SD)

Demographic	r-LH n=65	hMG n=57	P value
Age (Y)	35.2 ± 4.2	36.0 ± 4.4	0.412
Basal serum FSH (IU/L)	7.7 ± 1.5	7.2 ± 1.7	0.134
Basal serum estradiol (pmol/L)	167 ± 85	183 ± 77	0.292
Antral follicle count	18 ± 10	16 ± 6	0.200
Previous pregnancies	0.8 ± 1.2	1.0 ± 1.2	0.833
Previous full term pregnancies	0.1 ± 0.4	0.3 ± 0.8	0.274
Previous miscarriages	0.7 ± 1.1	0.7 ± 0.9	0.701
Previous IVF cycles at McGill	1.7 ± 0.9	1.9 ± 1.1	0.203
Previous IVF cycles elsewhere	0.4 ± 1.0	0.3 ± 0.9	0.543

Analysis performed with stepwise logistic regression.
r-LH; Recombinant human luteinizing hormone, hMG; Human menopausal gonadotropin, IVF; *In vitro* fertilization, and FSH; Follicle stimulating hormone.

There were similar rates for causes of infertility in subjects treated with the long GnRH agonist protocol that received either r-LH or hMG (P=0.469).

A comparison of the r-LH or hMG groups showed that the rates of male factor infertility were 54% (r-LH) and 39% (hMG), the unexplained infertility rates were 32% (r-LH) and 33% (hMG), the rates of endometriosis were 4% (r-LH) and 12% (hMG), and the anovulation rates were 2% in both groups. Tubal factor infertility did not occur in either group.

Table 2 shows treatment outcomes for the r-LH and hMG groups when treated with the long GnRH antagonist protocol. Patients treated with r-LH had a higher pregnancy rate per cycle start (P=0.0250) and implantation rate (P=0.001) after controlling for patient age, baseline FSH and estradiol levels, antral follicle count, previous pregnancies, full term deliveries and spontaneous abortions, number of previous IVF cycles, dose of FSH and LH administered, as well as days of stimulation. Patients treated with r-LH had a trend towards a higher clinical pregnancy rate per cycle start (P=0.0649). Patients that received r-LH compared to using hMG had more oocytes collected and more embryos created, even though the r-LH group used lower doses of FSH and LH. The number of days of stimulation did not differ between the r-LH and hMG groups.

Table 2: IVF cycle characteristics and treatment outcomes

	r-LH n=65	hMG n=57	P value
Total FSH dose (IU)	3207 ± 1300	4213 ± 1576	0.0001
Days of FSH	8.7 ± 2.5	9.0 ± 1.7	0.248
Total LH dose (IU)	1332 ± 587	1938 ± 1110 (obtained through hCG activity)	0.0001
Days of LH	7.9 ± 2.5	7.8 ± 2.6	0.997
Oocytes obtained	14.4 ± 6.3	11.0 ± 5.3	0.0100
Embryos obtained	7.9 ± 4.8	6.0 ± 3.7	0.0290
Percent of ICSI cases per group	72%	78%	0.663
Pregnancy rate per cycle start	49%	27%	0.0250
Clinical pregnancy rate per cycle start	39%	25%	0.0649
Implantation rate	62%	33%	0.001

Analysis performed with stepwise logistic regression.
IVF; *In vitro* fertilization, r-LH; Recombinant human luteinizing hormone, hMG; Human menopausal gonadotropin, FSH; Follicle stimulating hormone, LH; Luteinizing hormone, and ICSI; Intracytoplasmic sperm injection.

Discussion

In this study, there were lower FSH and LH doses required for stimulation in the long GnRH stimulation cycle with r-LH compared to hMG. r-LH treated subjects had larger numbers of oocytes and embryos obtained compared to hMG treated subjects. Pregnancy rates per cycle start and implantation rates were higher for patients in the r-LH group compared to the hMG group. There was a trend for increased clinical pregnancy rate in the r-LH group; however, this did not reach statistical significance when controlling for confounders. We observed these findings even after controlling for patient age, baseline FSH and estradiol levels, antral follicle count, previous pregnancies, full term deliveries, spontaneous abortions, and previous number of IVF cycles.

A systematic review and meta-analysis by Coomarasamy et al. (3) determined that the use of gonadotropins with LH as well as with FSH activity delivered as urinary hMG was shown to be superior to the use of r-FSH alone in long GnRH down-regulation protocols. They showed that the use of hMG was associated with a 4% increase in live birth rates compared to r-FSH alone. Other potential benefits to LH activity might also exist. Weghofer et al. (8) compared patients who underwent long protocol stimulation with either r-FSH or hMG. They found an improvement of embryonic ploidy in patients stimulated by hMG. However, the importance of the source of that LH activity should be further investigated. LH mediated activity can be administered in two forms, hMG and r-LH. LH activity in hMG is primarily achieved through hCG that acts as an LH analogue. There exist theoretical problems with hMG. For example, the risk of injection of prions through this urinary derived product, which may discourage patients and physicians from its use (9). As well, *in vitro* studies have demonstrated that r-LH and hCG result in different gene activation of the ovarian cumulous cells and endometrium (10). Therefore, r-LH may confer different beneficial effects than hMG. This difference in the endometrium may partially explain the increase in implantation seen with embryos achieved after r-LH as opposed to hMG treated cycles.

To date, few *in vivo* studies have been published. Hence, it is unclear which group of patients would benefit most from r-LH. Moro et al. (11), in a randomized controlled trial that enrolled patients over 35 years of age, found no benefit to r-LH

over highly purified hMG. A study conducted in the McGill Reproductive Center observed no benefits between subjects with extremely poor ovarian reserve (baseline follicle counts less than 6) who received r-LH and r-FSH compared to subjects who received hMG. However, r-LH was found to be advantageous in terms of pregnancy and clinical pregnancy rates compared to hMG in patients with good ovarian reserve. No distinction was made between the different IVF protocols used (long agonist versus microdose flair) (6). A study by Requena et al. (12) compared endocrine profiles of 50 oocyte donors that received either r-LH plus r-FSH together or hMG and urinary FSH. Although there were more oocytes retrieved in the r-LH plus r-FSH group, a lower proportion were in metaphase II. Serum steroid levels did not differ on the day of triggering. In recipients, the implantation and ongoing pregnancy rates were the same in both groups (46.1%). However, as the recipients were not subject to LH stimulation, the difference observed between these results and the current study could be related to the possibility that LH might have a beneficial effect at the level of the endometrium as well. Conversely, a multicenter randomized controlled trial performed in Italy assessed outcomes for two groups of patients who underwent IVF using a down-regulation protocol. The first group received r-FSH plus r-LH, whereas the second group only received urinary hMG. Both groups had the same pregnancy and implantation rates. A lower cost for the IVF cycle was noted in the hMG group, as they used less FSH (13).

Our data suggests that r-LH might be beneficial compared to hCG in terms of LH mediated activity in long GnRH agonist cycles. This was a retrospective study, hence, further studies should be undertaken to confirm these results. It would have been interesting to compare follitropin-alpha and r-LH versus hMG and follitropin-alpha. The number of patients treated with this protocol was too small for comparison and should be reassessed in future studies.

Conclusion

FSH plus r-LH may offer benefit compared to FSH plus hMG for ovarian stimulation in long GnRH-agonist protocols performed in good responders. This may occur through different stimulation of the ovarian cumulous cells or endometrium. Further studies, both larger and prospective, are needed to confirm these results.

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Original Article

Vitrification of Human Germinal Vesicle Oocytes: before or after *In Vitro* Maturation?

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Abstract

Background: The use of immature oocytes derived from stimulated cycles could be of great importance, particularly for urgent fertility preservation cases. The current study aimed to determine whether *in vitro* maturation (IVM) was more successful before or after vitrification of these oocytes.

Materials and Methods: This prospective study was performed in a private *in vitro* fertilization (IVF) center. We collected 318 germinal vesicle (GV) oocytes from 104 stimulated oocyte donation cycles. Oocytes were divided into two groups according to whether vitrification was applied at the GV stage (group 1) or *in vitro* matured to the metaphase II (MII) stage and then vitrified (group 2). In the control group (group 3), oocytes were *in vitro* matured without vitrification. In all three groups, we assessed survival rate after warming, maturation rate, and MII-spindle/chromosome configurations. The chi-square test was used to compare rates between the three groups. Statistical significance was defined at $P<0.05$ and we used Bonferroni criterion to assess statistical significance regarding the various pairs of groups. The Statistical Package for the Social Sciences version 17.0 was used to perform statistical analysis.

Results: There was no significant difference in the survival rate after vitrification and warming of GV (93.5%) and MII oocytes (90.8%). A significantly higher maturation rate occurred when IVM was performed before vitrification (82.9%) compared to after vitrification (51%). There was no significant difference in the incidence of normal spindle/chromosome configurations among warmed oocytes matured *in vitro* before (50.0%) or after (41.2%) vitrification. However, a higher incidence of normal spindle/chromosome configurations existed in the *in vitro* matured oocytes which were not subjected to vitrification (fresh oocytes, 77.9%).

Conclusion: In stimulated cycles, vitrification of *in vitro* matured MII oocytes rather than GV oocytes seems to be more efficient. This approach needs to be verified in non-stimulated fertility preservation cases.

Keywords: Vitrification, *In Vitro* Maturation, Meiotic Spindle, Fertility Preservation

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Introduction

The advent of vitrification in clinical practice has opened new possibilities and options for oocyte cryopreservation. Currently, oocyte vitrification is widely used in most *in vitro* fertilization (IVF) laboratories as a routine procedure (1-3). The high survival rates that follow warming have made oocyte banking feasible for heterologous use, fertility preservation for social reasons, in cases where women face the dangers of premature ovarian failure, as well as prior to chemo- or radiotherapeutic treatments (4-6). Vitrification of oocytes may easily serve as a rescue plan in IVF cycles when the male partner is not able to produce a semen sample at the time of oocyte retrieval. Under the above circumstances, a number of immature oocytes are often retrieved from a stimulated cycle. The majority of collected oocytes from *in vitro* maturation (IVM) cycles are immature and at the germinal vesicle (GV) stage (7). GV oocytes collected for fertility preservation in women who need an urgent onset of chemo- or radiotherapeutic treatments may be of great value since they can be either matured and cryopreserved or fertilized and cultured *in vitro* (8, 9). In such cases, the question is whether oocytes should be vitrified before or after IVM in order to maintain the highest developmental competence (10, 11).

Published data show conflicting results, mostly due to the multiple steps of this procedure and different outcomes of each step. Egerszegi et al. (12) have applied vitrification on GV or *in vitro* matured metaphase II (MII) pig oocytes. They reported a higher survival rate after vitrification-warming for MII compared to GV oocytes. However, both groups had similar maturation rates; most importantly, the GV vitrified group had better spindle configuration and F-actin integrity. The study concluded that vitrification at the GV stage was more advantageous in terms of cleavage and blastocyst formation compared to the *in vitro* matured MII oocytes. On the other hand, many studies that compared vitrification of immature GV oocytes and *in vitro* matured MII oocytes concluded that poor maturation and low fertilization rates were major problems associated with the vitrification of GV oocytes (13-18). It is widely accepted that oocytes which fail to mature *in vivo* under ovarian gonadotropin stimulation and human chorionic gonadotrophin (hCG) trigger are intrinsically abnormal. They present a high incidence of aneuploidies and low developmental competence compared to oocytes that have reached the MII stage at the moment of retrieval (19-22). Additionally, the probability of denuded GV oocytes to mature *in vitro* after vitrification and warming is significantly reduced due to the loss of oocyte-cumulus cell communication (23).

In the present study, the recruitment of immature oocytes from stimulated cycles of oocyte donors represented an experimental model that used easily available material and aimed towards the configuration of a methodology to rescue immature oocytes of significant value in natural or IVM cycles. We sought to investigate whether IVM might be more successful before or after vitrification. GV stage oocytes derived from stimulated cycles were subjected to IVM before or after vitrification/warming and monitored in terms of survival rate, maturation rate, and the status of spindle/chromosome configuration. The control group consisted of non-vitrified GV oocytes matured *in vitro*.

Materials and Methods

We conducted this prospective study from January 2013 until September 2014 in a private assisted reproduction unit. The Institutional Review Board (ref. no. 9/2012, 14 November, 2012) approved this study and we obtained informed consents from all couples that received eggs from their dedicated donors. The IVF Unit computerized database contains all patient characteristics as well as parameters related with stimulation protocol and gamete handling. These data are regularly recorded and revalidated on a monthly basis by specialized personnel in order to maintain data reliability. GV oocytes were obtained from controlled ovarian hyperstimulation oocyte donation cycles. Oocyte donors had a mean age of 26 ± 2 years. We randomly allocated the GV oocytes into three groups. Group 1 (GV vitrification) immature oocytes were first vitrified, subsequently warmed and matured *in vitro*. Group 2 (MII vitrification) immature oocytes underwent IVM and those that reached the MII stage were subsequently vitrified and warmed. Group 3 (no vitrification) GV oocytes were placed in maturation medium and served as the control group. We assessed MII spindle configuration by immunostaining in all three groups. MII oocytes that presented with partial or complete disorganization of the spindle poles or complete absence of the meiotic spindle were characterized as abnormal.

Ovarian stimulation and oocyte retrieval

We used a fixed 6-day gonadotropin-releasing hor-

mon (GnRH)-antagonist protocol (Orgalutran 0.25 mg, Organon) with 225 IU/day of recombinant follicle stimulation hormone (FSH, Puregon, Organon) that started on day 2 of the cycle for ovarian stimulation (19). The daily dose of recombinant FSH was adjusted according to the donor's ovarian response based on serum estradiol concentrations and the number and size of ovarian follicles. hCG (10000 IU, Pregnyl, Organon) was administered when three or more follicles >17 mm in mean diameter were present on ultrasound and a serum estradiol concentration of >1500 pg/ml. Donors at risk of hyperstimulation received an additional dose of GnRH-antagonist on the day before hCG administration (24). The retrieved oocytes were incubated for 2 hours in equilibrated Quinn's Advantage Fertilization (HTF, Sage, Copper Surgical, USA) supplemented with 5% human serum albumin (HSA, Sage, Copper Surgical, USA) at 37ºC and 6% CO_2. Afterwards, they were denuded by using 80 IU/ml hyaluronidase solution (Sage, Copper Surgical, USA).

In vitro maturation

We placed the GV oocytes in maturation medium (Sage, Copper Surgical, USA) supplemented with 75 mIU/ml FSH, 75 mIU/ml luteinizing hormone (LH), and 10% serum substitute supplement (SSS, Sage, Copper Surgical, USA) for 24-48 hours after oocyte denudation. Next, we determined oocyte maturation by the presence of the first polar body. The total time of incubation after oocyte retrieval was 26 hours until the first control and 50 hours until the second control, or 62 and 86 hours post hCG administration, respectively.

Oocyte cryopreservation

We used a closed carrier system (VitriSafe, VitriMed, Austria) for oocyte vitrification (25). All chemical substances were purchased by Sigma-Aldrich unless otherwise mentioned. A mixture of dimethyl sulphoxide (DMSO) and ethylene glycol (EG) was used in dilutions of 1.25%/1.25%, 2.5%/2.5%, 5%/5% and 10%/10% for the respective equilibration steps (ES1 to ES4) and 20%/20% for the vitrification step (VS), supplemented with 100 mg of Ficoll and 0.5 mol/l sucrose. For all solutions, the basal medium consisted of Quinn's Advantage Medium w/HEPES (Sage, Copper Surgical, USA) supplemented with 20% of SSS (Sage, Copper Surgical, USA). Briefly, the oocytes were placed for 3 minutes in each 50 µl ES1, ES2, and ES3 microdrop and for 6 minutes in a microdrop of ES4. Subsequently, oocytes were placed in a VS drop (100 µl) for approximately 60 seconds, which included the time needed to load the oocytes on the carrier, enclose them in a protective straw, and seal and plunge the oocytes into liquid nitrogen. During the cryopreservation steps, all solutions were maintained at room temperature (22ºC).

Oocyte warming

We warmed the oocytes by serially placing them into sequential step down sucrose solutions (1 mol/l, 0.75 mol/l, 0.5 mol/l, 0.25 mol/l, and 0.125 mol/l) in Quinn's Advantage Medium w/HEPES (Sage, Copper Surgical, USA) supplemented with 20% SSS (Sage, Copper Surgical, USA). Briefly, we cut the upper part of the protective straw and removed VitriSafe using an extractor tool. The tip of the carrier was immediately immersed in 1 ml of the 1 mol/l sucrose solution at 37ºC. After 1 minute, we placed the oocytes in 0.75 mol/l sucrose for an additional 1 minute, followed by 2 minutes in 0.5 mol/l, 2 minutes in 0.25 mol/l, and 1 minute in 0.125 mol/l sucrose. These steps were performed at room temperature. The oocytes that survived after warming with no signs of cytoplasmic degeneration or zona damage were cultured either in IVM medium (GV oocytes) or in Quinn's Advantage Fertilization (HTF) for MII oocytes (Sage, Copper Surgical, USA).

Spindle chromosome configuration analysis

Oocyte immunostaining was performed according to Chatzimeletiou et al. (26, 27) using a primary rat monoclonal antibody specific for α-tubulin (AbD Serotec, Oxford, UK) to visualize microtubules in combination with 4,6-diamidino-2-phenylindole (DAPI) to visualize DNA. Briefly, all oocytes were rapidly fixed in ice cold methanol, washed in Ca^{++}/Mg^{++}-free phosphate-buffered saline (PBS, Gibco BRL) that contained 2% bovine serum albumin (BSA), transferred into 10 ml drops of the primary antibody under mineral oil, and incubated at 4ºC for 1 hour. The oocytes were subsequently washed in PBS/BSA and transferred to 10 µl drops of the secondary antibody, which was highly cross-adsorbed Alexa Fluor 488 or 594 conjugates (Invitrogen, USA) that contained 1 ng/ml DAPI. After a one-hour incubation in the secondary antibody, the oocytes were washed in PBS/BSA, mounted on slides in

Vectashield antifade medium (Vector Laboratories, CA, USA) under a coverslip, and examined using a fluorescence microscope (Zeiss Axioskop) and/or laser scanning confocal microscope (Leica TCS-SP). We classified spindle abnormalities according to criteria previously described by Chatzimeletiou et al. (28). A spindle with barrel shaped poles and with chromosomes aligned at the equator was classified as normal. A spindle with one or two poorly defined or apparently absent poles, generally with misaligned chromosomes was classified as having an abnormal shape. Spindles with more than two poles were classified as multipolar. Finally, any chromosomes not aligned with the other chromosomes on the spindle were classified as lagging chromosomes which might occasionally result in chromosome loss.

