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INTERNATIONAL JOURNAL OF FERTILITY AND STERILITY

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# Contents

Review Article

# Spermatozoa: A Historical Perspective

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## Abstract

The 100,000th scientific article on the subject of spermatozoa was recently published. Numerous studies evaluated the characteristics of this important cell that led to tremendous discoveries. Since its first observation and description in 1677, many important characteristics have been described regarding this highly fascinating gamete. In this review, we intend to provide a historical account of the numerous milestones and breakthroughs achieved related to spermatozoa. We conducted a review of the literature by selecting the most important subjects with regards to spermatozoa. Since their discovery by van Leeuwenhoek, spermatozoa have been studied by scientists to better understand their physiology and process of interaction with their female counterpart, the oocyte, in order to treat and resolve infertility problems. Three centuries after van Leeuwenhoek's discovery, the 100,000th article about these cells was published. It is encouraging that sperm research reached this landmark, but at the same time it is clear that further research on male reproductive physiology and spermatozoa is required to shed more light on their function and pathology in order to reduce the number of unexplained infertility cases.

***Keywords:*** Fertility, History, Male Reproductive Physiology, Sperm

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## Introduction

Few cells have attracted the world's attention and intrigued scientists throughout history as much as the male gamete or spermatozoon. It is one of the most fascinating and important cells. A crucial part is involved in the fecundation process due to its role in the delivery of male genetic material and proteins to the oocyte at the time of fertilization. Despite the vital dependence of human life on the interaction between gametes, research on human and male reproduction is less than infectious diseases. This is perhaps due to the emergent and expansive nature of infections. Only 0.4% of all scientific papers published over the last 50 years have pertained to spermatozoa despite their discovery in 1677 by van Leeuwenhoek (1). van Leeuwenhoek did not act alone; his observations were made in the company of his assistant, Ham, which demonstrated the importance of scientific cooperation and communication.

In 2016, the 100,000th paper related to spermatozoa was published. To celebrate this significant milestone, we aim to highlight and discuss the most important events related to the history of this cell that travels from one individual to another in order to initiate and preserve life. We conducted an extensive evaluation of PubMed [National Center for Biotechnology Information (NCBI)] literature using the term "sperm" without any limitation on the date of publication. In addition, we determined the numbers of publications on another topic of major interest to the scientific community-viral infection caused by the human immunodeficiency virus (HIV). Furthermore, we evaluated the relationship between HIV and sperm articles in the last 30 years, and conducted a review of the literature about the most important findings. We selected and discussed milestones that pertained to spermatozoa.

### Historical review

In the database used for this analysis (PubMed), the number of results retrieved with the term "sperm" was much greater than when we used the term "spermatozoa".

The 100,000th article on HIV was published in the year 2000 (3,102,842 articles had been published when last accessed on January 8th, 2017, Fig.1). This milestone was already achieved 16 years before the 100,000th publication on spermatozoa (101,787 articles had been published when last accessed on January 8th, 2017).

During June 1843, according to PubMed, there were three manuscripts published. Two papers, one original and one editorial (2, 3), dealt with the presence of sperm in the hydrocele from three patients. The third paper was a letter that concerned sperm from a dromedary (4). In contrast, the first paper about HIV was published nearly

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140 years later in September 1982. This publication by the center for disease control (CDC) analyzed the reports received that pertained patients with the acquired immune deficiency syndrome (AIDS) (5).

It is important to note that since 1990 the relationship between articles published about HIV and sperm were similar (median 4.2-fold more for HIV, range: 3.4 to 4.8). In addition, there were significantly more (P<0.0001) numbers of papers published during the last three decades (1990-2016) in PubMed about HIV (median 10.4, range: 5.8 to 15.7) compared to spermatozoa (median 2.6, range: 1.7 to 3.6, Fig.1).

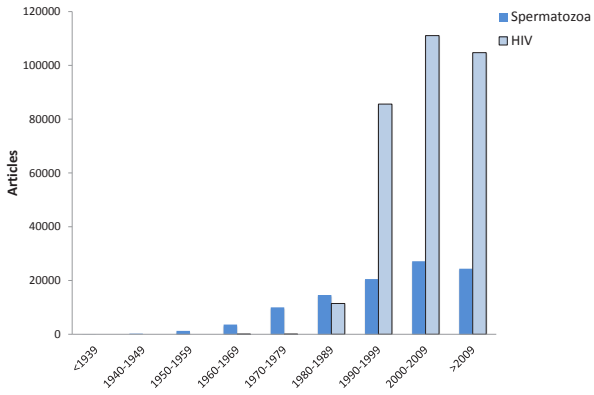


**Fig.1:** Number of articles published in relation to time on human immunodeficiency virus and spermatozoa.

Quantity does not necessarily imply quality. However, good quality manuscripts have been published about sperm and HIV. We intend to discuss a few of these publications to highlight some of the events that have marked the history of research on human reproduction (Table 1) from the perspective of the male gamete -the spermatozoon.

Important events related to spermatozoa include the advent of assisted reproduction, male contraception, and the effects of the interaction of sperm with microorganisms (viruses, bacteria, and fungi), among others. However, the story of the discovery of sperm has originated more than three centuries ago, in 1677. Without any scientific purpose and purely driven by human curiosity, van Leeuwenhoek and his assistant described animalculae (6) in human semen. It was not until 1776 that interest in the "new male gamete" began to focus on its physiology. Spallanzani noted that it became motionless when cooled by snow. Additionally, Spallanzani performed the world's first artificial insemination of a viviparous animal. He artificially and successfully inseminated a bitch, which led to the birth of a live puppy by using spermatozoa obtained directly from the reproductive tract of a dog that had died (7).

It is impossible to discuss sperm without referring to the environment in which it develops. Cooper (8) published a book "observations on the structure and diseases of the testis" in which he described the place of origin of sperm, i.e. the testicle. In the first chapter he cited: "The testes are contained within the scrotum, in which they are suspended at unequal heights, the left testis generally hanging lower than the right." The manuscript was not only limited to a description of this organ, but the author also recounted the anatomy, physiology, and alteration in the male reproductive system in general.

The development of spermatogenesis is connected to the testicular anatomy and the action of mainly two cell types: Leydig cells, first described by the German histologist Leydig in 1850 (9), and Sertoli cells, originally described by the Italian histologist Sertoli in 1865 (10). Leydig cells are the main source of testosterone, which is essential for spermatogenesis and sexual differentiation. One of the main targets for testosterone is the Sertoli cell that surrounds and nourishes germ cells during spermatogenesis.

The spermatozoon's physiology, as well as any alterations to it that could lead to infertility, prompted the development of techniques used to evaluate some important characteristics of sperm during the reproductive process. In 1866, Sims devoted a chapter of his book "Clinical Notes on Uterine Surgery" to describe the nature and properties of semen, artificial fertilization, and the conception period. Sims wrote: "If we take a drop of semen from the vagina immediately after sexual intercourse and place it under the microscope, we shall see the hurried movements of seemingly thousands of spermatozoa"; thus was born the post-coital test to "measure" the capability of sperm to penetrate a woman's cervical mucus. Huhner has described the details of this technique where it is known as the Sims-Huhner test (11).

Oocyte-sperm interaction and the interaction of sperm with the environment have been studied extensively from two perspectives: agglutination and chemotaxis. These phenomena were particularly studied in marine species such as sea urchins. Simple experiments combined with detailed observation allowed Lillie (12) to describe the production of sperm isoagglutinin by the ova of two marine species, *Arbacia punctulata* and *Nereis aibuhitensis* as early as 1912. Lillie's findings have shown that oocytes produce a substance which causes sperm agglutination for 3 to 5 minutes. The agglutination is totally reversible. However, it does not imply a return to the original physiological state, as the movement of spermatozoa gradually cease. After 10 minutes they no longer have the capability of fertilization. The researcher has concluded that sperm penetration is due to biochemical reactions and not solely mechanical properties of the cell. This biochemical response has been subsequently termed chemotaxis, a type of guidance system for spermatozoa to the oocyte. Sperm guidance may involve more than one chemoattractant and include thermotaxis, which is based on slight temperature differences. These guidance processes rely on the precise timing of $Ca^{2+}$ transients that control flagellar beating and the swimming trajectory. An asymmetric waveform is associated with turns or bends in the trajectory, whereas

more symmetric beating results in a straighter swimming path. Both the oocyte and cumulus cells secrete chemoattractants. A number of these molecules have been implicated in capacitation, hyperactivation, and eliciting the acrosome reaction (13).

Spermatozoa are highly specialized cells with specific energy requirements. Adenosine triphosphate (ATP), which constitutes the basis for supporting the key functions of the spermatozoa, is formed through the glycolysis and oxidative phosphorylation pathways. To date, a discrepancy has been reported as to which method of ATP production is primarily utilized by the spermatozoa for successful fertilization (14). McCarthy et al. (15) conducted one of the studies on sperm metabolism that explored the presence of glycolysis in semen in 1928. They reported that the sugar concentration in semen from 50 men decreased by 70 to 90% after one day of incubation.

In 1929, Macomber and Sanders (16) were the first to publish a paper on a reference value for sperm count based on the results from nearly 300 men. They showed that although pregnancy was possible with less than 60 million sperm/ml, the probability increased with sperm counts. During the same period, Carey and Hotchkiss, Mason, and Moench and Holt (17) reported the importance of sperm morphology assessment during evaluation of infertility cases based on their findings that correlated this parameter with pregnancy outcome. They also reported the initial reference values for morphology. They stipulated that: i. The number of abnormal sperm heads must not exceed 19 to 20% in a normal semen sample, ii. Impaired fertility could be assumed if sperm head abnormalities reach 20 to 23%, and iii. When the sperm head abnormalities were above 25%, clinical sterility usually prevailed.

In 1932, Baskin (18) described the first contraceptive vaccine to be directed against spermatozoa. In this study the author inoculated women with sperm and triggered the production of anti-sperm antibodies, which resulted in infertility. Only one of the 20 women in his study became pregnant. Of great significance was the fact that the immunization lasted for about one year and that the process appeared to be harmless for women. However, the most important criticism for this study was directed towards the antibodies produced, as these proved to be non-specific because they had the ability to bind to spermatozoa used for vaccination as well as to other individuals, and could react with other proteins in somatic cells.

Another important milestone was reached in 1942, when Lasley et al. (19) developed a staining technique to differentiate between live and dead sperm based on the fact that live ram sperm were impermeable to several stains.

The first suggestion that oxidative stress might play a role in the etiology of defective sperm function came from MacLeod, one of the pioneers of modern andrology. As early as 1943, MacLeod demonstrated that human spermatozoa rapidly lost their motility in oxygenated medium via mechanisms that could be recovered by the concomitant presence of catalase, a specific scavenger of hydrogen peroxide (reactive oxygen species) (20).

Oxidative stress is one of the major causes of defective sperm function and male infertility because it causes lipid peroxidation and disrupts the integrity of sperm DNA. This, in turn, can affect sperm motility and limit fertilizing potential as a result of collateral damage to proteins and lipids in the sperm plasma membrane. In addition, reactive oxygen species increase as a consequence of leukocyte infiltration. A variety of primary factors can initiate oxidative stress such as infection, age, obesity, and exposure to a variety of adverse environmental influences. Despite the resultant oxidative damage to the sperm's chromatin, once fertilization occurs, the oocyte can repair most of the damaged sperm DNA (21).

In 1947, Dan (22) explored the enigma of sperm entrance into the egg and discovered the acrosome reaction, "an event of exocytosis by which spermatozoa expose the devices essential for penetration through the egg coats and for fusion with the egg plasma membrane, and is species-specific and triggered by signals from the eggs or their appendices". Dan employed a very primitive model of electron microscopy to study and describe this phenomenon. His work demonstrated that many groundbreaking discoveries were based on careful observation of specific natural phenomena and not purely on the use of advanced technologies. Although the use of new technologies has allowed us to analyze problems in greater detail and from a different perspective, the best instrument for scientific research remains human curiosity and acute observation.

After the initial discovery of sperm and identifying the relationship of sperm with fertility, investigations started to focus more intensively on the process of sperm transfer to the oocyte. During the first *in vivo* fertilization trials in rabbits, it was observed that the sperm must remain in the fallopian tubes for six hours prior to ovulation, thereby pointing to a possible physiological change that the spermatozoa have to undergo in the female tract (23).

Spermatogenesis occurs in the testis, after which the sperm move to the epididymis for final maturation. Once ejaculated, sperm can undergo two phenomena, capacitation and the acrosome reaction, which thereby renders them able to fertilize oocytes (24). The metabolism of epididymal spermatozoa differs from that of ejaculated spermatozoa, thus underpinning the influence of the environment on their physiology. During the natural sequence of mammalian fertilization, the oocyte awaits the arrival of spermatozoa in the fallopian tubes after their relatively rapid transit from the vagina to the upper part of the tube. This time interval is for the spermatozoa to acquire fertilization capacity and not allow accumulation of a large quantity of spermatozoa, since the number of sperm present at the site of fertilization is relatively small (23). Once the first sperm penetrates the oocyte, its cortical granules undergo exocytosis and release their content into the perivitelline space. This leads to the modification of the zona pellucida and subsequently the blockage of

polyspermy. The second polar body is formed after the second mitotic division (25).

In the middle of the 20th century, researchers analyzed sperm structure to deduce phylogenetic relationships between taxa. Variations in the size, shape, and physiology of spermatozoa in the animal kingdom and their relation to the method of reproduction captivated the biologists of the time. Between 1940 and 1950, with the development of the electron microscope, ultra-thin dissection, and new staining techniques, researchers could observe the structure of spermatozoa from various species. One of the most interesting findings was made by Franzén (26), in 1955 and 1956. He suggested a causal relationship between sperm morphology and the mode of fertilization of the species. In 1950, Leuchtenberger and Schrader (27) reported that the acrosome contained the lytic enzyme hyaluronidase.

Soon after, it was suggested that sperm morphology related to pregnancy. Macleod and Gold (28) were the first to propose that a possible relationship existed between sperm motility and fertility. They performed a study on 2000 semen samples and reported that the average percentage of active cells was higher in the fertile group of proven fathers (n=1000) than in the group of infertile men (n=1000).

Spermatozoa undergo selection during their travel along the female reproductive tract. Different selection techniques have been developed *in vitro* to identify and choose sperm of higher quality (29-31). In 1958, Bhattacharya (29) reported that it was possible to separate rabbit sperm using colloid medium and centrifugation (gradient centrifugation method). The author claimed to be able to discriminate between X- and Y-bearing sperm with this technique. At a later stage (1971), Drevius (30) used bull semen to show that motile spermatozoa could swim upwards following centrifugation (swim-up method). When incubated for a specific period of time, only motile sperm were present in the supernatant. Another popular method used to increase the percentage of motile sperm was proposed by Tea et al. (31) in 1984 (migration-gravity sedimentation methodology), who used semen samples from normo- and asthenozoospermic individuals. During semen incubation in the Jondet tube, two built-in concentric tubes, and based on the migration ability of sperm and the sedimentation phenomena, motile human spermatozoa jumped into the central tube.

Amann and Katz (32), the pioneer of computer aided sperm analysis (CASA), in 1978 presented the first system to track movement. Initially, these systems were extremely expensive and the results were not reproducible. Presently, a number of different CASA systems have become available for sperm motion detection. CASA made the comprehension and assessment of various motility and kinematic parameters possible with great reproducibility and complete objectivity. However, the validity of the results is conditioned by the proper use of the CASA system. Recommendations include: i. The user of the system must be highly trained, especially in sample preparation, ii. Internal quality and external controls should be included, iii. The results should be compared directly with manual assessments, iv. The depth and temperature of the chamber should not affect sperm motility, and v. A permanent record of samples should be kept if reanalysis is required (33).

Yanagimachi et al. (34) developed the sperm penetration assay by using zona pellucida-free oocytes from hamsters. The authors showed that freshly ejaculated human spermatozoa were incapable of binding and penetrating zona-free hamster ova. However, after 4 or more hours of incubation, the sperm increased their ability to interact and penetrate these oocytes.

In 1979, a letter to the editor was published in The Lancet that informed the world about the birth of Louise Brown on July 25th, 1978. Her mother had a 9-year history of infertility. This pregnancy was achieved after laparoscopic recovery of an oocyte, IVF, and reimplantation of an embryo which was incubated for 2.5 days under laboratory conditions. This important achievement by Steptoe and Edwards (35) resulted in the first baby born through IVF.

In 1980, Rothman (36) has reported, for the first time, the postmortem retrieval of sperm. This procedure is important when the family requests sperm preservation after death to honor the wish of fatherhood by the deceased.

Bleil and Wassarman (37) examined the structure and function of the zona pellucida and subsequently described it in 1980 as a "relatively thick, translucent, acellular coat which surrounds the plasma membrane of fully grown mammalian oocytes". It was not until 1990 that they discovered the ZP3 protein, a sperm receptor localized on the surface of the egg's zona pellucida that serves as sperm receptor and acrosome reaction-inducer. They concluded that the sp56 sperm protein binds to ZP3 during adhesion of the gametes that precedes fertilization.

In 1980, Evenson et al. (38) reported an alternative method to assess semen quality. They have described the sperm chromatin structure assay (SCSA) as a highly useful test to determine the validity of male breeding by comparing the sperm chromatin of fertile and infertile men based on similar results previously observed in bulls. The basic semen analysis evaluates sperm count, motility and morphology, but there are numerous cases where these parameters are all within the "normal" range, yet the men are infertile. The SCSA test uses acridine orange dye, which emits a red fluorescence when it binds to single-stranded DNA and a green fluorescence when it binds to double-stranded DNA. The relationship between both types of fluorescence evaluates DNA heterogeneity and fragmentation, and can possibly account for the infertility of men who have a normal semen analysis (39). During the last decade, this test has been an important tool for the diagnosis of fertility due to the relation between a suc-

cessful pregnancy and DNA status.

During the previous century, it became generally accepted that assessment of the basic seminal parameters was the cornerstone for evaluation of male fertility. For this reason, the World Health Organization (WHO) published its first manual on the examination of human semen (WHO, 1980). Since then, the manual has been regularly updated with new versions published in 1987, 1992, and 1999; each edition has contained new proposals, the latest changes, and revised cut-off values for the semen variables. After rigorous scrutiny, the latest edition was published in 2010. This edition is believed to be one of the most comprehensive manuals to date because the authors have based updated semen variables on real life data which included men of proven fertility and secondly presented results as lower reference limits ($5^{th}$ percentile) (40).

In 1981, Yanagimachi (41) noticed that spermatozoa began to move in an extremely vigorous swimming pattern just prior to initiation of the acrosome reaction. This process was termed hyperactivation. The human sperm capacitation and acrosome reaction could be induced in vitro by the addition of albumin, which mediated cholesterol efflux and hyperactivation. This unique movement pattern is considered to give spermatozoa the strong thrusting power that allows them to penetrate the zona pellucida, and occurs on completion of the capacitation process (42).

The hypo-osmotic swelling test (HOS) was developed in 1984 to evaluate the functional integrity of the human sperm membrane. The principle of this technique is based on the fact that human spermatozoa "swell" under hypo-osmotic conditions due to the influx of water and subsequent membrane expansion. This technique is extremely important because membrane integrity is vital for sperm metabolism and the correct change in membrane properties required for a successful union with the female gamete. HOS can thus be used in the diagnosis of male infertility. Investigators have stated that any compounds that change osmolality and significantly enhance sperm swelling should be removed from IVF media (43).

Later, Kruger et al. (44) described the specific morphological characteristics of human spermatozoa in 1988. They stated that the following criteria should be met for a spermatozoon to be regarded as normal: the head (length: 5 to 6 μm and width: 2.5 to 3.5 μm) has to be oval with a well-defined acrosome (approximately 40 to 70% of the sperm head) and there should be no neck, midpiece, or tail defects. In addition, no cytoplasmic droplets should more than half the size of the sperm head. They concluded that morphologic features played a significant role in the probability of pregnancy.

In 1988, Hodgen et al. (45) published an editorial entitled "The Hemizona Assay (HZA): Finding Sperm that have the 'Right Stuff' ". In this editorial they reported the story of two infertile men who sought a new diagnostic method for their problem. To quote, they "needed quantifiable objective assessments of sperm quality having a high reliability, a low cost of operation, ease of application, a wide availability, and rapid results". In brief, we wished for a male-factor "litmus test". With the advent of IVF, they have ushered in the first direct laboratory assessment of a man's fertilization potential through the HZA. Tight binding of human spermatozoa to the human zona pellucida is an early critical event in gamete interaction, which is needed for fertilization and activation of development. In reality, this test only assesses the ability of sperm to interact with the egg *in vitro*. However, this binding step is believed to provide unique information that predicts the sperm's ultimate fertilizing potential (46). Briefly, a viable oocyte is micro-dissected into two equal parts. Subsequently, each matching half is exposed to the same concentration of sperm from the patient (test) and a proven father (control). The semen samples are prepared using swim-up separation to obtain motile sperm and co-incubation lasts 4 hours. The hemizonae are removed and technicians count the number of sperm tightly bound to the outer surface. The HZA index is calculated by a simple equation: number of test sperm bound divided by the number of control sperm bound ×100 (45).

The ability of sperm to act as vectors for foreign DNA was first published in 1971 by Brackett et al. (46) who used rabbit sperm and simian virus 40. However, it was only in 1989 when Lavitrano et al. (47) published the first sperm-mediated gene transfer in mice using a pSV2CAT plasmid that this methodology became more popular. Since then, successful *in vitro* uptake of exogenous DNA, including viral DNA, by sperm of different animal species has been reported.

In 1992, the landscape of the assisted reproduction technique (ART) was transformed with the first reports of intracytoplasmic sperm injection (ICSI). This technique allows the direct injection of a single spermatozoon into the ooplasm of metaphase-II oocytes, after which the embryos are placed in utero.

In the same year of ICSI development, Carlsen et al. (48) warned about a genuine decline in semen quality over a period of 50 years. They described a decrease in mean sperm count from $113\times10^6$/ml in 1940 to $66\times10^6$/ml in 1990, and in seminal volume from 3.40 ml to 2.75 ml among men without a history of infertility. This finding could be related to a concomitant increase in the incidence of genitourinary abnormalities, such as testicular cancer, and possibly cryptorchidism and hypospadias, which impact male gonadal function. The analysis was based on a total of 61 papers published between 1938 and 1990 that included data on 14947 men. Some researchers criticized the 'Carlsen study' based on the study design, the heterogeneity of the groups investigated, statistical evaluation, non-standardized methodology used for semen analysis, and variation in abstinence times. The authors of one specific manuscript who opposed this spermatic crisis article concluded with a sentence to motivate future research: "it must be acknowledged that the research group that led us

down the wrong path also demonstrated how we can get back on the proper research track" (49).

Sperm-specific ion channels or CatSper has been described in 2001 by Ren et al. (50). CatSper is a six-transmembrane-spanning repeat of the voltage-dependent $Ca^{2+}$ channels located in the principal piece of the sperm tail. The sperm from CatSper knockout mice showed poor motility and could not fertilize eggs with an intact zona pellucida. CatSper is considered as a target for the design of a possible contraceptive. The evaluation of the functionality of CatSper has also been considered useful in the detection of male infertility.

The identification of proteins in sperm was based principally on the use of anti-sperm antibodies, isolation and identification of individual protein spots from 2D gels and on infertility models in the mouse which involve mutations that affect sperm capacitation and motility. For example, to identify critical sperm-oocyte fusion antigens such as the IZUMO protein, Inoue et al. (51) used anti-sperm antibodies.

As already demonstrated, sperm physiology has been studied in humans as well as in various animal models that have contributed substantially to this scientific field. For example, the first systematic molecular study of sperm composition that utilized Drosophila melanogaster sperm samples was performed by Dorus et al. (52) and represented the first characterization of the eukaryotic cell content.

Additionally, in 2006, a novel gamete receptor known as beta 1,4-galactosyltransferase 1 (GalT) that mediates sperm adhesion to the zona pellucida, was described (53). Such key receptors considered vital for the fertilization process are not only important for infertility, but also for the development of new male contraceptive targets. The mechanisms that mediate sperm-egg binding in the mouse involve a GalT-ZP3-independent mechanism, which mediates initial sperm-egg adhesion followed by a GalT-ZP3-dependent interaction that contributes to acrosomal exocytosis.

One of the most rewarding moments in the history of sperm and sperm research occurred when Edwards was awarded the 2010 Nobel Prize in Physiology or Medicine for the development of IVF. In his biography, Edwards was described as competitive "all determined to win or, if not to win, to go down fighting". During his childhood, he developed an enduring curiosity about agricultural and natural history, in particular the reproductive patterns of some animals. This curiosity, through multiple experiments such as sperm labeling led to evaluation of the kinetics of spermatogenesis *in vivo* (54).

Spermatogenesis is one of the most complex and longest processes of sequential cell proliferation and differentiation known to man. Therefore, the *in vitro* production of functional spermatozoa has long been pursued. In 2011, the first successful attempt was reported. The authors produced spermatids and sperm by *in vitro* culturing and maintained spermatogenesis for more than 2 months. They subsequently performed round spermatid injection (ROSI) and obtained 12 offspring. These animals were proven fertile as examined by brother-sister mating. The researchers therefore demonstrated that the organ culture conditions, without a circulatory system as *in vivo*, could support complete spermatogenesis of mice. This finding will definitely contribute to the development of new diagnostic and therapeutic techniques for male infertility (55).