Primary and secondary outcomes

Primary outcomes included overall maturation rate, normal spindle configuration rate, and survival rate after warming. The maturation rate 62 hours after hCG administration was considered a secondary outcome. We defined the survival rate as the ratio of oocytes that survived after warming to the overall number of vitrified oocytes. The overall maturation rate was defined as the ratio of the number of final MII oocytes to the number of oocytes that underwent IVM. The maturation rate after 62 hours was defined as the ratio of MII 62 hours after hCG to the overall number of oocytes that underwent IVM. Finally, the normal spindle configuration rate was defined as the ratio of oocytes that had a normal spindle after IVM to the total number of oocytes that survived in each group.

Statistical analysis

The categorical variables were expressed as percentages. We used the chi-square test to compare rates between the three groups. Statistical significance was defined at $P<0.05$ while Bonferroni criterion was used to assess statistical significance regarding the various group pairs (group 1 versus group 2, group 1 versus group 3, group 2 versus group 3). Odds ratios (OR) with 95% confidence intervals (CI) were estimated for comparisons between pairs of groups.

Results

In this study, there were 318 oocytes (107 in group 1, 105 in group 2, and 106 in group 3). We compared the survival rate after warming between groups 1 and 2 as no vitrification was included in the protocol for group 3 oocytes. There was no significant difference between the two groups. The survival rate was 93.5% for group 1 (100/107 cases) and 90.8% for group 2 (79/87 cases, P=0.487, Table 1). Maturation rate significantly differed in groups 2 and 3 versus group 1. The overall maturation rate was 82.9% for group 2 (87/105) versus 51% for group 1 (51/100, OR: 4.6, 95% CI: 2.4-8.8, P<0.001). Similarly, the overall maturation rate significantly differed between groups 3 (81.1%) and 1 (51%, P<0.001). No significant difference existed between groups 2 and 3 (P=0.736, Table 1).

The maturation rate 62 hours after hCG significantly differed (P<0.001) among the study groups with 27.0% for group 1 (n=27), 60.0% for group 2 (n=63), and 67% for group 3 (n=71). Group 2 had a 4-fold increase compared to group 1 (OR: 4.06, 95% CI: 2.25-7.31, P<0.001), whereas group 3 had a 5-fold increase compared to group 1 (OR: 5.49, 95% CI: 3.01-9.98, P<0.001). There was no significant difference observed between groups 2 and 3 (P=0.529). Group 3 had a significantly different normal spindle configuration rate of 77.9% (67/86 cases) compared to 41.2% for group 1 (21/51 cases, P<0.001) and 50.0% for group 2 (35/79 cases, P<0.001). However, no significant difference existed between vitrification groups 1 (41.2%) and 2 (50.0%, P=0.797, Figs.1, 2).

Table 1: Main outcomes in the study groups

	Group 1 n=107	Group 2 n=105	Group 3 n=106	P value
Survival rate	93.5% (100/107)	90.8% (79/87)		0.487
Overall maturation rate	51% (51/100)	82.9% (87/105)	81.1% (86/106)	<0.001*
Maturation rate 62 hours after hCG	27% (27/100)	60% (63/105)	67% (71/106)	<0.001*
Normal spindle configuration	41.2% (21/51)	50.0% (35/79)	77.9% (67/86)	<0.001#

*; Statistical significance of the differences between group 1 versus group 2 and group 1 versus group 3 and #; Statistical significance of the difference between group 3 versus group 1 and group 3 versus group 2.

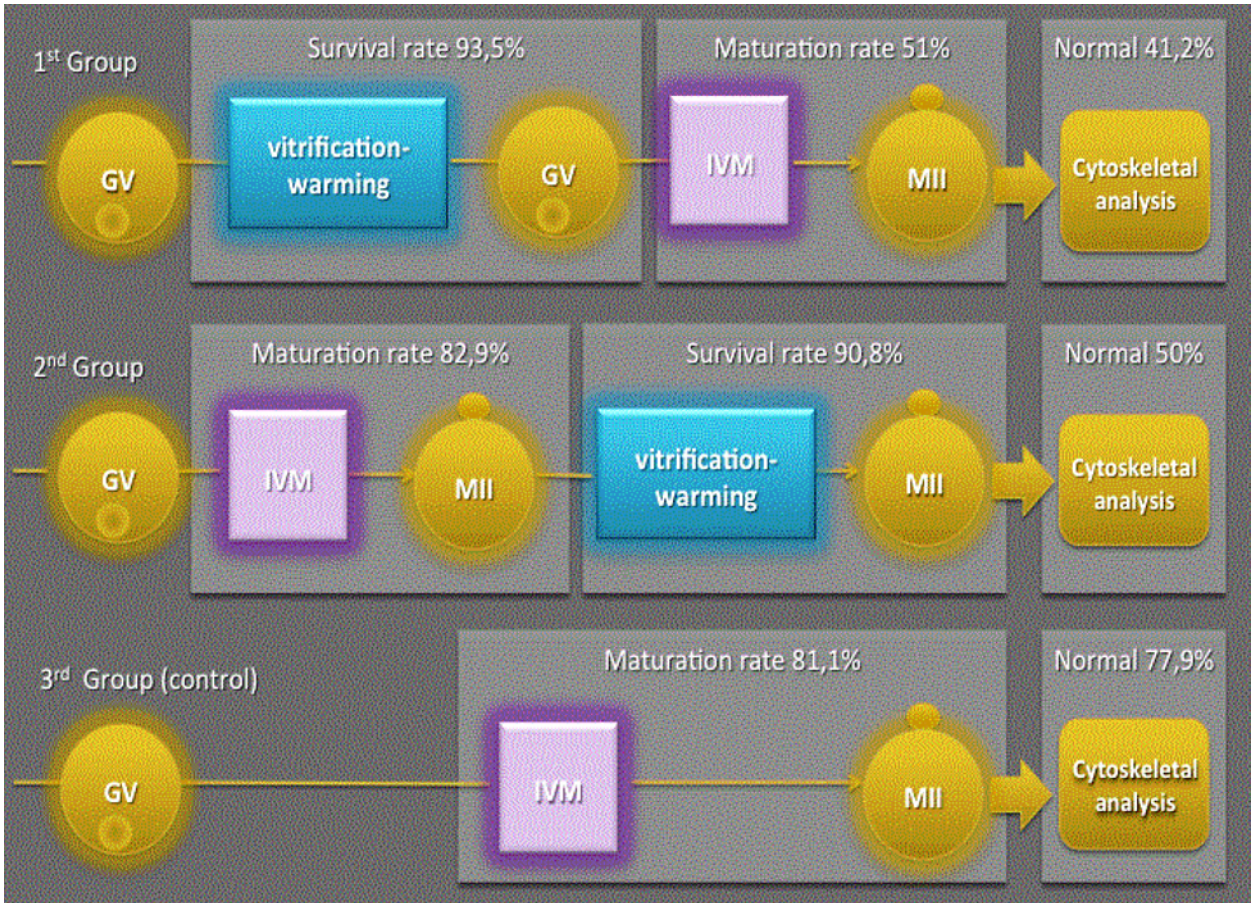

Fig.1: Graphical illustration of primary outcomes for the three study groups. GV; Germinal vesicle, IVM; *In vitro* maturation, and MII; Metaphase II.

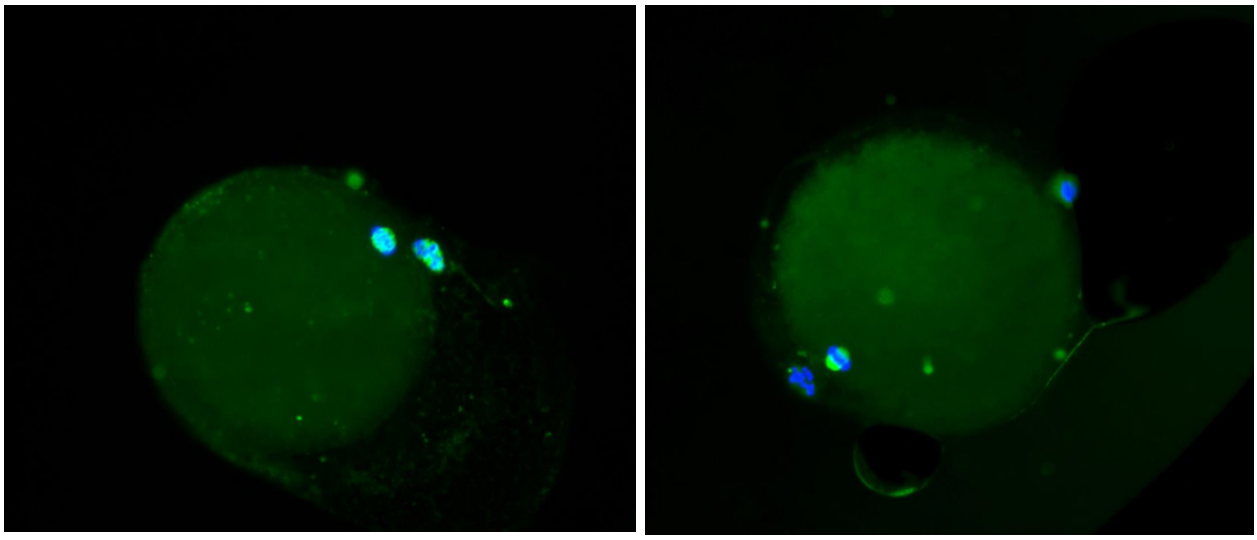

Fig.2: Spindle chromosome configuration in oocytes after *in vitro* maturation (IVM). **A.** Germinal vesicle (GV) oocyte cryopreserved and subsequently matured *in vitro* to metaphase II (MII) following warming. Note the normal spindle formation and **B.** GV oocyte cryopreserved and warmed following IVM. Note the normal spindle formation [α-tubulin stained is green and DNA labeling with 4,6-diamidino-2-phenylindole (DAPI) is blue].

Discussion

Results from previous studies indicate that vitrification of GV oocytes from IVM cycles is a feasible option (29-31). Various proposed protocols aim to optimize vitrification to keep synchronized the processess of nuclear and cytoplasmic oocyte maturation and maintain further, normal embryo development (32, 33). The immature oocytes assessed in the present study have been retrieved from stimulated cycles and were, by definition, of low developmental potential (34). However, they could still be used as a research model to investigate the influence of vitrification on immature human oocytes.

Our results, being in accordance with recent published data (10, 35), showed no significant difference in the survival rate of immature oocytes between vitrification at the GV (93.5%) and the MII (90.8%) stages, even with a closed vitrification protocol. However, the maturation process appeared to be compromised when intercepted by the vitrification/warming step. GV oocytes vitrified before IVM had a significantly lower maturation rate (51%) compared to oocytes that were *in vitro* matured before vitrification of note, approximately half of the GV oocytes that matured after vitrification needed a longer culture time in maturation media in order to reach the MII stage compared to those that underwent IVM before vitrification. These findings agreed with results from a similar study by Wang et al. (36). Data from both studies did not verify the hypothesis and general belief that the most appropriate time to vitrify oocytes would be at the GV rather than MII stage (12, 37). Although, at the GV stage, the chromatin is diffused in the diplotene state of prophase I and well protected by the nuclear membrane, which suggests that these oocytes are less vulnerable to the risk of chromosome missegregations. This study has demonstrated that GV vitrification significantly compromised the progress of oocyte maturation and rate of fertilization (6, 36).

The cooling and warming procedure seems to affect spindle/chromosome configurations regardless of the developmental stage of the vitrified oocytes (38-40). The cytoskeleton is quite sensitive to environmental changes, such as temperature, during *in vitro* manipulations (40). In the present study, abnormal spindle configurations have been equally found in oocytes from vitrification groups 1 and 2. Both groups presented a higher rate of abnormal configurations compared to the control group with no vitrification.

On the other hand, Hosseini et al. (41) reported that nucleo-cytoplasmic interactions which supported early embryonic development could be damaged during vitrification. Cytoplasmic, rather than nuclear insufficiencies, were generally the major cause of low developmental competence of embryos derived from vitrified oocytes. Lei et al. (42) provided evidence that the low developmental competence of vitrified *in vitro* matured oocytes might be attributed to a mitochondrial membrane dysfunction. The authors reported no significant change at the meiotic spindle configuration after vitrification, which conflicted with results of the present study and those reported by Chian et al. (43) who observed that vitrification was related with abnormal spindle and chromosome configurations in the warmed oocytes. Huang et al. (35) compared *in vitro* and *in vivo* matured mouse oocytes. They stated that the poor development of embryos after combined IVM and vitrification might be related to the rate of DNA fragmentation and toxicity of the cryoprotectants, but not to the chromosomal abnormalities. These researchers observed no increase in aneuploidies in the vitrification group. However, Vanderzwalmen et al. (44) reported that the intracellular concentration of cryoprotectants (and toxicity) during vitrification was much lower than the cryoprotectant concentration present in the vitrification solution and the intracellular concentration during slow freezing. Chatzimeletiou et al. (28) demonstrated that following vitrification, spindle abnormalities in human embryos increased. These abnormalities were mainly due to dehydration and mechanical stress sustained by the cells and not cryoprotectant toxicity. However, DNA repair mechanisms might be activated to rescue both oocytes and embryos following vitrification (45).

Despite the fact that embryos derived from *in vitro* matured, vitrified oocytes might be of lower developmental competence, they possibly provide a person's unique opportunity to fecundity. This attribute has tremendously increased their value

and the need to be processed in the best possible way. However, due to the lack of experience on utilization of embryos produced from such oocytes, extensive counseling and prenatal genetic testing should be considered.

Conclusion

The results of the present study indicated that in terms of survival rate, vitrification of either *in vitro* matured or GV oocytes was equally successful. However, the maturation process of the GV oocytes after vitrification and warming appeared to be compromised. Oocyte vitrification for fertility preservation would likely be more efficient if applied on *in vitro* matured MII oocytes. The present experimental data should be verified by further studies, preferably on non-stimulated cycles where most oocytes are collected at the GV stage.

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Original Article

Comparative Stepwise Pattern of Reactive Oxygen Species Production during *In Vitro* Development of Fertilized and Nuclear Transferred Goat Embryos

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Abstract

Background: A unique feature of embryo metabolism is production of reactive oxygen species (ROS). It is well established that during *in vitro* culture, ROS levels increase over normal ranges observed for embryos developed *in vivo*. This study evaluates and compares the stepwise pattern of ROS production during *in vitro* development of reconstructed goat embryos produced by zona-free method of somatic cell nuclear transfer (SCNT). Furthermore, the pattern of ROS production of SCNT embryos were compared with zona free embryos derived from *in vitro* fertilization (IVF).

Materials and Methods: In this experimental study, zona-free oocytes, SCNT and IVF embryos at different stages of *in vitro* development (2, 4, 8, 16-cells, morula, and blastocyst) were used for assessment of ROS production using 2, 7-dichloro dihydroflourescein diacetate (DCHFDA) probe and the result were presented as fold increase or decrease relative zona free oocytes.

Results: The relative level of ROS compared to metaphase-II (MII) oocytes insignificantly decrease during early stages post embryo reconstitution and regained its value by 8-cell and morula stage and, significantly increase compared to MII oocytes by blastocyst stage.

Conclusion: The pattern of ROS change in SCNT embryos is similar to zona free IVF derived embryos, except it decrease from two cell stage and regain its value at morula stage. The sudden rise in ROS at blastocyst stage, further emphasizes the special need of IVF and SCNT derived embryos during this stage of development.

Keywords: Somatic Cell Nuclear Transfer, Reactive Oxygen Species, Goat

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Introduction

The efficiency of blastocyst derived from *in vitro* embryo production (IVP) is around 30-40%, (1) and the birth rate per embryos transferred is around 35-45% in domestic animals (2), despite great advances achieved in this field, during the past two decades. These rates of *in vitro* and *in vivo* developmental competence are almost invariably lower for embryos produced by somatic cell nuclear transfer (SCNT) technique, probably because, SCNT embryos have altered gene expression and metabolism due to improper epigenetic reprogramming (3).

Through optimized of zona free SCNT procedure

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in goat, we were able to reach a cloning efficiency (live birth) of 28.6% per transfer or 6.9% per embryo transfer and live birth of 21.42% per transfer or 5.2% per embryo transfer (4) which is substantially higher than those previously reported in the literature for goat (5, 6). Despite high cloning efficiency obtain in this approach; we believe there is still room to further improvement. One approach is to increase reprogramming efficiency at epigenetic level (7) and an alternative approach is to improve the intervening techniques in zona free SCNT like oocyte maturation, activation protocols, single versus group culture, and culture condition (8).

Literature study reveal that across all the species studied, including goat, composition of embryo culture media have profound effects on *in vitro* and *in vivo* embryo development (8). In regard to this, a characteristic feature of embryos produced *in vitro*, is high production of reactive oxygen species (ROS) (9, 10) and goat is not of any exception, which to our knowledge has not been so far studied. During *in vitro* embryo culture, ROS level increases in a cell cycle dependent manner compared to the *in vivo* embryos at similar stages (9, 11). SCNT is a more complicated process in which oocyte is exposed to various media and high degrees of *in vitro* manipulation which may adversely affect oxidation-reduction (REDOX) state of developing embryo, a situation so called oxidative stress (11). Therefore, it is important to understand how different method of embryo production [*in vitro* fertilization (IVF vs. SCNT)] can effect ROS generation.

The association between increased generations of ROS with zygote genomic activation (ZGA), also known as maternal to embryo transition, was first reported by Nasr-Esfahani and Johnson (9) in mice as an *in vitro* effect which it is well established that early embryonic block, and the rise in generation of ROS during IVP are also maternally derived and are independent of paternal contribution (10). During SCNT, maternal chromosome is totally replaced with diploid nuclei of a somatic cell without any contribution by sperm. Therefore, it is interesting to know, how the absence of maternal and paternal chromosomes and presence of somatic cell nuclei affect pattern of ROS generation in developing SCNT embryos. Such information may reveal light on how cytoplasm may regulate production of ROS and may also help investigators to understand if, and what extent, antioxidant compounds could be used to improve the efficiency of SCNT reconstructed embryos. In farm animals, Dalvit et al. (12) and Ostad Hosseini et al. (13) studied patterns of ROS production during *in vitro* development of cattle and sheep embryos, respectively. But, there is no study on pattern of ROS production during *in vitro* development of goat IVF or SCNT embryos. Therefore, the aim of this study was to develop and compare patterns of ROS production during different stages of pre-implantation of zona free IVF and zona free SCNT embryos in goat.

Materials and Methods

This study was approved by the Ethical Committee of Royan Institute. In this experimental study, unless otherwise stated, all chemicals and media used in the present study were obtained from Sigma (St. Louis, MO, USA) and Gibco (Life Technologies, Rockville, MD, USA), respectively.

Oocyte *in vitro* maturation

Abattoir-derived ovaries were used for oocyte *in vitro* maturation (IVM) as described previously (4). In brief, cumulus oocyte complexes (COCs) were aspirated from antral follicles and cultured in maturation medium comprised of tissue culture medium199 (TCM199) supplemented with 10% fetal calf serum (FCS), Na-pyruvate (2.5 mM), L-glutamine (1 mM), penicillin (100 IU/mL), streptomycin (100 mg/mL), follicle-stimulating hormone (FSH, 10 mg/mL), luteinizing hormone (LH, 10 mg/mL), estradiol-17β (1 mg/mL), cysteamine (0.1 mM), epidermal growth factor (EGF, 100 ng/mL) plus insulin-like growth factor (IGF, 100 ng/mL) for 20-22 hours under mineral oil at 38.5°C, 5% CO_2, and maximum humidity.

Donor cell preparation

Ear biopsy of a healthy pre-pubertal female goat was taken, cut into 2-3 mm^2 fragments and cultured as explants in Dulbecco's Modified Eagle Medium F-12 (DMEM/F-12) containing 10% FCS and antibiotic (1% penicillin-streptomycin) at 37°C, 5% CO_2 in air. Cell started to shed out of the explants. Eventually, these cells proliferate to forms a confluent monolayer within 2-3 weeks. Obtained cells were used for investigation of fibroblast phenotype using differential immunostaining with anti-vimen-

tin (for fibroblasts) and pan-cytokeratin (for epithelial cells) antibodies (7). Confirmed fibroblasts at passages 3-5 were used for SCNT experiments. In order to provide a synchronized population of G0/G1, cells were first cultured at 2.5×104 cells/cm^2, and at the next day, the cells were washed thrice with phosphate buffer saline (PBS) before being cultured in medium that contained 0.5% FCS for 4-5 days. Serum starved cells were subsequently trypsinized and used for SCNT procedure.

Somatic cell nuclear transfer procedure

In vitro matured oocytes were denuded by vortexing in presence of 300 IU/mL hyalorunidase. Only good quality oocytes with homogenous cytoplasm and extruded first polar body were used for the experiments. The process of zona free enucleation was carried out as described previously by Nasr-Esfahani et al. (4). In brief, zona was removed by brief enzymatic digestion [5 mg pronase in 1 mL of Hepes-TCM199 (HTCM) for 1 minute] followed by incubation in TCM199 free of pronase and containing 20% FCS to neutralize the remaining enzyme. It has been demonstrated that goat matured oocytes revealed a cytoplasmic extrusion cone which is clearly visible upon zona removal (14). This extrusion is considered as a hallmark of MII spindle during enucleation. The cytoplasmic extrusion was gently aspirated into a 2-3 µm pipette and with a gentle touch against the blind needle (5-10 µm), the MII extrusion was separated from the oocyte. Successful enucleation was confirmed by staining the separated MII extrusion with H33342 (5 µg/ml, 5 minutes). During this procedure enucleated oocyte are not exposed to UV.

Nuclear transfer (NT) was carried out according to Hosseini et al. (15). In brief, individual fibroblasts were adhered to oocytes in medium containing 10 mg/ml phytohemagglutinin. Subsequently, the couplets were electrofused in 290 mOsm fusion buffer [0.3 M Mannitol, 100 µM $MgSO_4$, 50 µM $CaCl_2$, 500 µM hepes, 0.05% bovine serum albumin (BSA)]. The reconstructed oocytes were rested for 0.5 hours before being activated using ionomycin (5 µM, 1 minut) followed by incubation with 2 mM 6-DMAP for 2 hours. Reconstituted-activated oocytes were then cultured in groups of five to seven in modified synthetic oviductal fluid (mSOF) under mineral oil at 38.5°C, 5% CO_2, 5% O_2 and humidified air for 7 days in 20 µl wells.

***In vitro* fertilization procedure**

According to Forouzanfar et al. (16), matured COCs were washed in fertilization medium and groups of 20-25 COCs were transferred into 100 µl droplets of fertilization medium under mineral oil. Five straws of frozen spermatozoa were thawed at 37ºC for 1 minute, pooled and washed through Pure Sperm (Nidacon, Gothenburg, Sweden) gradient (40 and 80%) to separate the motile spermatozoa from the immotile by centrifugation [700 g for 15 minutes at room temperature (RT)]. Matured COCs were inseminated with a final concentration of two million sperm per ml. The inseminated COCs were incubated for 22 hours in 5% CO_2 in humidified air at 38.5°C. Twenty-two hours after insemination, cumulus cells attached to oocytes were mechanically removed via pipetting. Then, the zona was removed by brief enzymatic digestion as described above. The presumptive zygotes were then cultured in groups of five to seven as described for SCNT embryos.

Reactive oxygen species measurement

The process of ROS measurement was as described previously (13). In brief, stock solutions of 2, 7-dichloro dihydroflourescein diacetate (DCH-FDA, Sigma D6883, 5 mM) were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C in dark. For each experiment, 5 µM working solution were prepared by dilution in TCM199 containing 1 mg/ mL poly vinyl alcohol (PVA). To measure ROS levels, 15-30 embryos per replicate derived from zona free IVF or SCNT were pooled from different stages of embryo development (2-cells, 4-cells, 5-8-cells, and greater than 8-cells, morula and expanded blastocysts). Zona free metaphase-II (MII) oocytes were also simultaneously assessed. Samples were incubated with 5 µM of DCFHDA in TCM199 for 30 minutes in incubator. Samples were then washed in TCM199, placed in 5 µl droplets covered by mineral oil and then immediately exposed to UV light of a fluorescent microscope (Olympus, IX71, Japan) and observed using filter sets (excitation wavelength: 450-490 nm, emission wavelength: 515-565 nm). Digital images of individual oocyte or embryo were taken with a high sensitive camera (DP-72, Olympus, Japan). Background, positive and negative controls were taken to account for fluorescence or inter-experimental variations. Fluorescent intensity of each taken im-

age was assessed by Image J (National Institute of Mental Health, Bethesda, MD, USA). To reduce variations and possible errors, when comparison between different groups was required, experiments were designed so that oocytes and embryos from each group were available for assessment at the same period. To further minimize inter-experimental variation, the relative fluorescence intensity of each embryonic stage to the mean intensity of MII-oocytes in the same experiment was calculated according to the below formula:

The relative intensity is defined as the difference in the intensity of embryos from the mean intensity of MII-oocytes/mean intensity of MII-oocytes. It is important to note that for assessment of ROS at each embryonic stage at least three replicates were carried out. For each replicate at least 30 to 80 embryos and 55 to 95 oocytes were assessed.

Statistical analysis

Percentages data were transformed by ArcSin and analyzed by one way ANOVA model of SPSS version 17 (SPSS, Science, Chicago, IL, USA). Differences were compared by the Tukey multiple comparison post hoc test. All data are expressed as mean ± SEM and differences were considered as significant at $P<0.05$.

Results

Reactive oxygen species measurement

Figure 1 shows fluorescence images of goat MII-oocyte, zona free SCNT and IVF embryos at different stages of pre-implantation embryo development following staining with DCFHDA for ROS measurement. As depicted, irrespective of embryo production method, fluorescence intensity increased as the embryo progressed toward blastocyst stage.

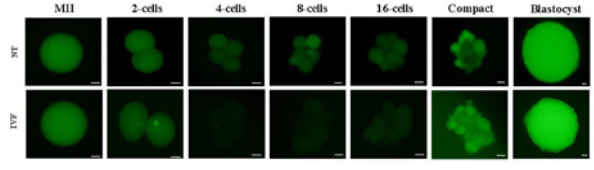

Fig.1: Representative fluorescence images of goat zona free MII-oocyte, IVF and SCNT-derived embryos at different stages of preimplantation embryo development. Oocytes were stain with DCFHDA for ROS measurement. Bar represents 25 µm.
MII; Metaphase-II, IVF; *In vitro* fertilization, SCNT; Somatic cell nuclear transfer, DCFHDA; 2, 7-dichloro dihydroflourescein diacetate, ROS; Reactive oxygen species, and NT; Nuclear transfer.

Figure 2 shows the mean relative intensities of embryos relative to MII-oocytes at different stages of development in zona free IVF and SCNT derived embryos. As shown, in zona free IVF embryos, the mean relative ROS levels significantly decreased at 4 and 8- cell stages relative to the mean intensity of MII-oocytes and then began to rise by 16 cell stage which resulted in a significant increases by compact and blastocyst stages relative to all the earlier stages. Moreover, the relative increase at the blastocyst stage was significantly higher than compact embryos.

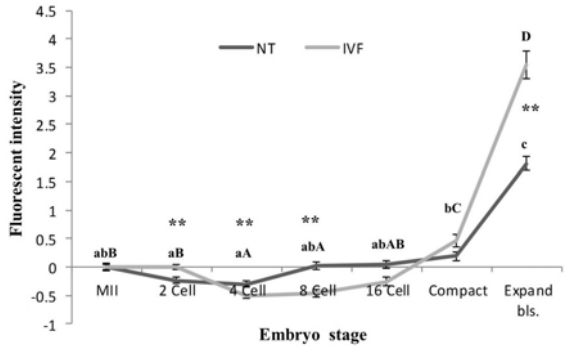

Fig.2. Comparative analysis of the mean ROS level of MII oocytes, zona free IVF and SCNT embryos assessed using the DCHFDA probe. ROS; Reactive oxygen species, MII; Metaphase-II, IVF; *In vitro* fertilization, SCNT; Somatic cell nuclear transfer, DCFHDA; 2, 7-dichloro dihydroflourescein diacetate, NT; Nuclear transfer, a-c; Different letters showed significant differences within the NT derived embryos, A-D; Different letters showed significant differences within the IVF derived embryo, **; In each embryo stage showed significant differences between NT and IVF groups.

The trend of ROS production in SCNT embryos appear to follow the same trend as those of zona free IVF embryos. In zona free IVF embryos the decrement in ROS occur after 2-cell stage while in zona free SCNT reconstructs, the decrements being at earlier stage (post reconstruction). Subsequently, the increment in ROS production in zona free IVF embryos begin at around 8-cell stage while the increment in zona free SCNT embryo begin at around 4-cell stage. Therefore, despite similar trend of ROS production, a significant difference between the two groups were observed at 2- (lower in SCNT group), 4- (higher in SCNT group) and 8- (higher in SCNT group) cell stages. A significant difference was also observed at blastocyst stage. The degree of ROS production was significantly higher in blastocyst derived from zona free IVF embryos in comparison to zona free SCNT reconstructs.

During this study, unlike the zona free IVF embryos, in some of the SCNT embryos one or more blastomeres showed higher fluorescence intensity compared to other blastomeres (Fig.3).

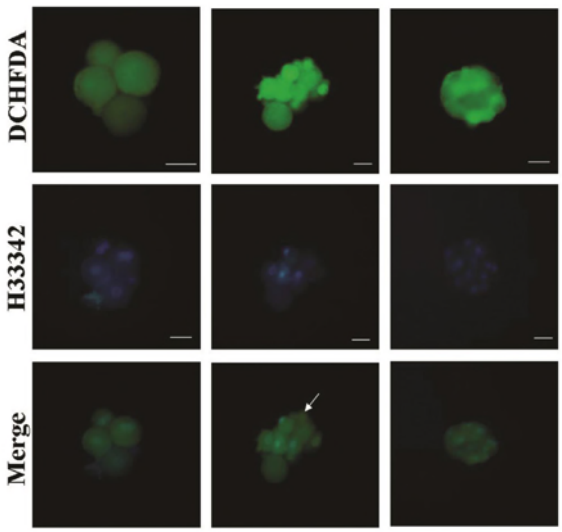

Fig.3: Confirmation of presence of nuclei in cloned embryos. During the SCNT procdure, some embryos may become fragmented and providing blastomeres without nuclei. Therefore, combined staining with nuclear dye (H33342) and DCHFDA was carried out to investigate this phenomenon. As show, except for one blastomere without nucleus (arrow), all the other blastomeres with high intensity for DCHFDA had nuclei. Bar represents 50 µm. SCNT; Somatic cell nuclear transfer and DCFHDA; 2, 7-dichloro dihydroflourescein diacetate.

Also, it has been reported that some blastomeres, due to asymmetrical division, are non-nucleated. In order to understand if this phenomenon has any relation to intensive ROS levels within blastomeres, the SCNT embryos with non-uniformed ROS staining were also stained with viable chromatin dye (H33342, 5 µg/ml for 5 minutes) and no relation was observed between ROS intensity with presence or absence of chromatin in each blastomeres. As shown in Figure 3, except for one blastomere without nucleus (arrow), all the other blastomeres with high ROS intensity had nuclei.

Discussion

The results of this study showed that the relative ROS production in zona free IVF derived embryos decreased following fertilization, began to rise at around zygote genomic transition (ZGA) which occur around 8-16 cell stage (17), and substantially increased from compaction to the blastocyst stage. The overall trend of ROS pattern in developing SCNT embryos was similar to zona free IVF embryos, except for the time of ROS raise that apparently took place at earlier stages (4-8 cell stage) in SCNT embryos. The increase in relative ROS production around the ZGA in consistent with the previous reports in other species [mice: Nasr-Esfahani and Johnson (9)], [bovine: Dalvit et al. (12)], [sheep: Ostad Hosseini et al. (13)]. During ZGA maternal stores of RNA become gradually depletion and embryo begins to rely on its own genome transcription. The earlier raise of ROS in SCNT embryos might be related to difference in mRNA clearance, mitochondrial activation, or the depletion of antioxidant capacity (like glutathione (GSH) content) during SCNT or genomic reprogramming which needs further investigations (18).

Although, the pattern of ROS production is species-specific, it has been established that the stage of ZGA in developing embryos of mice and other animals investigated so far, coincides with a sharp increase in ROS level (19, 20). In this regard, a number of studies have shown that antioxidant supplementation of culture medium, particularly around the peak of ROS production, improves developmental competence of embryos (13, 21-23), thus suggesting a link between REDOX state and ZGA arrest embryos. In agreement, it is frequently reported that when first embryonic division commences, majority of the cleaved embryos may progressed to the stage which coincides with ZGA irrespective of their initial quality. Therefore, arrest around the ZGA period is considered the bottleneck of *in vitro* embryo development (18). In this sense, this study for the first time in the goats show that the increase in ROS production also occurs around the ZGA stage, and therefore, supplementation of antioxidant around 8 to 16 -cells stages and after that when ROS level substantially increases, may improve *in vitro* development of goat embryos.

In accordance with previous reports in other species, (9, 12, 13), we also observed a substantial raise in ROS at compaction and blastocyst stage in both groups. This rise in ROS production, is very likely to be related to a switch from anaerobic to aerobic glycolysis, since the ATP production becomes dependent on Krebs cycle after ZGA while before this stage ATP production is mainly dependent on glycolysis. The degree of ROS production was significantly higher in IVF compared to SCNT derived blastocysts and this

is likely due to higher quality and metabolic activity of IVF derived embryos, but this conclusion needs further validation. Another interesting observation in this study was the higher ROS production in some nucleated blastomeres of SCNT embryos which was rarely seen in IVF derived embryos. The reason for this difference remains to be elucidated.

Conclusion

This study for the first time described the pattern of ROS production in reconstructed embryo derived from SCNT procedure and in zona free goat embryo. The results showed two major time points of increased ROS production. The first raise in ROS production was observed during ZGA and the second raise took place during the period of blastocyst formation. These results may emphasize the special need of SCNT and zona free IVF derived embryos to external source of antioxidants during these two critical stages of development which in turn may affect the efficiency of embryo production from these two techniques.

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Original Article

Kisspeptin: A Potential Factor for Unexplained Infertility and Impaired Embryo Implantation

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Abstract

Background: Kisspeptin (KP) is a neuropeptide that causes the release of the gonadotropin releasing hormone, which controls hypothalamo pituitary ovarian axis and exerts a number of peripheral effects on reproductive organs. The primary objective of this study was to compare baseline KP levels in females with different types of infertility and identify possible correlations with risk of failure to conceive, preclinical abortion and pregnancy after intracytoplasmic sperm injection (ICSI).

Materials and Methods: A longitudinal cohort study was carried out from August 2014 until May 2015 by recruiting 124 female patients undergoing ICSI, after obtaining ethical approval from the Australian Concept Infertility Medical Center. Cause of infertility due to male, female and unexplained factors was at a frequency of 32 (24%), 33 (31%) and 59 (45%) among the individuals respectively. KP levels were measured by ELISA assay before the initiation of the ICSI treatment protocol. Outcome of ICSI was categorized into three groups of non-pregnant with beta-human chorionic gonadotropin (β-hCG)<5-25 mIU/ml, preclinical abortion with β-hCG>25 mIU/ml and no cardiac activity, and clinical pregnancy declared upon confirmation of cardiac activity. Results based on cause of infertility and outcome groups were analyzed by one-way ANOVA.

Results: Females with unexplained infertility had significantly lower levels of KP when compared with those with male factor infertility (176.69 ± 5.03 vs. 397.6 ± 58.2, P=0.001). Clinical pregnancy was observed in 28 (23%) females of which 17 (71%) had a female cause of infertility. In the non-pregnant group of 66 (53%) females, common cause of infertility was unexplained 56(85%). A weak positive correlation of KP levels with fertilized oocytes and endometrial thickness was observed (P=0.04 and 0.01 respectively).

Conclusion: Deficiency of KP in females with unexplained infertility was associated with reduced chances of implantation after ICSI.