**Table 1:** Milestones in the history of spermatozoa

| Year | Author | Milestone | Reference |
|---|---|---|---|
| 1677 | Howards | Sperm cell discovery | (6) |
| 1776 | Mann and Lutwak-Mann | Spermatozoa temporarily immobilized by cooling | (7) |
| 1830 | Cooper | Observations on the structure and diseases of the testis | (8) |
| 1850 | Leydig | Leydig cells | (9) |
| 1866 | Huhner | Post-coital test | (11) |
| 1888 | Ebner | Sertoli cells | (56) |
| 1878 | Dudley et al. | Spermine | (57) |
| 1912 | Lillie and Kaupp et al. | The production of sperm iso-agglutinine by ova | (12, 13) |
| 1929 | Macomber and Sanders | The spermatozoa count | (16) |
| 1932 | Baskin | Temporary sterilization by injection of human spermatozoa | (18) |
| 1940 | Charny | First testicular biopsy | (58) |
| 1942 | Lasley et al. | Staining method for sperm viability | (19) |
| 1943 | MacLeod | The first suggestion that oxidative stress might play a role in the etiology of defective sperm function | (20) |
| 1948 | Pegg | Cryopreservation | (59) |
| 1950 | Dan | Acrosome reaction | (22) |
| 1951 | MacLeod and Gold | First seminal parameters proposed-fertility standard | (28) |

**Table 1:** Continued.

| | | | |
|---|---|---|---|
| 1951 | Chang and Austin | Capacitation | (23, 25) |
| 1951 | Tulloch | Varicocele as a cause of infertility | (60) |
| 1953 | Bunge and Sherman | Living child after insemination with semen from a sperm bank | (61) |
| 1953 | Kleegman | Donor insemination | (62) |
| 1958 | Bhattacharya | Density gradient | (29) |
| 1969 | Baccetti and Afzelius | The First International Symposium on Spermatology | (63) |
| 1971 | Drevius | Swim-up | (30) |
| 1978 | Amann and Katz | Computer aided sperm analysis (CASA) | (32) |
| 1976 | Greep and Chang | Distinguished andrologist award | (64) |
| 1976 | Yanagimachi et al. | Sperm penetration assay (SPA) | (34) |
| 1978 | Steptoe and Edwards | Development of *in vitro* fertilization (IVF) | (35) |
| 1980-2010 | World Health Organization (WHO) | Range of arbitrary threshold values for normal human semen | (40) |
| 1980 | Bleil and Wassarman | Structure and function of the zona pellucida | (37) |
| 1980 | Evenson et al. | Sperm chromatin structure assay (SCSA) | (38, 39) |
| 1980 | Rothman | Postmortem sperm retrieval | (36) |
| 1981 | Yanagimachi | Hyperactivation | (41, 42) |
| 1984 | Jeyendran et al. | Hyposmotic swelling test (HOS) | (43) |
| 1984 | Tea et al. | Migration-gravity sedimentation method | (31) |
| 1988 | Kruger et al. | Sperm morphologic features | (44) |
| 1988 | Hodgen et al. | Hemizona assay (HZA) | (45) |
| 1989 | Lavitrano et al. | Methods for sperm-mediated gene transfer | (65) |
| 1990 | Bleil and Wassarman | ZP3-binding protein | (37) |
| 1992 | Palermo et al. | First intracytoplasmic sperm injection (ICSI) | (66) |
| 1992 | Carlsen et al. | Evidence for decreasing quality of semen during past 50 years. | (48) |
| 1994 | Ogura et al. | Birth of normal young after electrofusion of mouse oocytes with round spermatids | (67) |
| 1995 | Wilcox et al. | Probability of conception | (68) |
| 1996 | Stief et al. | Sildenafil | (69) |
| 2001 | Ren et al. | Sperm-specific ion channel - CatSper | (50) |
| 2006 | Shur | Identification of novel gamete receptors - beta1,4-galactosyltransferase-I | (70) |
| 2010 | Johnson | Nobel Prize for development of IVF | (54) |
| 2011 | Sato et al. | *In vitro* production of functional sperm | (55) |

## Conclusion

In this journey through the history of sperm, we have observed how human curiosity prompted the description of natural phenomena and the attempts made to try to explain these phenomena. Strictly speaking, it is impossible to discover facts of nature that have been in existence for thousands of years. We therefore clarify and define the use of the word ‘discover’ as "looking with new eyes", which encompasses acute observation, critical thinking, relating ideas, and questioning those events that we encounter on a daily basis as part of the miracle of life. Advances in modern technology allow us to find answers to rephrased Pleaquestions and analyse them from a new perspective in our attempt to explore and understand complex functional and molecular processes.

Physiological knowledge of each human organ system and cell allows us to understand not only how the human body works, but also where and how alterations, specifically in the male reproductive tract, may affect the continuation of life. Understanding sperm biology allows for a significant impact on infertility. For example, by increasing the chance of fertilizing the oocyte with enhancing the male gamete’s presence at the site of fertilization. New technologies allow us to investigate unexplained male infertility and possibly lead to the discovery of new and novel treatment solutions, thereby reducing the prevalence of cases of idiopathic infertility.

Finally, this review reminds us that the greatest advantage of recollecting history is that it can solve old problems with new methodologies that understand their nature. We can conclude that despite numerous advances and new insights published in these 100,000 articles, more research on human reproduction, especially on spermatozoon, are needed to help reduce the overall numbers of patients who suffer from infertility.

As authors of this review and seeing the historical development of this magnificent cell, we thank the researchers who are part of this journey in knowledge for their incred-

ible contributions and invite new researchers to remember that curiosity is the best human attribute. We apologize to authors whose important contributions we could not cite due to limitations in reference number.

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## Author's Contributions

J.P.S., S.S.d.P., W.D.C.M.; Were responsible for idea, design and outline of content of the paper. All authors read and approved the final manuscript.

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Systematic Review

# Prevalence of *Chlamydia trachomatis* in Pregnant Iranian Women: A Systematic Review and Meta-Analysis

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## Abstract

Several studies have been conducted regarding the prevalence of *Chlamydia trachomatis*, *Mycoplasma hominis*, and Ureaplasma urealyticum in pregnant Iranian women. However, it is necessary to combine the previous results to present a general assessment. We conducted the present study based on systematic review and meta-analysis studies according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA). We searched the national and international online databases of MagIran, IranMedex, SID, MedLib, IranDoc, Scopus, PubMed, ISI Web of Knowledge, and Google Scholar search engine for certain MeSH keywords until June 16, 2017. In addition, heterogeneity, sensitivity analysis, subgroup analysis, and publication bias were performed. The data were analyzed using random-effects model and Comprehensive Meta-Analysis version 2 and P value was considered lower than 0.05. The prevalence of *Chlamydia trachomatis* in 11 surveyed articles that assessed 2864 pregnant Iranian women was 8.74% [95% confidence interval (CI): 5.40-13.84]. The prevalence of *Chlamydia trachomatis* was estimated 5.73% (95% CI: 2.09-14.73) and 13.55% (95% CI: 11.23-16.25) by enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR), respectively which the difference was not significant (P=0.082). The lowest and highest prevalence of *Chlamydia trachomatis* was estimated in Tehran province [4.96% (95% CI: 2.45-9.810)] and Ardabil province [28.60% (95% CI: 20.61-38.20)], respectively. This difference was statistically significant (P<0.001). Meta-regression for the prevalence of *Chlamydia trachomatis* based on year of the studies was significant with increasing slope (P=0.017). According to the systematic review, the prevalence of *Mycoplasma hominis* and Urea plasma urealyticum indicated 2 to 22.8% (from 4 articles) and 9.1 to 19.8% (from 3 articles), respectively. There was no evidence of publication bias (P value for Begg and Eggers' tests was 0.161 and 0.173, respectively). The prevalence of *Chlamydia trachomatis* is high among pregnant Iranian women. Screening pregnant women as part of preventive measures seem necessary considering the potential for maternal and fetal complications.

***Keywords:*** *Chlamydia trachomatis*, Meta-Analysis, *Mycoplasma Hominis*, Pregnant Women, *Ureaplasma Urealyticum*

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## Introduction

Pregnancy is a serious period in women´s lives, which is related to physiological changes, such as weakening the immune system (1, 2). Reproductive tract infections are one of the most serious public health issues in developed and developing countries (3). *Chlamydia trachomatis* is one of the most common sexually transmitted diseases worldwide (4). Colonization of *Chlamydia trachomatis* in the reproductive tract of pregnant women causes complications such as infertility, chronic pelvic pain, ectopic pregnancy, premature rupture of membranes (PROM), prematurity, spontaneous abortion, and perinatal mortality (5, 6). The prevalence of *Chlamydia trachomatis* infection is currently increasing throughout the world. The treatment costs of *Chlamydia trachomatis* infection is estimated to be more than 2 million US dollars. Diagnostic costs are much lower than treatment costs. Therefore, timely diagnosis and screening can decrease the prevalence of reproductive tract infections and reduce treatment costs of this disease (7).

The level of immunity in the body decreases during pregnancy (2). A weak immune system increases the risk factor for the entrance of infectious agents into the vagina. Ureaplasma urealyticum and *Mycoplasma hominis* are genital mycoplasmas that can be detected in the lower genitourinary tract of sexually active women as a result of colonization of the genital tract through sexual contact (5). These microorganisms can affect each part of the urogenital system and cause infection (8).

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These microorganisms have an important role in infections and potential complications during pregnancy. Therefore, it is necessary to be aware of prevalence patterns to plan and screen pregnant women for these microorganisms. Several studies have been conducted in Iran to determine the prevalence of *Chlamydia trachomatis*, *Mycoplasma hominis*, and *Ureaplasma urealyticum* in pregnant Iranian women (9-21). Combining the previous results to present a general assessment seems necessary. A review of all relevant documents and presenting a general assessment based on systematic review and meta-analysis studies can provide a more detailed picture of the dimensions of this problem in pregnant women (22-24). Therefore, we have conducted the present meta-analysis women from Iran.

## Materials and Methods

### Study protocol

We conducted the present study based on systematic review and meta-analysis studies according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (24). To avoid bias, two researchers conducted independent searches, selection of studies, quality assessment, and data extraction. In case of dispute, the case was referred to a third researcher. The final agreement was reached as a general discussion.

### Search strategy

We searched national online databases such as MagIran, IranMedex, SID, MedLib, and IranDoc, in addition to the international databases Scopus, PubMed, ISI Web of Knowledge, and Google Scholar search engine till June 16, 2017. To maximize the comprehensiveness of the search, we used MeSH keywords with all possible combinations with "OR" and "AND" in the English databases: 'Epidemiology', 'Prevalence', 'Chlamydia', 'Ureaplasma', 'Mycoplasma', 'Sexually transmitted diseases', 'Reproductive tract infections', 'Pregnant women', 'Pregnancy', 'Gestational', and 'Iran'. At the end of the search, the titles of the collected articles were entered into EndNote™ software to find similar articles.

### The studied population

The studied population included pregnant Iranian women. The positive result for *Chlamydia trachomatis* was determined by enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR). The positive results for *Mycoplasma hominis* and *Ureaplasma urealyticum* were determined by PCR (25, 26).

### Inclusion and exclusion criteria

Inclusion criteria of this study consisted of a reference to the prevalence of *Chlamydia trachomatis*, *Mycoplasma hominis*, and *Ureaplasma urealyticum* in pregnant Iranian women, either in Persian or English. Exclusion criteria were: non-random sample size; irrelevance; limited information such as failure to report disease prevalence; review articles, case reports, and editorials; duplicate articles; and failure to diagnose based on laboratory results.

### Quality assessment

In the next step, researchers assessed the quality of articles according to the modified Newcastle Ottawa Scale (NOS) for cross-sectional studies (27) that consisted of 8 sections in 4 categories, including selection, comparability, exposure assessment, and outcome. This scale ranges from 0 to 9 point. The minimum acceptable score was 7.

### Data extraction

All included articles were prepared for data extraction by a pre-prepared checklist. The checklist included the author's name, year of the study, the location of the study, study design, sample volume, mean age, quality score and the prevalence of *Chlamydia trachomatis*, *Mycoplasma hominis*, and *Ureaplasma urealyticum*.

### Statistical analysis

The variance of each study was estimated according to the binomial distribution. We used the Q test and $I^2$ index to assess the heterogeneity of the studies (28). Studies with heterogeneity greater than 75% fell into the category of high heterogeneity. If the $I^2$ index was lower than 25%, the heterogeneity was low; between 25-75% indicated medium heterogeneity, and higher than 75% indicated high heterogeneity. Due to the significance of the $I^2$ index, we used the random effects model for the meta-analysis (29, 30). Sensitivity analysis was conducted by deleting every single study from meta-analysis. Subgroup analysis based on province, diagnostic test, year of studies and meta-regression based on years and diagnostic test were used to detect heterogeneity of papers with the subject of *Chlamydia trachomatis*. Egger and Beggs' tests were used to assess publication bias. The data were analyzed using Comprehensive Meta-Analysis (CMA) version 2. P values were considered less than 0.05.

## Results

### Search results and characteristics of the eligible studies

We located 240 relevant studies in the systematic review. There were 229 studies omitted due to the following reasons: duplicate studies [120]; irrelevance [68]; lack of epidemiological data in the article [10]; non-Iranian sample size [17]; failure to report disease prevalence [2]; controlled sample size [8]; and review articles, case reports and editorials [4] (Fig.1). Finally, 11 qualified studies for *Chlamydia trachomatis* (9-16, 20, 21) entered the meta-analysis process. In addition, 4 and 3 qualified studies for *Mycoplasma hominis* (17-20) and *Ureaplasma urealyticum* (16-18), respectively entered the systematic review process (Table 1). The mean age of the pregnant women belong to the qualified studies was estimated 27.45 years old (95% CI: 26.03-28.88).

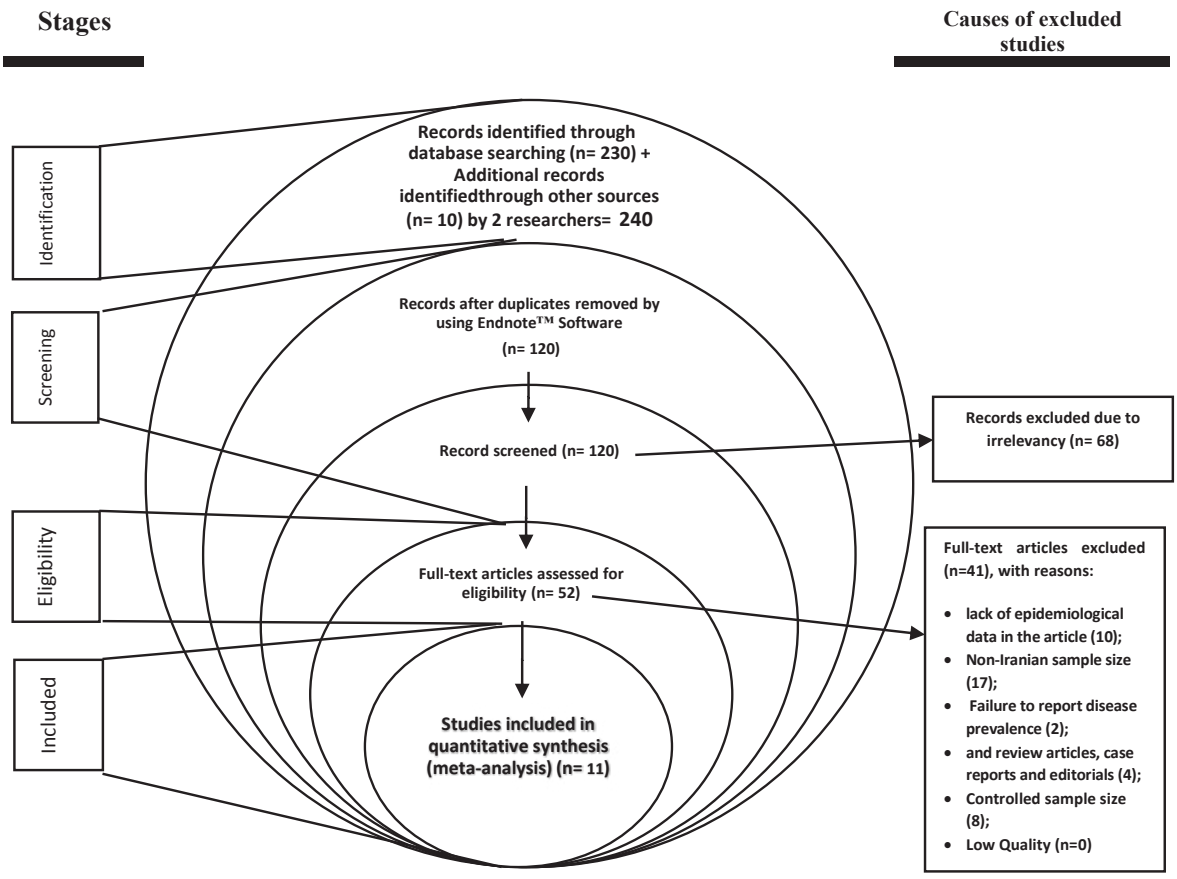


**Fig.1:** Study flow diagram.

**Table 1:** Characteristics of 13 studies on *Chlamydia trachomatis*, *Mycoplasma hominis*, and *Ureaplasma urealyticum* in pregnant Iranian women

| Reference | First author | Place | Year | Sample size | Prevalence (%) | | | Test |
|---|---|---|---|---|---|---|---|---|
| | | | | | *Chlamydia trachomatis* | *Mycoplasma hominis* | *Ureaplasma urealyticum* | |
| (9) | Sohrabi et al. | Ahwaz | 2005 | 79 | 10.1 | | | ELISA |
| (10) | Rashidi et al. | Tehran | 2008 | 225 | 11.1 | | | PCR |
| (11) | Khezerdoust et al. | Tehran | 2006 | 667 | 3.3 | | | ELISA |
| (11) | Khezerdoust et al. | Tehran | 2006 | 447 | 2.2 | | | ELISA |
| (12) | Chamani Tabriz et al. | Tehran | 2003 | 340 | 11.2 | | | PCR |
| (13) | Behrozi and Badamee | Tehran | 1994 | 400 | 2.75 | | | ELISA |
| (14) | Ahmadi et al. | Sanandaj | 2012 | 218 | 17.43 | | | PCR |
| (15) | Mobasheri et al. | Ardal | 2010 | 85 | 4.7 | | 19.8 | ELISA |
| (16) | Rohi et al. | Ardabil | 2010 | 100 | 28.6 | | 15 | PCR |
| (17) | Sobouti et al. | Tehran | 2010 | 165 | | 15 | 9.1 | PCR |
| (18) | Azizmohammadi et al. | Tehran | 2015 | 350 | | 2.8 | | PCR |
| (19) | Mohseni et al. | Tonekabon | 2012 | 44 | | 22.7 | | PCR |
| (20) | Haghighi Hasanabad et al. | Sabzevar | 2010 | 196 | 14.8 | 2.04 | | PCR |
| (21) | Sisakht et al. | Yasuj | 2010 | 107 | 14.02 | | | PCR |

### Prevalence of *Chlamydia trachomatis*

We assessed 11 surveyed articles that had a sample size of 2864 pregnant Iranian women and determined the prevalence to be 8.74% (95% CI: 5.40-13.84), and high heterogeneity was estimated between studies (P<0.001, $I^2$=92.32%) for *Chlamydia trachomatis* (Fig.2A). The lowest prevalence pertained to the study by Khezerdoust et al. (11) in Tehran (2.2%), whereas the highest prevalence was reported by Rohi et al. (16) in Ardebil (28.6%). Sensitivity analysis indicated that the pooled results were robust (Fig.2B).

**A**

| Study name | Event rate | Lower limit | Upper limit | Z-Value | p-Value | Relative weight |
|---|---|---|---|---|---|---|
| Sohrabi A, 2007 | 0.1010 | 0.0513 | 0.1893 | -5.855 | 0.000 | 8.42 |
| Rashidi BH, 2009 | 0.1110 | 0.0761 | 0.1591 | -9.804 | 0.000 | 9.51 |
| Khezerdoust S, 2009 1 | 0.0330 | 0.0218 | 0.0496 | -15.583 | 0.000 | 9.48 |
| Khezerdoust S, 2009 | 0.0220 | 0.0118 | 0.0406 | -11.768 | 0.000 | 8.80 |
| Chamani Tabriz L, 2006 | 0.1120 | 0.0826 | 0.1502 | -12.040 | 0.000 | 9.71 |
| Rohi E, 2011 | 0.2860 | 0.2061 | 0.3820 | -4.134 | 0.000 | 9.46 |
| Haghighi HM, 2011 | 0.1480 | 0.1048 | 0.2049 | -8.702 | 0.000 | 9.57 |
| Behrozi R, 1999 | 0.0275 | 0.0153 | 0.0490 | -11.662 | 0.000 | 8.92 |
| Ahmadi A, 2016 | 0.1743 | 0.1295 | 0.2305 | -8.713 | 0.000 | 9.68 |
| Mobasheri M, 2016 | 0.0470 | 0.0177 | 0.1187 | -5.872 | 0.000 | 7.33 |
| Jahangiri Sisakht A, 2017 | 0.1402 | 0.0863 | 0.2196 | -6.513 | 0.000 | 9.11 |
| | 0.0874 | 0.0540 | 0.1384 | -8.890 | 0.000 | |

Statistics for each study — Event rate and 95% CI

**Random effects model, heterogeneity: P<0.001; $I^2$=92.32%**

0.00 0.25 0.50

**B**

| Study name | Point | Lower limit | Upper limit | Z-Value | p-Value |
|---|---|---|---|---|---|
| Sohrabi A, 2007 | 0.0861 | 0.0514 | 0.1408 | -8.360 | 0.000 |
| Rashidi BH, 2009 | 0.0849 | 0.0494 | 0.1422 | -8.031 | 0.000 |
| Khezerdoust S, 2009 | 0.0970 | 0.0616 | 0.1497 | -8.861 | 0.000 |
| Khezerdoust S, 2009 | 0.0996 | 0.0630 | 0.1539 | -8.676 | 0.000 |
| Chamani Tabriz L, 2006 | 0.0847 | 0.0484 | 0.1439 | -7.811 | 0.000 |
| Rohi E, 2011 | 0.0765 | 0.0479 | 0.1199 | -9.808 | 0.000 |
| Haghighi HM, 2011 | 0.0823 | 0.0480 | 0.1374 | -8.224 | 0.000 |
| Behrozi R, 1999 | 0.0977 | 0.0610 | 0.1528 | -8.540 | 0.000 |
| Ahmadi A, 2016 | 0.0807 | 0.0475 | 0.1338 | -8.438 | 0.000 |
| Mobasheri M, 2016 | 0.0917 | 0.0556 | 0.1475 | -8.336 | 0.000 |
| Jahangiri Sisakht A, 2017 | 0.0831 | 0.0491 | 0.1371 | -8.379 | 0.000 |
| | 0.0874 | 0.0540 | 0.1384 | -8.890 | 0.000 |

Statistics with study removed — Event rate (95% CI) with study removed

0.00 0.25 0.50

**Fig.2:** Forest plot. Prevalence of *Chlamydia trachomatis* in pregnant Iranian women. **A.** Overall estimate and **B.** Sensitivity analysis.

**Subgroup analysis of *Chlamydia trachomatis* prevalence based on diagnostic test, year of studies and province**

The prevalence of *Chlamydia trachomatis* by enzyme-linked immunosorbent assay (ELISA) was 5.73% (95% CI: 2.09-14.73), and for polymerase chain reaction (PCR) it was 13.55% (95% CI: 11.23-16.25). The difference was not significant (P=0.082). The prevalence of *Chlamydia trachomatis* sub-grouped by year of study (2005 to 2009 versus 2010 to 2014) was statistically significant (P=0.016, Table 2). The prevalence of *Chlamydia trachomatis* was estimated based on the province, and the lowest prevalence was estimated in Tehran province [4.96% (95% CI: 2.45-9.81)] whereas the highest prevalence estimated in Ardebil province [28.60% (95% CI: 20.61-38.20)]. This difference was statistically significant (P<0.001, Table 2).

**Meta-regression for the prevalence of *Chlamydia trachomatis***

Meta-regression for the prevalence of *Chlamydia trachomatis* based on year of studies was significant (meta-regression coefficient: 0.110, 95% CI: 0.019-0.201, P=0.017) and also based on diagnostic test was not significant (meta-regression coefficient: 0.093, 95% CI: -0.038-1.910, P=0.059) (Fig.3).

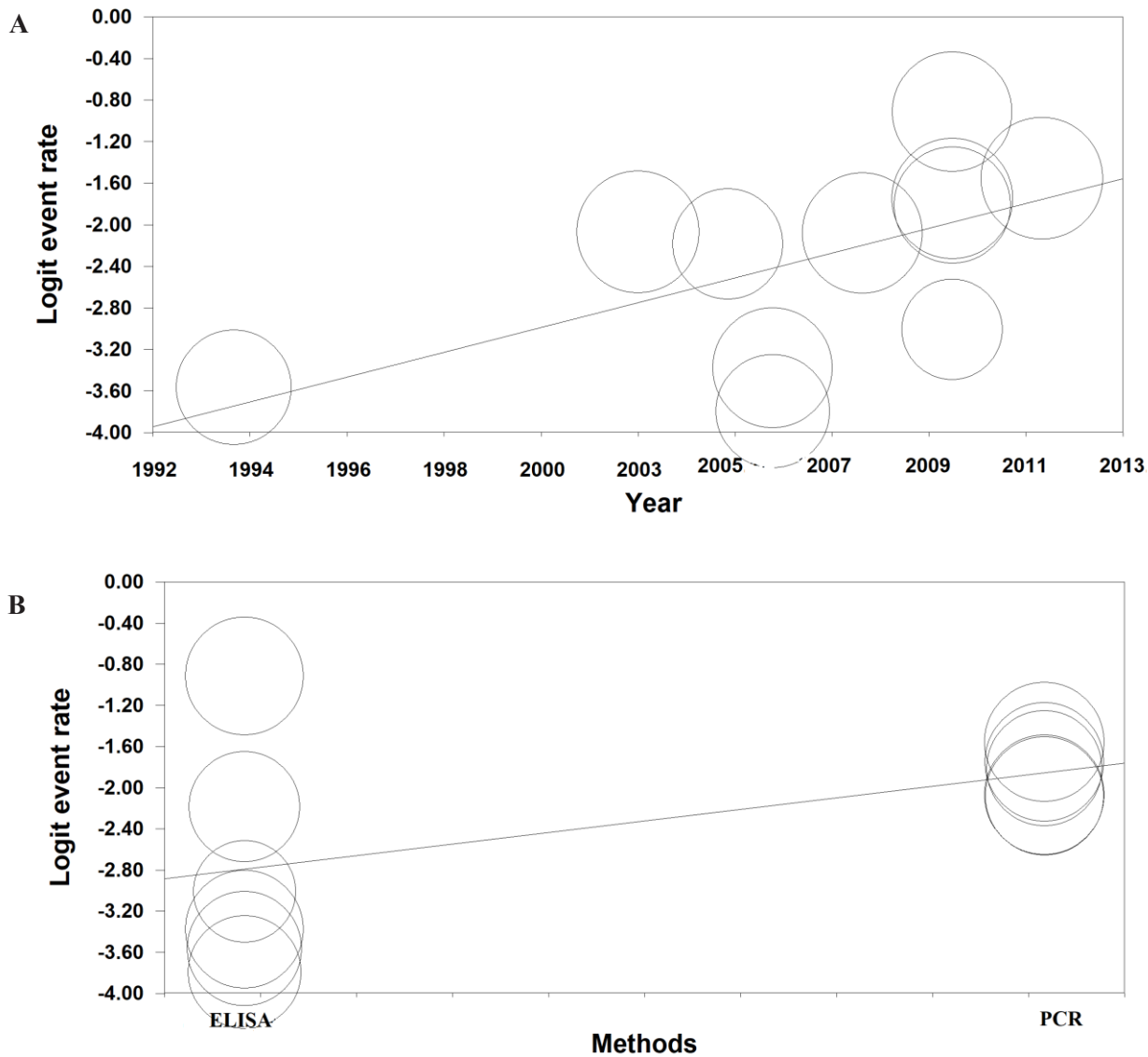


**Fig.3:** Meta-regression of prevalence of *Chlamydia trachomatis* in pregnant women. **A.** Based on year of studies and **B.** Based on diagnostic test (Larger circles indicate larger sample size).

**Table 2:** Prevalence of *Chlamydia trachomatis* in pregnant women in Iran according to diagnostic test, year of studies and province

| Variable | | Studies (n) | Sample size (n) | Prevalence (%) | 95% CI | $I^2$ (%) | P value (heterogeneity) |
|---|---|---|---|---|---|---|---|
| Diagnostic test | ELISA | 6 | 1778 | 5.73 | 2.09-14.73 | 94.81 | <0.001 |
| | PCR | 5 | 1086 | 13.55 | 11.23-16.25 | 31.11 | 0.214 |
| | | Subgroup differences: Q value=3.029, df=1, P=0.082 | | | | | |
| Year of studies | 2005-2009 | 4 | 1418 | 5.41 | 2.40-11.73 | 90.25 | <0.001 |
| | 2010-2014 | 5 | 706 | 15.67 | 10.47-22.78 | 78.05 | 0.001 |
| | | Subgroup differences: : Q value=5.76, df=1, P=0.016 | | | | | |
| Province | Khuzestan | 1 | 79 | 10.10 | 5.13-18.93 | - | - |
| | Tehran | 5 | 2079 | 4.96 | 2.45-9.81 | 92.19 | <0.001 |
| | Ardebil | 1 | 100 | 28.60 | 20.61-38.20 | - | - |
| | Razavi Khorasan | 1 | 196 | 14.8 | 10.48-20.49 | - | - |
| | Kurdistan | 1 | 218 | 17.43 | 12.95-23.05 | - | - |
| | Chaharmahal and Bakhtiari | 1 | 85 | 4.7 | 1.77-11.87 | - | - |
| | Kohgiloyeh and Boyerahmad | 1 | 107 | 14.02 | 8.63-21.96 | - | - |
| | | Subgroup differences: : Q value=32.88, df=6, P<0.001 | | | | | |

CI; Confidence interval, $I^2$; Heterogeneity in Meta-analysis, ELISA; Enzyme-linked immunosorbent assay, and PCR; Polymerase chain reaction.

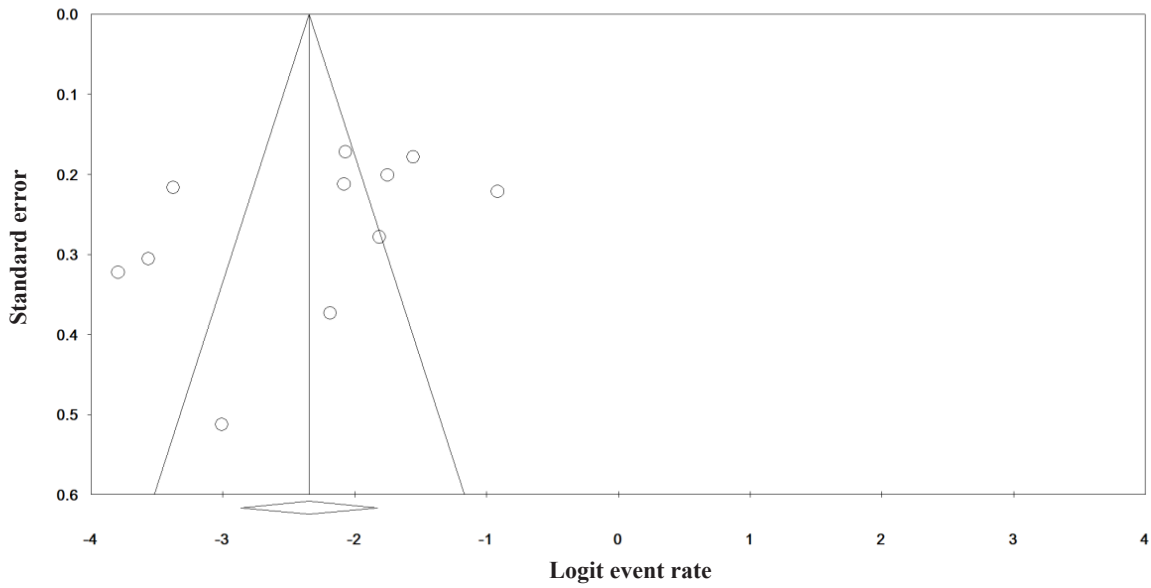


**Fig.4:** Publication bias in the studies for the prevalence of *Chlamydia trachomatis*.

### Prevalence of *Mycoplasma hominis* and *Ureaplasma urealyticum*

The systematic review results of *Mycoplasma hominis* and *Ureaplasma urealyticum* indicated the prevalence of 2% to 22.8% (from 4 articles) (17-20) and 9.1 to 19.8% (from 3 articles) (16-18), respectively.

### Publication bias

Funnel plot for the prevalence of *Chlamydia trachomatis* did not reveal significant publication bias (P value for Begg and Eggers' tests was 0.161 and 0.173, respectively) (Fig.4).

## Discussion

Awareness of the prevalence pattern and screening for diseases that affect the health of the mother and fetus is necessary during pregnancy. The present study is the first systematic review to assess the prevalence of *Chlamydia trachomatis*, *Mycoplasma hominis*, and *Ureaplasma urealyticum* in pregnant Iranian women.

We have determined the prevalence of *Chlamydia trachomatis* in 11 surveyed articles that had a sample size of 2864 pregnant Iranian women to be 8.74% (95% CI: 5.40-13.84). The prevalence of this disease is 10.1% in China (31), 10.5% in Saudi Arabia (32), and 35% in India

(33). However, the results of the present study are similar to those reported in Scotland (34).

The prevalence of *Chlamydia trachomatis* in some studies was higher than the present study (35, 36). On the other hand, the results of other studies were in the same range as the present study (37, 38), though not exactly identical. Inconsistent results with the present study might be due to cultural differences, social and religious norms, and mean age of the studied populations. The high prevalence of this infection in women age 20 and above might be due to early onset of sexual intercourse, numerous pregnancies, and the use of oral contraceptives (39, 40). Women with vaginal secretions and inflammatory changes in cervical cytology are more prone to infection and should be examined by their gynecologists (41). Several documents have demonstrated that dysuria, vaginal discharge, and lower abdominal pain may be clinical symptoms of this infection (41, 42), which are more common in pregnant women.

Age (particularly 18-27 years) and socioeconomic conditions such as an urban residence or low income (43) are among the risk factors for *Chlamydia trachomatis* in pregnant women. A study in Japan has reported a significantly high prevalence of *Chlamydia trachomatis* in primiparous pregnant women (44).

Recent studies report a significant relationship between *Chlamydia trachomatis* infection to preterm delivery (5, 45). The importance of *Chlamydia trachomatis* for midwives is due to the ability of this microorganism to cause urethritis, cervicitis, preterm births, PROM, and neonatal infections as the baby passes through the birth canal, in addition to abortion, maternal mortality, and stillbirth. Repeated screening tests in the first prenatal examination and during the third trimester of pregnancy, along with successful treatment with erythromycin can reduce the complications of pregnancy according to the American College of Obstetricians and Gynecologists (5, 6, 45, 46).

These bacteria can be easily detected by cell culture and serological methods that use micro-immunofluorescence techniques, ELISA, the complement fixation test (CFT), antigen detection methods, molecular methods (DNA hybridization, nucleic acid amplification techniques), and direct cytological methods (Giemsa, Gimenez, and hematoxylin stains) (25, 26). The best and most cost-effective method to determine whether the infection during pregnancy is acute or chronic the ELISA test (47, 48). In the present study, we have noted that the prevalence of *Chlamydia trachomatis* according to PCR results was not significantly more than ELISA (P=0.082).

The prevalence of *Mycoplasma hominis* in pregnant Iranian women from 4 studies was 2 to 22.8% (17-20). The prevalence of *Ureaplasma urealyticum* in Iranian pregnant women was 9.1 to 19.8% (16-18). Meta-analysis was not performed on the prevalence of *Mycoplasma hominis* and *Ureaplasma urealyticum* because of a scanty number of studies. Therefore, we recommend conducting more research in this area for future studies. *Mycoplasma hominis* had the following prevalence in other countries: 3.7% (Poland), 31.5% (Portugal), 11.2% (Japan). *Ureaplasma urealyticum* had the following prevalence in other countries: 29.8% (Poland), 27.8% (Portugal), and 8.7% (Japan) (49-51). These results did not agree with the present study, which might be due to cultural differences, social and religious norms, and the mean age of the studied populations.

*Mycoplasma hominis* is isolated from the vaginal secretions of 15-70% of women. *Ureaplasma urealyticum*is is isolated from the vaginal secretions of 40-95% of women (8, 52-56). *Mycoplasma hominis* and *Ureaplasma urealyticum*is are transferred to the fetus during pregnancy or normal vaginal delivery. They are often associated with cervicitis, vaginitis, pyelonephritis, pelvic inflammatory disease, postpartum septicemia, uterine infections, meningitis, PROM, postpartum fever, preterm delivery, and low weight premature birth (8, 57, 58). Most premature births for women with these two infections happen before the 34th week of pregnancy (59, 60). However, no significant relationship has been found between these infections and adverse effects on pregnancy in some studies (51). Studies conducted on *Mycoplasma hominis* have demonstrated that it caused adnexal lesions but not salpingitis (53). *Ureaplasma urealyticum* is the main cause of non chlamydial and nongonococcal urethritis, chorioamnionitis, cervicitis, vaginitis, sepsis and preterm delivery. Moreover, it may cause pneumonia, meningitis and even death of the infant as the baby passes through the birth canal (56). The role of *Ureaplasma urealyticum* has not been specified (54). PCR is often used to diagnose this infection in Iran. However, the molecular technique has also been used in some studies in Iran (61). Therefore, considering the fact that failure to diagnose, prevent, and treat these infections leads to dangerous complications, it is necessary to identify these bacteria, particularly in pregnant women (53, 62).

Factors that increase the prevalence of prenatal infections in women include young age (adolescents and young adults); use of an intrauterine device (IUD); low level of education, unemployment, and low income; multiple sex partners; not using a condom, diaphragm or spermicide; lack of attention to individual health care for both men; and women and smoking, alcohol consumption, and drugs (25, 63, 64).

Several meta-analysis studies in Iran have focused on other infections in pregnant women and reported the following results: prevalence of urinary tract infection (11.2%) (65), hepatitis B (2%) (66, 67), and Helicobacter pylori (45.9%) (68, 69). According to Ahmadi et al. (70), the prevalence of urogenital mycoplasmas in the male population was 11.1% (95% CI: 7.4-16.4) and 12.8% (95% CI: 9.8-16.5) in females, which was high. Hence, *Chlamydia trachomatis* is one of the most common infections in pregnant Iranian women. Determining the causes of these infections and methods of prevention should be

among the medical priorities for pregnant Iranian women to ensure the health of the next generation.

The limitations of the study included the failure to search using a combination of words in internal databases due to low sensitivity and the inability to perform further subgroup analysis because of the limited number of studies.

Future case-control studies to determine the role of various risk factors in Iranian societies seems necessary.

## Conclusion

The high prevalence of reproductive tract infections among pregnant Iranian women necessitates screening these women as a preventive measure. Therefore, timely recognition and treatment of this disease can prevent maternal and fetal complications.

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## Author's Contributions

M.A., Gh.B., A.M., M.H.Y.K., W.K., Z.T., A.S., Sh.A.; Participated in study design, data collection and evaluation, drafting. M.H.Y.K., M.A.; Statistical analysis. M.A., Gh.B., M.H.Y.K., Sh.A.; Scientific review and helping in discussion. All authors performed editing and approving the final version of this paper for submission, also participated in the finalization of the manuscript and approved the final draft.

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Original Article

# Evaluation of Influencing Factors on Tubal Sterilization Regret: A Cross-Sectional Study

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## Abstract

**Background:** The aim of this study is to evaluate the menstrual pattern, sexual function, and anxiety, and depression in women with poststerilization regret, and potential influencing factors for regret following tubal ligation (TL) in Iranian women.

**Materials and Methods:** In this cross-sectional study, 166 women with TL were subdivided into two groups including women with poststerilization regret (n=41) and women without poststerilization regret (n=125). They were selected from a health care center in Guilan province (Iran) during 2015-2016. Menstrual blood loss was measured using the Pictorial Blood Loss Assessment Chart (PBLAC) and through a self-administered questionnaire. In addition, sexual function was assessed by the Female Sexual Function Index (FSFI), and psychological distress was measured by employing the Hospital Anxiety and Depression Scale (HADS). Student's t test and Chi-square test were used to reveal the statistical differences between the two groups. We used logistic regression to determine the influencing factors associated with regretting sterilization.

**Results:** Women with poststerilization regret had more menorrhagia (78 vs. 57.6%, P=0.03) than those who did not regret sterilization. A significant difference was found in sexual dysfunction in orgasm (P=0.02), satisfaction (P=0.004), pain (P=0.02), and total FSFI scores (P=0.007) between the two groups. Also, there was a significant difference between the two groups in anxiety, depression and total scores HADS (P=0.01). In the logistic regression model, age of sterilization [odds ratio (OR=2.67), confidence interval (CI): 1.03-7.81, P=0.04)], pre-sterilization counseling (OR=19.92, CI: 6.61-59.99, P<0.001), score of PBLAC (OR=1.01, CI: 1.004-1.01, P=0.001), the number of days of bleeding (OR=1.37, CI: 1.01-1.99, P=0.04), and the length of menstrual cycles (OR=0.91, CI: 0.84-0.99, P=0.03) were significantly associated with regretting sterilization.

**Conclusion:** Complications due to sterilization are the main causes of regret; therefore, it is necessary to pay due attention to mentioning the probable complications of the procedures such as menstruation disorders, sexual dysfunction, and anxiety and depression in women during pre-sterilization counseling.

***Keywords:*** Anxiety, Menstrual Cycle, Regret, Sexual Dysfunction, Tubal Ligation

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## Introduction

Permanent contraception method is a greatly desired and frequently used contraceptive option for women around the world who desire never to become pregnant (1). Tubal ligation (TL) is the most prevalent contraceptive method practiced in many countries, chosen by those women who no longer want children or have decided to limit the size of family (2). Despite the wide popularity of this method, more recent evidence suggests that some TL women may regret their decision after sterilization during the ensuing years (3).

An increasing number of women have shown post-TL regret for their decision to undergo TL. Some studies have revealed that the prevalence of post-sterilization regret ranges from 0.9% to 26% (4, 5). Regret rates among sterilized women in Iran vary from 6 to 12.5% of all sterilized women (6). Definition of regret after sterilization may be different in the various studies, and this may bias the rates reported (7). The term "regret" is commonly associated with the feeling of sadness, pain, hurt, affliction, anxiety and displeasure, some authors have considered "clearly regretful" only for the women who obviously show their desire and intention to undergo surgery for reversal of sterilization (ROS) (8). The interval from post-sterilization regret and eventual request for ROS varies in different studies (9, 10).

Some factors such as sterilization age, the death of chil-

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dren, the number of children at the time of TL, remarriage, changes in socioeconomic status, and lack of information about surgical sterilization contribute to post-sterilization regret (3). If the factors associated with post-sterilization regret could be identified prior to TL, the feeling of regret by TL women could be prevented (7).

Although TL is particularly common in the developing than in the developed countries, recently the majority of studies on post-sterilization regret have been carried out on women in the developed countries (7). Research on sterilization regret in Iran is limited; hence, the overall objective of this pioneering study is to identify the potential influencing factors on regret following TL in Iranian women, and also to evaluate the menstrual pattern, sexual function, and anxiety, and depression in women regretting sterilization.

## Materials and Methods

In this cross-sectional study, first, a pilot study was conducted on 20 women. Then, using the appropriate formula with α set at 0.05 and 1-β at 0.95, it was found that a sample size of 40 women was needed for each group. This cross-sectional study was conducted on women (aged 20-40 years) undergone TL. They were selected from a health care center in Guilan province (Iran) during 2015-2016.

Satisfaction with TL was evaluated in response to questions such as "Do you think TL as a permanent method of birth control was a good choice for you?" Those who answered 'no' were further asked: "Do you regret for deciding to undergo sterilization?" If the answer was a consistent yes, then this group was considered to have regretted the decision (women regretting sterilization group). Further questioning continued to seek reasons for regret. This included interrogation regarding menstrual irregularities, depression and anxiety, sexual dysfunction, and having desire to have more children. The last question was: "Have you ever requested that your sterilization is reversed?" Possible responses included "no" and "yes" (11).

A total of 238 women were enrolled in the study; 166 women were eligible for inclusion, and 72 women were excluded from the study. The final analysis was conducted on 41 women regretting the decision, and 125 women not regretting the decision.

The inclusion criteria were free of any gynecological diseases, free of chronic diseases, include diabetes, hypertension, thyroid and cardiovascular diseases, not being in the postmenopausal period, not using antidepressants, not having the history of sexual abuse, not having the history of menstrual disorders before TL, not being a cigarette smoker, not having the history of operative gynecology except caesarean section and TL, and not doing breastfeeding.

We compared the distribution of demographic and obstetrical characteristics, menstrual disorder, sexual function, and depression and anxiety between the two groups. This study was performed after obtaining approval from our Institutional Review Board (IRB # 1056668). All women participated voluntarily and provided a signed informed consent.

### Measures

Menorrhagia is defined as a Pictorial Blood Loss Assessment Chart (PBLAC) score of ≥100 (12). A validated PBLAC was also used for evaluating the Menstrual Blood Loss (MBL) (13). Participants used this chart to keep diaries on the amount of daily menstrual bleeding by marking the number of clots, and the amount of staining on each pad or tampon. Everyone completed their charts for one menstrual cycle, and all patients used the same sanitary products.

The participants' sexual function was evaluated and compared by using the Female Sexual Function Index (FSFI) questionnaire. This standardized questionnaire is a validated, 19 items, self-administered, and a screening tool that measures six aspects of sexual function (desire, arousal, lubrication, orgasm, satisfaction, and pain). Each question describes the status of sexual function during the last 4 weeks. The full-scale score range is from 2.0 to 36.0, with higher scores associated with a lesser degree of sexual dysfunction (14). In this study, we used the Persian version translated by Mohammadi et al. (15). A score <3.3 in the desire domain, score <3.4 in arousal and orgasm, score <3.8 in satisfaction and pain, score <3.7 in lubrication, and total score <28 were considered as sexual dysfunction.

The Hospital Anxiety and Depression Scale (HADS) was used to assess depression and anxiety. The instrument has two subscales including anxiety (HADS-A) and depression (HADS-D). The HADS is a self-administered instrument consisting of 14 questions. The instrument has two subscales including anxiety (seven items) and depression (seven items). All items rate from 0 to 3. Sum scores <8 indicate normal range; scores 8-10 reflect mild alterations and scores ≥11 indicate clinical relevance of symptoms (16). A study on Persian version of the HADS has shown that this scale has a satisfactory reliability and validity for measuring psychological symptoms in Iranian patients (17).

The study was approved by the Tarbiat Modares Ethical Committee and all subjects signed a written informed consent.

### Statistical analysis

All statistical analyses were performed by the SPSS software (version 20.0, SPSS Inc., Chicago, IL, USA). Student's t test and Chi-square test were used to reveal the statistical differences between the two groups, after adjusting for women's age (at the time of data collection), age at the time of sterilization, partner's age, education levels, BMI. We used logistic regression to determine the influencing factors associated with regretting sterilization. Women's age (at the time of data collection), age at the time of sterilization, pre-sterilization counseling,

PBLAC score, the number of days of bleeding days, the length of menstrual cycles, total score of FSFI, and total score of HADS were included in the regression analysis as continuous variables. Odds ratio (OR) at 95% confidence interval (CI) were also calculated for each factor. P<0.05 were considered to be statistically significant.

## Results

The mean duration of TL was 4.6 ± 1.2 years. The demographic and reproductive of participants are shown in Table 1. Both groups were not significantly different in terms of age (at the time of data collection), partner's age, menarche age, BMI, parity, educational level, previous contraceptive use, and the method of delivery. There were significant differences in pre-sterilization counseling between the two groups. Of 166 women who completed the questionnaires, 34.9% did not receive any pre-sterilization counseling from a physician or a healthcare worker (Table 1).

Regret declined as the age of the TL women increased. There was a significant difference between women with poststerilization regret and those who did not regret sterilization. Post TL regret was found in those aged less than 30 years (age at the time of sterilization) and those above the age of 30 (P=0.01, Table 1). Our participants did not have a history of remarriage or the death of children.

### Menstrual pattern status in the two groups of study after adjusting

Table 2 displays the findings regarding the participants' menstruation disorders. There was a significant difference between the two groups in PBLAC score for menstrual loss between the two groups. The mean score of PBLAC was significantly higher in the women with poststerilization regret compared to their counterparts who did not regret sterilization (214.21 ± 116.08 vs. 126.24 ± 72.46, P<0.001) (Table 2). The women regretting sterilization had more menorrhagia (78 vs. 57.6%, P=0.03) than those who did not regret sterilization. There is a significant difference between the two groups in the length of menstrual cycles (P=0.005), and also in the number of days of bleeding (P<0.001, Table 2).

**Table 1:** Comparison of demographic and personal characteristics between two groups

| Parameter | Regret n=41 | | Non-regret n=125 | | P value |
|---|---|---|---|---|---|
| | Mean ± SD | n (%) | Mean ± SD | n (%) | |
| Women's age (Y) | 36.06 ± 3.20 | | 35.95 ± 4.40 | | 0.10** |
| Partner's age (Y) | 40.51 ± 4.84 | | 41.21 ± 4.13 | | 0.36** |
| Age of menarche (Y) | 12.78 ± 1.72 | | 12.64 ± 1.23 | | 0.57** |
| Age of sterilization (Y) | | | | | |
| ≤30 | | 19 (46.3) | | 30 (24) | 0.01* |
| >30 | | 22 (53.7) | | 95 (76) | |
| Parity | 2.36 ± 0.58 | | 2.32 ± 0.56 | | 0.65** |
| BMI (Kg/m$^2$) | 28.00 ± 5.92 | | 27.96 ± 4.62 | | 0.97** |
| Education level | | | | | |
| Primary school | | 13 (31.7) | | 24 (19.2) | |
| Completed high school | | 12 (29.3) | | 50 (40) | 0.20* |
| University | | 16 (39) | | 51 (40.8) | |
| Method of delivery | | | | | |
| Normal vaginal delivery | | 10 (24.4) | | 31 (24.8) | 0.12* |
| Caesarean section | | 31 (75.6) | | 94 (75.2) | |
| Previous contraceptive method used | | | | | |
| Pill | | 4 (9.8) | | 7 (5.6) | |
| Condom | | 31 (75.6) | | 104 (83.2) | 0.51* |
| Other*** | | 6 (14.6) | | 14 (11.2) | |
| Pre-sterilization counseling | | | | | |
| No | | 35 (85.4) | | 23 (18.4) | <0.001* |
| Yes | | 6 (14.6) | | 102 (81.6) | |

BMI; Body mass index, *; Chi-square test, **; t test, and ***; This category included withdrawal and natural family planning or the rhythm method.

**Table 2:** Comparison of changes in menstrual function between two groups

| Parameter | Regret n=41 | | Non-regret n=125 | | OR adjusted (95% CI) | P value* |
|---|---|---|---|---|---|---|
| | Mean ± SD | n (%) | Mean ± SD | n (%) | | |
| Menstrual cycle length (day) | 25.34 ± 6.81 | | 29.01 ± 5.94 | | 0.89 (0.83-0.96) | 0.005 |
| Duration of bleeding menstrual (day) | 7.41 ± 1.91 | | 6.30 ± 1.35 | | 1.64 (1.24-2.16) | <0.001 |
| Menstrual irregularities | | 13 (31.7) | | 20 (16) | 2.14 (0.91-5.05) | 0.07 |
| Menorrhagia | | 32 (78) | | 72 (57.6) | 2.44 (1.04-5.69) | 0.03 |
| PBLAC score | 214.21 ± 116.08 | | 126.24 ± 72.46 | | 1.01 (1.006-1.01) | <0.001 |

BMI; Body mass index, OR; Odds ratio, CI; Confidence interval, and *; P values are adjusted for women's age (at the time of data collection), age at the time of sterilization, education levels, BMI.