Keywords: Infertility, Ovarian Stimulation, Intracytoplasmic Sperm Injection, Kisspeptin

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Introduction

Infertility has been recognized as a disease that requires timely diagnosis, recognition and treatment in terms of assisted reproduction. The prevalence of infertility ranges from 7 to 20% in populations across the globe (1). In 30% of infertile couples, male factors are diagnosed as the cause of infertility (2). Regarding females, structural causes of infertility include tubal damage due to pelvic inflammatory disease, endometriosis and

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uterine anomalies (3). The dysfunction of the hypothalamic pituitary ovarian axis (HPO) is also thought to be a key player resulting in infertility in many such situations. However, infertility in the presence of normal semen parameters, ovulatory concentration of serum progesterone in mid-luteal phase, tubal patency and a normal uterine cavity is often diagnosed as unexplained infertility (4). Assisted reproductive technology (ART) comprise *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) with the latter shown to have a higher success rate (5). With both ARTs, higher failure is observed in couples with unexplained infertility as compared with other infertile subgroups, suggesting a need to explore other key factors that may play a role even in the absence of known abnormalities (6). Furthermore, studies linking unexplained infertility with higher rate of failure to implant after ART highlight the role of interleukins and pro-inflammatory factors in assisting the invasion of the uterine wall (7). In general, the subfertile population is at a greater risk of preclinical abortion. Likewise, its frequency prevails between 30 and 50% of cases who receive ART. Both decreased receptivity of the uterus and chromosomal abnormality in the fetuses are thought to trigger this response, however, other factors that may lead to loss of pregnancy between the time of implantation and onset of menses still remains unknown (8).

Recently, the role of serum kisspeptin (KP) in regulating HPO axis and maturity of oocytes has been aptly investigated (9). KP, an RF-amide peptide coded by the *KISS1* gene, was initially believed to be a tumor suppressor gene. The KP receptor, previously known as GPR54, plays an important role in maintaining fertility in both human and other animal species (10). About 40% of gonadotrophin releasing hormone (GnRH) neurons express the GPR54 receptors (11). Neurons located in the hypothalamus secrete KP, thus triggering KP receptors (GPR54) on the GnRH neurons and leading to the secretion of GnRH (9). Besides this, KP is triggered at the onset of puberty and relays information about energy stores of the body to the central nervous system (CNS) by modulating negative and positive feedback of gonadal steroids. Aside from its wide distribution in the brain, KP receptors are highly expressed in the pancreas, placenta, uterus, small intestine, kidney, lung, liver and heart (12). Studies have also suggested other roles for Kisspeptin such as the emergence of KP as a key regulator of the central mammalian reproductive axis along with its role in placentation and pregnancy. This has lead to the exploration of its probable therapeutic role in treating certain forms of infertility (9, 13). We therefore aimed to study the levels of baseline serum KP in infertile females with various types of infertility and examine whether it correlates with the risk of failure to conceive, preclinical abortion and pregnancy after assisted reproduction by ICSI.

Materials and Methods

A longitudinal cohort study was carried out from August 2014 to May 2015, after Ethical approval was obtained from the Australian Concept Infertility Medical Center. One hundred and twenty-four female patients were recruited after receiving their written informed consent to participate in the study. Females were between 20 and 40 years of age (mean of 32.16 ± 4.8 years), had a mean body mass index (BMI) of 24.19 ± 2.3 kg/m^2, were segregated into three groups on the basis of their infertility and all were recommended for ICSI. Cause of infertility due to male, female and unexplained factors was at a frequency of 32 (26%), 33 (27%) and 59 (47%) respectively. The first group consisted of those with a male factor infertility (e.g varicocele, prior surgeries and semen abnormalities). The second group comprised females with diagnoses of uterine fibroids (n=2), endometriosis (n=20) and tubal blockade (n=11). The third group comprised 59 patients with unexplained infertility. Females with metabolic disorder (polycystic ovaries) and endocrine disorders such as thyroid dysfunction and abnormal prolactin levels were excluded from the study. Couples diagnosed with both male and female infertility factors were excluded from the study.

Serum samples were collected on the second day of the menstrual cycle before commencement of down-regulation of ovaries. KP was measured using the KISS-1-ELISA Kit (Shanghai, China). The analytical sensitivity of the KP kit was 10.16 ng/L and intra- and inter-assay coefficients of variation was less than 10 and 12% respectively.

Treatment protocol

Down-regulation of ovaries by 1 mg of subcutaneous buserelin acetate (Suprefact, Sanofi) on day 21 of previous menstrual cycle was followed by controlled ovarian stimulation with Gonal-f (Merck Sereno) from the second day of periods. Confirmation of maturity of follicles to ≥18 mm in diameter was assessed by transvaginal scan (TVS) and cycles were cancelled when follicles failed to develop in response to gonadotropin stimulation. Endometrial thickness was gauged on the day of ovulation induction in the mid sagittal plane by two-dimensional ultrasound with a 7.5-MHz vaginal probe (Hitachi EUB 525, Hitachi, Japan). Oocytes were retrieved from mature follicles (20 mm in diameter), 36 ± 1 hours after injection of human chorionic gonadotropin (hCG, Ovitrelle 250), on the 14th, 15th or 16th day of stimulation. Semen samples were retrieved by masturbation and sperms were then immobilized by 7% polyvinyl pyrrolidone after which microinjection was performed (Leica DMIRB, Leica Microsystems, Wetzlar, Germany). Finally, the microinjected oocytes were incubated for 16-18 hours at 37°C, 6% CO_2 and 5% O_2.

Outcome measure

Patients were categorized on the basis of β-hCG concentrations on the 14th day after egg collection and on the basis of sonographic evidence of an intrauterine gestational sac 14 days after β-hCG measurement. Females with beta hCG<25 mIU/ml were declared as non-pregnant, pre-clinical abortion was labelled on a beta hCG>25 m IU/ml but with no cardiac activity on ultrasound while clinical pregnancy was confirmed based on higher levels of beta hCG along with intrauterine gestational sac and cardiac activity on TVS. The implantation rate (IR) was calculated as the number of pregnancies per embryo transferred (14), clinical pregnancy (CP) was established by the presence of an intrauterine gestational sac confirmed by TVS per number of patients at the start of cycle (15).

Statistical analysis

Data were analyzed by SPSS version 21 (IBM statistics, Chicago, IL) and was expressed as mean ± SD/standard error of mean wherever appropriate. Analyses were undertaken for the whole group and independently in sub-groups according to the infertility factor. Variation in KP levels in females with different types of infertility and different outcomes after ICSI was compared by Analysis of variance (ANOVA) and Tukey's Post-hoc test. Pearson's correlation coefficient was used to test correlation between KP levels and the study variables. In all cases a P<0.05 was considered significant.

Results

Table 1 summarises the characteristics of the study population with respect to type of infertility. Significant difference in KP levels amongst these groups was observed (P<0.001). Post-hoc analyses of KP levels revealed significantly reduced levels in female and unexplained infertility factor sub-groups when compared with those with a male factor infertility (P=0.035 and P<0.001, respectively).

Table 1: Clinical variables in different types of infertility

Variable	Male infertility n= 32	Female infertility n=33	Unexplained infertility n=59
Kisspeptin (ng/L)	397.6 ± 58.2*^	257.11 ± 24.4*	176.69 ± 5.03
No of oocytes fertilized	5.8 ± 0.2	6.02 ± 0.22	5.83 ± 0.16
Endometrial thickness (mm)	8.3 ± 0.6	9.90 ± 3.2*	6.74 ± 0.36
No of transferred embryos	1.5 ± 0.1*	1.37 ± 0.11*	1.83 ± 0.06
Number of gestational sacs	0.3 ± 0.1^	0.75 ± 0.13*	0.102 ± 0.05
Implantation rate	19.27 ± 6.35	44.9 ± 6.74	5.08 ± 2.88

Blood collected on the second day of cycle before initiation of stimulation.
Data expressed as mean ± SD, except kisspeptin levels expressed as mean ± SEM.
Results compared by One-Way ANOVA, significant difference (P<0.05) on Post-hoc analysis with unexplained infertility is expressed as * while that between male infertility and female infertility factors is expressed as ^.

Table 2: Stratification of outcomes based on type of infertility

Variable	Male infertility			Female infertility			Unexplained infertility		
	Non pregnant n=6	Preclinical abortion n=18	Clinical pregnancy n=8	Non pregnant n=4	Preclinical abortion n=12	Clinical pregnancy n=17	Non pregnant n=56	Preclinical abortion n=0	Clinical pregnancy n=3
Serum KP (ng/L)	352.8 ± 15.9	384.2 ± 38.0	461.1 ± 43.0*	163.1 ± 21.5	164.9 ± 2.0	203.3 ± 12.0*	171.2 ± 3.6	0 (0%)	278.9 ± 24.9*
Oocyte maturation rate	81.8 ± 11.5	93.4 ± 3.7	89.4 ± 5.4	100 ± 0.0	98.4 ± 1.0	97.7 ± 1.0	75.9 ± 1.7	0 (0%)	85.4 ± 0.6
Fertilization rate	63.9 ± 8.7	79.7 ± 2.7	74.1 ± 5.0	82.0 ± 1.6	83.0 ± 1.4	81.3 ± 1.2	59.9 ± 1.4	0 (0%)	76.9 ± 0.5
Implantation rate	0	0	77.0 ± 8.8	0	0	87.2 ± 5.0	0	0 (0%)	3

Data expressed as mean ± SD, except Kisspeptin (KP) levels which are expressed as mean ± SEM. Highest level of KP was observed in clinical pregnancy group in all stratifications (expressed as *).

After ICSI, 66 (53%) patients failed to conceive, 30 (24%) reported with preclinical abortions while 28 (23%) were confirmed as clinically pregnant. Mean ± SEM values of KP were 87.24 ± 14.94 ng/L in non-pregnant females and 215.11 ± 34.14 ng/L in pre-clinical abortion while it was 296.23 ± 12 ng/L in patients with clinical pregnancy (Fig.1).

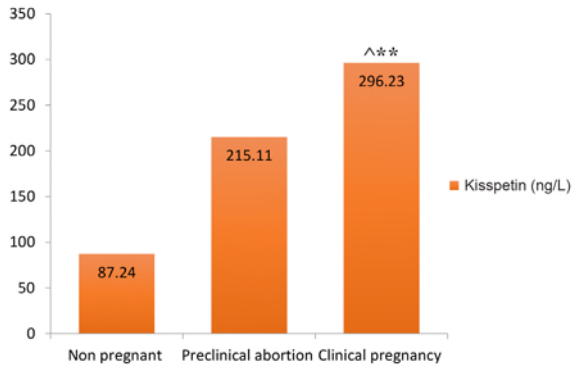

Fig.1: Mean levels of Kisspeptin (ng/L) in groups segregated on the bases of ICSI outcomes.
ICSI; Intracytoplasmic sperm injection, **; Clinical pregnancy group versus non-pregnant P<0.001, and ^; Clinical pregnancy versus pre-clinical abortion P<0.05. Error bars represent SEM.

Although variation in KP levels between non-pregnant and pre-clinical abortion groups was marginally significant (P=0.044), it was found to be highly significant between non-pregnant and clinically pregnant females (P<0.001). The outcomes of ICSI treatment based on type of infertility are presented in Table 2. Highest number of clinical pregnancies was achieved in the group of infertile females with diagnosed cause of infertility (51%), however, only 5% of patients with unexplained infertility got pregnant. A weak but significant positive correlation were observed between KP levels and number of fertilized oocytes (r=0.18, P=0.04) and thickness of the endometrium (r=0.27, P=0.01).

Discussion

With the advancement of ART, couples have been able to conceive, nevertheless, the outcome is associated with a limited success rate (14). The treatment procedures are equally offered to all females suffering from known and unknown factors that disrupt effective functioning of reproductive organs. We observed the lowest levels of KP in females who had unexplained infertility which is consistent with the hypothesis that mutation in the 5′ untranslated region (UTR) (deletion, rs5780218) in Exon 1 of KISS1 may disrupt HPO axis, reduce KP levels and likely to be one of the reasons of unexplained infertility (16). Cytokines play an active role in folliculogenesis, ovulation, fertilization and implantation (17). KP is known to work as a pro-inflammatory cytokine alongside tumor necrosis factor α and regulates trophoblast cell invasion, leading to implantation (18, 19). Many studies have linked unexplained infertility with low implantation rates while highlighting the role of interleukins and pro-inflammatory factors in helping the invasion of the uterine wall (7). In our study, reduced KP levels in females with unexplained infertility was associated with inadequate egg maturation, reduced fertilization, thinner endometrial lining and failure of implantation of the blastocyst. This is probably the basis by which injection of a single dose of KP-54 triggers egg maturation, fertilization and implantation of blastocyst in infertility treatment procedures (20, 21).

Fayazi et al. (22) discovered the presence of a KP/KISS1R signaling pathway in the uterus of 4-day pregnant mouse with a slight expression of KP signaling in the uterus of non-pregnant mice. KP expression thus has a maintenance basal level in peripheral reproductive organs, which is up-regulated at the initiation of pregnancy. A low level of KP in non-pregnant females in our study emphasizes its role in placentation and implantation. This observation is in accordance with the researchers previous work with the difference that KP was estimated after the suppression of HPO axis (23). Preclinical abortion after ICSI is subject to a number of factors including the etiology of infertility (8). The preclinical abortion was observed in 24% of the study population, which mainly comprised females who had uterine fibroids, endometriosis and tubal blockade. These results are contradictory to those in studies that observed similar pregnancy loss with different types of infertility (24). We nonetheless observed that women with preclinical abortions had a higher KP level than patients who failed to conceive. This may be explained by the existence of a circadian KP expression in a full-term human placenta of healthy women that regulates trophoblastic invasion and probably pregnancy maintenance (25).

During the menstrual cycle, secretory changes in the endometrium account for coordinated signal exchange between hormonally primed endometrium and functional embryo for the implantation of embryo. This is made possible by the interplay of hormones and cytokines, and studies have demonstrated that increased endometrial thickness is associated with higher pregnancy rates (26). A cut-off value of 8mm thickness was considered to be optimal for embryo implantation after ICSI in the female population of Pakistan (17). We observed that a thicker endometrial lining was associated with a higher implantation rate when compared with females who could not conceive at all. Moreover, thickness of the endometrium was at its lowest in patients with unexplained infertility, who also had minimum levels of serum KP.

The prevalence of infertile couples with unexplained factors is estimated at 30% (27). To the best of our knowledge, this is the first study that supports association of reduced KP in unexplained infertile females with reduced fertilization of oocytes, implantation of blastocyst and hence conception. Improving the success rate after IVF and ICSI for couples with unexplained infertility is thus what further work should be aimed at. This study is uni-centric, has a small sample size and included infertile couples with both male and female factors, which all may bias the observed correlation between KP levels and unexplained infertility. In addition, we did not stratify the male infertility factor based on sperm parameters including count, motility and morphology. This may be a confounding factor for low implantation rate in females with diagnosed male infertility factor.

Conclusion

Low level of KP in females with unexplained infertility before the initiation of the ICSI protocol was seen. One of the factors causing unexplained infertility may thus be low KP levels. The level of KP has an impact on fertilization of oocytes, preparation of endometrial beds for implantation of embryo and hence successful pregnancy after ICSI. This observation urges the need of further studies at genetic and molecular levels to define and explain the role of KP for preservation of conception and continuation of pregnancy in females with unexplained infertility.

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Original Article

Evaluation of Relationship between Body Mass Index with *Vitamin D Receptor* Gene Expression and Vitamin D Levels of Follicular Fluid in Overweight Patients with Polycystic Ovary Syndrome

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Abstract

Background: Polycystic ovary syndrome (PCOS) is the most common endocrine disorder associated with reproductive disorders and metabolic dysfunctions including insulin resistance. The roles of vitamin D in the regulation of metabolic modulations specifically involving insulin and reproduction processing are introduced. In addition, obesity appears to be closely associated with severity of PCOS. The present study is to evaluate the effect of body mass index (BMI) on vitamin D levels in follicular fluid and *vitamin D receptor* (*VDR*) expression levels in granulos cells.

Materials and Methods: A comparative study was conducted on 80 women with average age of 20-35 years referred for *in vitro* fertilization (IVF). Patients were divided into four groups, and serum levels of testosterone and insulin resistance (IR) were evaluated at the puncture time. Also, vitamin D levels of follicular fluid were evaluated. *VDR* gene expression was assayed by quantified-polymerase chain reaction (PCR) technique. Correlations were evaluated with calculation of the Spearman coefficient, and also independent relationships were assessed by means of multiple regression analysis.

Results: Vitamin D levels of follicular fluid decreased in PCOS patients compared with non-PCOS. Also, over-weight individuals had lower vitamin D levels compared with normal-weight patients. Vitamin D levels of follicular fluid were highly correlated with BMI ($r=-0.51$, $P<0.01$). Homeostatic model assessment-IR (HOMA-IR) values were significantly higher in women of PCOS/overweight and PCOS/normal weight in comparison with women of non-PCOS/normal weight ($P<0.01$). The gene expression data of *VDR* in granulosa cells were significantly lower in the PCOS/overweight group compared with the non-PCOS/normal weight ($P<0.01$).

Conclusion: The findings indicated significant differences in *VDR* gene expression in granulosa cells and vitamin D of follicular fluid in PCOS/overweight patients.

Keywords: Polycystic Ovary Syndrome, Vitamin D, Granulosa Cells, *Vitamin D Receptor*, Follicular Fluid

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Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder that can affect 8% to 10% of women in their reproductive age (1, 2). PCOS is associated not only with reproductive disorders, but also with significantly increased risks of metabolic dysfunctions, including insulin resistance (IR) (3), dyslipidemia (4), systemic inflammation (5), increased oxidative stress (6), and endothelial dysfunction (7). It can be noticed that PCOS, as a heterogeneous androgen excess disorder with varying degrees of reproductive and metabolic abnormalities, is determined by the interaction of multiple genetic and environmental factors (8).

Several studies have revealed that certain metabolic disturbances such as IR and hyperinsulinemia are majour defects in the majority of PCOS patients (9, 10). Emerging data characterize serious roles for vitamin D in biological processes, including regulation of cellular growth (11), differentiation, and metabolic modulations specifically involving insulin action (12, 13). Among the many physiologic processes influenced by vitamin D, serious roles in reproductive physiology are submitted (14). Biological actions of vitamin D are intermediated through *vitamin D receptor* (*VDR*) gene expression which is a member of the steroid/thyroid nuclear hormone receptor superfamily, displayed in calcium-regulating tissues, intestines (12), the skeleton (15), parathyroid glands (16), and reproductive tissues including ovary, uterus, placenta (17), testis (18), and granulosa cells (19, 20).