### Sexual function status in the two groups of study after adjusting

Evaluation of the two groups by FSFI showed that all mean values were lower in the women with poststerilization regret. The differences of scores in the two groups were statistically significant in the domains of orgasm (OR= 0.68, CI:0.49-0.94, P=0.02), satisfaction (OR=0.59, CI:0.41-0.84, P=0.004), pain (OR=0.72, CI:0.54-0.95, P=0.02), and total FSFI scores (OR=0.88, CI:0.88-0.96, P=0.007) (Table 3).

The women regretting with poststerilization regret had more sexual dysfunction in the domains of satisfaction (48.8 vs. 30.4%, P=0.03), pain (48.8 vs. 28%, P=0.01), and total FSFI scores (63.4 vs. 40.8%; P=0.01) than the other group (data not shown).

### Anxiety and depressive status in the two groups of study after adjusting

The mean scores of anxiety and depression were found to be higher in the women with poststerilization regret compared to their counterparts who did not regret sterilization, and the differences between the two groups were statistically significant on anxiety scale (OR=1.14, CI:1.03-1.27, P= 0.01), depression scale (OR=1.14, CI:1.02-1.27, P=0.01), and total HADS scores (OR=1.09, CI:1.02-1.16, P= 0.01) (Table 3).

Of the women regretting sterilization, 61% (n=25) demonstrated elevated HADS anxiety scores (i.e. HADS anxiety subscale ≥11), and 17.1% (n=7) showed higher HADS depression scores (i.e. HADS depression subscale ≥11). Finally, 35 women (85.4%) in the women regretting sterilization group scored above the cut-offs (≥11) for both anxiety and depression (data not shown).

### Reasons for sterilization, regret, and reversal

The reason for requesting sterilization in the majority of women was the higher effectiveness of sterilization (36.8%) as compared to other methods. Other reasons were having enough children or having no desire for more children (35.5%), and unsatisfied with other contraceptive methods for their many side effects (27.7%).

**Table 3:** Scores and total scores for the domain subgroups of sexual function and HADS between two groups

| Parameter | Regret n=41 (Mean ± SD) | Non-regret n=125 (Mean ± SD) | OR adjusted (95% CI) | P value* |
|---|---|---|---|---|
| FSFI | | | | |
| Desire | 2.83 ± 0.78 | 3.11 ± 0.76 | 0.66 (0.39-1.10) | 0.11 |
| Arousal | 3.16 ± 1.04 | 3.49 ± 0.89 | 0.70 (0.45-1.07) | 0.10 |
| Lubrication | 3.68 ± 1.28 | 4.06 ± 1.08 | 0.80 (0.56-1.14) | 0.22 |
| Orgasm | 3.55 ± 1.42 | 4.17 ± 1.13 | 0.68 (0.49-0.94) | 0.02 |
| Satisfaction | 3.82 ± 1.14 | 4.49 ± 1.13 | 0.59 (0.41-0.84) | 0.004 |
| Pain | 3.81 ± 1.65 | 4.41 ± 1.19 | 0.72 (0.54-0.95) | 0.02 |
| Total score | 20.87 ± 5.91 | 23.75 ± 4.52 | 0.88 (0.88-0.96) | 0.007 |
| HADS | | | | |
| Anxiety score | 11.39 ± 4.06 | 9.48 ± 3.67 | 1.14 (1.03-1.27) | 0.01 |
| Depression score | 7.97± 3.68 | 6.12 ± 3.49 | 1.14 (1.02-1.27) | 0.01 |
| Total score | 18.97 ± 6.75 | 15.56 ± 6.07 | 1.09 (1.02-1.16) | 0.01 |

FSFI; Female Sexual Function Index, HADS; Hospital Anxiety and Depression Scale, BMI; Body mass index, OR; Odds ratio, CI; Confidence interval, and *; P values are adjusted for women's age (at the time of data collection), age at the time of sterilization, education levels, BMI.

No significant difference was found between the women regretting sterilization and the women not regretting sterilization in reasons for requesting sterilization (data not shown).

When the women with poststerilization regret were asked to state reason(s) for regret, 43.9% (n=18) had both menorrhagia, anxiety and depression, 19.5% (n=8) reported having sexual problems, menorrhagia, and anxiety and depression after the operation, 14.6% (n=6) had anxiety and depression, 12.2% (n=5) simply wanted to have another child, and about 9.8% (n=4) regretted the operation because it brought about only menorrhagia.

Requesting ROS after TL was 3% (5 women). The reasons for requesting ROS after TL involve both menorrhagia, and anxiety and depression (n=2), both sexual problems and menorrhagia (n=1), desire for having more children (n=1), and only menorrhagia (n=1). Age at the time of sterilization can affect desire for ROS. Women younger than 30 years at the time of sterilization were more likely to request reversal than those who were above 30 years old (80 vs. 20%, P=0.02) (data not shown).

There were significant differences in pre-sterilization counseling between the women requested reversal and those who did not. Of the 5 ROS women, nobody did receive any pre-sterilization counseling from a physician or a healthcare worker (P=0.005) (data not shown).

Finally, in order to build a prediction model and to find the most important factors predicting with poststerilization regret, we used a logistic regression model in a backward manner. The results of fitting the logistic regression model to the data (Table 4). Only significant results are presented in Table 4.

**Table 4:** Logistic regression analysis of 166 women for regretting sterilization

| Parameter | OR (95% CI) | P value* |
|---|---|---|
| Age of sterilization (Y) | | |
| ≤30 | 2.67 (1.91-7.81) | 0.04 |
| >30 | 1† | |
| Pre-sterilization counseling | | |
| No | 19.92 (6.62-59.90) | <0.001 |
| Yes | 1† | |
| PBLAC score | 1.01 (1.004-1.07) | 0.001 |
| Number of days of bleeding | 1.37 (1.01-1.99) | 0.04 |
| Length of menstrual cycles | 0.91 (0.83-0.99) | 0.03 |
| Constant | 0.012 | 0. 01 |

OR; Odds Ratio, CI; Confidence interval, †; Reference category, *; P value logistic regression, and women's age (at the time of data collection), age at the time of sterilization, parity, pre-sterilization counseling, PBLAC score, number of days of bleeding, length of menstrual cycles, total score of FSFI, and total score of HADS were included in the regression analysis as continuous variables. Only significant results are presented.

In the logistic regression model, age of sterilization (OR=2.67, CI: 1.91-7.81, P=0.04), pre-sterilization counseling (OR=19.91, CI: 6.62-59.90, P<0.001), score of PBLAC (OR=1.01, CI: 1.004-1.07, P=0.001), and the number of bleeding days (OR=1.37, CI: 1.01-1.99, P=0.04) were significantly associated with poststerilization regret. However, the length of menstrual cycles (OR=0.91, CI: 0.83-0.99, P=0.03) was negatively related to regretting sterilization regret (Table 4).

## Discussion

Tubal ligation is chosen by the women who have decided to limit the size of their families or those who are sure that no longer want to have children (18). The goal of this study was to evaluate the menstrual pattern, sexual function, and anxiety and depression, in women regretting sterilization, and also identify the potential influencing factors for regret following TL. To our knowledge, none of the several recent studies on Iranian population have investigated the influencing factors for regret after TL.

The present results indicated that women with poststerilization regret were more likely to experience an increase in menorrhagia when compared with the other group. We found a significant increase in PBLAC score for menstrual blood loss in the women regretting sterilization when compared with the other group. Also the women regretting sterilization were more likely to experience a shortening of the duration of menses and an increase in the number of days with bleeding. The term "Post-TL Syndrome" (PTLS) has been used variously to include menstrual disorders, dysmenorrhea, premenstrual distress, and miscellaneous other conditions like menopausal syndrome, feeling of regret, and need for recanalization (19). Wilcox et al. (20) reported that menstrual irregularities were not more prevalent among the women regretting their sterilization than those not regretting their sterilization. Malhotra et al. (21) mentioned that menstrual irregularities and dysmenorrhea did not influence regret.

Our findings suggest that menorrhagia were more common in the women regretting sterilization in comparison to the women not regretting sterilization. The relationship between regretting sterilization and menstrual disorders is a complex process influenced by multiple factors including psychological and cultural conditions, as well as behavior, ethnicity, climate, and religion.

It was revealed that regret following TL may be an influencing factor of women's sexual dysfunction. In the present study, the prevalence of FSD in the women regretting TL was 63.4% in comparison with 40.8% in the other group. Warehime et al. (22) suggested a relationship between sexual dysfunction and post-sterilization regret in women with TL. Shah and Hoffstetter (23) reported that TL had a positive impact on sexual function unless women regretting sterilization.

"Making a decision about sterilization is difficult for both women and men, as it means ending fertility. As negative biological and psychological issues may occur after vaginal surgeries including loss of sexual function, the same negative effects after TL could be expected". The negative effects of PTLS on general health status and the sexual function have not been described yet (24).

"In societies such as Iran, the picture a woman holds of herself is to a high degree dependent on her fertility and motherhood ability. It conveys to women feelings of satisfaction, perfection, and value. Despite the women's volunteer participation in the sterilization programs, the cultural factors, which are rooted in their deep unconscious layers, seem to produce in some of them an unattractive and imperfect picture after a while; this can be reflected in the appearance of sexual dysfunction and regret from this operation" (25).

The results of the present study showed that anxiety and depression were more common in the women regretting sterilization in comparison to the other group. Kelekçi et al. (26) reported that anxiety and depression scores in the women regretting sterilization were significantly higher than in the control group. Regret following TL may contribute to higher anxiety and depression scores after the procedure, and may pointedly affect the psychological health of women. In addition, they suggested that women with high anxiety and depression scores must be given time to consider their decision on fertility state, and should be provided psychological support during this time. A high preoperative anxiety and depression score may be an essential factor in future post-TL regret and high anxiety and depression scores (27). Counseling before sterilization, especially in younger women, needs to cover alternative birth control methods, irreversibility, and failures of the procedure. It should future elicit information about the couples' psychosocial and marital dynamics, the somatic or psychological symptoms, as well as women's sexual history and menstrual history (28).

The psychological status of women prior to the sterilization technique may be important in predicting future satisfaction and success of an operation. Depression is one of the most prevalent illnesses in women, which significantly affects their quality of life. It has been thought that anxiety and depression are related to cyclical hormonal changes, which may be affected by TL (26).

In the present study, the prevalence of requesting ROS after TL was 3%. Age at the time of sterilization can affect desire for ROS. Women younger than 30 years old at the time of sterilization were more likely to request reversal than women above the age of 30 (80 vs. 20%). There were significant differences in pre-sterilization counseling between the women requested reversal versus those not requested reversal. From 5 ROS women, nobody did receive any pre-sterilization counseling from a physician or healthcare workers.

Previous studies have indicated several factors predicting desire for ROS (3). Age at the time of sterilization can affect desire for ROS. Women with TL at younger ages were significantly more likely to experience regret than older TL women (7). Rogayeh et al. (6) reported that 2.7% of the sterilized women requested a reversal surgery. Overall, recent evidence suggests that the younger the women are when they undergo TL, the more likely they are to regret their decision, to request for information regarding ROS, or to obtain a ROS (7).

Young age at sterilization, pre-existing emotional disorders, new marriage, and a history of unreliable contraceptive method increased the likelihood of a request for ROS. Women requesting TL should be advised that TL is permanent if no further tubal surgery is performed and that ROS is feasible but involves expensive operation (2).

In this study, we aimed to examine various parameters that might predict the incidence of regretting sterilization in women with TL. Age at the time of sterilization, pre-sterilization counseling, score of PBLAC, and the number of days of bleeding was evaluated as predictors for regretting sterilization. In addition, the length of menstrual cycles was negatively associated with regretting their decision. We found a significant difference in the age at the time of sterilization under 30 years between the women regretting versus those not regretting their sterilization. Young age at sterilization was the strongest predictor for post-sterilization regret (3, 7). Rogayeh et al. (6) reported that post-sterilization regret rate did not increase for the women under 30 or younger when sterilized.

Additionally, the other factor significantly associated with regret is counseling before sterilization (29). This result supports the recommendation that women should be fully informed about the TL procedure and its complications, and have access to other contraceptive options before being sterilized. If factors that lead to regret could be identified prior to sterilization, some of this regret may be prevented (7). It was revealed that there were significant differences in pre-sterilization counseling between the two groups. The women regretting sterilization group had lower pre-sterilization counseling than in the other group. In our study, pre-sterilization counseling was only reported by 14.6% of the women regretting sterilization. With respect to personality and adaptability differences in facing the changes, pre-sterilization counseling has an important role to play in psychological and psychosexual aspects of women health promotion (6). This finding is consistent with the results of many similar studies conducted in other countries (6, 21). Allyn et al. (28) reported a positive correlation between the degree of contentment with pre-sterilization counseling and the level of satisfaction with being sterilized. It was also found that only 11.3% of under 25 women at the time of surgery completely regretted their sterilization. The ethnic backgrounds of the participants and the number of living children at the time of TL had no significant effect on their satisfaction with sterilization.

More knowledge about the potential influencing factors, which have a strong association with regretting the sterilization decision and are easily identifiable before sterilization, is definitely valuable in counseling to avoid regrets. Motivators who may not be doctors in our society need to be informed of the influencing factors prior to counseling women for TL (21).

Although post-sterilization regret is hardly avoidable,

certain groups such as women under 30 years need specific counseling before taking such a major decision like TL (21).

There are potential limitations to consider in interpreting the results of this study. First, expressing post-sterilization regret is generally an attitudinal measure for which there is no standardized definition. For this reason, these rates may overestimate true sterilization regret. Second, we could not assess the method of TL because women did not have information about the type of sterilization.

## Conclusion

The results showed that complications due to sterilization are the main causes of post-sterilization regret. Therefore, it is necessary to pay due attention to mentioning the probable complications of the procedure such as menstruation disorders, sexual dysfunction, and anxiety and depression in women during the pre-sterilization counseling; post-sterilization counseling is also encouraged for increasing satisfaction rate in these volunteers.

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## Author's Contributions

S.Z., Sh.J.S., E.M.; Contributed to conception and design. S.Z., Sh.J.S., E.M., A.K.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. S.Z., E.M.; Were responsible for general supervision. S.Z., Sh.J.S.; Drafted and revised the manuscript. All authors have read and approved the final manuscript.

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Original Article

# Sexual Behaviors and Its Predictors among Iranian Women Living in Kashan City 2017: A Cross-Sectional Study

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## Abstract

**Background:** Women's sexual well-being has been the center of attention in the field of sexology. Study of sexual behavior and investigating its predictors are important for women's health promotion. This study aimed to explore the components of women's sexual behaviors and their possible associations with demographic variables.

**Materials and Methods:** This study was a cross-sectional study (descriptive and analytic) that was conducted in Kashan city, Iran. A National Sexual Behavior Assessment Questionnaire was completed by 500 women of 15 to 49 who referred to the public health centers. To analyze the data, R software was used, ANOVA or Kruskal-Wallis (for parametric or nonparametric data, respectively) were used to compare outcomes among different groups. In order to evaluate the correlation between the subscales, the Pearson correlation coefficient was used.

**Results:** From all participants, 31.8% obtained high scores in the sexual capacity, 21.2% had high scores in sexual motivation and 0.2% had high scores in sexual function. In sexual script component, 86.2% of women who held traditional beliefs toward sexual behaviors; the majority (91.5%) of women believed in mutual and relational sexuality, 83.4% believed in androcentricity (male-dominated sexuality). Pearson correlation test showed a significant positive correlation between sexual capacity, motivation, function and sexual script. Linear Regression model showed that sexual capacity is associated with women's education and age of her spouse. Sexual function and sexual motivation were significantly associated with the age of subjects' spouses.

**Conclusion:** In this study, subjects had low scores in sexual performance while higher scores were achieved in sexual capacity and motivation. This discrepancy can be attributed to the role of sexual scripts dominating the participants' sexual interactions in this study. We suggest gender-specific and culturally-sensitive education should become a part of women's health programs in Iran.

***Keywords:*** Iran, Sexual Behaviors, Women

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## Introduction

In recent years, researchers have focused on the topic of sexual well-being (1-4). In this regard, sexual behaviors as the main aspect of one's sexuality have been investigated in order to comprehensively assess one's sexuality-related needs (5-7). Though there has been extensive research on sexual well-being, attention to women's sexual well-being is increasing (8-10) because gender-specific approaches have become center of focus in sexuality education programs (11, 12). Gender-specific approaches are seminal; particularly in societies where sexuality is dominantly gendered (13).

According to the literature, sexual behaviors do not only refer to erotic behaviors but they involve gender role as well as reproductive and life enhancing behaviors. Hilbert pointed out three main factors namely, sexual capacity (i.e. what person can do), sexual motivation (i.e. what person wants to do); and sexual performance (i.e. what person does), which all affect human's sexual behaviors (14). Human sexual behavior includes all physical-biological aspects, life experiences, knowledge, attitudes and behaviors, and is influenced by several factors such as physical, physiological, interpersonal and cultural issues (15).

Despite the increasing attention towards sexual well-being of women in particular, it is noteworthy that not only

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primary sexual health assessment is being ignored, but also basic sex education is completely absent in females' health programs in Iran (15, 16). This should be considered a major shortcoming since sexual behaviors are highly regarded as essential elements of women's capacities that influence their marital happiness (17, 18). In some studies presented the discourses and symbolic representations through which the women stated their own experiences about womanhood with focusing on sexuality in the context of marriage (19-21). They explicitly reveled a strong link between women's sexuality and marital happiness:

"Despite many opportunities available for frank discussion on sexual topics in the context of marriage, penetrating the individuals' silence was much harder than I expected. Perhaps, I had underestimated the power of the marriage. The crucial point is that open discussion on sexuality and conceptualization of actual sexual activity, were possible only within the context of marriage"(19).

This argument is also confirmed by results of several Iranian empirical research (15, 22). These studies have demonstrated that a positive evaluation of one's sexual behavior is closely associated with her/his overall sexual well-being. Given the facts that sexuality is considered as an integral part of women's well-being and their marital happiness, sexual behaviors should be evaluated by a comprehensive sexual well-being assessment, in practice; however, this step has not yet been taken in Iranian clinical settings. This current situation entails the challenge that sexual health care and sexual well-being of women are simply disregarded when developing policies, making decisions and delivering care fora general promotion of subjective well-being and quality of life among women and families.

Despite extensive studies, there are some challenges (i.e. the approach that should be used for assessment of sexual behaviors is not clear) in studies undertaken in the field of women's sexual behaviors (17).

A study on women's sexual behavior was conducted by Avasti et al. (23) in India. In this study, vaginal sexual activity was the most common form of sexual activity and oral sex had the lowest prevalence; also, 27% of women reported one problem (such as headaches) or more following having sex. Another study done by Hashemi et al. (24) investigated certain sexual behaviors in women of reproductive age. The results of this study indicated that although oral and anal sex is taboos in Iran, but they have a high prevalence among the couples.

We used this survey to examine women's sexual behaviors by exploring various aspects of their sexual behaviors and also investigating possible associations between sexual behaviors and demographic variables. The purpose of this study was to explore the components of sexual behaviors and their possible associations with the demographic variables.

## Materials and Methods

This study was a cross-sectional survey (descriptive and analytic) aimed to identify and analyze sexual behaviors among women of reproductive age in Kashan, Iran from June to October 2015. A random sample of 500 women of reproductive age who referred to health centers (14 centers) in Kashan and had the inclusion criteria according to random numbers table was included in this survey. Based on Shirpak et al. (16) study, considering d=1, $\alpha$: 5 %, $\sigma$: 10/9, sample size was calculated 500.

Inclusion criteria included having Iranian nationality and the ability to read and write, and being within the age range of 15-49 years and married as well as having an active sex life. Exclusion criteria comprised of unwillingness to answer questions because of the high number of questions, as well as having medical and surgical conditions.

Research tool in this study was women's sexual behavior assessment questionnaire developed by Qoreshi et al. (25). Psychometric properties of this questionnaire had been assessed in Iranian women of reproductive age. Theoretical framework of this tool is based on the Hilbert's classification of sexual behaviors. This questionnaire was designed in four Likert sub-scales that were consisted of thirty-three items. The first sub-scale (ten questions) measures sexual capacity. The second sub-scale that consists of nine questions is the sub-scale of sexual performance. The third subscale that has the highest number of items (eleven questions) deals with sexual motivation and the fourth sub-scale (three questions) is sexual script. Sexual script includes guides for sexual thinking, feeling and behavior in different situations (14); here, three scripts were considered based on these questions: the religious-sexual script, the right relational script and the male-dominated script.

Each question has a value of zero to five; So, the minimum and maximum scores for sexual capacity are 0 and 50, for sexual motivation are 0 and 45, for sexual function are 0 and 55 and for sexual script are 0 and 15, respectively. Each of these sub-groups was divided into three parts (<33%, 34-66%, and>67%) based on the obtained scores.

After obtaining written informed consents, the questionnaire was completed by the participants. To analyze the data, R software was used for descriptive statistical analysis to describe the participants and The Kolmogorov-Smirnov test was performed to test the normality of continues variables. Data on capacity, motivation and performance variable were found normally distributed, but not the script variable. Moreover, ANOVA was used to compare outcomes among different groups in terms of capacity, motivation and performance variables and Kruskal-Wallis was used for comparing the means of script variable.

In order to evaluate the correlation between the subscales, the Pearson correlation coefficient was used. In addition, we applied the linear regression model to examine the effect of variables on sexual capacity, function and motivation. Also, Bonferroni method was used the post-hoc test with alpha error correction. For checking whether the data fit the model, we examined the normality of residuals. In addition, $R^2$ values were mentioned for each of the models ($R^2$ was 0.66 for capacity, 0.78 for motivation

and 0.58 for performance). For modeling, first we evaluated the relationship of each risk factor (the age of spouse, level of education and other demographic variable) with the outcome variables, variables with a P<0.3 were used in the regression model. The final model was obtained using backward method considering the P<0.05.

This study was approved by Ethic Committee of Tehran and Shahroud University of Medical Sciences in 2015 (IR.SHMU.REC.2015.44 and TUMS 34262-159-01-96). It should be mentioned that we received informed consent from all participants.

## Results

More than half of the women participated in this study (51.9%) were in the age range of 26 to 35 years old. The majority of women (82.8%) were homemaker; also, the majority of them (39.9%) had a high-school education. About half of the women (54.6%) had a middle economic level. The mean duration of marriage was 10.2 ± 7.7 (Table 1).

**Table 1:** Demographic characteristics of individuals participated in this study

| Variable | n (%) |
|---|---|
| Education | |
| Elementary | 129 (25.7) |
| High school | 200 (40) |
| University | 172 (34.4) |
| Employment status | |
| Homemaker | 414 (82.8) |
| Employed | 86 (17.2) |
| Level of education of the spouse | |
| Elementary | 147 (29.3) |
| High school | 179 (35.7) |
| University | 175 (35.0) |
| Employment status of the spouse | |
| Self-employed | 266 (53.2) |
| Worker | 104 (20.9) |
| Employee | 117 (23.3) |
| Retired | 13 (2.7) |
| Socioeconomic status | |
| Very bad | 22 (4.4) |
| Bad | 57 (11.5) |
| Intermediate | 273 (54.6) |
| Good | 113 (22.7) |
| Very good | 35 (7.1) |
| Age (Y), mean ± SD (30.5 ± 7.3) | |
| 15-25 | 148 (29.6) |
| 26-35 | 260 (51.9) |
| 36-45 | 92 (18.5) |
| Age of spouse (Y), mean ± SD (34.8 ± 7.9) | |
| 20-25 | 48 (9.6) |
| 26-35 | 254 (50.7) |
| 36-45 | 150 (30) |
| 46-55 | 42 (8.4) |
| >56 | 7 (1.3) |
| Duration of marriage (Y), mean ± SD (10.2 ± 7.7) | |
| ≤- 5 | 174 (34.8) |
| 6-10 | 128 (25.5) |
| 11-15 | 91 (18.2) |
| >15 | 108 (21.6) |

The scores of sexual behavior in terms of different subgroups. Most women obtained a medium score in terms of sexual capacity and sexual motivation but a low score for sexual function (<18.3 out of 55) (Table 2).

**Table 2:** The scores of sexual behavior according to the subgroups

| Subgroups of sexual behavior (minimum score-maximum score) | n (%) | Mean ± SD |
|---|---|---|
| Sexual capacity (0-50) | | |
| <16.6 | 39 (7.8) | 29.4 ± 8.1 |
| 16.7-33.2 | 302 (60.4) | |
| 33.3- 50 | 159 (31.8) | |
| Sexual performance (0-55) | | |
| <18.3 | 317 (63.4) | 16.5 ± 6.6 |
| 18.4-36.6 | 182 (36.3) | |
| 36.7-55 | 1 (0.2) | |
| Sexual motivation (0-45) | | |
| <15 | 28 (5.5) | 25.7 ± 6.6 |
| 16-30 | 366 (73.3) | |
| 31-45 | 106 (21.2) | |
| Sexual script (0-15) | | |
| <5 | 28 (5.5) | 12.2 ± 3.4 |
| 6-10 | 366 (73.3) | |
| 11-15 | 106 (21.2) | |
| Sexual behavior (0-165) | | |
| <55 | 29 (5.7) | 85 ± 1.6 |
| 56-110 | 442 (88.6) | |
| 111-165 | 29 (5.7) | |

Pearson correlation test showed a significantly positive correlation between the scores of sexual capacity, motivation and sexual (P<0.001). This means with an increase in one score, the scores of other domains also increase.

The majority of participants agreed with three questions of sexual script (Table 3). Question 1 measured the sexual-religious pattern and Question 2 was a correct belief regarding sexual relationship but question 3 was a wrong belief about the sexual relations between a husband and a wife. Pearson correlation test showed a significant relationship between each of the questions of script and sexual function (P<0.001), sexual motivation and sexual capacity; in this regard, there was a positive and significant relationship between sexual behavior and questions 1 and 2 and a significantly negative relationship between sexual behavior and question 3.

The results of the regression model that assessed the association between sexual capacity, motivation and performance and independent variables (Table 4). As shown in this Table, education and the age of spouse had a significant effect on mean score of sexual capacity. Sexual function had a positive and significant relationship only with the age of spouse and women's sexual function declined with increasing age of their husbands. Age of woman and age of spouse had a significant correlation with sexual motivation so that with increasing age of women, sexual motivation increased while it declined with increasing age of the husband.

**Table 3:** Distribution of answers to questions of sexual script

| Numbers of questions | Items | Mean ± SD | I don't know n (%) | None n (%) | Low n (%) | Medium n (%) | High n (%) | Very high n (%) |
|---|---|---|---|---|---|---|---|---|
| 31 | Sexual-religious idea | 3.9 ± 1.4 | 28 (5.9) | 19 (4) | 19 (4) | 57 (12) | 106 (22.3) | 247 (51.9) |
| 32 | A right idea regarding sexual relationship | 4.3 ± 1.1 | 7 (1.4) | 11 (2.3) | 24 (4.9) | 35 (7.2) | 115 (23.6) | 296 (60.7) |
| 33 | Traditional andmale-centered idea | 3.9 ± 1.5 | 24 (4.9) | 44 (9) | 13 (2.7) | 46 (9.4) | 97 (19.9) | 264 (54.1) |

*; P<0.05 was considered significant statistically.

**Table 4:** The results of the regression model (outcome: sexual capacity, performance, motivation)

| | Standardized coefficients | | Unstandardized coefficients | P value |
|---|---|---|---|---|
| | Beta | Std. error | Beta | |
| Sexual capacity | | | | |
| Education | | | | |
| (constant) | 29.416 | 1.528 | - | <0.001 |
| Elementary (base line) | - | - | - | - |
| High school | 2.854 | 1.094 | 0.172 | 0.000 |
| University | 4.392 | 1.152 | 0.259 | <0.001 |
| Age of spouse (Y) | | | | |
| 20-25 (base line) | - | - | - | - |
| 26- 35 | -2.064 | 1.411 | -0.127 | 0.14 |
| 36-45 | -4.300 | 1.507 | -0.239 | 0.000 |
| >45 | -4.021 | 1.907 | -0.144 | 0.03 |
| Sexual performance | | | | |
| Age of spouse (Y) | | | | |
| (constant) | 18.405 | 1.043 | - | <0.001 |
| 20-25 (base line) | - | - | - | - |
| 26- 35 | -1.763 | 1.146 | -0.138 | 0.12 |
| 36-45 | -2.560 | 1.206 | -0.184 | 0.034 |
| >45 | -4.090 | 1.465 | -0.196 | 0.000 |
| Sexual motivation | | | | |
| Age (Y) | | | | |
| (constant) | 26.598 | 1.098 | - | <0.001 |
| 20-25 (base line) | - | - | - | - |
| 26- 35 | -0.565 | 1.325 | -0.044 | 0.67 |
| 36-45 | -3.383 | 1.572 | -0.236 | 0.030 |
| >45 | -7.610 | 2.438 | -0.322 | 0.000 |
| Age of spouse (Y) | | | | |
| 15-25 (base line) | - | - | - | - |
| 26-35 | 1.224 | 0.970 | 0.094 | 0.20 |
| 36 -45 | 7.012 | 2.930 | 0.194 | 0.01 |
| >45 | 2.450 | 1.531 | 0.138 | 0.11 |

*; P<0.05 was considered significant statistically.

## Discussion

The main objective of this study was to explore the components of sexual behaviors and their possible associations with demographic variables. Our results showed that the majority of women participating in this study obtained a medium score for sexual capacity and sexual motivation but a low score for sexual function. Different aspects of sexual behavior showed a significant correlation with some demographic characteristics of women.

Our results are consistent with the results reported by Qoreshi et al. (25) as they found women's passive sexual function and argued that women's not using their sexual capacity is compatible with the male-centered theory in which women are considered as passive parts versus men as active parts.

Since the most important factor of an individual's sexual function is his/her sexual capacity (14), it was found that women do not optimally use their sexual capacity. This can be explained by the role of sexual script dominating the participants' sexual interactions.

In this study, most participants' sexual behaviors were originated from right and wrong sexual scripts. The first question (i.e. "giving positive response to the husband's sexual demands will leads to rewards") is a religious -sexual question suggesting that women's sexual script can be affected by religious beliefs. Merghati Khoi et al. (19) also showed that religion is a main factor in women's sexual self-understanding, and sexual obedience is known as a factor in women's chastity and self-esteem.

This emphasizes the importance of religion in sex education. Women's sexual behaviors seem to change through women's empowerment and educational interventions based on religious norms. Addis et al. (26) showed that patients complain about lack of sex education compatible with culture and religious. In our study, it was identified that the sexual-religious script has a relationship with sexual function, sexual capacity and sexual motivation. Shirpak et al. (16) also found the effect of religion on people's sexual behaviors.

The second question of sexual script (i.e. "giving a positive response to the husband's sexual demands creates more intimacy between couples and peace in the family.") is also a right idea regarding sexual relationship that reinforces the relationship between couples. Merghati khoie et al. (19) believed that this emotional connection is an important factor in women's sexual satisfaction and that should be considered by health care providers. In our study, this script was related to sexual function, sexual motivation and sexual capacity that has been also demonstrated by other studies (25).

But, the third question of sexual script (i.e. "giving a positive response to the husband's sexual demands prevents the husband's extramarital relationships.") represents a traditional cultural scenario created by a restricted and male-dominated sexual script. In the qualitative study of Shirpak et al. (15), it was indicated that although many Iranian women have low desire for sex, they do not refuse their husband's sexual requests. Hashemi et al. (24) showed that one of the reasons that women could not refuse their husbands' sexual demands was the issue of obedience and women's obligation to comply with their husbands' demands, and the other reason was women's fear of their husbands' extramarital affairs. In our study, the limited and male-dominated sexual script had a negative relationship with women's sexual behaviors, which was supported by the study of Qoreshi et al. (25).

In this study, there was a significant relationship between sexual script and women's sexual behaviors. The results of this study indicated the role of social and cultural determinants in shaping sexual behaviors, which is consistent with the social construction theory (14).

In this study, sexual capacity had a significant association with education level and the age of spouse but it was not associated with woman's job, economic status and the number of children. In Addis's study, sexual satisfaction was correlated with age, so that young women were more sexually active and had more sexual satisfaction. In this study, the researcher found that sexual satisfaction reduced with increasing ages (26). Rahmani et al. (1) believed that the cause of decreased sexual satisfaction with increasing age is the interference of sexual activity with other tasks, such as taking care of children, job, etc. Our study identified that education is related to sexual behavior. They also concluded that sexual satisfaction has a relationship with the level of education and the higher the level of education of husbands, the higher women's sexual satisfaction. In the study of Alavi et al. (27) also, with increasing levels of education, sexual function was improved; authors believed that this occurred as educated people participated in educational programs. In general, in our study, sexual behaviors varied according to women's demographic characteristics. Billy e al. (28) also showed that people's sexual behaviors vary according to demographic and social characteristics.

Herbenick et al. (29) in their study on sexual behavior of women living in the United States, showed that sexual behaviors markedly varied among women and masturbation was reported to have high prevalence in women aging 18-39 years. In that study, women reported sex with same-sex partners and oral and anal sex in their recent sexual activities. Sexual behaviors in our study was different from other studies that shows that sexual behavior and sexual orientation vary from one society to another due to differences in socio-cultural structure of each society that is consistent with the theory of social construction. According to this paradigm, all human experiences are sociocultural products. This is also true about the issues related to human sexuality; Masters et al. (14) explained the factors affecting sex and noted that in addition to the biology, social and cultural factors have pronounced influences on sex.

Laws and Scwartz (30) also applied social constructionism to explain sexual behaviors in women. They believed that exclusive experiences such as menarche, pregnancy, childbirth, sex, inability to have sex, and sexual dysfunction, in addition to biological basis, were affected by other events.

Finally, premarital sex education is suggested to be implemented for young women to enable them to identify their sexual capacity and sexual motivation, correct the deterrent attitudes and strengthen the reinforcing attitudes for having safe sex. Suitable sexual behaviors can increase marital satisfaction, maintain the foundation of family and ultimately promote the public health.

## Conclusion

This study results showed that the participants' sexual function is not in at a good level; however, sexual capacity and sexual motivation are reported to be at an acceptable level. This might be due to the role of sexual scripts dominating the participants' sexual interactions in this study. Premarital education can familiarize women with different dimensions of sexual motivation, sexual capacity and sexual function by taking into account cultural-sexual scenarios. Finally, we suggest assessment of sexual behaviors in other age groups such as adolescents and designing an educational program for promotion of sexual behaviors.

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## Author's Contributions

Z.K., E.M.K.; Had full access to all of the study results and takes responsibility for the integrity of data and the accuracy of data analysis. Z.K., E.M.K., S.A.A.; Participated in the study conception and design. Acquisition of data was done by Z.K., analysis of data was conducted by F.A. and finally interpretation of data was conducted by Z.K., Z.G., R.M. and E.M.K. All authors were involved in drafting the manuscript or revising it critically for important intellectual content, and approved the final version to be submitted for publication.

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Original Article

# The Association between ABO and Rh Blood Groups and Risk of Endometriosis in Iranian Women

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## Abstract

**Background:** Endometriosis is a common gynaecological disease that affects quality of life for women. Several studies have revealed that both environmental and genetic factors contribute to the development of endometriosis. The aim of this study was to investigate the distribution of ABO and Rh blood groups in Iranian women with endometriosis who presented to two referral infertility centers in Tehran, Iran.

**Materials and Methods:** In this case-control study, women who referred to Royan Institute and Arash Women's Hospital for diagnostic laparoscopy between 2013 and 2014 were assessed. Based on the laparoscopy findings, we categorized the women into two groups: endometriosis and control (women without endometriosis and normal pelvis). Chi-square and logistic regression tests were used for data analysis.

**Results:** In this study, we assessed 433 women, of which 213 patients were assigned to the endometriosis group while the remaining 220 subjects comprised the control group. The most frequent ABO blood group was O (40.6%). The least frequent blood group was AB (4.8%). In terms of Rh blood group, Rh+ (90.1%) was more frequent than Rh- (9.9%). There was no significant correlation between ABO (P=0.091) and Rh (P=0.55) blood groups and risk of endometriosis. Also, there was no significant difference between the two groups with regards to the stage of endometriosis and distribution of ABO and Rh blood groups (P>0.05).

**Conclusion:** Although the O blood group was less dominant in Iranian women with endometriosis, we observed no significant correlation between the risk of endometriosis and the ABO and Rh blood groups. Endometriosis severity was not correlated to any of these blood groups.

***Keywords:*** ABO and Rh Blood Groups, Endometriosis, Women

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## Introduction

Endometriosis is a chronic gynaecological disorder which severely impacts the quality of life of affected women (1). Endometriosis is defined as the presence of endometrial tissue outside the uterine cavity. The frequency of endometriosis in reproductive age women is 5-10%, whereas for infertile women it is 50% (2). This disease can lead to severe pain, inability to perform daily activities, and negatively affect family relationships, confidence, social functioning, sexual and mental health. The pathogenesis and mechanisms of endometriosis, which lead to the immigration of endometrial cells to areas outside the uterine cavity, has not been determined thus far. Several studies have suggested that endometriosis is caused by the interaction of genetic and environmental factors (2-4).

Identifying risk factors, especially in the severe form of endometriosis, can shorten the time between onset of symptoms and diagnosis. Proposed risk factors include low body mass index (BMI), family history or personal

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history of endometriosis, and dysmenorrhea (5-7). Moini et al. (8) have reported that age, BMI, duration of the menstrual cycle, history of abortion, dyspareunia, pelvic pain, and a family history of endometriosis are independent risk predictors for each type of endometriosis.

On the other hand, several studies have shown a correlation between ABO and Rh blood groups with infectious and noninfectious diseases. In addition, the distribution of ABO and Rh blood groups varies worldwide because of geographical and ethnic differences (9-13).

A biological explanation for the correlation between main blood groups and certain diseases has not been stated. Genomic studies have shown that single variations at the ABO locus and ABO blood groups were associated with pro-inflammatory cytokine tumour necrosis factor (TNF) receptor levels and adhesion molecules such as intercellular adhesion molecule (ICAM) and E-selectin (14-16). ABO and Rh blood groups have been reported as genetic risk factors for tumours and tumour-like diseases (17). The reason for this relationship remains unclear; however, an important assumption is that the ABO and Rh blood group antigens on the tumour cells facilitate tumour cell movement. Although several studies have assessed the relationship between ABO and Rh blood groups with endometriosis, their results were controversial (18-21).

The aim of present study was to identify any association between ABO and Rh blood groups with endometriosis. Identification of an association between ABO and Rh blood groups with endometriosis could assist with diagnosis at the early stages of this disease in at-risk women.

## Materials and Methods

In this case-control study, we assessed women who underwent diagnostic laparoscopy secondary to unexplained infertility or tubal ligation at Royan Institute and Arash Women's Hospital between 2013 and 2014. The Ethics Committee of Royan Institute approved this study and all study participants signed an informed consent form. Inclusion criteria consisted of women with endometriosis or normal pelvis according to laparoscopic findings. Endometriosis was confirmed according to ASRM classification based on the features of the lesions during laparoscopy and pathology reports. A total of 433 women were assigned to the endometriosis and control (women without endometriosis and normal pelvis) groups. In the endometriosis group, we determined the stage of endometriosis according to ASRM classification (22) based on type and distribution of lesions, adhesion or existence of endometrioma.

ABO and Rh blood group typing were performed before laparoscopy at laboratories located in Royan Institute and Arash Women's Hospital. A questionnaire was completed for each woman.

### Statistical analysis

Statistical analysis was performed by using the Statistical Package for the Social Sciences (SPSS) version 20. The chi-square, t test, one-way ANOVA, and logistic regression tests were used for data analysis. Ordinal logistic regression was used to examine the relationship between the ABO and Rh blood groups to stages of endometriosis. Data are presented as number (percent) or mean ± SD. $P<0.05$ were considered significant.

## Results

In this study there were 433 participants. According to laparoscopy findings, 213 women with endometriosis were placed in the endometriosis group and 220 subjects with normal pelvis comprised the control group.

The 433 enrolled women had a mean age of 30.74 ± 5.97 years. The mean age of the endometriosis groups was 30.43 ± 5.81 years and the control group was 31.05 ± 6.11 years, which was not statistically significant [P=0.27, odds ratio (OR): 0.98, 95% confidence interval (CI): 0.95-1.01].

The average BMI in the endometriosis group was 23.48 ± 3.38 kg/m$^2$. The BMI of the control group was 25.88 ± 4.40 kg/m$^2$. Statistical analysis showed a significant inverse association between BMI and endometriosis (P<0.001, OR: 0.85, 95% CI: 0.80-0.90). The OR indicated that each unit increase in BMI decreased the risk of endometriosis.

In this study, the most common ABO blood group was O with a frequency of 40.6%. There was no significant association between ABO blood groups and endometriosis (P=0.091), although the risk of endometriosis in the O blood group was lower than the other blood groups. The Rh+ blood group was present in 390 women (90.1%). There was no significant correlation between Rh blood group and risk of endometriosis (P=0.55, Table 1).

Among demographic variables, regression analysis indicated that a positive family history of endometriosis in first-degree relatives had a significant association with the risk of endometriosis (P=0.004, OR: 8.85, 95% CI: 2.01-38.98). The risk of endometriosis in women whom first-degree relatives (mother, sister) suffered from endometriosis was approximately 9 times more than women without a positive family history (Table 2).

In this study, the most common symptoms of endometriosis were pain during menstruation (73.7%) and infertility (56.3%). There was no significant association between the different stages of endometriosis (I-IV) and frequency of ABO and Rh blood groups (Table 3).

One-way ANOVA was used to compare mean age and BMI of women in different stages of endometriosis. Mean age and BMI did not significantly differ in the different stages of endometriosis (Table 4).

**Table 1:** The frequency of ABO and Rh blood groups in the endometriosis and control groups

| Blood groups | Endometriosis group n (%) | Control group n (%) | OR (95% CI) | P value |
|---|---|---|---|---|
| A | 73 (34.3) | 73 (33.2) | 1.316 (0.84-2.04) | 0.091 |
| B | 50 (23.5) | 40 (18.2) | 1.645 (0.98-2.74) | |
| AB | 14 (6.5) | 7 (3.2) | 2.63 (1.01-6.83) | |
| O | 76 (35.7) | 100 (45.4) | Reference level | |
| Rh+ | 190 (89.2) | 200 (90.9) | Reference level | 0.55 |
| Rh- | 23 (10.8) | 20 (9.1) | 1.21 (0.64-2.27) | |

OR; Odds ratio and CI; Confidence interval.

**Table 2:** Relation between history of endometriosis in first-degree relatives with the risk of endometriosis

| Family history of endometriosis | Endometriosis group n (%) | Control group n (%) | OR (95% CI) | P value |
|---|---|---|---|---|
| No | 197 (92.5) | 218 (99.1) | Reference level | 0.004 |
| Yes | 16 (7.5) | 2 (0.9) | 8.85 (2.01-38.98) | |

OR; Odds ratio and CI; Confidence interval.

**Table 3:** Association of severity of endometriosis with ABO and Rh blood groups

| Blood group | Control group n=220 | Endometriosis group n=213 | | | | P value[c] | P value[b] | OR (95% CI)[a] |
|---|---|---|---|---|---|---|---|---|
| | | Stage 1 | Stage 2 | Stage 3 | Stage 4 | | | |
| A | 73 (33.2) | 6 (27.3) | 7 (30.4) | 27 (36.5) | 33 (35.1) | 0.053 | --- | Reference level |
| B | 40 (18.2) | 4 (18.2) | 2 (8.7) | 19 (25.7) | 25 (26.6) | | 0.292 | 1.297 (0.799-2.106) |
| AB | 7 (3.2) | 3 (13.6) | 3 (13) | 1 (1.4) | 7 (7.4) | | 0.310 | 1.542 (0.668-3.557) |
| O | 100 (45.4) | 9 (40.9) | 11 (47.8) | 27 (36.5) | 29 (30.9) | | 0.127 | 0.723 (0.477-1.096) |
| Rh+ | 200 (90.9) | 20 (90.9) | 20 (87) | 65 (87.8) | 85 (90.4) | 0.871 | --- | Reference level |
| Rh- | 20 (9.1) | 2 (9.1) | 3 (13) | 9 (12.2) | 9 (9.6) | | 0.680 | 1.130 (0.630-2.031) |

Values are given as number (%). [a]; Results are reported based on the odds ratio (OR) obtained from ordinal logistic regression, [b]; P value based on ordinal logistic regression, and [c]; P value based on Fisher's exact test.

**Table 4:** The frequency of different stages of endometriosis, age, and body mass index (BMI) in the endometriosis group

| Stage of endometriosis | n (%) | Age (Y) Mean ± SD | BMI (kg/m$^2$) Mean ± SD |
|---|---|---|---|
| Stage 1 | 22 (10.3) | 31.59 ± 4.53 | 23.65 ± 3.38 |
| Stage 2 | 23 (10.8) | 29.83 ± 4.98 | 23.72 ± 2.78 |
| Stage 3 | 74 (34.8) | 30.64 ± 5.89 | 23.17 ± 3.61 |
| Stage 4 | 94 (44.1) | 30.14 ± 6.22 | 23.62 ± 3.36 |
| Total | 213 (100) | 30.43 ± 5.81 | 23.48 ± 3.38 |
| P value | - | 0.696 | 0.812 |

## Discussion

Several studies have shown that the distribution of Rh and ABO blood groups can depend on ethnicity and change in a geographic area over time (23-28). In the present study, we have determined that O was the most common ABO blood group and the least frequent group was AB. The most common Rh blood group was Rh+. These findings agreed with studies in Iran conducted by Pourfathollah et al. (29) and Hosseini et al. (30).

Although the result of our study showed that the risk of endometriosis in women with blood group O was lower than other ABO blood groups, this difference was not statistically significant. The risk of endometriosis in all ABO blood groups was similar, which was consistent with studies by Daliri et al. (31) in Fars Province, Southern Iran, Kim and his colleague (32) in Korea, and Borghese et al. (33) in France. However, these results differed from findings reported by Demir et al. (21) in Turkey and Matal-

liotakis et al. (20). They reported that the highest risk of endometriosis was among women with blood group A and the lowest risk was seen in women with blood group O.

In the present study, the most frequent Rh blood group was Rh+. There was no significant relationship observed between Rh and the risk of endometriosis, which was consistent with another study in Iran by Daliri et al. (31). However, Borghese et al. (33) in France reported that the chance of developing endometriosis was double in Rh- women and Demir et al. (21) reported that the risk of endometriosis in Rh+ women was significantly higher than the control group.

The results of our study showed that the different stages of endometriosis and ABO and Rh blood groups did not have a significant relationship, which was consistent with findings by Demir et al. (21) and Matalliotakis et al. (20).

The limitations of this study included difficulties in finding women with normal pelvis during laparoscopy and low sample size based on the study power of 0.56. The power of this study indicated the need to conduct a study with a larger sample size.

## Conclusion

This study has shown no significant association between the main blood groups (ABO and Rh) and the risk of endometriosis. It seems that Iranian women with the O blood group are less likely to develop endometriosis compared to women with other blood groups. However, this finding should be confirmed with a larger study population. In addition, the severity of endometriosis was not correlated to any of these blood groups.

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## Author's Contributions

A.M., F.M., E.A.; Conception and design of study. M.T., L.D., R.H.; Acquisition of data. M.R.A., A.M., F.M., E.A.; Analysis and/or interpretation of data. F.M., E.A.; Drafting the manuscript. A.M., F.M., E.A..; Revising the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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Original Article

# Subtelomeric Rearrangements in Patients with Recurrent Miscarriage

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## Abstract

**Background:** The subtelomeric rearrangements are increasingly being investigated in cases of idiopathic intellectual disabilities (ID) and congenital abnormalities (CA) but are also thought to be responsible for unexplained recurrent miscarriage (RM). Such rearrangements can go unnoticed through conventional cytogenetic techniques and are undetectable even with high-resolution molecular cytogenetic techniques such as array comparative genomic hybridization (aCGH), especially when DNA of the stillbirth or families are not available. The aim of the study is to evaluate the rate of subtelomeric rearrangements in patients with RM.

**Materials and Methods:** In this cross-sectional study, fluorescent in situ hybridization (FISH), based on ToTelVysion telomeric probes, was undertaken for 21 clinically normal couples exhibiting a "normal" karyotype with at least two abortions. Approximately 62% had RM with a history of stillbirth or CA/ID while the other 38% had only RM.

**Results:** FISH detected one cryptic rearrangement between chromosomes 3q and 4p in the female partner of a couple (III:4) [46,XX,ish t(3;4)(q28-,p16+;p16-,q28+)(D3S4559+,D3S4560-,D4S3359+; D3S4560+, D4S3359-,D4S2930+)] who presented a history of RM and family history of ID and CA. Analysis of the other family members of the woman showed that her sisters (III:6 and III:11) and brother (III:8) were also carriers of the same subtelomeric translocation t(3;4)(q28;p16).

**Conclusion:** We conclude that subtelomeric FISH should be undertaken in couples with RM especially those who not only have abortions but also have had at least one child with ID and/or CA, or other clinically recognizable syndromes. For balanced and cryptic anomalies, subtelomeric FISH still remains the most suitable and effective tool in characterising such chromosomal rearrangements in RM couples.

***Keywords:*** Chromosomal Aberration, Fluorescent In Situ Hybridization, Intellectual Disability, Translocation, Spontaneous Abortion

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## Introduction

IRecurrent miscarriage (RM), one of the most frustrating problems faced by both patients and clinicians, is recently defined by the American Society for Reproductive Medicine as the miscarriage of two or more consecutive pregnancies in the first or early second trimester of gestation (1).

When conventional cytogenetic techniques are used, balanced chromosomal anomalies are detected in about 5% of RM cases (2). Such rearrangements may result in meiotic errors and chromosomal nondisjunction, leading to the production of unbalanced gametes. The resulting unbalanced chromosomal constitution in gametes may lead to the birth of malformed children, RM or infertility (3). Several studies have shown that the presence of stillborn and/or live born malformed children, in addition to spontaneous abortion, increases the probability for a couple that one partner is a carrier of a balanced translocation (4, 5). Cryptic subtelomeric translocations, which would be missed by conventional techniques (6), may also be frequent in such cases.

In the past decade, subtelomeric rearrangements have been shown to be implicated in congenital malformations and intellectual disabilities (ID) (7, 8). Therefore, high resolution cytogenetic techniques such as subtelomeric fluorescent in situ hybridization (FISH) screening and array comparative genomic hybridization (aCGH) were developed and became reference tools for rearrangement screening in ID and

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congenital abnormalities (CA) cases (9). This need for technological advancement was due to cryptic anomalies being missed by conventional cytogenetic techniques because of their small size and similar banding patterns. Furthermore, because of their quantitative pattern, even revolutionary tools such as aCGH and multiplex ligation-dependent probe multiplex amplification (MLPA) cannot detect balanced rearrangements. Interestingly, only a few studies have proven the usefulness of subtelomeric FISH screening in these cases (7, 10, 11).

Nevertheless, the effectiveness of such a tool in RM cases is still unclear and the exact incidence of such rearrangements remains uncertain. In this study, we screened subtelomeric regions in 21 couples having experienced two or more spontaneous abortions with or without stillbirth and/or children with CA to examine the rate of cryptic subtelomeric translocations in RM.