Additionally, investigations of animals have confirmed the role of calcium in oocyte maturation and its impact on the resumption and progression of follicular development. Furthermore, disturbances in calcium regulation can be responsible for follicular arrest (21). Previous study have suggested the functions of vitamin D in reproduction (22). They have also indicated that *VDR* regulates more than 3% of the human genome, including genes that are crucial for glucose metabolism. *VDR* is a transcription factor regulating the transcription of other downstream genes in many tissues that are crucial for glucose metabolism (23, 24). On the other hand, calcium fluxes and regulation of intracellular calcium stores are essential in the regulation of insulin secretion by b-cells. Therefore, vitamin D and *VDR* gene are important factors in calcium regulation and control of b-cell functions, respectively. This is further supported by the fact that low vitamin D levels are associated with IR and that they can induce type-2 diabetes in PCOS patients (25). However, the exact mechanisms underlying the association of vitamin D and IR are not fully understood. One complication of PCOS is obesity which appears to be closely associated with severity of the disease phenotype (26).

In Iran, more than half of the patients with PCOS are either overweight or obese (27). It is well known that obesity influences the phenotypic expression of PCOS and might play a significant role in the pathophysiology of hyperandrogenism, severity of insulin resistance, and also chronic anovulation (28). Increased adiposity is associated with several abnormalities of sex steroid metabolism and results in increased androgen production and suppression of sex hormone binding globulin (SHBG) (29). Thus, obesity may affect vitamin D levels in healthy women and PCOS patients. The present study aimed to evaluate the effect of obesity on vitamin D levels in follicular fluid and *VDR* expression levels in granulosa cells. The results of this research may contribute to the diagnosis and treatment of overweight PCOS patients.

Materials and Methods

The current comparative study was carried out on 80 women of 20 to 35 years old and who referred to Alzahra-Hospital of Tabriz, Iran for *in vitro* fertilization (IVF). Before entering the study, all the patients provided written consent forms. This study was approved by the Ethics Committee of Tabriz University of Medical Sciences (code: 5/4/2781). Weight and height of all the patients were measured, and then the body mass index (BMI, Kg/m^2) was calculated by dividing weight by height square. The patients were divided into two groups based on BMI categories, using the specified criteria by the World Health Organization (WHO): the normal weight and overweight groups with the BMI of 18.5-24.9 and 25-29.9, respectively. The control group consisted of 40 non-PCOS patients (20 normal weights and 20 over-weights) who had referred for IVF due to tubal and/or male infertility or even ovulatory volunteers with normal ovaries. The PCOS group included 40 patients (20 normal weights and 20 over-weights) who had referred for IVF. These patients were recognized based on Rotterdam Consensus criteria and had at least two of the three following criteria: ovulatory disturbance,

hyerandrogenism, and more than twelve 2 to 9 mm follicles in each ovary. The exclusion criteria were as follows: history of menstrual disorders such as cycle length either less than 25 days or more than 35 days, patients with other endocrine disorders or neoplastic causes of hyperandrogenemia such as androgen-secreting tumors (serum testosterone levels above 0.6 ng/mL), congenital adrenal hyperplasia, and Cushing's syndrome.

Ovarian stimulation

For pituitary down-regulation, the patients were treated with 0.5-1 mg subcutaneous (SC) injection of gonadotropin releasing hormone (GnRH) agonist (Lucrin, Abbott Pharmaceuticals, Kurnell, Australia), depending on the age of every woman. When at least three follicles reached a diameter of almost 17 mm and the levels of peripheral plasma estradiol concentrations were ≥3 nmol/l, 5000-IU of human chorionic gonadotropin (hCG, Profasi, Serono, Aubonne, Switzerland) was given as a single IM injection. Thirty six hours after hCG administration, the oocytes were retrieved and collected with sterile Pasteur pipettes, and then the remainder of the follicular fluid was poured into 50 ml sterile falcon tubes for subsequent isolation of granulosa cells. The follicular fluid was centrifuged, and vitamin D levels were measured in the supernatant. 25-OH vitamin D was estimated by chemiluminescence enzyme immunoassay (IDS, Boldon, UK). The blood samples were obtained before the operation for subsequent biochemical analyses. These samples were analyzed for follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, prolactin, insulin, and glucose levels.

Granulosa cells collection

The freshly collected follicular fluid samples were then centrifuged at 3000 rpm for 1 minute at 4°C; afterwards, 4 ml of phosphate buffer saline (PBS) was added to the pallet. After mixing, the solution was placed on 50% Percoll gradient (Amersham Pharmacia Biotech, Uppsala, Sweden). The sample was centrifuged at 700 rpm for 30 minutes to remove red blood cells. After the centrifugation, the granulosa cells were placed between PBS and Percoll solutions. The cells were harvested by gentle pipetting, and also washed several times with PBS, and used for RNA extraction and cDNA synthesis. The total RNA of the collected samples was the recommended protocol of manufacture. In brief, 1 ml of RNX plus was added to the sample in a clean RNase-free tube. The sample was homogenated via gentle up and downing and then was incubated for 5 minutes at room temperature. After adding chloroform (200 µl), the mixture was incubated at room temperature for 5 minutes and was centrifuged at 12,000 rpm for 15 minutes at 4°C. The aqueous phase containing RNA was transferred to a clean RNase-free tube. This solution was put on ice, and 500 µl of ice isopropanol was added to it; the sample was then incubated for 30 minutes at -80°C. Afterwards, the tube was centrifuged at 12,000 rpm for 15 minutes at 4°C, and the supernatant was discarded. The pellet, including the total RNA, was washed using 75% ethanol and was centrifuged at 7,500 rpm for 8 minutes. After drying ethanol, the RNA pellet was re-suspended in 50 µl or less of DEPC-treatment water. The concentration of total RNA was calculated based on OD 260/280 ratio measurements as a means to address the purity of RNA.

To confirm the integrity of the extracted RNA, it was electrophoresed. The genomic DNA was removed from the extracted RNA by adding RNase free DNase I (Thermo, Fermentase). The cDNA was synthesized with Moloney murine leukemia virus reverse-transcriptase (MMLV-RT, 200 U/µl, Sigma-Aldrich Co., UK) according to the manufacture's protocol. As soon as the RNA was isolated from the granulosa cells, the reverse transcriptase reactions were performed on all the samples to generate cDNA.

To quantify the mRNA expression levels of *VDR* gene in the granulosa cells, real-time polymerase chain reaction (RT-PCR) was performed on a Bio-Rad iQ5 system (Bio-Rad Laboratories, Hercules, USA), using EVA-Green quantitative PCR mix kit (Sinaclon, Tehran-Iran). *GAPDH* gene was used as reference standard gene for all analyses to control the amount of the synthesized cDNA. PCR reactions were carried out in triplicate for each sample, and then the mean of the three readings was taken as fold-induction value. Fold change (X) values were calculated, using $X=2^{-\Delta\Delta Ct}$ equation, in which ΔCt represents the difference between the Ct values of the target genes and the Ct values of the reference standard genes, and ΔΔCt describes the difference between ΔCt value of each sample for each target gene and the average ΔCt of the reference standard gene. The sequence of PCR primers for amplifying *VDR* and *GAPDH* genes is provided in

Table 1. IR was estimated, using the homeostatic model assessment-IR (HOMA-IR) method. In addition, HOMA-IR was calculated as the product of the fasting plasma insulin value (mU/mL) and the fasting plasma glucose value (mg/dL). Insulin levels were also estimated by ELISA kit (Siemens, Erlangen, Germany) according to the manufacture's recommendations.

Statistical analysis

All statistical procedures were run, using SPSS-16 software (SPSS Inc., Chicago, IL), and P<0.05 was considered statistically significant. Normal distribution of data was evaluated through the one-sample Kolmogorov-Smirnoff test. The comparisons of the means were performed by one-way ANOVA and the general linear model multi-variance by post-hoc analysis for pairwise comparisons. Correlations were evaluated by calculating the Spearman coefficient, and independent relationships were assessed via multiple regression analysis.

Results

Table 1 shows the variables measured in PCOS patients. The statistical Kolmogorov-Smirnoff test proved a normal distribution for the measured parameters. The multiple regression analysis was applied to examine the relationship between obesity and testosterone, HOMA-IR levels, vitamin D levels of follicular fluid, and *VDR* gene expression on gramulosa cells. The results demonstrated that vitamin D levels of follicular fluid decreased in PCOS patients and over-weight individuals compared with non-PCOS and normal-weight patients. The results also revealed that 25 OH-D levels of follicular fluid were highly correlated with BMI (r=-0.51, P<0.01). In addition, HOMA-IR values were significantly higher in the women in PCOS/overweight and PCOS/normal weight than group those in non-PCOS/normal weight group (P<0.01). However, the difference in HOMA-IR values between the women in non-PCOS/overweight and non-PCOS/normal weight group was not significant (P=0.1, Table 1).

Table 1: Applied primer sequences for quantitative polymerase chain reaction (PCR)

Gene name	Primer sequence (5´-3´)	Accession number
VDR	F: ATACCAGGATTCAGAGACCTC R: TACTTGTAGTCTTGGTTGCCAC	NM_000376.2
GAPDH	F: CGATGCGGCGGCGTTATTC R: TCTGTCAATCCTGTCCGTGTCC	NM_002046.3

Table 2: Clinical and biochemical characteristics of studied women

	PCOS/Overweight n=20	PCOS/Normal weight n=18	Non-PCOS/Overweight n=19	Non-PCOS/Normal weight n=20
Follicle number	12.7 ± 3[b]	17.2 ± 4.2[c, d]	7.2 ± 1.2	9.1 ± 2
Age (Y)	29 ± 4.9	28.1 ± 4.1	28.1 ± 2.3	28.9 ± 4.2
BMI (kg/m^2)	28.4 ± 2.7[a]	23 ± 1.9[d]	28.1 ±2.1[e]	22.5 ± 2
LH (IU/L)	7.4 ± 4.5[a, b]	9.2 ± 6.5[c, d]	4.8 ± 2.1[e]	6.5 ± 3
FSH (IU/L)	5.6 ± 1.7[b]	5.9 ± 1.7[c, d]	7.1 ± 2.5	7.2 ± 2.3
LH/FSH	1.4 ± 0.9[b]	1.6 ± 0.8[c, d]	0.9 ± 0.1	1.0 ± 0.2
Insulin (µmol/L)	24.7 ± 9.6[a]	11.6 ± 2.5[c]	19.6 ± 1.8[e]	13.7 ± 2.7
Glucose (mg/dl)	131.9 ± 30.5	111.96 ± 14.5[c]	120.46 ± 32[e]	95.4 ± 14.3
HOMA-IR	7.3 ± 1.4[a]	5.8 ± 0.9[c, d]	2.5 ± 1.1	2.3 ± 0.4
Testosterone (ng/ml)	1.9 ± 0.4	2.0 ± 0.7	1.7 ± 0.4	1.2 ± 0.5
Vitamin D (nmol/ml)	1.6 ± 0.9[a, b, c]	4.5 ± 1.7[d]	5.2 ± 1.8[e]	7.1 ± 1.3

Values are referred as mean ± SD. P<0.05 was considered statistically significant.
[a]; Significant differences between polycystic ovary syndrome (PCOS)/overweight and PCOS/normal weight, [b]; Significant differences between PCOS/overweight and Non-PCOS/overweight, [c]; Significant differences between PCOS/normal weight and Non-PCOS/normal weight, [d]; Significant differences between PCOS/normal weight and Non-PCOS/overweight, [e]; Significant differences between Non-PCOS/normal weight and Non-PCOS/overweight, BMI; Body mass index, LH; Luteinizing hormone, FSH; Follicle-stimulating hormone, and HOMA-IR; Homeostatic model assessment-insulin resistance.

Table 2 represents the correlation between age, BMI, and HOMA-IR and the number of follicles, serum testosterone levels, follicular fluid vitamin D levels, and *VDR* expression levels of granulosa cells. Furthermore, there was a considerable positive correlation between HOMA-IR and BMI (r=0.43, P<0.05). The results indicated that testosterone levels in PCOS/overweight patients were not substantially higher than those in non-PCOS/overweight patients (P=0.2). Similarly, there was not a significant correlation between BMI and testosterone (r=0.09, P>0.05, Table 3).

Table 3: Relationship between age, body mass index (BMI) and homeostatic model assessment-insulin resistance (HOMA-IR) with the studied variables

	Age	BMI	HOMA-IR
Follicle number	0.01	- 0.16	0.34
Testosterone	0.04	0.09	0.18
Vitamin D	- 0.12	- 0.51**	- 0.28
VDR	0.02	- 0.43*	- 0.41*

*; indicates significant difference from control at P<0.05 and **; Indicates significant difference from control at P<0.01.

Quantitative RT-PCR results exhibited a lower *VDR* gene expression in PCOS patients compared to the control group (Fig.1).

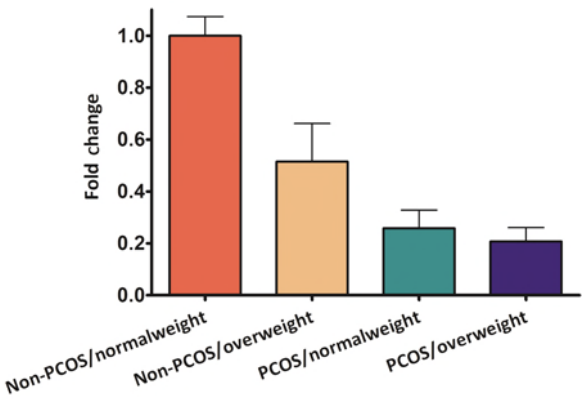

Fig.1: The fold change in gene expression patterns of *VDR* in polycystic ovary syndrome (PCOS)/overweight, PCOS/normal weight, non-PCOS/overweight, compared with non-PCOS/normal weight individuals. Expression levels are given as fold change compared with non-PCOS/normal weight samples. The error bars represent the 95% confidence intervals.

The fold change in the expression of the target gene, *VDR*, was normalized to *GAPDH* in the PCOS/overweight, PCOS/normal weight, and non-PCOS/overweight groups. Afterwards, its expression in the control group, non-PCOS/normal weight, was analyzed. Melting curve analysis confirmed the specificity of the PCR (data not shown). A negative strong correlation was found between *VDR* expression levels and BMI (r=-0.43, P<0.05), using Spearman statistical test (Table 3). The gene expression data of VDR in granulosa cells were significantly lower (three times) in PCOS/overweight group in comparison with non-PCOS/normal weight (P<0.01). The level of *VDR* expression in PCOS/overweight group was similar to that of *VDR* expression in PCOS/normal weight group.

Discussion

Two PCOS related complications are obesity and IR. Studies which show low vitamin D levels are associated with IR, and administration of vitamin D may ameliorate insulin sensitivity; however, the mechanisms of this effect are not clear (30). Moreover, studies demonstrate that insulin plays a significant role in the regulation of renal 1-a-hydroxylase activity and serum 1,25(OH) 2D3 levels in response to parathyroid hormone (PTH), while 1,25(OH) 2D3 is observed to act like a genomic stimulator of the insulin response in the control of glucose transport (31). Therefore, vitamin D may exert a positive effect on insulin action by stimulating the expression of insulin receptor, thus stimulating insulin responsiveness for glucose transport. Additionally, vitamin D responsive element is present in the promoter of the human insulin gene, and the transcription of insulin gene is activated by 1,25(OH) 2D3 (32). In accordance with the previous study, the current results confirmed that the majority of PCOS patients had vitamin D deficiency. There was a significant negative correlation between BMI increase and vitamin D levels of follicular fluid. It should be noted that the overweight PCOS individuals had lower vitamin D levels in their follicular fluid in comparison with other patients. Recent reports have indicated possible mechanisms for lower serum 25-OH-D3 associated with obesity. IR and obesity are also related to a reduction in growth hormone (GH) secretion in PCOS patients. This could be accounted for decreased levels of 1,25(OH) 2D3 because GH significantly increases renal 1-a-hydroxylase expression and, consequently, serum 1,25(OH) 2D3 concentrations (33). The findings revealed

a negative correlation between vitamin D levels of follicular fluid and IR. Although this negative correlation was not substantial, its value level was imperative. Studies have also shown that obesity has been consistently associated with vitamin D deficiency, and this fact is supported in the present study by the negative correlation of weight and BMI with vitamin D level in follicular fluid (34). Hence, obesity can affect the decline of vitamin D concentrations in PCOS patients. On the other hand, vitamin D receptors are present in the majority of body cells, such as granulosa cells, and can influence the inhibition of cell proliferation and the induction of cell differentiation. Hence, vitamin D levels of follicular fluid and vitamin D receptors in granulosa cells may play an important role in proliferation and differentiation of granulosa and theca cells (35).

The relationship between allelic variation of VDR in pancreatic island and insulin secretion and glucose tolerance indicates a role for vitamin D in the pathogenesis of IR (36). Furthermore, vitamin D administration can improve insulin sensitivity and decrease insulin level (37). Accordingly, it is logical to state that in PCOS patients with increased BMI, vitamin D levels and *VDR* gene expression decrease, but IR increases (19). The findings indicated a significant negative correlation between *VDR* gene expression and HOMA-IR, which is in agreement with previous reports (38).

Based on the recent research, 25(OH) D levels are correlated with androgen levels in men, and one might speculate on an association of vitamin D with androgen. The underlying mechanisms, however, remain to be explored. The current study showed a negative correlation between vitamin D levels of follicular fluid and serum testosterone levels. Although this negative correlation is not significant, it is imperative. Vitamin D levels and intracellular calcium stores may regulate serum androgen levels of PCOS patients. As a result, vitamin D has a biologically plausible role in female reproduction, including the regulation of insulin secretion (39), androgen synthesis (40), proliferation of granulosa cells, and oocyte differentiation (41). In the previous studies, reduced vitamin D levels of serum in PCOS individuals were introduced as a possible factor affecting the reproductive disorders and metabolic disturbances of these patients (39). Overweight or obesity is one of the most important characteristics of PCOS individuals that may be employed as a powerful predictor of decreasing vitamin D levels. Previous studies have revealed the association of vitamin D status which improved IVF outcome. Women with higher levels of 25(OH) D in serum and follicular fluid were significantly more likely to achieve clinical pregnancy following IVF. On the other hand, high vitamin D levels were significantly associated with improved parameters of controlled ovarian hyper-stimulation (42). The present study revealed that the incidence of PCOS was associated with lower vitamin D levels of follicular fluid and decreased level of *VDR* gene expression in granulosa cells, which was more dominant in the PCOS patients with obesity.