## Materials and Methods

This cross-sectional study was undertaken in the Department of Cytogenetic and Reproductive Biology at Farhat Hached University Teaching Hospital (Sousse, Tunisia). We selected 21 clinically normal couples, from 01/07/2012 to 31/07/2013, based on the inclusion criteria of having at least two abortions and exhibiting "normal" karyotypes. These couples had normal endocrine function and had no medically assisted procreation attempt in the study period. The women had normal ovarian function, normal genital organs and had no anterior toxic exposure, trauma, radiotherapy, chemotherapy, chronic diseases or medications. The local Ethics Board approved the present study (IRB00008931) and all patients gave informed consent for this study.

### Fluorescent in situ hybridization

R banding karyotyping on peripheral blood lymphocyte cultures was carried out systematically. FISH based on Vysis ToTelVysion Multi-Color FISH Probe Kit (Abbott® Molecular Inc., Des Plaines, USA) was performed for the 21 couples according to the standard protocol. This kit contained 41 TelVysion probes which were specific to subtelomeric regions of chromosomes 1-12 and 16-20, subtelomeres of the q arm of acrocentric chromosomes and pseudo-autosomal region subtelomeres (Xp/Yp and Xq/Yq). For each chromosome, we analyzed at least ten cells and in case of translocations, more metaphases were considered.

## Results

Among the 21 selected couples, only one cryptic subtelomeric translocation was found in the female partner of the 21st couple who were referred to the Obstetrics and Gynecology Department and had a history of two abortions. The pedigree of this couple is illustrated in Figure 1. Around 62% had RM with history of stillbirth or CA/ID and the other 38% had only RM. Using i. TelVysion 3p Spectrum Green (D3S4559) and TelVysion 3q Spectrum Orange (D3S4560) for chromosome 3, and ii. TelVysion 4p Spectrum Green (D4S3359) and TelVysion 4q Spectrum Orange (D4S2930) for chromosome 4, subtelomeric FISH analysis of this patient (III:4) showed a subtelomeric translocation between the long and short arms of chromosomes 3 and 4 respectively. Her chromosomal formula was 46,XX,ish t (3;4) (q28-,p16+;p16-,q28+) (D3S4559+, D3S4560-, D4S3359+; D3S4560+, D4S3359-, D4S2930+).

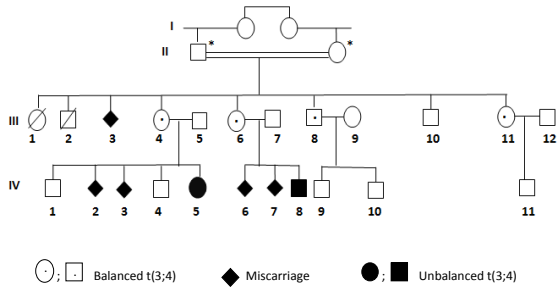


**Fig.1:** Family pedigree. The black arrow points to the couple number 21 (III: 4 and III: 5) in this study. *; Not available for analysis.

The partial karyotype of this patient (III:4) showed apparently normal banding pattern of chromosomes 3 and 4 (Fig.2). FISH results are shown in Figure 3. Investigation of other family members of III:4 showed that her sisters (III:6 and III:11) and brother (III:8) were also carriers of the same translocation t(3;4)(q28;p16).

With this molecular information ignored by the family, two years later, patient III:4 requested consultation after giving birth to a daughter (IV:5) with congenital malformations and ID. The classical cytogenetic analysis of IV:5 showed that she inherited the same translocation in its unbalanced form (46, XX, ish der (4), t(3;4)(q28;p16) from her mother (results not shown).

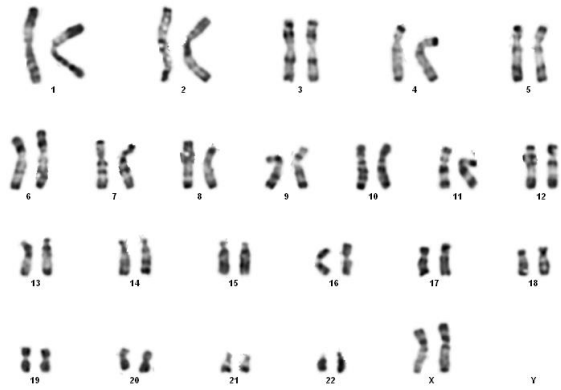

**Fig.2:** Karyotype of the mother (III: 4).

A review of the literature of screening for subtelomeric rearrangements is summarized in Table 1.

**Table 1:** Review of studies screening subtelomeric regions in patients with recurrent miscarriage

| Study | Number of studied cases | Number of subtelomeric rearrangements | Number of miscarriages | Malformed/ stillborn child | Translocation | Technique |
|---|---|---|---|---|---|---|
| Wakui et al. (12) | 10 | 5 | 2 or more | + | 46, XY, t (7;16)(q36; q22) | Dual-colour subtelomere FISH |
| | | | | + | 46, XX, t (4; 7)(q35; p15.3) | |
| | | | | + | 46, XX, t(5; 10)(p15.1; p13 | |
| | | | | - | 46,XY,t(1;5)(q42;q33) | |
| | | | | + | 46,XY,t(7;13)(q36.2;q34) | |
| Giardino et al. (13) | 1 family with 2 female carriers | 1 | 2 | + | 46, XX, t(2;16)(q37.3;q24.3) | Multi-subtelomere FISH using the CytocellMultiprobe-T system |
| Benzacken et al. (14) | 114 | 0 | 2 or more | NM | - | Multi-subtelomere FISH using the CytocellMultiprobe-T system |
| Fan and Zhang (15) | 80 | 0 | 4 or more | NM | - | Multi-subtelomere FISH using the CytocellMultiprobe-T system |
| Joyce et al. (16) | 2 | 2 | 2 | + | 46,XX,t(11;17)(p15.5;p13.3) | Dual-colour subtelomere FISH |
| | | | 4 | + | 46,XX, t(11;17)(p15.5;p13.3) | FISH analysis with telomere-specific probes |
| Yakut et al. (17) | 10 | 2 | 5 or more | - | 46,XY,ish t(3q; 10p) | Subtelomere specific FISH probe |
| | | | 5 or more | - | 46,XX,ish t(20p;?Dp) | Multi-subtelomere FISH using the CytocellMultiprobe-T system |
| Jalal et al. (18) | 53 | 0 | Multiple | NM | - | |
| Bruyere et al. (19) | 1 | 1 | 3 | + | 46,XX, t(2;17) (q37.2; q25) | Multi-subtelomere FISH using the CytocellMultiprobe-T system |
| Cockwell et al. (20) | 100 | 1 | 7 | - | 46,XX t(3:10)(q29;p15.3) | ToTelVysion Multi-Color FISH |
| Monfort et al. (21) | 36 | 1 | 7 | - | 46,XX,t(2;3) | ToTelVysion Multi-Color + Miller-Dieker probe |
| Primerano et al. (22) | 1 | 1 | 2 | + | 46,XX. t(5;17) | ToTelVysion Multi-Color FISH |
| Present study | 42 | 1 | 2 | + | 46, XX, t(3;4)(q28;p16) | |
| Total | 448 | 15 | | 5- | | |
| | | | | 10+ | | |

**+; Exist, -; Does not exist, and NM; Not Mentioned.**

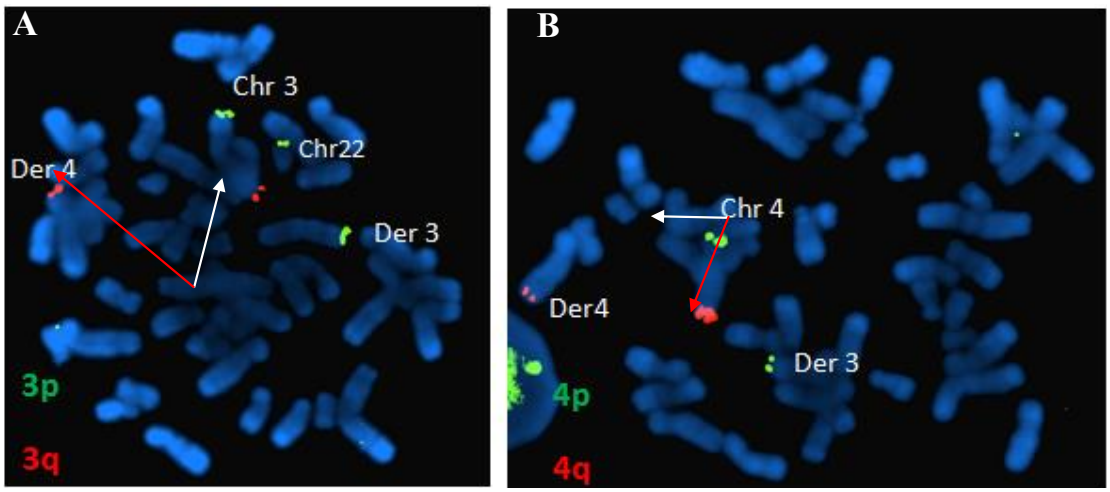


**Fig.3:** FISH on metaphase spread prepared from the women's (III:4) blood with Mix 3 and Mix 4 from Vysis ToTelVysion Kit. **A.** Mixture 3: chromosome 3 (p spectrum green and q spectrum orange) and chromosome 22 (q spectrum green + spectrum orange), LCI bcr (22q11) spectrum aqua. The red signal of the 3q telomere probe is observed on the normal 3q chromosome (white arrow) and on the short arm of the derivative chromosome 4 (red arrow) and **B.** Mixture 4: chromosome 4 (p spectrum green and q spectrum orange) and chromosome 21 (q spectrum green+spectrum orange), LCI bcr (21q22) spectrum aqua. Similarly, as expected, the green signal of the probe specific for telomere 4p is observed on normal chromosome 4 (white arrow) and derivative 3 (red arrow).

## Discussion

Humans are characterized by a high rate of embryonic failure at the early stages of development. RM, stillbirths and the birth of children with multiple CA remain the most spectacular varieties of this reproductive failure. The cause of RM remains elusive in approximately 50% of cases, although many studies have attempted to identify the underlying mechanism (23). Interestingly, it has been shown that an unknown proportion of parents who appear chromosomally normal on conventional cytogenetic analysis may carry cryptic subtelomeric rearrangements following malsegregation or recombination at gametogenesis, giving rise to segmental aneuploidy and thus resulting in RM (20, 24). This missingness is because of the same banding pattern at telomeric regions as well as their small size at the 500-550 band level karyotype resolution (19, 25).

The purpose of the present study was to examine whether RM is associated with subtelomeric rearrangements. Among the 42 individuals tested, one female showed a cryptic translocation between the 3q and 4p arms with distal breakpoints near the telomeres. Consistently, her affected daughter (IV:5) showed inheritance of the same translocation in its unbalanced form (46, XX, der 4,t(3;4)(q28;p16)) based on classical cytogenetic analysis. In this family, it was important to consider that not only a higher risk of RM was observed, but also congenital anomalies were present in subsequent pregnancies of carriers of cryptic rearrangements. Depending on the type of the reciprocal translocation, it has been estimated that the recurrent risk varies from1 to 50% (26, 27). The recurrent risk of the present patient for subsequent pregnancies was estimated to be 25%. Accordingly, genetic counseling should be mandatory after the diagnosis of a cryptic reciprocal translocation. The affected couple should be well informed about subsequent abortions, risks of transmission of the aberration, as well as giving birth to malformed children.

We reviewed the literature and identified eleven studies which had screened for subtelomeric regions in patients with RM. By combining these studies and our present report, 15 out of 448 patients showed subtelomeric translocations based on different sets of telomeric probes. This give a total rate of 3.34% for carriers of cryptic translocations while this rate was 4.76% in this study. Interestingly, these carriers not only had a history of RM, but also had a history of giving birth to children with ID and/or CA, or a clinically recognizable syndrome. This shows the importance of detailing the family history in improving diagnosis and suggesting the appropriate tool of exploration for precision in genetic counseling.

As previously mentioned, both conventional karyotyping and more advanced techniques such as aCGH and MLPA have limitations in detecting subtle genomic aberrations including balanced rearrangements (28, 29). These limitations have been overcome by using subtelomeric FISH. However, the high cost of subtelomeric FISH (due to expensive consumables) makes the clinical application of this technique unjustifiable for all couples presenting with a history of only RM and aCGH seems to be more practical. However, for couples with RM and a family history of stillbirth or children with CA or ID, subtelomeric FISH should be mandatory as it represents the most efficient approach in diagnosing RM couples than any other molecular assay.

## Conclusion

Identification of cryptic subtelomeric translocations is an active area of investigation. This study emphasizes the importance of screening these types of balanced rearrangements with subtelomeric FISH particularly in couples with RM. We conclude that subtelomeric FISH analysis should become mandatory for couples with RM and familial family history of stillbirth or children with CA or ID. This has a great impact when DNA or abortion products are no longer available. In fact, these products are not always accessible due to incompatibility with life of the congenital anomalies besides, in some cases, patient refusal to be tested. In addition, miscarriages and ID remain a somewhat offensive and delicate subject for parents, which makes the recruitment of patients more difficult in familial cases of RM. Furthermore, subtelomeric FISH is required to exclude any cytogenetic cause before searching for other spermatic factors such as sperm aneuploidy, sperm DNA integrity, chromatin packaging and semen parameters as we previously reported. Through this study, we highlight the importance of early clinical identification of such cases toward a more efficient diagnosis of subtelomeric translocations in RM cases.

## Acknowledgements

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## Author's Contributions

S.M.-Z.; Contributed to conception and design. A.H., W.S.; Contributed to all experimental work, analysis, and interpretation of data. F.E.A., A.C., M.B.; Contributed to data collection. S.M.-Z., M.K., Am.S.; Were responsible of the consultation. S.M.-Z., A.S.; Were responsible for overall supervision. A.H., W.S.; Drafted the manuscript, which was revised by S.M.-Z. All authors read and approved the final manuscript.

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Original Article

# Hypothalamic *KiSS1/GPR54* Gene Expressions and Luteinizing Hormone Plasma Secretion in Morphine Treated Male Rats

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## Abstract

**Background:** The inhibitory effects of morphine and the stimulatory influence of kisspeptin signaling have been demonstrated on gonadotropin releasing hormone (GnRH)/luteinizing hormone (LH) release. Hypothalamic kisspeptin is involved in relaying the environmental and metabolic information to reproductive axis. In the present study, the role of kisspeptin/GPR54 signaling system was investigated on relaying the inhibitory influences of morphine on LH hormone secretion.

**Materials and Methods:** In this experimental study, 55 wistar male rats weighing 230-250 g were sub-grouped in 11 groups (in each group n=5) receiving saline, kisspeptin (1 nmol), peptide234 (P234, 1 nmol), morphine (5 mg/kg), naloxone (2 mg/kg), kisspeptin/P234, morphine/naloxone, kisspeptin/morphine, kisspeptin/naloxone, P234/morphine or P234/naloxone respectively. Blood samples were collected via tail vein. Mean plasma (LH) concentrations and mean relative *KiSS1* or *GPR54* mRNA levels were determined by radioimmunoassay (RIA) and real time reverse transcriptase-polymerase chain reaction (RT-PCR), respectivwely.

**Results:** Morphine significantly decreased mean plasma LH concentration and mean relative *KiSS1* gene expression compared to saline; while it did not significantly decrease mean relative *GPR54* gene expression compared to saline. Naloxone significant increased mean LH level and mean relative *KiSS1* gene expression compared to saline; while it did not significantly increase mean relative *GPR54* gene expression compared to saline. Injections of kisspeptin plus morphine significantly increased mean LH concentration compared to saline or morphine, while simultaneous infusions of them significantly declined mean plasma LH level compared to kisspeptin. In kisspeptin/naloxone group mean plasma LH level was significantly increased compared to saline or naloxone. Co-administration of P234/morphine significantly decreased mean LH concentration compared to saline.

**Conclusion:** Down regulation of *KiSS1* gene expression may be partly involved in the mediating the inhibitory effects of morphine on reproductive axis.

***Keywords:*** *GPR54*, *KiSS1*, Luteinizing Hormone, Morphine

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## Introduction

kisspeptin/GPR54 signaling pathway has a therapeutic potential, as regulator of gonadotropin releasing hormone (GnRH)/luteinizing hormone (LH) release and gonadal steroid hormone secretions. G protein-coupled receptor, GPR54, is expressed in GnRH neurons and normal pubertal development, while sexual function is also dependent to normal actions of it (1, 2). Reproductive process is disrupted by the mutations of GPR54 receptor or kisspeptin genes (3). Kisspeptin analogues are introduced as endogenous ligand for GPR54 receptor and four types of kisspeptin (kisspeptins 10, 13, 14 and 54) have similar affinity to this receptor. They induce puberty and peripheral or central injections of them increase the GnRH/LH release and plasma gonadal steroid (1-4). Infusions of peptide 234 (P234) also block the stimulatory effects of kisspeptin on LH secretion (5).

Opioids suppress the reproductive process, resulting in hypogonadotropic hypogonadism (HH) dominantly via inhibiting the hypothalamus-pituitary-gonadal (HPG) axis rather than direct effects on pituitary or testes (6). Morphine, as an alkaloid extracted from poppy plant, is extremely used as drug abuse and drugs for the suppressing pain. Injections of morphine decrease the secretion of GnRH and LH via binding to opioid μ-type receptors (6-8). However, Aloisi and her colleague reported that morphine treatment may play a role in declining the mean plasma testosterone level by increasing peripheral testosterone metabolism in testes, liver and hypothalamus (9). It has also been found that naloxone, acting as the antagonist of μ-opioid receptor, blocks the influences of morphine on the HPG axis. In contrast, it induces puberty and improves the GnRH/LH as well as gonadal hormone

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secretions in males and females of different species (10).

Opioids receptors are not directly expressed on GnRH neurons and they exert their inhibitory influences on the reproductive axis via different interneurons pathways (11). In addition, several studies have established that kisspeptin has a crucial role in relaying the central or peripheral information to the reproductive axis (12-16). In order to the significant importance of physiological action of kisspeptin/GPR54 signaling pathway for controlling GnRH/LH release and considering the clinical overuse of opioid drugs, the present study aimed to investigate that if the level of kisspeptin/GPR54 signaling system activity may be partly involved in the morphine- induced decline of LH mean plasma levels.

## Materials and Methods

In this experimental research, three months old male wistar rats (n=55), weighing 230-250 g (provided by the Center of Neuroscience Research of Shahid Beheshti University, Tehran, Iran), were housed in the cages under controlled temperature (22 ± 2°C) and light (12 hours light/dark cycle). Animals had always free access to food and water. All procedures for the maintenance and use of experimental animals were executed with the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication No. 80-23, revised 1996, Iran) and were approved by the Ethical Committee of Neuroscience Research Center of Shahid Beheshti University of Medical Sciences (Tehran, Iran).

### Intra cerebral ventricular cannulation and injections

Animals were anesthetized by intraperitoneal (IP) injections of a mixture of ketamine and xylezine (ketamine 80 mg/kg bodyweight+xylezine 10 mg/kg bodyweight), a 22-gauge stainless cannulae was implanted in the third cerebral ventricle according to coordinates of Paxinos and Watson Atlas [anterior posterior (AP)=-2.3, midline (ML)=0.0, dorsoventral (DV)=6.5] (17). After one week, 55 rats were divided into 11 groups (5 in each group), receiving drugs as mentioned in the Table 1.

**Table 1:** Received drugs (name and dose) in each groups (n=5)

| Groups | |
|---|---|
| 1 | Saline (3 µl, ICV)/saline (200 µl, SC) |
| 2 | Kisspeptin (1 nmol/3 µl, ICV)/saline (200 µl, SC) |
| 3 | P234 (1 nmol/3 µl, ICV)/saline (200 µl, SC) |
| 4 | Kisspeptin (1 nmol/1.5 µl, ICV)+P234 (1 nmol/1.5 µl, ICV)/saline (200 µl, SC) |
| 5 | Saline (3 µl, ICV)/morphine (5 mg/kg, 200 µl, SC) |
| 6 | Saline (3 µl, ICV)/naloxone (2 mg/kg, 200 µl, SC) |
| 7 | Saline (3 µl, ICV)/naloxone(2 mg/kg, 100µl, SC)+morphine (5 mg/kg, 100 µl, SC) |
| 8 | Kisspeotin (1 nmol/3 µl, ICV)/morphine (5 mg/kg, 200 µl, SC) |
| 9 | Kisspeptin (1 nmol/3 µl, ICV)/naloxone (2 mg/kg, 200 µl, SC) |
| 10 | P234 (1 nmol/3 µl, ICV)/morphine (5 mg/kg, 200 µl, SC) |
| 11 | P234 (1 nmol/3 µl, ICV)/morphine (5 mg/kg, 200 µl, SC) |

**ICV; Intra cerebral ventricular and SC; Subcutaneously.**

Kisspeptin10 (Ana Spec Co., USA) and P234 (Phoenix Pharmaceuticals Inc., USA) were dissolved in distilled water and injected intra third cerebral ventricle by using Hamilton micro syringe at 09:00- 9:30. Morphine sulfate (Temad Co., Iran) and naloxone hydrochloride (Toliddaru Co., Iran) were dissolved in distilled water and injected SC by an insulin syringe at 09:00-9:30. In simultaneous groups, naloxone was injected 15 minutes before morphine injections. The time of blood sampling as well as kisspeptin, naloxone or morphine doses was chosen based on our laboratory and other previous studies reporting the stimulatory or inhibitory effects of these drugs on the reproductive axis, respectively (2, 3, 9, 10).

### Hormone assays

Blood samples were collected in a volume of 0.5 cc at 60 minutes following the injections via tail vein. Heparin was added to the samples to prevent clotting. Blood samples were immediately centrifuged for 15 minutes at 3000 rpm and the plasma samples were stored at -20°C until assayed for LH concentration. Mean plasma LH concentration was measured by using rat LH kit and the method of the radioimmunoassay (RIA, Institute of Isotopes Co, LT'D, Hungary). Sensitivity and intraassay of the kit were 0.09 ng/ml and 4.61%, respectively.

### Microdissections and total RNA extraction

Four hours after injections, the rats were sacrificed by decapitation and the brains were immediately autopsied. The brains were placed ventral side up, anterior coronal slices were cut from 1 mm anterior to optic chiasm. The slices were then dissected laterally up to the hypothalamic sulci and posterior coronal slices were cut posterior to the mammillary bodies (17). The samples were frozen by liquid nitrogen and stored at -80°C for determination of mRNA levels. Total RNA was isolated from individual frozen samples using the acid guanidinium thiocyanate-phenol-chloroform extraction method, according to PureZol manufacturer instruction (Bio RAD, USA). The quantification of each RNA sample was performed by measuring absorbance at 260 nm. The *GAPDH* gene was used to normalize the values obtained for each sample.

### RNA analysis by real-time reverse transcriptase polymerase chain reaction

Changes in the gene expression levels were determined by using the Corbett Real-Time PCR detection system Rotorgene 6000 (Qiagen Ltd, Germany). Total RNA (100 ng) was treated by DNaseI to remove residual genomic DNA according to manufacturer instruction (Thermo Scientific Inc., USA). Then, total RNA was further amplified in triplicate by using SYBR green I as fluorescent dye and one step quantitative reverse transcriptase RT-qPCR Master Mix Plus for SYBR Green I kit in a final volume of 25 µl according to manufacturer instruction (Eurogentec CO, USA). The PCR cycling conditions were as follows: reverse transcriptase step 48ºC for 30 minutes, 95ºC for

10 minutes, followed by 40 cycles of denaturation at 95ºC for 15 seconds, annealing at 54ºC (*KiSS1*), 54ºC (*GPR54*) and 58ºC (*GAPDH*) for 15 seconds and extension at 72ºC for 40 seconds. Specific oligo nucleotide sequences for sense and antisense primers were used as following:
*KiSS1-*
F: 5′-AGCTGCTGCTTCTCCTCTGT-3′
R: 5′-AGGCTTGCTCTCTGCATACC-3′ (18)
*GPR54-*
F: 5′-GGTGCTGGGAGACTTCATGT-3′
R: 5′-AGTGGCACATGTGGCTTG-3′ (18)
*GAPDH-*
F: 5′-AAGAAGGTGGTGAAGCAGGCATC-3′
R: 5′-CGAAGGTGGAAGAGTGGGAGTTG-3′ (19).

*KiSS1*, *GPR54* and *GAPDH* amplified product lengths were 151, 72 and 112 base pairs, respectively. To ensure the specification of RT-qPCR products the melting curve for fragments were generated by the Rotorgene 6000 program and the PCR products were evaluated in 1.5% agarose gel electrophoresis. Calculation of the relative expression levels of targeted cDNAs were conducted based on the cycle threshold ($C_t$) method. The Ct for each sample was calculated using the Corbett Real-Time PCR detection system software with an automatic fluorescence threshold (Rn) setting. Accordingly, fold expression of target mRNAs over the reference values was calculated by the equation $2^{-\Delta\Delta Ct}$.

### Statistical analysis

The results are presented as mean ± SEM. The data were analyzed by using SPSS software (version 16) and the one- way ANOVA followed by post hoc Tukey test. In all cases, statistical significance was defined by P<0.05.

## Results

Kisspeptin increased significantly the mean plasma LH concentration by 1.71 times compared to saline. P234 decreased mean plasma LH concentration by 0.12 compared to saline; however this decrease was not statistically significant. Simultaneous injection of kisspeptin and P234 increased the mean plasma LH concentration by 0.29 times compared to saline, while this increase was not statistically significant. In addition, injection of P234 solely or simultaneous injection of kisspeptin and P234 decreased significantly mean plasma LH concentration respectively by 0.67 or 0.52 times compared to kisspeptin.

Morphine decreased significantly mean plasma LH concentration by 0.48 times compared to saline. Mean plasma LH concentration increased significantly following naloxone injection by 0.48 times compared to saline. Simultaneous injection of naloxone and morphine increased mean plasma LH concentration by 0.17 or 1.24 times compared to saline or morphine, respectively. This increase was not statistically significant compared to saline, while it was statistically significant compared to morphine.