Conclusion

There are fundamental and significant differences in *VDR* gene expression in granulosa cells and vitamin D of follicular fluid in PCOS/overweight patients. Further investigations on larger sample populations are required to confirm that changes in the expression of *VDR* and vitamin D level of follicular fluid influence the development of different appearances of PCOS.

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Original Article

Tumor Necrosis Factor Alpha -308 G/A Single Nucleotide Polymorphism and Risk of Sperm Abnormalities in Iranian Males

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Abstract

Background: Signaling molecules such as cytokines regulate spermatogenesis during the maturation of germ cells and sperm apoptosis. Tumor necrosis factor alpha (TNFα) is one of the most-documented cytokines that is involved in spermatogenesis. We investigated the association of the TNFα -308 G/A single nucleotide polymorphism with sperm abnormalities in Iranian males.

Materials and Methods: This case-control study included 180 infertile men who referred to Yazd Research and Clinical Center for Infertility and 100 healthy normospermic controls. Infertile men were classified into four groups of azoospermia (n=91), oligospermia (n=26), teratospermia (n=30) and asthenoteratospermia (n=33). After sperm analysis, DNA was extracted from blood and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was carried out for the genotyping of TNFα-308 G/A.

Results: The A allele was significantly associated with sperm abnormality in our population [(P<0.001, odds ratios (OR) 95% confidence interval (CI)=2.31]. In addition, the A allele was also associated with azoospermia (P<0.001, OR (95% CI)=2.484), oligospermia (P=0.005, OR (95% CI)=2.51) and teratospemia (P<0.001, OR (95% CI)=3.385) but not with asthenoteratospermia (P=0.623).

Conclusion: Our data suggest that this single nucleotide polymorphism (SNP) maybe associated with the risk of sperm abnormality in infertile men of Iranian origin.

Keywords: Infertility, Cytokines, Tumor Necrosis Factor Alpha, Polymorphism, Restriction Fragment Length Polymorphism

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Introduction

It is estimated that about 15% of couples globally suffer from infertility. Male infertility constitutes 50% of causes among which genetic factors are mainly responsible (1, 2). Other causes of male infertility maybe related to post-testicular obstruction, endocrine dysfunction and vascular abnormalities (3). During the last decade, it has become clear that some signaling molecules that mediate the intercellular communication and integration have an important role in the hormonal regulation of germ cell maturation in testis. Cytokines are mediator molecules that are involved in this regulation and as a result have an important impact on spermatogenesis (4, 5). Among cytokines, tumor necrosis factor alpha (TNFα) is not only the

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most studied molecule but also the most potent in germ cell apoptosis, peritubular cell secretion and regulation of spermatogenesis (6). Its receptors are present in Sertoli and Leydig cells, allowing TNFα to regulate secretion from these cells (7). Some studies have shown a negative association of TNFα plasma levels with sperm motility and morphology (8, 9). The effect of TNFα on testosterone production, which has a direct impact on male infertility, has also been reported in some experimental models (10, 11).

The *TNFα* gene as a single copy gene is located on chromosome 6p21.3 within the major histocompatibility complex (MHC) gene cluster (12). Gene variation such as single nucleotide polymorphisms (SNPs) in *TNFα* gene can alter TNFα production. Several SNPs including -308 G/A, -1031 T/C, -863 C/A, -857 C/T, -575 G/A, -376 G/A, -244 G/A and -238 G/A in the promoter region of the gene have been investigated (13). The -308 G/A SNP in the promoter region of *TNFα* has been implicated to increase promoter activity, leading to an increased production of TNFα in blood (14, 15). Some studies have reported a negative association of TNFα with sperm motility and morphology (9, 16, 17). Zalata et al. (18) showed the association of the -308 G/A SNP with increased seminal caspase-9 and decreased sperm motility, count, morphology, acrosin activity and seminal a-glucosidase. Shukla et al. (19) showed that there is a strong association between this SNP and male infertility in the Indian populations of Uttar Pradesh.

In this study, given that TNFα is an important regulator of steroidogenesis and may affect spermatogenesis, we investigated the association of the TNFα -308 G/A SNP with different kinds of sperm abnormality in infertile males of Iranian origin.

Materials and Methods

This case-control study included 180 infertile males as the case group and 100 healthy normospermic individuals as the control group. The case individuals were recruited from Yazd Research and Clinical Center for Infertility from September 2012 until August 2013. They were divided based on sperm abnormality into azoospermia (n=91, AZ group), oligospermia (n=26, OL group), teratospermia (n=30, T group) and asthenoteratospermia (n=33, AT group) groups. This study was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran. Written informed consent was obtained from each individual. All semen analysis and clinical examinations were done according to the World Health Organization guidelines (20).

Tumor necrosis factor alpha -308 polymorphism genotyping

Genomic DNA was extracted from whole blood samples using the salting out method. We used the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method for genotyping of TNFα -308G/A. The F: 5'-AGGCAATAGGTTTTGAGGGCCAT-3' and R: 5'-TCCTCCCTGCTCCGATTCCG-3' primers were used to amplify a107 bp fragment of the TNFα promoter that included this SNP. PCR was carried out in a total volume of 25 µl containing 3-5 µl genomic DNA, 1 µl of each primer (10 µM) and 12.5 µl of PCR Master Mix (Cinnagen, Iran) and dH_2O. The condition of DNA amplification was an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of 94°C for 40 seconds, 60°C for 1 minute and 72°C for 40 seconds, and a final extension step at 72°C for 5 minutes and hold at 4°C. Subsequently, the PCR products were digested with NcoI restriction enzyme (14 hours at 37°C) and the specific bands were identified using 2% agarose gel electrophoresis in 1X Tris/Borate/EDTA (TBE) buffer and visualized under the ultraviolet (UV) light. When digested, the PCR fragment was cleaved into two fragments with sizes 87 bp and 20 bp.

Statistical analysis

The frequency of alleles and genotypes were compared with a 2×2 contingency table using Chi-squared and Fisher's exact test. Fisher's exact test was used when sample sizes were small in each category. We considered $P<0.05$ as a statistically significant and 95% confidence interval (CI) for calculating odds ratios (OR).We used the SPSS statistical software (version 20, SPSS Inc., Chicago, IL, USA) for all statistical analyses.

Results

In this study, PCR-RFLP was able to identify both alleles efficiently at position -308 in the promoter region of *TNFα* gene (Fig.1).

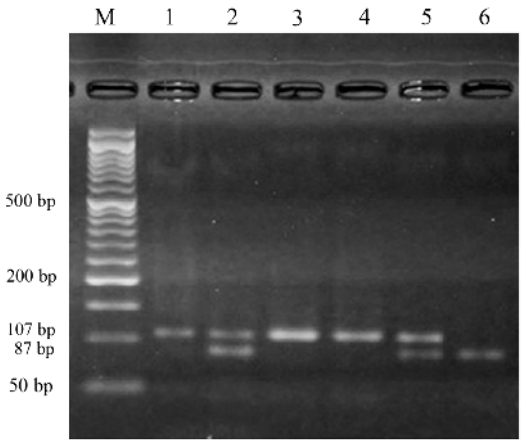

Fig.1: The results of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of *TNFα* -308 polymorphism. Lane M shows the molecular weight marker, Lanes 1, 3 and 4 show the AA genotype. Lanes 2 and 5 show the GA genotype. Lane 6 shows the GG genotype.

Table 1 shows the related parameters of each group. The frequencies of alleles and genotypes and their association with the studied group are listed in Table 2. Ancestral genotype GG and allele G were taken as reference. Statistical analysis showed that there is a significant association between this SNP and the AZ, O and T patient groups but not with the AT group. The frequency of the AA genotype was 13% in the healthy normospermic (N) group, 27.4% in the AZ group [OR (95% CI)=2.535], 26.9% in the O group [OR (95% CI) =2.97], 30% in the T group [OR (95% CI)=2.86] and 15.2% in the AT group. The AG genotype was identified in 22% of the N group, 32.9% of the AZ group [OR (95% CI)=1.74], 34.6% of the O group [OR (95% CI)=1.87], 43.3% of the T group [OR (95% CI)=2.71] and 24.2% of the AT group.

Table 1: The sperm parameters in the case group

Group	Sperm parameter	Mean ± SD
Azoospermia	Sperm count (10^6/mL)	0
Oligospermia	Sperm count (10^6/mL)	6.6 ± 2.3
Asthenoteratospermia	Motility (grades a+b)%/Morphology (% normal forms)	10.7 ± 5.8/4.7 ± 2.8
Teratospermia	Morphology (% normal forms)	6.9 ± 3.4

Table 2: The frequencies of alleles and genotypes of the -308 G/A SNP in the *TNFα* promoter in the azoospermic, teratospermic, Asthenoteratospermic and Oligospermic groups

Genotype-allele/ group	Normospermic (%) n=100	Azoospermic (%) n=91	P; OR (95% CI)	Oligospermic (%) n=26	P; OR (95% CI)
AA	13 (13%)	25 (27.4%)	0.018; 2.535 (1.2-5.3)*	7 (26.9%)	0.040; 2.97 (1.076-8.22)*
AG	22 (22%)	30 (32.9%)	0.010; 1.74 (0.916-3.20)*	9 (34.6%)	0.206; 1.87 (0.736-4.787)
GG*	65 (65%)	36 (39.5%)	Ref.	10 (38.5%)	Ref.
AA+AG	35 (35%)	55 (60%)	0.001; 2.837 (1.57-5.107)*	16 (61%)	0.024; 2.971 (1.22-7.240)*
A	48 (24%)	80 (43.9%)	<0.001; 2.484 (1.604-3.845)*	23 (44.2%)	0.005; 2.51 (1.32-4.7)*
G**	152 (76%)	102 (56%)	Ref.	29 (55.8%)	Ref.
		Teratospermic (%) n=30		**Asthenoteratospermic (%) n=33**	
AA	13 (13%)	9 (30%)	0.049; 2.86 (1.083-7.599)*	5 (15.2%)	0.772; 1.195 (0.392-3.648)
AG	22 (22%)	13 (43.3%)	0.033; 2.71 (1.143-6.428)*	8 (24.2%)	0.812; 1.135 (0.449-2.864)
GG*	65 (65%)	8 (26.7%)	Ref.	20 (60.6%)	Ref.
AA+AG	35 (35%)	22 (73%)	<0.001, 5.107 (2.061-12.657)	13 (39%)	0.679; 1.207 (0.537-2.714)
A	48 (24%)	31 (51.7%)	<0.001; 3.385 (1.85-6.17)*	18 (27.3%)	0.623; 1.18 (0.632-2.23)
G**	152 (76%)	29 (48.3%)	Ref.	48 (72.7%)	Ref.

***TNFα*; Tumor necrosis factor alpha, SNP; Single nucleotide polymorphism, OR; Odds ratios, CI; Confidence interval, *; Significant P<0.05. Ancestral genotypes GG* and alleles G** were taken as reference.**

Discussion

Genetic variation such as SNPs in *TNFα* promoter region may affect its expression. There are several studies that have investigated the association of *TNFα* SNPs with different diseases such as colorectal cancer, pre-eclampsia, prostate cancer and Crohn's disease (21, 22). The basic knowledge about the crucial role of TNFα in spermatogenesis is based on the study by Suh et al. (23) in which male TNFα knockout mice showed delayed spermatogenesis, reduced testis weight and sperm count in comparison with wild-type mice.

In this study, we analyzed the TNFα -308 G/A SNP to identify its possible association with sperm abnormality in Iranian males. To the best of our knowledge, this study has not been undertaken in Iranian males. Our findings indicate that this SNP is significantly associated with azoospermia, oligospermia and teratospermia. In other words, this SNP is among many genetic factors that may lead to a decreased count of sperm and abnormal morphology in our cases.

Similar to our study, Tronchon et al. (13) also found a positive association of the TNFα -308 A allele with oligospermia and teratospermia. Zalata et al. (18) also observed an increased frequency of TNFα -308 GG genotypes in fertile males compared with the infertile group in the Egyptian population. In the Indian population, consistently, the frequency of the AA genotype was higher in infertile individuals rather than fertile subjects, and higher level of apoptosis and necrosis levels were observed in infertile males, likely due to increased levels of reactive oxygen species (19). In contrast, Kurz et al. (24) found no association of TNFα -308 C>T and -863 C>A SNPs with sperm abnormalities (asthenozoospermia and oligo-asthenoteratozoospermia) in the Australian population. In the Greek population, Lazaros et al. (25) also found no association between -863 C>A and semen quality. The differences between the results of studies can be related to different number of studied individuals and different studied population with subgroups and ethnicities.

Based on the results of previous studies, TNFα is known to affect spermatogenesis by changing the structure of the blood-testis barrier and apical ectoplasmic specialization of Sertoli cells, which may lead to abnormal spermatogenesis (26). Moreover, it affects the Fas ligand system and germ cell apoptosis which have important roles in the germ cell maturation and normal spermatogenesis (27, 28). Binding of the TNFα molecule with its type 1 receptor activates signaling molecules in the transduction pathway, in which adaptor proteins interact with conserved death domains. The adaptor proteins increase activation of caspase-8 causing the release of cytochrome c from mitochondria. This is followed by the configuration of a high molecular weight complex (apoptotic protease activating factor-1, cytochrome C, and caspase-9) that activates caspase-3 and causes cell death (29, 30). Our study certainly has its own limitations and further association studies with more polymorphisms and individuals may provide more representative data on the association of *TNFα* variation with different sperm abnormalities in the Iranian population.

Conclusion

Our study shows that there is a positive association between TNFα -308 G/A SNP and different sperm abnormalities in the Iranian population. Given that the A allele leads to increased expression of TNFα, anti-TNFα agents could be a useful treatment for male infertility.

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Original Article

Sperm Retrieval in Patients with Klinefelter Syndrome: A Skewed Regression Model Analysis

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Abstract

Background: The most common chromosomal abnormality due to non-obstructive azoospermia (NOA) is Klinefelter syndrome (KS) which occurs in 1-1.72 out of 500-1000 male infants. The probability of retrieving sperm as the outcome could be asymmetrically different between patients with and without KS, therefore logistic regression analysis is not a well-qualified test for this type of data. This study has been designed to evaluate skewed regression model analysis for data collected from microsurgical testicular sperm extraction (micro-TESE) among azoospermic patients with and without non-mosaic KS syndrome.

Materials and Methods: This cohort study compared the micro-TESE outcome between 134 men with classic KS and 537 men with NOA and normal karyotype who were referred to Royan Institute between 2009 and 2011. In addition to our main outcome, which was sperm retrieval, we also used logistic and skewed regression analyses to compare the following demographic and hormonal factors: age, level of follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone between the two groups.

Results: A comparison of the micro-TESE between the KS and control groups showed a success rate of 28.4% (38/134) for the KS group and 22.2% (119/537) for the control group. In the KS group, a significantly difference ($P<0.001$) existed between testosterone levels for the successful sperm retrieval group (3.4 ± 0.48 mg/mL) compared to the unsuccessful sperm retrieval group (2.33 ± 0.23 mg/mL). The index for quasi Akaike information criterion (QAIC) had a goodness of fit of 74 for the skewed model which was lower than logistic regression (QAIC=85).

Conclusion: According to the results, skewed regression is more efficient in estimating sperm retrieval success when the data from patients with KS are analyzed. This finding should be investigated by conducting additional studies with different data structures.

Keywords: Klinefelter Syndrome, Sperm Retrieval, Logistic Regression

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Introduction

The most common chromosomal abnormality due to non-obstructive azoospermia (NOA) is Klinefelter syndrome (KS) which occurs in 1-1.72 out of 500-1000 male infants (1-3). This genetic abnormality can develop meiotic nondisjunction due to a 47,XXY genotype in the majority of cases (4). KS is characterized by infertility, elevated luteinizing hormone (LH) levels, elevated follicle stimulating hormone (FSH), normal or reduced testosterone, normal or increased height, muscle weakness and reduced strength, increased facial hair, osteoporosis, obesity, increased thromboembolic risk, dyslipidemia, and low glucose tolerance. In addition there are slight deficits in very specific domains of cognition, but without an increase in the occurrence of mental retardation (5). Although the typical karyotype is 47,XXY, chromosomal mosaics are specified with 46XY/47,XXY and complements with multiple X chromosomes such as 48XXXY.

It is important to evaluate the chances for sperm retrieval among KS patients and compare them to azoospermia with normal karyotype by using a model which provides more efficient results for researchers. When researchers have more efficient results they can choose the best method to handle this problem and patients may not need to undergo invasive surgeries (6). On the other hand, it can also increase the chances for success and decrease patient expenses. Logistic and probit regressions have already been applied to analyze binary outcome data. The models are fit using a symmetric distribution function. However, the use of such distributions may give biased results in some cases. The purpose of this study is to apply the skewed regression model, as a new regression model, (7) to analyze data collected by microsurgical testicular sperm extraction (micro-TESE) among azoospermic patients with and without non-mosaic KS syndrome who referred to Royan Institute due to infertility. This type of regression has a further parameter, the skewness parameter, which increases its flexibility. This parameter controls the skewness of the link function. Finally, we have compared results from the two models (logistic regression and skewed regression) according to statistical criteria.

Materials and Methods

This cohort study enrolled 134 patients with KS and 537 patients without KS. Patients were referred to Royan Institute, a referral infertility clinic in Tehran, Iran between 2009 and 2011. We individually matched patients and controls according to disease duration, time of surgery, and surgeon in order to avoid confounding factors such as surgical skills and age.

Clinical evaluation

The 47,XXY karyotype was confirmed by G-banding of more than 30 peripheral blood lymphocytes. Semen analysis was performed according to World Health Organization (WHO) guidelines to evaluate sperm parameters. At least two analyses confirmed azoospermia in each patient. Blood samples were taken in the morning to measure FSH, LH, and testosterone levels.

Microsurgical testicular sperm extraction

Microsurgical testicular sperm extraction (Micro-TESE) was performed on the patients under general anesthesia as described by Schlegel (8). The procedure was considered successful when sperm were retrieved from the patients.

Statistical modeling

Independent binary regression model

The binary logistic regression model, as previously introduced (9), is appropriate for independent outcomes. When y_1, y_2, …,y_n are a set of n observations of binaryoutcomes such as success and failure in sperm retrieval, which are independent of each other; p_i is the probability of success forpatient "i"; x_1, x_2,…, x_n are independent predictors; and β_1, β_2, …, β_k are equal to regression coefficients, then, the binary regression model can be written as follows:

$$p_i=F(x_i'\beta) \qquad i=1, \ldots, n \qquad \text{(Model 1)}$$

where: F(.) is acumulative distribution function. If F is the distribution function of normal and logistic, then the above model will be alogistic and probit regression model, respectively.

Correlated binary regression model with a symmetric link

Assume that a binary outcome "Y_{it}" (t=1,2,e T)

is measured for a particular person during time T. As the outcomes for one person are correlated, in order toevaluate the relationship betweenindependent variables and theoutcome, the earlier model is no longer appropriate. There are different methods to model a relationship between a correlated binary outcome and a set of independent variables. In this study, we have focused on a mixed generalized linear model with a simplified form as follows:

$$p_{it}=F(x_{it}' \beta+b_i) \qquad \text{(Model 2)}$$

where b_i is a random effect with normal distribution in this formula. Adding this term into model 1 enables the observations to be considered independently. In model 2, which is similar to the independent model, symmetrical logistic or normal distribution functions are assumed for F.

Correlated binary regression model with an asymmetric link

Asymmetric links are used for regression models to enable better data fit. In this study, the dependent regression model as previously introduced by Chen et al. (7) is explained. This model uses a hidden variable structure, which has been introduced by Albert and Chib (10). Binary outcome can be created by a desired cut-off point as zero on a continuous latent variable with a mixed structure. The hidden variables can have distributions such as the logistic or normal. Symmetric or a symmetric distributions can be produced for the variable through a mixed structure. Various asymmetric links also can be produced in this way. The most important property of Chen's model is that it considers the problem of "skewness", which controls amount of skewness of a link. The link will be symmetric if skewness is zero.

Data were analyzed by R software (version 3.2.2) using the BB package and GEE pack for asymmetric and symmetric models, respectively. The logistic regression model with the symmetric and asymmetric link was fit to the data and we compared the goodness of fit of the two models by quasi Akaike's information criteria (QAIC) (11). The level of 0.05 was considered significant.

Results

The mean age ± SD of patients with KS was 32.64 ± 0.64 years (range: 22-49) and the mean age of participants without the KS was 34.11 ± 0.27 years. The mean serum level for FSH was 34.5 mlU/m Lin KS patients and 22.6 mlU/mL in those without KS. Patients with KS had a mean serum testosterone level of 2.65 ng/mL; those without KS had a mean testosterone level of 4.04 ng/mL. Of 134 patients with KS, 38 patients had sperm retrieved; out of 537 participants, 119 had sperm retrieved. The sperm retrieval rate was 28.4 in KS patients and 22.2 in patients without KS (Table 1).

We performed a marginal effect logistic regression analysis with serum FSH, LH, testosterone, age, and patient groups to determine an association between the probability of sperm retrieval and the covariates during micro-TESE. Adjusted association from the model showed that probability of retrieving sperm during micro-TESE did not differ between the two groups (control and KS) after adjusting for the covariates and could not be predicted by any of the variables. On the other hand, the results obtained from skewed logistic regression showed that the probability of retrieving sperm was not the same for patients with and without KS (P=0.01). QAIC for logistic regression with the symmetric link was equal to 85, whereas for an asymmetric link it was 74. The results of comparing the regression model are shown in Table 2. The proportion of sperm retrieval was almost equal between patients with and without KS.

Table 1: Clinical characteristics of patients and controls subdivided according to Micro-TESE outcome

Factor	Klinefelter syndrome			Control		
	Total	Success	Failure	Total	Success	Failure
Age	32.64[a] ± 0.48	30.0[b] ± 0.65	33.68 ± 0.6	34.11 ± 0.27	34.6 ± 0.55	33.93 ± 32
FSH (mIU/ml)	34.52[a] ± 1.4	34.69 ± 2.52	34.44 ± 1.68	22.6 ± 0.83	23.54 ± 1.65	22.22 ± 0.96
LH (mIU/ml)	17.89[a] ± 1.34	17.0 ± 1.94	18.28 ± 1.24	8.83 ± 0.41	9.22 ± 0.85	8.67 ± 0.47
T (ng/ml)	2.65 ± 0.22	3.4[b] ± 0.48	2.33 ± 0.23	4.04 ± 0.51	3.52 ± 0.29	4.2 ± 0.67

FSH; Follicle-stimulating hormone, LH; Luteinizing hormone, T; Testosterone, [a]; Significant compared to the control group, and [b]; Significant compared with failures in the Klinefelter syndrome (KS) group. Data are presented as mean ± SD.

Table 2: Results of logistic regression and skewed regression comparing sperm retrieval between KS and control groups after adjusting for LH, FSH, age and testosterone

Parameter	Logistic regression[a] (95% CI)	P value	Skewed regression[a] (95% CI)	P value
Group				
Control	Reference		Reference	
Klinefelter	0.35 (-0.92 – 0.21)	0.22	-0.49 (-0.79 – -0.19)	0.01
LH (mIU/mL)	-0.01 (-0.04 – 0.02)	0.51	-0.02 (-0.03 – 0.01)	0.26
FSH (mIU/mL)	0.01 (-0.01 – 0.03)	0.34	0.03 (-0.01 – 0.07)	0.28
Testosterone (ng/mL)	-0.001 (-0.03 – 0.03)	0.95	-0.002 (-0.03 – 0.01)	0.48
Age	-0.01 (-0.05 – 0.03)	0.66	-0.01 (-0.04 – -0.02)	0. 4

FSH; Follicle-stimulating hormone, LH; Luteinizing hormone, CI; Confidence interval, [a]; Significant compared to the control group, and [b]; Significant compared with failures in the Klinefelter syndrome (KS) group.

Discussion

The present study has compared sperm retrieval in azoospermia patients with and without KS by two regression models. Sperm from KS patients is typically retrieved by conventional TESE and micro-TESE techniques. Although both techniques have successfully retrieved sperm in 44% of patients, a comparison between conventional TESE and micro-TESE showed that micro-TESE had a higher success rate (55%) compared to conventional TESE (44%) (12). Sperm retrieval rate in men with KS by micro-TESE ranged from approximately 21-72% (13-19). A study by Madureira et al. (20) reported a sperm recovery rate of 38.5% among non-mosaic KS patients. Possibly the rate of sperm retrieval in the current non-mosaic KS series (28.4%) could be compared with those previously reported. A pilot study proposed that sperm retrieval rates in adolescents with KS could be compared with those reported in older men (21).

In some studies it was found that the sperm recovery rate in men with KS reduced with increased age, however there were no effects on serum FSH, LH, or testosterone levels (22-25). Medical and ethical issues were mentioned related to sperm retrieval in adolescent males with KS in a study by Okada et al. (26). Whether previous testosterone treatment, could be considered or not be as a barrier in the sperm retrieval. In our study, skewed logistic regression showed that only younger men had a higher sperm recovery rate compared to older men which suggested that aging could reduce successful sperm recovery in men with KS. In the KS group, a comparison of laboratory parameters between men with successful sperm retrieval and those with failed sperm retrieval showed a significantly higher testosterone level in patients with successful sperm retrieval (3.4 ± 0.48 ng/ml) compared to those with failed sperm retrieval (2.33 ± 0.23 ng/ml). In patients without KS no difference existed between age, FSH, LH, and testosterone levels in patients who experienced successful sperm retrieval compared to those with failed sperm retrieval. Our study reported higher sperm retrieval rates in patients with KS compared to the control group, which could be related to confounder distribution in the two groups and their associations with the outcome. The skewed regression was more powerful than logistic regression to detect this relation. This finding differed from previous studies (19, 22, 23). This result has shown that KS patients can be hopeful for sperm detection and subsequent pregnancy outcomes.

We used logistic regression with symmetric and asymmetric links for data analysis. To the best of our knowledge, this was the first study that applied a regression model with asymmetric link in reproduction research and in this group of infertile patients. The incorrect use of asymmetric link instead of an asymmetric link could lead to a poor fit and result in biased estimates of the regression coefficients. Chen's model that has been used in

this study was more flexible than common models. Therefore, the QAIC value was lower and has shown a better fit than logistic regression with a symmetric link (27, 28). In addition, sometimes it is not possible to control all confounding factors. Hence, the confounding effects should be adjusted by regression model. Thus, a better fit model can provide a more accurate result. The importance of selecting a more appropriate model is specified in such studies more than ever.

Conclusion

Our findings have revealed that the logistic regression model with an asymmetric link is more flexible and a better fit than the conventional regression model. Since this is the first time a skewed regression has modeled data from infertility studies, we recommend that additional studies and analyses be conducted to evaluate how well this model fits a set of observations. It is also should be notice, sperm could be retrieve of non-mosaic KS as well as without KS patients.

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Original Article

Evaluation on Hope and Psychological Symptoms in Infertile Couples Undergoing Assisted Reproduction Treatment

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Abstract

Background: This study evaluated hope, depression, anxiety, and stress among three groups of infertile couples.

Materials and Methods: This cross-sectional study consisted of three groups of infertile couples-candidates for oocyte donation (n=60), embryo donation (n=60), and normal infertile (n=60). Participants included couples seen at Royan Institute, Tehran, Iran between 2013-2014 who were at least 18 years of age and could read and write in Persian. Participants provided demographic and general characteristics and completed the Persian version of the Adult Trait Hope Scale (hope, agency and pathway) and Depression, Anxiety, and Stress Scale (DASS). Data was analyzed by the paired t test, ANOVA, ANCOVA and Pearson correlation tests using SPSS statistical software.

Results: Overall, 180 infertile couples participated in the three groups. There was a significant higher mean score for hope in husbands compared to wives in the normal infertile group (P=0.046). Husbands in the normal infertile group also had a significantly higher mean score for pathway (P=0.032). The frequency of anxiety significantly differed in female subjects (P=0.028). In the normal infertile group, the anxiety distribution significantly differed between wives and husbands (P=0.006). There was a significantly different stress frequency in male subjects (P=0.048). In the embryo donation group, stress significantly differed between wives and husbands (P=0.002). In the normal infertile group, stress also significantly differed between wives and husbands (P=0.05).

Conclusion: The results have suggested that hope might be important in reducing psychological symptoms and psychological adjustment in those exposed to infertility problems who follow medical recommendations, which accelerates recovery. It is recommended to hold psychological counseling sessions (hope therapy) during reproduction cycles.

Keywords: Hope, Depression, Anxiety, Stress, Infertility

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Introduction

Infertility is an unpleasant, unexpected experience for individuals. According to a population-based study in Iran, the overall prevalence of lifetime primary infertility among couples is 17.3% (1). Infertility is associated with tremendous negative psychological and mental burdens on both infertile men and women, in addition to somatic and sexual disorders (2). The typical, common psychological problems that result from infertility are anxiety, depression, low self-confidence, stress, distress, and lower marital and sexual satisfaction (3, 4). When infertility treatment takes a long time or in the event of treatment failure, infertile patients are more likely to encounter hopelessness. The loss of hope to have a child is important because hope is one of the main psychological needs (5). In other words, hope generates self-confidence and internal positive feelings toward solving an existing problem. Hope has been defined by Snyder et al. (6) as "a reciprocally derived sense of successful

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agency (goal-directed determination) and pathways (planning of ways to meet goals)". What can be deduced is that agency is the perception that one can reach his/her goals, whereas pathways is known as the perception that one can find alternative routes to reach these goals should the need arise (7). It is expected that people with high levels of hope can think about pathways to reach their goals, deal with diseases better, and generate additional coping strategies (8) so that fear, anxiety, and fatigue occur less often in these individuals (9, 10). Hence, psychologists should find hope and belief issues in patients in order to facilitate the treatment process during a life crisis, because this is considered an important part of patient treatment (10).

To the best of our knowledge, no studies have examined the utility of Snyder's theory of hope in patients who suffer from infertility. This study aimed to investigate the levels of hope as conceptualized by Snyder in infertile couples who undergo infertility treatment. Specifically, we sought to determine whether hope would be significantly positively or negatively related to major psychological symptoms commonly experienced by infertile patients (i.e., anxiety, stress, and depression).

Materials and Methods

We conducted this cross-sectional study on infertile couples who referred to Royan Institute, a referral infertility clinic in Tehran, Iran between 2013 and 2014. The study sample consisted of three groups of infertile couples-candidates for oocyte donation (n=60), candidates for embryo donation (n=60), and normal infertile (n=60). The inclusion criteria were aged 18 years or older, a history of infertility, and ability to read and write in Persian.

The Ethical Committee of Royan Institute approved the study. Aims of the study and the confidentiality of the data were clearly explained for all participants. We reassured all eligible individuals that acceptance or refusal to participate in the research had no influence on their treatment procedures. Voluntary completion of the questionnaire was considered as written informed consent. Participants completed three questionnaires. First, the demographic questionnaire included age (years), sex (male or female), educational levels (under diploma, diploma and academic), duration of marriage (years), and duration of treatment (months). Second, participants completed the Adult Trait Hope Scale published in 1991 by Snyder et al. (11). This 12 item self-report instrument contains two subscales: agency and pathway. These components include a sense of personal agency related to goal attainment (4 items) and the ability to recognize/generate pathways to reach a goal (4 items). This questionnaire also includes 4 items which do not belong to either of the above dimensions. The items are answered by an 8-option Likert scale. Each subscale has a score that ranges from 4 to 32. Hope is scored from 8 to 64, so that higher scores indicate a higher level of agency, pathway, and hope, respectively. We have used the Persian version of hope (Snyder), validated in 2011 by Kermani et al. (12). This version had a Cronbach's alpha coefficient for reliability of 0.86 and an ICC equal to 0.81. Couples completed the Depression, Anxiety, and Stress Scale (DASS), developed in 1998 by Antony et al. (13). DASS includes 21 items and 3 subscales - anxiety, depression, and stress. The anxiety scale evaluated autonomic arousal, situational anxiety, and subjective experience of anxious affect. The stress scale assessed difficulty relaxing, nervous arousal, and becoming easily upset or agitated, irritable, or over-reactive and impatient (14-16). Each subscale includes 7 items and is categorized according to normal, slight, severe, and very severe. The Persian version of DASS-21 was validated in 2007 by Samani and Joukar (17). The Cronbach's alpha coefficient to test reliability was 0.81, 0.74, and 0.78, respectively for anxiety, depression and stress. The ICCs equaled 0.80, 0.76, and 0.77, respectively.

We used the SPSS statistical software package (SPSS Inc, Chicago, IL, USA), version 22.0 for statistical analyses. Continues variables were expressed as mean ± SD and categorical variables as number (percentage). Normality of the variables was verified by the Kolmogorov-Smirnov test. The relationship between individual independent variables (demographic, and duration of treatment and marriage) and dependent variables (hope, agency, pathway, anxiety, depression, and stress) were assessed with Pearson correlation coefficient and we conducted the paired t test (between wives and husbands), ANOVA (between groups of infertile in hope subscales), and chi-square test (between groups of infertile in depression, anxiety and stress). Moreover, the mutual effects of demo-

graphic characteristics, hope, and groups studied were evaluated using ANCOVA. P<0.05 was considered statistically significant.

Results

During the study period, 180 infertile couples participated. The mean age was 32.94 ± 4.74 years in men and 29.39 ± 5.09 years in women. Approximately 72 (40%) men and 60 (33.3%) women had academic educations. The mean duration of marriage was 6.07 ± 4.13 years in the couples. The mean duration of treatment was 25.73 ± 25.13 months. The demographic and fertility characteristics of the participants are given in Table 1.

As seen in Table 2, husbands had a significantly higher mean score for hope compared to wives in the normal infertile group (P=0.046). There was no significant difference in the mean score for hope in male and female patients between groups. There was no significant difference in the mean score for agency between wives and husbands in each group. The mean score for agency did not significantly differ in male and female patients between groups. In the normal infertile group, the husbands had a significantly higher mean score for pathway (P=0.032). There was no significant difference observed in the mean score for pathway in male and female patients between groups.

Table 1: Demographic and general characteristics of the infertile couples (n=180)

		Oocyte donation [mean ± SD or n (%)]	Embryo donation [mean ± SD or n (%)]	Normal infertile [mean ± SD or n (%)]	P value*
Age (Y)	Male	33.05 ± 5.40	32.77 ± 4.51	33.02 ± 4.42	0.912
	Female	30.10 ± 5.41	29.05 ± 5.09	29.02 ± 4.77	0.544
P value**		<0.000	<0.000	<0.000	
Education	Male				<0.000
	Female				0.006
Under diploma	Male	8 (13.3)	34 (56.7)	7 (11.7)	
	Female	12 (20)	26 (43.3)	12 (20)	
Diploma	Male	26 (43.3)	13 (21.7)	20 (33.3)	
	Female	22 (36.7)	23 (38.3)	25 (41.7)	
Academic	Male	26 (43.3)	13 (21.7)	33 (55)	
	Female	26 (43.3)	11 (18.3)	23 (38.3)	
P value**		0.437	0.180	0.019	
Marital duration (Y)		5.37 (3.94)	6.69 (4.27)	6.15 (4.09)	0.003
Treatment time (Months)		25.52 (20)	28.97 (31.10)	22.72 (22.73)	0.272

*; Test for several independent groups and **; Paired test.

Table 2: Hope and its subscales in study couples and groups

		Oocyte donation (mean ± SD)	Embryo donation (mean ± SD)	Normal infertile (mean ± SD)	P value*
Hope	Male	52.38 ± 6.52	51.82 ± 6.86	53.93 ± 6.36	0.148
	Female	51.78 ± 7.17	50.72 ± 6.00	51.17 ± 7.17	0.735
	P value**	0.614	0.440	0.046	
Agency	Male	26.05 ± 3.71	26 ± 2.86	26.69 ± 3.91	0.335
	Female	25.85 ± 3.52	25.62 ± 3.73	25.43 ± 4.37	0.911
	P value**	0.470	0.902	0.126	
Pathway	Male	26.33 ± 3.55	25.82 ± 3.87	27.28 ± 3.44	0.090
	Female	26.17	24.87 ± 3.47	25.73 ± 3.75	0.210
	P value**	0.735	0.318	0.032	

*; Test for several independent groups and **; Paired test.