Co-administration of kisspeptin/morphine increased significantly mean plasma LH concentration by 0.73 or 2.32 times compared to saline or morphine, respectively.

Additionally, co-administration of kisspeptin/morphine decreased significantly mean plasma LH concentration by 0.37 times compared to kisspeptin. Co-administration of kisspeptin/naloxone increased significantly mean plasma LH concentration by 2.12 or 5.04 times compared to saline or naloxone, respectively.

Moreover, LH concentration was increased in kisspeptin/naloxone group by 0.16 times compared to kisspeptin group, although this increase was not statistically significant. Co-administration of P234/morphine decreased mean plasma LH concentration by 0.5, 0.1 or 0.4 times compared to saline, morphine or P234, respectively. This decrease was statistically significant compared to saline or P234 (P<0.05, Fig.1), while it was not statistically significant in comparison with morphine. Co-administration of P234/naloxone increased mean plasma LH concentration by 0.18 times compared to saline, but this increase was not statistically significant. Furthermore, co-administration of P234/naloxone decreased mean plasma LH concentration by 0.21 times compared to naloxone, while this decrease was not statistically significant (Fig.1).

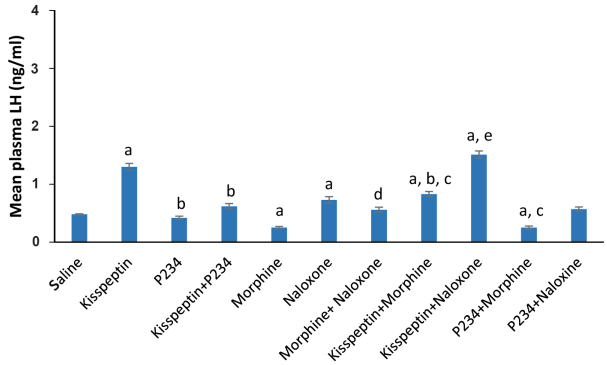


**Fig.1:** Effects of kisspeptin (1 nmol), P234 (1 nmol), 5 mg/kg morphine (MOR), 2 mg/kg naloxone (NAL) or co-administration of kisspeptin/morphine, kisspeptin/naloxone, P234/morphine or P234/naloxone on mean plasma LH concentration, in comparison with a; Saline, b; Kisspeptin, c; P234, d; Morphine, and e; Naloxone. Data are presented as mean ± SEM, P<0.05 and n=5 in each group.

In addition, results showed that morphine induced a significant decrease in *KiSS1* mRNA expression levels in the hypothalamic samples compared to saline, naloxone or morphine plus naloxone injected groups. So that morphine decreased significantly mean relative *KiSS1* gene expression by 0.89, 0.93 or 0.85 times compared to saline, naloxone or morphine plus naloxone, respectively. Naloxone increased significantly mean relative KiSS1 gene expression by 0.68, 14.27 or 1.21 times compared to saline, morphine or morphine+naloxone respectively.

In animals receiving naloxone+morphine, the mean relative *KiSS1* gene expression was decreased by 0.24 or 0.54 times compared to saline or naloxone, respectively. This decrease was not statistically significant compared to saline, while it was statistically significant compared to naloxone. Additionally, injections of naloxone+morphine

increased significantly the mean relative *KiSS1* gene expression by 5.9 times compared to morphine (P<0.05, Fig.2). The mean relative *GPR54* gene expressions were not significantly influenced by the injections of morphine, naloxone or morphine+naloxone compared to saline group. Moreover, a significant decrease or increase was not observed on the *GPR54* mRNA levels between different groups (Fig.3).

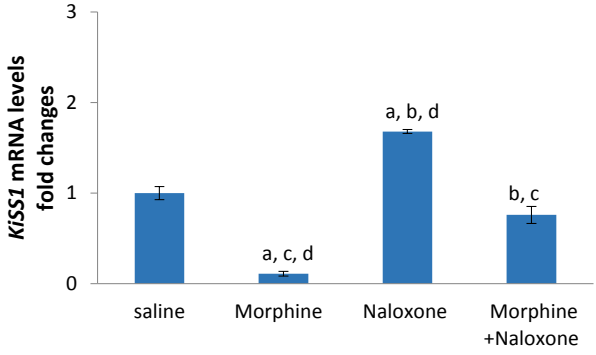


**Fig.2:** Effects of morphine (5 mg/kg), naloxone (2 mg/kg) or simultaneous injections of morphine and naloxone (n=5 in each group) on *KiSS1* mRNA expression in the hypothalamus of male rats. The cDNA amplified from *GAPDH* mRNA was used to normalize corresponding *KiSS1* results. The results are presented as mean ± SEM. In all cases P<0.05 was considered to be statistically significant. a; Compared to saline, b; Compared to morphine, c; Compared to naloxone, and d; Compared to morphine+naloxone.

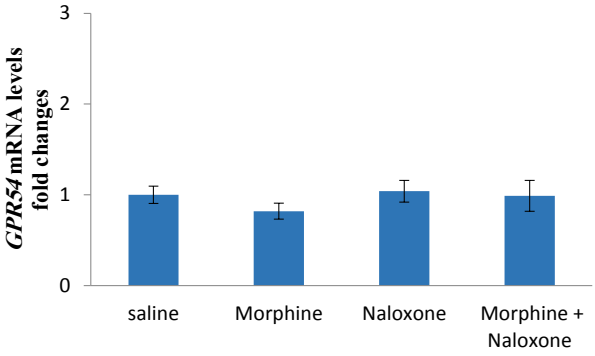


**Fig.3:** Effects of morphine (5 mg/kg), naloxone (2 mg/kg) or simultaneous injections of morphine and naloxone (n=5 in each group) on *GPR54* mRNA expression in the hypothalamus of male rats. The cDNA amplified from *GAPDH* mRNA was used to normalize corresponding *GPR54* results. The results are presented as mean ± SEM. In all cases P<0.05 was considered to be statistically significant.

## Discussion

The results showed that subcutaneous injection of naloxone or central injection of kisspeptin increased significantly the mean plasma LH concentration compared to saline, while subcutaneous injection of morphine significantly decreased it, in comparison with saline. These results are consistent with the other researches which established the stimulatory effects of naloxone (10), kisspeptin (1-5) or inhibitory effects of morphine on the sexual hormone secretions (6-9) and introduced them as important key regulators for controlling the HPG axis in the male and females of different species.

In our previous studies, we showed that interaction of morphine/kisspeptin play a role in the regulating of mean plasma testosterone concentration in male rats (8). In this work, our results indicated that morphine injection attenuates the stimulatory effects of kisspeptin on mean plasma LH concentrations anf injection of kisspeptin+naloxone exerts an additive stimulatory effect on mean levels of LH, compared to naloxone. The precise molecular and central mechanisms underlying the effects of opioids on the reproduction neuroendocrine axis is not clear yet.

However previous researches demonstrated that endogenous opioids, exogenous opiates (e.g. morphine) or their receptor antagonists influence the release of LH and subsequently gondal steroid hormones via indirect regulation of the hypothalamic GnRH release (11). However Kappa opioid receptors have been found on hypothalamic kisspeptin neurons of arcuate nucleus (ARC) (20), but mu opioid receptors mediating the physiological effects of β-endorphin or morphine (21) are widely expressed in the brain stem and thalamic nuclei and lower levels expression of them has been reported in hypothalamus or GnRH neurons. Different signaling pathways supposed to be involved in mediating opioids indirect effects on the hypothalamic GnRH-producing neurons, which we could point to noradrenergic, dopaminergic or GABAergic neurons (11).

It is well established that more than 80% GnRH neurons express GPR54 receptor and hypothalamic *KiSS1* has been proposed as key molecular conduit for relaying a number of peripheral or central signals including steroid hormones, fasting, ghrelin, leptin or photoperiod into the GnRH system (12-16). Therefore we examined the effects of morphine/naloxone injections on *KiSS1/GPR54* mRNA levels to investigate that if the opioids and kisspeptin pathways may interact to each other in controlling the HPG axis.

The results showed that morphine significantly downregulated the *KiSS1* mRNA levels and naloxone blocked the inhibitory effect of morphine on *KiSS1* mRNA expression. But *GPR54* mRNA levels were not significantly influenced by morphine or naloxone injections. For the first time in reproductive axis, we investigated the effects of morphine/naloxone on *KiSS1/GPR54* mRNA levels and no study has previously been performed to compare this point in any species. However morphine may take part in the regulating of kisspeptin synthesis partly via other brain interneurons or peptides. It has been revealed that ghrelin system negatively influences the gonadal axis (22-24). It has also been reported that co-administration of naloxone with ghrelin restores mean LH concentration and pulse frequency in rats (23). Moreover, ghrelin inhibits and delays the LH response to naloxone in men (24). Changes in the hypothalamic KiSS-1 system have been reported in situations of negative energy balance, which are linked to the suppressed gonadotropin secretion. Studies reported that intravenous injection of ghrelin or fasting, accompanying with increased ghrelin levels, results in a significant decrease in *KiSS1* gene mRNA level in

the rat brain (16-18). Because GnRH pulse generator and kisspeptin neurons are located in the medio basal hypothalamus in which the ghrelin receptor is also expressed (25). Our studies have also shown that morphine increases hypothalamic ghrelin gene expression in male rats (data not published). Thus, central opioid system may down-regulate *KiSS1* gene expression partly via up-regulating ghrelin levels.

There is a close relationship between hypothalamus-pituitary-adrenal (HPA) and HPG axis activities. Corticotrophin-releasing factor (CRF), synthesized by hypothalamic neurons, is a potent inhibitor of the GnRH pulse generator. Central administrations of CRF decrease the GnRH concentration in hypophyseal portal system and mean plasma LH/sex steroid concentrations (26-28). While suppression of LH secretion, by CRF injection, or a variety of stressful stimuli, increasing the CRF/cortisol secretions, can be reversed by CRF antagonists (29). The previous studies have reported that injections of opioid increase CRF/ACTH release and pretreatment of the animals with opioid antagonists especially μ-type receptor antagonists abolish the inhibitory effects of CRF on GnRH/LH release, suggesting that the CRF-induced inhibition of gonadotropin secretion is mediated by opioids (27). Recently Kinsey-Jones et al. (30) showed that CRF or corticosterone injections as well as both acute and chronic stressors down-regulate *kiSS1/GPR54* mRNA levels in rat hypothalamic nuclei. So, a possible functional interaction between the opioid and CRF/corticosterone systems could be considered in regulating kisspeptin/GPR54 signaling system. Leptin, the hormone which is mainly secreted by adipose tissue, may be involved.

Leptin is a stimulatory factor for controlling reproduction process and it improves secretion of LH hormone via projecting direct or indirect signals including kisspeptin neurons to GnRH ones (31). Studies demonstrated that kisspeptin mRNA levels are extremely lower in leptin gene knocked-out mice compared to normal ones and infusion of leptin reverse the results in these animals. They contributed to the down-regulation of HPG axis activity to declined arcuate kisspeptin levels (13). Many other studies confirmed the mediatory role of kisspeptin/GPR54 signaling pathway for exerting leptin effects on GnRH/LH release (31, 32). There is also an inverse relationship between plasma β-endorphine (endogenous ligand for mu receptor) and leptin level. It has been established that β-endorphine contains lipolytic properties and it plays an important role in decreasing body weight via declining leptin secretion (33). So, it is proposed that suppressing leptin signaling might partly be involved in the inhibitory effects of mormhine on *KiSS1* gene expression.

However for first time our results showed that down-regulation of kisspeptin pathway may have a role in the inhibitory effects of morphine on HPG axis. To better understand mechanisms of opioid-induced hypogonadism via affecting kisspeptin/GPR54 signaling system, in further studies we could examine the effects of injection of other opiates including methadone, codeine or endogenous opioid such as β-endorphine on hypothalamic *KiSS1/GPR54* mRNA levels. In addition, the interactions of morphine and effect of inhibitory/stimulatory factors involved in the regulation of reproduction including leptin, alpha melanocyte stimulating hormone (αMSH) or CRF should be investigated on kisspeptin/GPR54 signaling pathway and HPG axis activity.

## Conclusion

Subcutaneous injection of morphine attenuates the stimulatory effects of third cerebral ventricular injection of kisspeptin on mean plasma LH levels. Kisspeptin+naloxone exerts an additive stimulatory effect on mean plasma levels of LH compared to naloxone. Additionally, morphine significantly down-regulates the hypothalamic *KiSS1* levels and naloxone blocks the inhibitory effect of morphine on *KiSS1* mRNA expression. The *GPR54* mRNA levels were not significantly influenced by morphine or naloxone injections. These results suggest that down-regulation of the kisspeptin signaling pathway might partly be involved in opioid-induced infertility.

## Acknowledgements

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## Author's Contributions

H.K., F.M.; Participated in study design, data evaluation, conducted molecular experiments, and statistical analysis. M. J.; Contributed to conception ad study design. All authors read and approved the final manuscript.

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Original Article

# *IL-1α* C376A Transversion Variant and Risk of Idiopathic Male Infertility in Iranian Men: A Genetic Association Study

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## Abstract

**Background:** IL-1α produced by Sertoli cells is considered to act as a growth factor for spermatogonia. In this study, we investigated the association of the C376A polymorphism in IL-1α with male infertility in men referring to the Kashan IVF Center.

**Materials and Methods:** In this case-control study, 2 ml of blood was collected from 230 fertile and 230 infertile men. After DNA extraction, the C376A variant was genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). In addition, the molecular effects of the C376A transversion were analysed using bioinformatics tools.

**Results:** A significant association was observed between the homozygous genotype CC with male infertility [odds ratio (OR)=1.97, 95% confidence interval (CI)=1.14-3.41, P=0.016)]. Carriers of C (AC+CC) showed a similar risk for male infertility (OR=1.78, 95% CI=1.06-2.99, P=0.030). Also, allelic analysis showed that the C allele is associated with male infertility (OR=1.43, 95% CI=1.09-1.88, P=0.011). In sub-group analysis, we found that the AC genotype is associated with asthenozoospermia (OR=2.38, 95% CI=1.03-5.53, P=0.043). In addition, carriers of C were at high risk for asthenozoospermia (OR=2.25, 95% CI=1.01-4.10, P=0.047). Also, C allele was significantly associated with oligozoospermia (OR=1.44, 95% CI=1.01-2.06, P=0.049) and non-obstructive azoospermia (OR=1.67, 95% CI =1.04-2.68, P=0.034). Finally, in silico analysis showed that the C376A polymorphism could alter splicing especially in the acceptor site.

**Conclusion:** This is the preliminary report on the association of *IL-1α* C376A polymorphism with male infertility in the Kashan population. This association shows that the *IL-1α* gene may be a biomarker for male infertility, and therefore needs additional investigations in future studies to validate this.

***Keywords:*** Genetic Polymorphism, *Interleukin-1α*, Male Infertility, Spermatogenesis

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## Introduction

Male infertility is a multifactorial syndrome that affects up to 12% of men (1). Male factors are responsible for 40-50% of total infertility cases (2). In more than 70% of cases, there is a conclusive reason including varicocele, aneuploidies, infectious diseases and post-testicular obstruction, however, in less than 30% of infertile males, the cause of their infertility is unknown and are thus diagnosed as idiopathic (3, 4).

Environmental, lifestyle, physiological and genetic factors are involved in male infertility (5-7). From numerous genetic factors that are essential for normal spermatogenesis, cytokines play an important role (8). These are regulatory peptides which regulate testicular and glandular function (9).

Human seminal plasma contains several cytokines including IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-11, IL-12, IL-13, IL-17, IL-18, IL-23, TNFα, IFN-γ, TGFα, TGFβ (8). One of the most important gene sets involved in fertility is the interleukin-1 (*IL-1*) gene family which encodes regulatory cytokines playing multifaceted roles in the male reproductive system. For example, they may act as growth factors and are involved in physiological protection, germ cell proliferation and differentiation, regulation of junctions and steroidogenesis (10, 11).

The *IL-1* gene family members include *IL-1α* (OMIM: 147760), *IL-1β* (OMIM: 147720) and *IL-1RA* (OMIM: 147679), all of which are located on chromosome 2q14 (12). IL-1α is secreted from seminiferous epithelium and is known as a growth factor for immature Sertoli cells and

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spermatogonia (13).

Single nucleotide polymorphisms (SNPs), by altering the structure of genes involved in spermatogenesis, may affect gene expression, mRNA structure and protein function, and may therefore lead to male infertility (14-16). Therefore, evaluating SNPs in the *IL-1* gene family could be considered as an interesting research topic. A SNP (C376A; rs2071376) has been found to have a high frequency in the *IL-1α* gene. The association of this SNP with some disorders has been investigated in different studies including cancers (17, 18), systemic sclerosis (19), periodontitis (20), endometriosis (21) and keratoconus (22). The association between the C376A SNP and idiopathic male infertility has, however, not been reported. In this study, we investigated the association between the *IL-1α* C376A SNP and idiopathic male infertility in an Iranian population as a preliminary project. Also, we evaluated the functional effects of C376A on *IL-1α* using bioinformatics tools.

## Materials and Methods

### Subjects and inclusion criteria

In this cross-sectional study, a total of 460 samples comprising 230 infertile men (with mean age of 30.93 ± 5.47) and 230 fertile men (with mean age of 32.12 ± 5.52) selected among individuals attending the Kashan Infertility Centre (Shahid Beheshti Hospital, Kashan, Iran). Infertile patients were defined as 'idiopathic' and selected based on andrological examination. Patients with previous testis trauma, obstruction of the vas deferens, infectious and chronic diseases, hypogonadotropic hypogonadism, abnormal hormonal profile (Luteinizing, Follicle Stimulating, and testosterone hormones) and abnormal karyotype or Y chromosome microdeletions were excluded from the study. According to the World Health Organization (WHO) 1999 criteria, the patient sub-groups were determined (23) and the subjects were categorized into non-obstructive azoospermia (n=51) without spermatozoa in the ejaculated semen, oligozoospermia (n=95) with sperm concentration less than 20 million/ml, and asthenozoospermia (n=84) with progressive sperm motility less than 50%.

The control group was randomly selected from healthly men referred to the Kashan Infertility Centre. They had normal sperm parameters, had no history of chronic and familial diseases and had at least one offspring. Finally, a total of 2 ml of whole blood was collected from all males into EDTA-K3 containing tubes and were stored in -20°C for further usage. Written informed consent was obtained from all case and control subjects. The study was approved by the Medical Research Ethics Committee of the Kashan University of Medical Sciences (IR.KAUMS.REC.1394.6).

### Single nucleotide polymorphism genotyping

Total genomic DNA was isolated from whole blood by using a DNA extraction kit (Bioneer, Korea). Purified DNA was stored at -20°C for further use. The *IL-1α* C376A SNP was genotyped by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. For this purpose, forward and reverse primers flanking the SNP were designed based on the complete sequence of *IL-1α* by the Oligo7 software (Molecular Biology Insights, Inc., Cascade, CO, USA).
The sequences of the primers were:
5′-ATGCTAAAATTACCGTGATTCT-3′
5′-AGATCAATGGAATAAATGGATG-3′ respectively.

The PCR was carried out in a total volume of 20 µl containing 10µl pre-mix (CinnaGen, Iran), 0.35 µM of each forward and reverse primers, and 3 µl of template DNA. PCR cycling conditions were an initial denaturation step at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 56.9°C for 1 minute and extension at 72°C for 1 minute along with a final extension at 72°C for 5 minutes. PCR products were then digested with the *BstYI* restriction enzyme (CinnaGen, Iran). For this purpose, approximately 0.1 µg of the PCR product was incubated with 5 units of *BstYI* at 37°C for 16 hours. Finally, *BstYI* was inactivated by incubation at 65°C for 20 minutes. The digested fragments were separated on a 1% agarose gel stained with DNA Green Viewer (CinnaGen, Iran) and visualised under the UV light. To verify PCR-RFLP results, 2% of samples were sequenced randomly. PCR product recovery kit (Roche Applied Science, Mannheim, Germany) was used to purify the PCR product (368 bp in length). Direct sequencing of the purified PCR products was undertaken by Bioneer (Daejeon, Korea). Chromas (version 2.33) was used to check the chromatograms.

### Statistical analysis

The difference in frequencies of genotypes and alleles between the case and control groups was analyzed by Chi-square test. For association analysis, the odds ratios (ORs) and 95% confidence intervals (95% CI) were estimated by a binary regression logistic test. A two-tailed p-value less than 0.05 ($P<0.05$) was considered significant. All analyses were conducted in the SPSS software (SSPS Inc., IBM Corp, Armonk, NY, USA) version 19.

### In silico analysis

Bioinformatics tools were used to analyze the influence of the *IL-1α* C376A intronic SNP on RNA structure and splicing pattern. The effect on RNA structure and splicing was assessed with RNAsnp online server (24) and NetGene2 (25) respectively. Finally, reported interactions of IL-1α with other molecules were obtained from the BioGRID interactome database (26).

## Results

### Polymerase chain reaction-restriction fragment length polymorphism and DNA sequencing

Results of PCR-RFLP showed that 368 bp fragment was fully digested into 114 bp and 254 bp fragments in some

samples, showing the efficiency of the method used. The samples with two, three and one fragments were identified as CC, AC, and AA genotypes respectively (Fig.1A). The data from direct sequencing also confirmed the results of PCR-RFLP (Fig.1B).

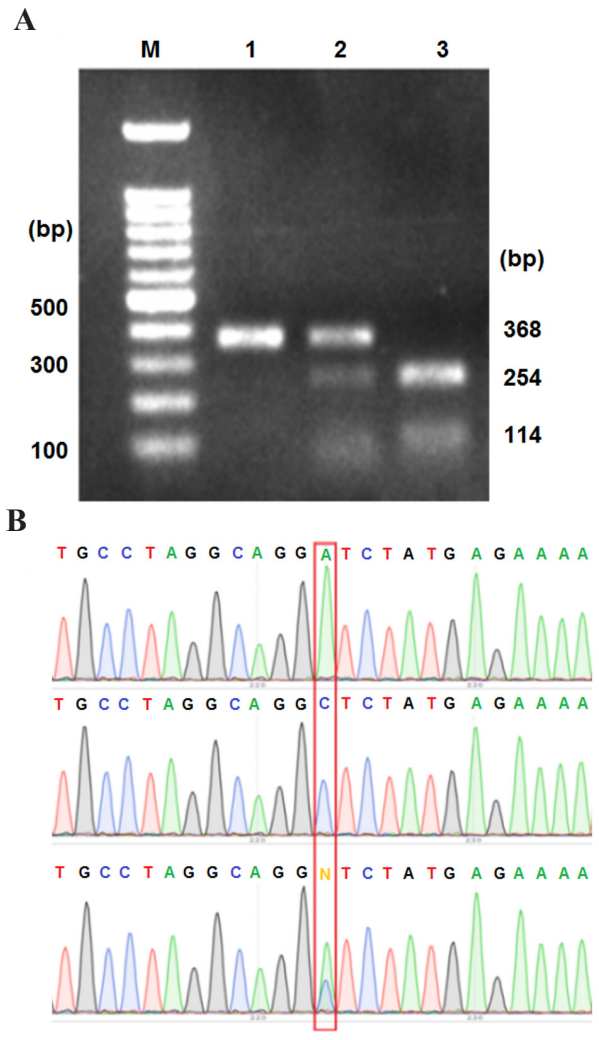


**Fig.1:** Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing results. **A.** The M, 1, 2 and 3 lanes show the 100 bp DNA ladder, and the AA, AC and CC genotypes, respectively and **B.** Partial sequence of *IL-1α* flanking the single nucleotide polymorphism (SNP) (red box).

## *IL-1α* C376A distribution

In this study, the genotype and allele frequencies of the *IL-1α* C376A SNP were compared between the infertile and healthy groups (Table 1). We observed a significant association between the homozygous genotype CC with male infertility (OR=1.97, 95% CI=1.14-3.41, P=0.016). Carriers of C (AC+CC) were at a similar risk for male infertility (OR=1.78, 95% CI=1.06-2.99, P=0.030). Also, allelic analysis showed that the C allele is associated with infertility (OR=1.43, 95% CI=1.09-1.88, P=0.011). In sub-group analysis, we found that the AC genotype is associated with asthenozoospermia (OR=2.38, 95% CI=1.03-5.53, P=0.043). In addition, there was a significant association between carriers of C and asthenozoospermia (OR=2.25, 95% CI=1.01-4.10, P=0.047). Also, C allele was significantly associated with oligozoospermia (OR=1.44, 95% CI=1.01-2.06, P=0.049) and non-obstructive azoospermia (OR=1.67, 95% CI=1.04-2.68, P=0.034).

## In silico analysis

Functional consequence of the C376A transversion on RNA structure was evaluated. However, no significant effect on RN (distance: 0.0191, P=0.686) was observed (Fig.2). Minimum free energy of normal RNA was equal to -81.80 kcal/mol but increased to -80.50 kcal/mol for the variant allele. The data from NetGene2 revealed that the C370A SNP alters the *IL-1α* splice site pattern on the direct strand (+ strand) especially for the acceptor splice pattern (Fig.2). The BioGRID interactome showed that *IL-1α* has 17 gene-gene interactions (Fig.3).