As shown in Table 3, the distribution of depression (normal, slight, medium, severe, and very severe) significantly differed in male subjects among all groups (P=0.01). The frequency of anxiety (normal, slight, medium, severe, and very severe) significantly differed in female subjects (P=0.028). The normal infertile group had a significantly different distribution for anxiety between wives and husbands (P=0.006). The frequency of stress (normal, slight, medium, severe, and very severe) significantly differed in male subjects (P=0.048). In the embryo donation group, stress significantly differed between wives and husbands (P=0.002). In the normal infertile group, stress also significantly differed between wives and husbands (P=0.05).

Table 3: Depression, Anxiety, and Stress Scale (DASS) and its subscales in studied couples and groups

		Oocyte donation n (%)	Embryo donation n (%)	Normal infertile n (%)	P value*
Depression	Male Female				0.010 0.716
Normal	Male Female	37 (61.7) 34 (56.7)	27 (45) 26 (43.3)	38 (63.3) 31 (51.7)	
Slight	Male Female	2 (3.3) 7 (11.7)	11 (18.3) 15 (25)	11 (18.3) 9 (15)	
Medium	Male Female	11 (18.3) 12 (20)	17 (28.3) 14 (23.3)	5 (8.3) 13 (21.7)	
Severe	Male Female	7 (11.7) 4 (6.7)	5 (8.3) 2 (3.3)	3 (5) 4 (6.7)	
Very severe	Male Female	3 (5) 3 (5)	0 (0) 3 (5)	3 (5) 3 (5)	
P value**		0.914	0.797	0.091	
Anxiety	Male Female				0.231 0.028
Normal	Male Female	23 (36.3) 24 (40)	25 (41.7) 26 (43.3)	31 (51.7) 20 (33.3)	
Slight	Male Female	3 (5) 7 (11.7)	4 (6.7) 8 (13.3)	7 (11.7) 2 (3.3)	
Medium	Male Female	13 (21.7) 15 (25)	17 (28.3) 11 (18.3)	13 (21.7) 17 (28.3)	
Severe	Male Female	9 (15) 2 (3.3)	10 (16.7) 3 (5)	5 (8.3) 12 (20)	
Very severe	Male Female	0 (0) 0 (0)	0 (0) 0 (0)	0 (0) 0 (0)	
P value**		1.000	0.938	0.006	
Stress	Male Female				0.048 0.831
Normal	Male Female	31 (51.7) 30 (50)	48 (80) 29 (48.3)	40 (66.7) 31 (51.7)	
Slight	Male Female	8 (13.3) 9 (15)	3 (5) 13 (21.7)	8 (13.3) 6 (10)	
Medium	Male Female	11 (18.3) 10 (16.7)	8 (13.3) 10 (16.7)	5 (8.3) 13 (21.7)	
Severe	Male Female	7 (11.7) 7 (11.7)	1 (1.7) 4 (6.7)	5 (8.3) 5 (8.3)	
Very severe	Male Female	3 (5) 4 (6.7)	0 (0) 4 (6.7)	2 (3.3) 5 (8.3)	
P value**		0.860	0.002	0.050	

*; Test for several independent groups and **; Paired test.

Table 4: Correlation between Depression, Anxiety, and Stress Scale (DASS) subscales and hope

	Depression		Anxiety		Stress	
	r	P value	r	P value	r	P value
Agency	-0.319*	<0.000	-0.252*	<0.000	-0.272*	<0.000
Pathway	-0.187*	<0.000	-0.203*	<0.000	-0.155*	<0.000
Hope	-0.276*	<0.000	-0.249*	<0.000	-0.228*	<0.000

*; P<0.05 and r; Pearson correlation coefficient.

Table 5: ANCOVA results regarding the differences among the studied groups

Variable	Source	Sum of squares	DF	Mean square	F
Hope	Sex	73.167	1	73.167	1.702
	Age	105.917	1	105.917	2.464
	Education	14.444	1	14.444	0.336
	Marital duration	14.969	1	14.969	0.348
	Treatment time	152.952	1	152.952	3.558
	Group	47.850	2	23.925	0.556

*; P<0.05, DF; Degree of freedom, and F; F value.

Additionally, bivariate correlations were conducted among the subscales of the DASS and Adult Trait Hope Scale. Agency negatively and significantly correlated with depression, anxiety, and stress (P<0.001). Pathway and hope showed negative, significant correlations with depression, anxiety, and stress (P<0.001, Table 4). Results of the ANCOVA test showed that regardless of demographic variables, the mean differences in hope did not significantly differ between study groups (Table 5).

Discussion

To the best of our knowledge, this was the first study that measured two components of the Adult Trait Hope Scale, agency and pathway, in Iranian infertile patients undergoing assisted reproduction treatment according to Snyder's theory. This was the first study that included both infertile men and women (couples). We classified the study subjects into three groups, oocyte donation, embryo donation, and normal infertile, because the main hypothesis was that a difference existed in hope subscales among these groups and between wives and husbands. Mainly, we hypothesized that those who undergo donation (either embryo or oocyte) could show different hope and other psychological properties compared to other infertile participants. However, many studies have investigated hope in the context of chronic diseases, such as cancer (7, 8). The results of these studies have revealed that hope physiologically and emotionally helped patients tolerate the crisis of the disease (18, 19). Hope is considered an essential element in a chronically ill patients' life and has a high impact on their adaptation to the disease. Patients who have high levels of hope alleviate psychological tensions better through application of more efficient coping strategies such as reevaluation and problem solving, which affect various stages of the disease process (16, 18, 19). For many infertile patients, the effect of infertility and notably of medical therapy is a considerable emotional stress. It has been shown that infertile women undergo more tension, anxiety, depression, self-reproach, and suicide (9, 20). However, another study revealed that hope was one of the main effective factors for successful IVF (21).

Our study also confirmed the findings that husbands had more hope than wives in the normal infertile group. Further investigation into the two components of hope indicated in the normal infertile group a significantly higher mean score of pathway in husbands. The results of this investigation supported findings extracted from other studies that showed strong inverse relations between hope and psychological symptoms in patients who suffer from chronic diseases. Berendes et al. found

an association between higher levels of hope and lower levels of depression among cancer patients (8). These findings agreed with prior research where higher hope was related to less depression in mixed cancer populations (22, 23). Our study also indicated reverse correlations between subscales of DASS and the Adult Trait Hope Scale. Increasing levels of hope resulted in anxiety reduction (24). Some researchers also reported that hope was accompanied by reductions in depression symptoms (8, 25). Studies on the effect of psychological and consultative interventions on the psychological disorders and pregnancy outcomes in infertile couples have shown that psychological therapy effectively reduced anxiety and depression, and increased pregnancy rates (16). A positive psychological treatments, hope therapy, can enhance infertile women's general health and subsequently improve family health. Therefore, hope therapy is recommended for infertile individuals to be offered with assisted reproductive techniques in order to enhance the quality of life and help these individuals cope with their problems (9, 16).

Our study had several limitations. First, an inherent limitation of this study might be its generalizability. We relied on patients who presented to only one center, a referral clinic for infertility treatment in Iran where patients throughout the country come to this center. Second, the cross-sectional nature of the study only allowed for correlations, but not conclusions on causality.

Conclusion

Our study was the first study to examine Snyder's construct of hope in a sample of infertile couples. The results suggest that hope may be important in reducing psychological symptoms and psychological adjustment in those with infertility problems who follow medical recommendations more efficiently through better behavioral patterns, which would accelerate recovery. We suggest that psychological counseling sessions (hope therapy) be offered during reproduction cycles.

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Short Communication

Relationship between *Chlamydia-Ureaplasma-Mycoplasma* Genital Detection with Semen Concentration and Motility among Greek Men

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Abstract

One hundred and seventy two men at the State of Thessaly, Greece, inquiring semen analysis were enrolled in the study in order to investigate the incidence of *Chlamydia*, *Ureaplasma* and *Mycoplasma* (C-U-M) genera in respect to total sperm number (TSN), progressive motility (grades a and b) and total motility (grades a, b and c). Putative relation of C-U-M acquirement with sexual behavior was also investigated. Incidence of C-U-M among non-oligozoospermic and oligozoospermic men was similar. No correlation of C-U-M carriage to either oligozoospermia or asthenozoospermia was found. The tested semen parameters were negatively correlated to the age of sexual intercourse initiation and positively correlated to the number of sex partners. Early age of sexual intercourse initiation or high number of sexual partners was not statistical significantly correlated to C-U-M acquirement. Overall, TSN and motility (either progressive or total) were not influenced by the presence of C-U-M genera in a sample of Greek population undergoing semen evaluation. To distinguish the role of C-U-M in male infertility and clarify the so far controversial scarce literature, large control case studies are needed using nucleic acid amplification techniques to detect these pathogens.

Keywords: Polymerase Chain Reaction, Oligozoospermia, Asthenozoospermia, Azoospermia, Infertility

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Up to date, epidemiological studies have shown that infertility affects approximately 10% of the couples, whereas in 50% of this incident, a male infertility associated factor is found (1). A thorough sexual/medical history as well as hormonal/physical examination may reveal the cause of male infertility in 70% of infertile men. Of the remaining 30%, the vast majority of infertile males may carry genetic abnormalities such as Robertsonian translocations, Y chromosome micro/macrodeletions, aneuploidy as in Klinefelter syndrome or mutations in the cystic fibrosis transmembrane regulator gene (2).

Among the factors that might influence male fertility, infections of the lower genital tract have been the least investigated field. Effects of urogenital tract pathogens on sperm concentration and motility have still remained unclear and further studies are needed to evaluate their influence on male fertility. Particularly, the *Chlamydia*, *Ureaplasma* and *Mycoplasma* (C-U-M) genera are considered sexually transmitted pathogens (although U. and M. are also suggested to constitute normal flora) that might cause chronic urogenital tract infections. Their asymptomatic carriage as well as the low specificity and sensitivity of conven-

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tional microbiological methods for C-U-M detection, compared to nucleic acid amplification techniques such as polymerase chain reaction (PCR) (3), might explain the still poorly evaluation of the roles of these pathogens in male fertility. We have recently shown the incidence of C-U-M in Greek population and the prevalence of *U.* spp. as well as the potential pathogenicity of *U. urealyticum* (4). The aim of the present study was to investigate the presence of C-U-M among Greek men in respect to TSN, sperm motility and sexual behavior in order to investigate the putative effect of these pathogens in semen parameters that might influence conception.

From August 2013 until July 2015, a total of 172 men at the State of Thessaly, Greece, participated in the study. All participants enrolled in the study for semen analysis either inquired a microbiological evaluation (mainly due to clinical manifestations of genital tract infection or preventive screening), or were reported as being infertile after at least a 12-month unprotected sexual intercourse and failure to impregnate their wives. Informed consent was obtained from all participants and each individual filled in a questionnaire regarding demographic data, medical record and sexual history. Males with an underlying pathology (e.g. varicocele and hormone deficiency) to which oligo-azoo- or asthenospermia could be attributed were excluded. Duplicate samples from individuals, even if surgical or medical treatment was applied, were not included in the study. Eventually, participants were divided into two groups according to TSN and irrespectively to reason of enrollment in order to study the prevalence of C-U-M in respect to sperm concentration and motility.

Semen samples of all participants were collected into sterile nontoxic recipients by masturbation after 3 to 5 days of sexual abstinence. All participants were evaluated according to the guidelines of World Health Organization (WHO) 2010 Semen Analysis Reference Limits (5), and TSN, progressive motility (grades a and b) and total motility (grades a, b and c) were determined. All semen samples were investigated for DNA of C-U-M using Amplisens *C.trachomatis/U./M.genitalium*-MULTIPRIME-FRT and Amplisens *M. hominis*-FRT diagnostic CE-IVD PCR kits (Amplisens, Slovak Republic). These kits provide *in vitro* nucleic acid amplification qualitative tests for the detection of DNA of C. trachomatis, *U.* spp. (*U. parvum* and *U. urealyticum*), and *M. genitalium* simultaneously, as well as *M. hominis*, in the clinical material using real-time hybridization-fluorescence detection. They are intended for *in vitro* diagnostic use and are Conformite Europeene (CE) marked. C-U-M DNA extraction was performed using DNA-Sorb-AM Nucleic Acid Extraction Kit (Amplisens, Slovak Republic) which includes Internal Control-FL (IC) reagent in order to test both efficacious DNA extraction and putative inhibition of PCR. Analyses were performed at BIOGONIDIAKI Center of Infertility Investigation and Genetic Research, Volos, Greece that serves for the broad region of the State of Thessaly.

Mean and median values as well as SD were calculated and are given as mean ± SD or median. Unpaired t test, Fisher's exact test, odds ratios (ORs), 95% confidence intervals (CI), as well as simple linear regression analysis (Pearson r) were performed using statistical software Statistical Package for the Social Sciences (SPSS, SPSS Inc., USA). Statistically significant difference was defined as the $P<0.05$.

Sixty-eight participants of the study had TSN≥39 millions (control group) and 104 were oligo- azoospermic with TSN<39 millions (study group). In the control group, mean age was 36.66 ± 5.96, mean age of sexual initiation was 18.19 ± 3.15 and median number of sexual partners was 6. In the study group, mean age was 37.42 ± 5.50, mean age of sexual initiation was 19.11 ± 3.69 and median number of sexual partners was 6. Mean TSN of the 68 males of the control group was 258.59 ± 197.32 millions. Progressively motile spermatozoa, more than 32%, were present in 46 participants (67.65%) and totally motile spermatozoa, more than 40%, were present in 63 (92.65%). C-U-M DNA was present in 10 participants (14.71%). Particularly, 8 (11.76%) were positive for *U.* spp., two (2.94%) for *M. hominis* and two (2.94%) were found to co-carry *U.* spp. and *M. hominis*. Mean TSN of the 104 males of the study group was 13.69 ± 12.67 millions, which was shown to be statistically significant different ($P<0.0001$) from the TSN of the control group using unpaired t test. Progressively motile spermatozoa, more than 32%, were present in 20 participants (19.23%), and totally motile spermatozoa, more than 40%, were present in 40 (38.46%). C-U-M DNA was present in 10 partici-

pants (9.62%). Particularly, one (0.96%) was positive for *C. trachomatis*, seven (6.73%) for U. spp. and two (1.92%) for *M. hominis* (Table 1). Of note, no M. genitalium-positive participant was found in either the control or the study group.

Table 1: Prevalence of the detected microorganisms among the participants of the study

Species	Participants with TSN	
	≥39 millions of spermatozoa n (%)	<39 millions of spermatozoa n (%)
C. trachomatis	0 (0.00)	1 (0.96)
U. spp.	8 (11.77)	7 (6.73)
M. hominis	2 (2.94)	2 (1.92)
U. spp.	2 (2.94)	0 (0.00)
M. hominis		

TSN; Total sperm number, *C. trachomatis*; *Chlamydia trachomatis*, *U. spp.*; *Ureaplasma species*, and *M. hominis*; *Mycoplasma hominis*.

Fisher's exact test and calculation of ORs showed no higher probability of C-U-M carriage in men with low TSN (<39 million of spermatozoa) (P=0.17, OR=0.53 with 95% CI=0.21-1.29) or low sperm motility [progressive motility less or equal to 32% (P=0.15, OR=0.50 with 95% CI=0.20-1.23) and total motility less or equal to 40% (P=0.49, OR=0.66 with 95% CI=0.26-1.72), respectively]. There was no relation between C-U-M acquirement with age of sex initiation (cut off value more than and less or equal to 18) (P=1.00, OR=1.11 with 95% CI=0.45-2.74) or number of sex partners (cut off value more than and less or equal to 6) (P=0.82, OR=0.90 with 95% CI=0.37-2.20). Linear regression analysis showed negative correlation between age of sexual intercourse initiation with TSN (Pearson r=-0.918), sperm progressive motility (Pearson r=-0.666) and sperm total motility (Pearson r=-0.686). Also, positive correlations were shown for the number of sex partners with TSN (Pearson r=+0.493), sperm progressive motility (Pearson r=+0.125) and sperm total motility (Pearson r =+0.387).

In our study, no correlation of C-U-M carriage to either oligo-azoospermia or asthenospermia was found. It should be noted that when the participants of the study were clustered not according to TSN but according to sperm concentration (semen conc.≥or <15 millions/ml, respectively), the statistical analysis showed no variances. Similar negative and positive correlation coefficients in regression analysis and P>0.05 in chi-square tests were obstructed (statistical analysis and statistical data not shown). Our findings are in accordance with Al-Sweih et al. (6) who found no statistically significant correlation of C-U-M carriage to male infertility (although other parameters such as leukocytospermia were shown to be influenced) studying a total of 127 infertile and 188 fertile men in Kuwait with a PCR-based detection protocol. Our results also comply with Gdoura et al. (7) who did not associate C-U-M carriage with either oligozoospermia or asthenozoospermia, although they found a higher prevalence of *C. trachomatis* among male partners of infertile couples. On the contrary, Liu et al. (8) correlated *U. urealyticum* infection to oligozoospermia in 621 Chinese infertile men using culture based microbiological procedures though. Similarly, Lee et al. (9) found that progressive motility and vitality were significantly lower in *U. urealyticum* positive men, while low total motility and total motile sperm count were significantly related to the presence of *M. hominis*. However, they tested 50 infertile couples and 48 fertile couples with Mycofast Evolution 2 commercial kit (International Microbio, France), a culture medium based assay for the detection of *U. urealyticum* and *M. hominis*. The controversial results reported on the current scarce literature are probably related to the diversity of the detection methods used.

Under the scope of a possible correlation of sexual behavior to C-U-M acquirement that might influence semen quality, we found that semen parameters were correlated negatively to the age of sexual intercourse initiation and positively to the number of sex partners. In other words, the earlier a man initiated his sexual life and the more sexual partners he had, the better tested semen parameters he appeared to have. Moreover, the early age of sexual intercourse initiation or the high number of sexual partners was not statistical significantly and correlated to C-U-M acquirement.

In conclusion, TSN and sperm motility seem not to be influenced by the presence of C-U-M genera in a sample of Greek men undergoing semen evaluation. Although prevalence of C-U-M in our sample was low [as was expected due to a lately published large-scale study from Central Greece (4)], C-U-M appeared to be equally spread to both

groups of the study which further highlights the doubtful role of C-U-M in the tested semen parameters. Furthermore, early onset of sexual intercourse and high number of sexual partners were not correlated with C-U-M acquirement. To distinguish the role of C-U-M in male infertility and clarify the so far controversial scarce literature, larger case control studies are needed using nucleic acid amplification techniques to detect these pathogens, as recent reviews have suggested (10).

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International Journal of Fertility and Sterility (Int J Fertil Steril)
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