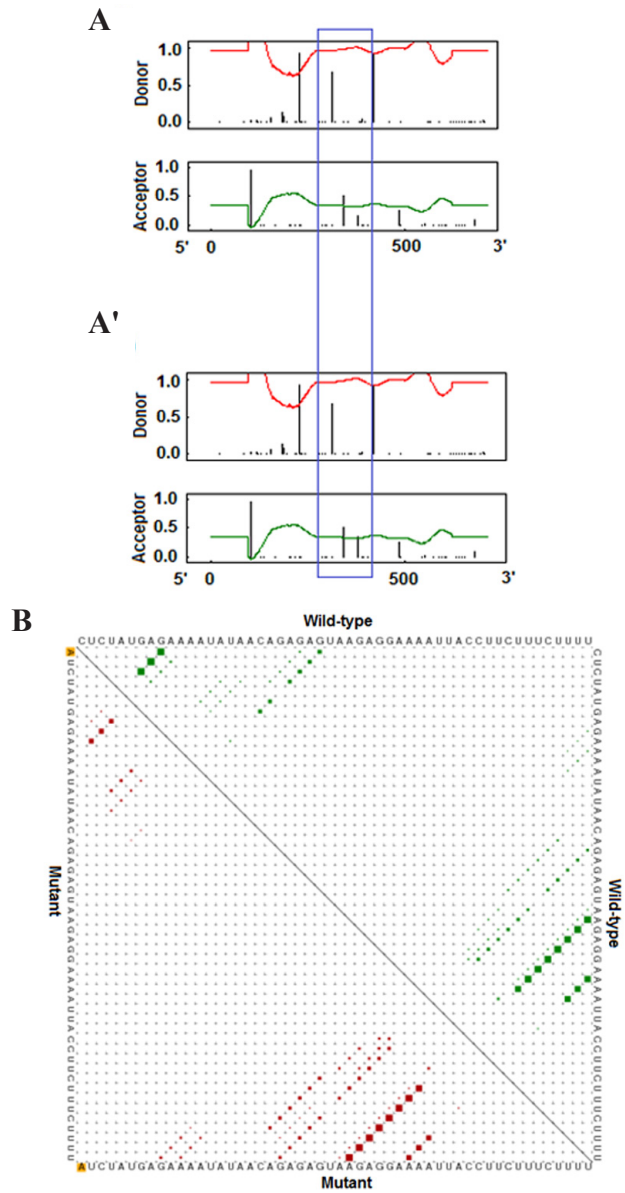


**Fig.2:** Results of NetGene2 and RNAsnp. **A.** Splice sites prediction by NetGene2 when nucleotide A is present at the C376A position, **A'.** Splice sites pattern after the C substitution at the C376A position. Some changes were observed after the substitution especially in the acceptor site (the differences between the splice patterns are shown by the blue box), and **B.** The presumptions of variant and ancestral sequences are introduced in lower and upper triangle of the plots respectively. The single nucleotide polymorphism (SNP) is highlighted by yellow color.

**Table 1:** Allelic and genotypic distribution of the *IL-1α* C376A SNP

| Genotype/ Allele | n (%) | | | | | OR (95% CI) | | | | P value | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control n=230 | All cases n=230 | Oligo n=95 | Asteno n=84 | NOA n=51 | Total | Oligo | Asteno | Azo | Total | Oligo | Asteno | Azo |
| AA | 44 (19.13) | 27 (11.74) | 14 (14.74) | 8 (9.52) | 5 (9.80) | - | - | - | - | - | - | - | - |
| AC | 90 (39.13) | 87 (37.83) | 30 (31.58) | 39 (46.43) | 18 (35.29) | 1.58 (0.90-2.74) | 1.05 (0.51-2.17) | 2.38 (1.03-5.53) | 1.76 (0.61-5.05) | 0.113 | 0.901 | 0.043 | 0.293 |
| CC | 96 (41.74) | 116 (50.43) | 51 (53.68) | 37 (44.05) | 28 (54.90) | 1.97 (1.14-3.41) | 1.67 (0.84-3.33) | 2.12 (0.91-4.93) | 2.57 (0.93-7.09) | 0.016 | 0.146 | 0.081 | 0.069 |
| AC+CC | 186 (80.87) | 203 (88.26) | 81 (85.26) | 76 (90.48) | 46 (90.20) | 1.78 (1.06-2.99) | 1.37 (0.71-2.64) | 2.25 (1.01-4.10) | 2.18 (0.82-5.80) | 0.030 | 0.348 | 0.047 | 0.127 |
| A | 178 (38.70) | 141 (30.65) | 58 (30.53) | 5 5(32.74) | 28 (27.45) | - | - | - | - | - | - | - | - |
| C | 282 (61.30) | 319 (69.35) | 132 (69.47) | 113 (67.26) | 74 (72.55) | 1.43 (1.09-1.88) | 1.44 (1.01-2.06) | 1.30 (0.89-1.88) | 1.67 (1.04-2.68) | 0.011 | 0.049 | 0.172 | 0.034 |

**SNP; Single nucleotide polymorphism, OR; Odds ratio, Oligo; Oligozoospermia, Asteno; Asthenozoospermia, and NOA; Non-obstructive azoospermia. Significant differences between the case and control groups are shown in bold type.**

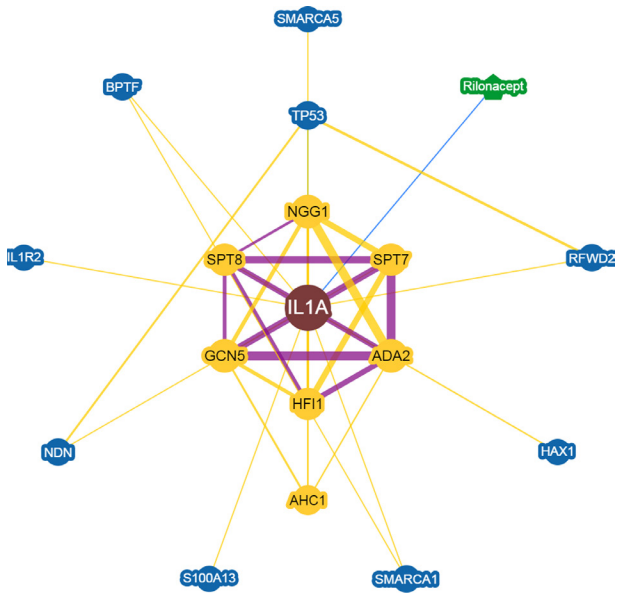


**Fig.3:** Network of human *IL-1α* interactions based on BioGRID. *IL-1α* interacts with 17 other molecules. Purple and yellow lines show interactions detected by genetic and physical experiments respectively.

## Discussion

In this study, we examined the association of the *IL-1α* C376A SNP with male infertility in an Iranian population (Kashan, Iran) as a pilot study. Our study revealed that not only the CC genotype was associated with male infertility, but also the C allele showed significant association. In addition, carriers of the C allele were at almost two-fold risk for male infertility. Sub-group analysis revealed that AC genotype and carriers of C were associated with asthenozoospermia. Also, the C allele was significantly associated with oligozoospermia and non-obstructive azoospermia. Therefore, *IL-1α* C376A is a potential genetic risk factor for male infertility, although further studies of different ethnicities in Iran and other populations are required to obtain a more accurate picture. After Hardy-Weinberg equilibrium (HWE) calculation in the control group, we found a highly significant deviation. However, the case group showed no deviation. even though it does not necessarily need to follow HWE due to the inherent sampling bias in cases. The deviation from HWE in the control group (normozoospermic men) could also be due to the selection bias (27) given that not all men in the general population will be fertile.

Spermatogenesis is a dynamic process in which many factors are necessary for creating and regulating balance in this process. For example, growth factors and cytokines are essential for development of functional spermatozoa (28, 29). Interleukin-1 is produced by epithelia of seminiferous tubules and acts as a physiological paracrine/autocrine factor on testicular cells and required for immunological protection (30). There is a probable mechanism that in the absence of testosterone, followed by increased cell apoptosis, spermatogenesis is finally reduced (31, 32). The second probable mechanism is excess reactive oxygen species (ROS).

The presence of the associated SNP and the consequent change in the amount of interleukin along with excess production of ROS may reduce sperm motility. One of the reasons for reduced sperm motility may be DNA damage and lipid peroxidation of sperm membrane (33). Also, increased ROS with oxidizing DNA or proteins, enzyme inhibition, cell death and apoptosis of sperm may cause the oligozoospermia phenotype (34, 35). Due to these possible mechanisms, the association of the *IL-1α* SNP with some abnormalities in sperm parameter may be explained. SNPs could change the gene expression pattern (14), mRNA structure (36, 37), splicing pattern (38) and protein function (39, 40). In silico tools, which can predict the damaging effects of SNPs, were therefore used especially that *IL-1α* C376A is an intronic SNP and may affect RNA structure and splicing. Although we found no evidence for C376A to affect RNA structure, we observed a predicted effect on splicing alteration. Therefore, the association of this SNP may be due to this effect. In this study, there were various limitations including gene-environment and gene-gene interactions that must be consid-

ered in subsequent studies. Also, lack of in vitro studies such as investigating the effect of the SNP on *IL-1α* gene expression and isoform formations due to splicing alterations is another limitation of this study.

## Conclusion

Our study suggests that the *IL-1α* C376A SNP may increase the risk of male infertility up to two-fold. Since this is the first study, future studies with larger sample sizes in different ethnicities and populations is warranted given the variable environmental factors in different geographic regions.

## Acknowledgements

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## Author's Contribution

M.K., H.N.; Planned and supervised the study upon which the current subset project was based. M.K., T.Z.-B.; Developed the outline for the current study and supervised the analysis of the samples. T.Z.-B.; Write the manuscript and M.K., H.N. revised the paper. M.K., H.N., T.Z.-B., A.A.T.; Contributed to data analysis and prepared the manuscript. All authors reviewed and approved the final manuscript.

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Original Article

# Flaxseed Can Reduce Hypoxia-Induced Damages in Rat Testes

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## Abstract

**Background:** Hypoxia causes detrimental effects on the structure and function of tissues through increased production of reactive oxygen species that are generated during the re-oxygenation phase of intermittent and continuous hypobaric hypoxia. This study was carried out to evaluate the effects of flaxseed (Fx) in reducing the incidence of hypoxia in rat testes.

**Materials and Methods:** In this experimental study, 24 adult Wistar rats were randomly divided into four groups: i. Control group (Co) that received normal levels of oxygen and food, ii. Sham group (Sh) that were placed in hypoxia chamber but received normal oxygen and food, iii. Hypoxia induction group (Hx) that were placed in hypoxia chamber and treated with normal food, iv. Hypoxia induction group (Hx+Fx) that were placed in hypoxia chamber and treated with 10% flaxseed food. Both the Hx and Hx+Fx groups were kept in a hypoxic chamber for 30 days; during this period rats were exposed to reduced pressure (oxygen 8% and nitrogen 92%) for 4 hours/day. Then, all animal were sacrificed and their testes were removed. Malondialdehyde (MDA) and total antioxidant capacity (TAC) levels were evaluated in the testis tissue. Tubular damages were examined using histological studies. Blood samples and sperm were collected to assess IL-18 level and measure sperms parameters, respectively. All data were analyzed using SPPSS-22 software. One way-ANOVA or Kruskal-Wallis tests were performed for statistical analysis.

**Results:** A significant difference was recorded in the testicular mass/body weight ratio in Hx and Hx+Fx groups in comparison to the control (P=0.003 and 0.027, respectively) and Sh (P=0.001 and 0.009, respectively) groups. The sperm count and motility in Hx+Fx group were significantly different from those of the Hx group (P=0.0001 and 0.028, respectively) .Also sperm viability (P=0.0001) and abnormality (P=0.0001) in Hx+Fx group were significantly different from Hx group.

**Conclusion:** This study therefore suggests that the oral administration of flaxseed can be useful for prevention from the detrimental effects of hypoxia on rat testes structure and sperm parameters.

***Keywords:*** Flaxseed, Hypoxia, Rat, Sperm, Testis

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## Introduction

Hypoxic conditions can be found in many situations such as high altitude, diving, and chronic obstructive pulmonary disease (COPD). Globally, COPD is considered as a leading cause of death and disability (1). Hypoxic conditions result in lower levels of circulating oxygen (2) and 4-week exposure to hypoxia produces systemic hypoxia in rats as manifested by pulmonary hypertension, and increased right ventricular systolic pressure (3). These hypoxic signs present special challenges to homeostasis because of their effects on sympathetic outflow and vascular smooth muscle.

It is generally accepted that chronic systemic hypoxia, whether due to high altitude or imposed experimentally by a hypoxic or hypobaric chamber, induces physiological adaptations that help to compensate the impaired $O_2$ transport to tissues. Enhancing red blood cell production (e.g.by administration of erythropoietin (Epo) has been shown to modulate the ventilatory response to reduced oxygen supply and critically help the organism to cope with increased oxygen demand (4). Exposure to hypoxia has been associated with an increase in the production of reactive oxygen species (ROS) that are generated during the re-oxygenation phase of intermittent and continuous hypobaric hypoxia and contribute to physiological responses (5) such as pulmonary hypertension and vasoconstriction as well as neomuscularization and thickening of the media and adventitia of pulmonary arterioles.

Weight loss due to exposure to chronic hypoxia may reflect multiple changes in cardiovascular function, hormone production, energy metabolism, and other aspects of cellular and systemic physiology (4). ROS may cause cell membrane damage, and prevent the maintenance of ionic gradient which can lead to detrimental effects on structure and function of tissues (6, 7), impairment in ATP production and tissue inflammation. Oxidative stress (OS)

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refers to an imbalance between generation of ROS and the ability of endogenous antioxidant systems to scavenge ROS, where ROS overwhelms antioxidant capacity (5, 8).

Furthermore hypoxic condition increases the levels of inflammatory cytokine such as IL-1β, IL-18 and tumor necrosis factor-alpha (TNF-α) (9). Also, hypoxia increases levels of lipid peroxidation-while reduces glutathione reductase activity and number of epididymal sperm (10). Evident changes observed following hypoxia-induced lipid peroxidation have been reported (11). These changes are partially attenuated by supplementation of antioxidants such as melatonin and ascorbate but there is no report about the effect of flaxseed on male reproductive system affected by hypoxia. The major components of flaxseed are the essential n-3 fatty acid, α-linolenic acid (ALA), lignans such as secoisolariciresinol diglucoside (SDG) and carbohydrates such as mucilages containing arabinoxylans. ALA is orally bioavailable and may be stored or converted into longer chain n-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and other bioactive lipid metabolites (12). SDG is metabolized to the mammalian lignans, enterodiol and enterolactone, in the intestine (13); recent research has demonstrated the ability of lignans to scavenge hydroxyl radicals suggesting a potent antioxidant activity for lignans. lignans are biologically active phytochemicals with anticancer and antioxidant potential (14). Docosahexaenoic acid has been shown to increase sperm motility in men (15).

Improvement of vascular endothelial cell function, enhancement of vascular reactivity and compliance, modulation of lipid metabolism and reduction of inflammatory cytokine production have been noted as the underlying mechanisms through which poly unsaturated fatty acid (PUFA) exert their beneficial effects (16) . In mammalian sperm, lipids especially n-3 fatty acids are dominantly present. Previous studies have shown that n-3 fatty acids are also present in human sperm (15). Their protective mechanisms include induction of anti-inflammatory transcriptional pathways , reducing the intracellular $Ca^{2+}$ levels, suppression of vascular proliferation, and improvement of cell membrane integrity (17). Little information is available regarding the effect of dietary flaxseed supplementation on male rats' reproductive system following exposure to hypoxia. The objective of the present study was to investigate the effect of flaxseed supplementation on testes structure and sperm parameters of hypoxic rats.

## Materials and Methods

In this experimental study, 24 male Wistar albino rats (270-300 g, 12-weeks-old) were purchased from Pharmacy Faculty of Tehran University of Medical Sciences, Tehran, Iran. Animals were allowed to have access to food and water. Also, they were kept under 12- hour periods of light and darkness at 23 ± 2°C. All procedures were carried out in accordance with the guidelines of the Iranian Council for use and care of animals and approved by Ethics Committee of Tehran University of Medical Sciences.

### Experimental design

The rats were randomly divided into 4 groups: control (Co), sham (Sh), hypoxia (Hx) and hypoxia+flaxseed (Hx+Fx). Hypoxic rats were kept in a hypoxic chamber with a reduced pressure (oxygen 8% and nitrogen 92% for 4 hours/day for 30 days). The reason for using 8% oxygen is that the rats are capable to survive at this level of hypoxia which allows us to measure the patho-physiologic variables in them (18).

Control group (Co) was kept under normoxia and had free access to standard food and water. Sham group (Sh) was maintained in a hypoxia chamber (but not under hypoxia) receiving normal oxygen and food. Hypoxia group (Hx) was exposed to hypoxia 4 hours/day and fed with normal food. Hx+Fx group: 10% Fx was added to the normal food of Hx+Fx group after the first hypoxic exposure.

### Testis index

At the end of the experimental period, each rat was weighed and sacrified. Then, the right testis was removed and weighed. The testicular mass relative to body weight was determined on day 42 using the following equation: (testicular/body weight ratio)*100=(%).

### Detection of IL-18 levels

At the end of each experiment, blood samples were collected from the left ventricle. Blood was centrifuged at 1000 g for 15 minutes and serum was separated for biochemical analysis. IL-18 levels in serum samples were quantified by an ELISA kit (zell Bio-GmbH, Germany) according to the manufacturer's instructions.

### Histological procedure

At the end of the experiment, rats were weighed and sacrificed and their right testis was removed. The right testicular (internal spermatic) vein drained directly into the right common iliac vein in 77.4%, and into the inferior vena cava in 22.6% of the animals. The left testicular vein drained into the left common iliac vein in all animals, but in 90.3% of rats there was also an accessory branch of the testicular vein draining into the left renal vein (19). Testes were placed in Bouin's solution for 24 hours at room temperature. Later, they were processed, sectioned and stained with H&E technique. On slices with 5- µm thickness, the morphometric assessment of seminiferous tubules was performed. The tubular diameters and germinal epithelial thickness of seminiferous tubules that were sectioned transversely were evaluated using light microscopy (20). In this way, the slides were studied at ×100 magnifications, and in different fields of testis tissue, 20 tubules from each specimen were studied. The analyses were carried out on images were taken using LABOMED digital camera (LABOMED, USA). Then, the images were processed by the image analysis system software of Image J (ImageJ U. S. National Institutes of Health, Bethesda, Maryland, USA). Finally, the scale bar was added to the images (21).

### Sperm sampling

The caudal epididymis was used for sperm analysis. Briefly, epididiymal sperms were collected by slicing the caudal epididymis in 1 ml of Minimum Essential Medium-α (MEM-α) medium (P/N 22561-021, Gibco, CA, USA) after that 9-ml medium was added and samples were incubated for 10 minutes to allow the sperms to swim into the medium. The epididymis was then processed for further analysis.

### Sperm count

To enumerate the spermatozoa, the heads of spermatozoa were counted. For sperm counting, a hemocytometer device was used. Here, 50 µl of the suspension was mixed with an equal volume of 2% formalin. Then, 10 µl of this diluted suspension was transferred to a Neubauer chamber. The sperms were counted under light microscopy at ×400 (22).

### Sperm morphology

A part of sperm sample was used for preparing smears to evaluate the sperm morphological abnormalities. For this purpose, 10 µL of suspension was spread onto a glass slide and allowed to air-dry at room temperature to prepare a smear. The smears were then stained with Diff-Quik stain and 200 sperms were then examined under light microscopy at ×400 (22).

### Sperm viability assay

In order to study the sperm viability, 10 µl of sperm suspension was mixed with 2 µl Eosin-y 0.05%. Slides were prepared and incubated for two minutes at room temperature before evaluation at ×400 magnifications using light microscopy. Two hundred sperms were counted for each sample. Dead sperms appeared pink and live sperms were not stained (22).

### Sperm motility

One to two drops of the sperm suspension were placed on a glass slide and motile sperms were counted immediately using light microscopy (22).

### Tissue preparation for enzyme assay

Rat testes were rapidly removed and manually homogenized in cold phosphate buffer (pH=7.4) and debris was removed by centrifugation at 3500 g for 10 minutes. Then, 50 mg of supernatant was homogenized in 10 volumes of $KH_2PO_4$ (100 mmol) buffer and was centrifuged at 12,000 g for 30 minutes at 4ºC. The supernatant was collected and used for enzymes and MDA levels studies (23).

### Measurement of total anti-oxidant capacity and lipid peroxidation

Total antioxidant capacity was measured based on the absorbance of the 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS+) radical cation. The pre-formed radical monocation ± of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen-donating antioxidants. The influences of both the concentration of a given antioxidant and duration of reaction on the inhibition of the radical cation absorption are taken into account when determining the antioxidant activity (24). A common method for measuring MDA, referred to as the thiobarbituric acid-reactive-substances (TBARS) assay, is based on its reaction with Thiobarbituric acid (TBA) followed by reading the absorbance at 532 nm. Thiobarbituric acid substance assay is a method to quantify malondialdehyde concentration by spectrophotometry (25).

### Statistical analyses

Data were statistically analyzed using SPSS-22 (IBM crop., Armonk, NY, USA) software. All data were expressed as mean ± standard errors of mean (SEM), median and interquartile range (IQR). At first, the normality of variables was checked using the Kolmogorov-Smirnov test. Then, for analyzing the differences among four groups of study, one way-ANOVA test and Tukey-post hoc test were chosen if the distribution of data were normal (for sperm parameters, testicular/body weight ratio, diameter of seminiferous tubules, MDA level and TAC). Otherwise, nonparametric test of Kruskal-Wallis was carried out (for thickness of the germinal epithelium). The statistical significance level was set at 0.05.

## Results

### Model confirmation

Using one way-ANOVA test, serum levels of IL-18 were compared to confirm state of hypoxia. Tukey post hoc test showed a significant difference in serum levels of IL-18 in rat exposed to 30-days hypoxia (0.08 ± 0.05 pg/ml) compared to control (0.51 ± 0.08 pg/ml, P=0.0001) and Sham (0.52 ± 0.08 pg/ml, P=0.0001) groups (Fig.1).

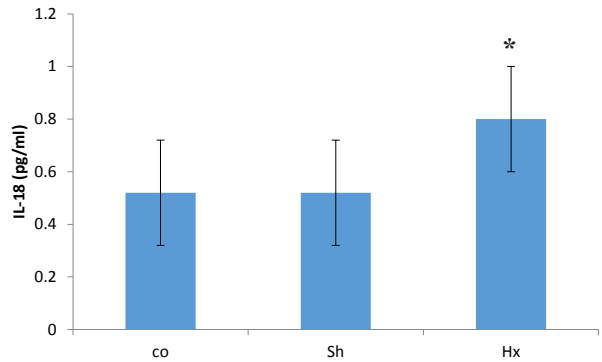


**Fig.1:** Effects of hypoxia on serum levels of IL-18 (pg/ml) in rats following hypoxia. *; P<0.05 compared to control and sham groups, Co; Normal group that received normal oxygen levels and normal food, Sh; Sham group maintained in hypoxia chamber with normal oxygen levels and food, and Hx; Animals were exposed to hypoxia and received normal food.

### Effects of flaxseed on the body weight and testicular mass/body weight ratio in rats with hypoxia

The effect of oral Fx on the testicular/body weight ra-

tio was evaluated in rats after hypoxia. According to the ANOVA test, the testicular mass/body weight were significantly different in the studied groups (P=0.0001, Fig.2). A significant difference was observed in the testicular mass/body weight of Hx (0.54 ± 0.01%) and Hx+Fx (0.56 ± 0.1%) groups compared to control (0.6 ± 0.1%, P=0.003 and P=0.027, respectively) and sham (0.61 ± 0.1%, P=0.001 and P=0.009, respectively) groups (Fig.2).

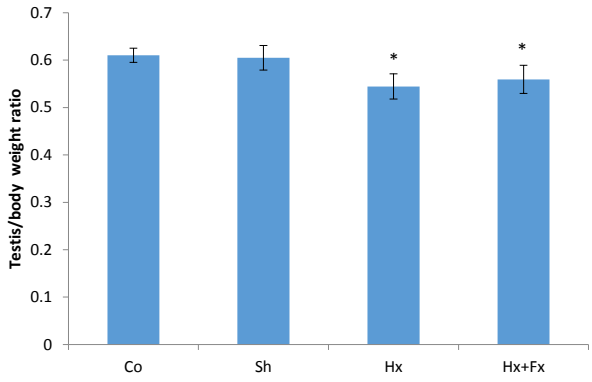


**Fig.2:** Effects of oral flaxseed on testicular mass/body weight ratio in rats following hypoxia.
*; P<0.05 compared to control and sham groups, Co; Normal group that received normal oxygen levels and normal food, Sh; Sham group maintained in a hypoxia chamber with normal oxygen levels and food, Hx; Animals were exposed to hypoxia and received normal food, and Hx+Fx; Animals were exposed to hypoxia and treated by normal food supplemented with 10% Fx.

## Effects of flaxseed on sperm parameters in rats exposed to hypoxia

The effects of oral Fx on sperm parameters were evaluated in rats after hypoxia. The mean sperm count was significantly different in the studied groups (P=0.0001, Fig.3). A significant difference (P=0.0001) was observed in the sperm count between Hx+Fx group (73.02 ± 1.93) and the Hx group (55.12 ± 3.84) (control=71.78 ± 0.22 and Sham=64.06 ± 6.14) (Fig.3). Moreover, the mean sperm motility was significantly different among the studied groups (P=0.025, Fig.3). A significant difference was found in sperm motility between Hx group (74.76 ± 2.27%) and the control (82.35 ± 1.59%, P=0.032) and sham (80.47 ± 0.67%, P=0.041) groups (P<0.05, Fig.3). Also, a significant difference was observed in the sperm motility between Hx+Fx group (83.04 ± 1.52%) and the Hx group (P=0.028, Fig.3). Based on ANOVA test, a significant difference was found in sperm viability between Hx group (60.8 ± 0.85%) and control (83.31 ± 2.5%, P=0.0001) and sham (82.92 ± 1.5%, P=0.0001) groups (Fig.3) and a significant difference was observed in the sperm viability between Hx+Fx group (85.67 ± 1.33%) and the Hx group (P=0.0001, Fig.3). The mean sperm abnormality was significantly different among the studied groups (P=0.0001, Fig.3). A significant difference was seen in sperm abnormality between Hx group (41 ± 1%) and control (17 ± 1.1%, P=0.0001) and sham (16 ± 1.3%, P=0.0001) groups (Fig.3) and a significant difference was observed in the sperm abnormality between Hx+Fx group (14 ± 1.2%) and Hx group (P=0.0001, Fig.3).

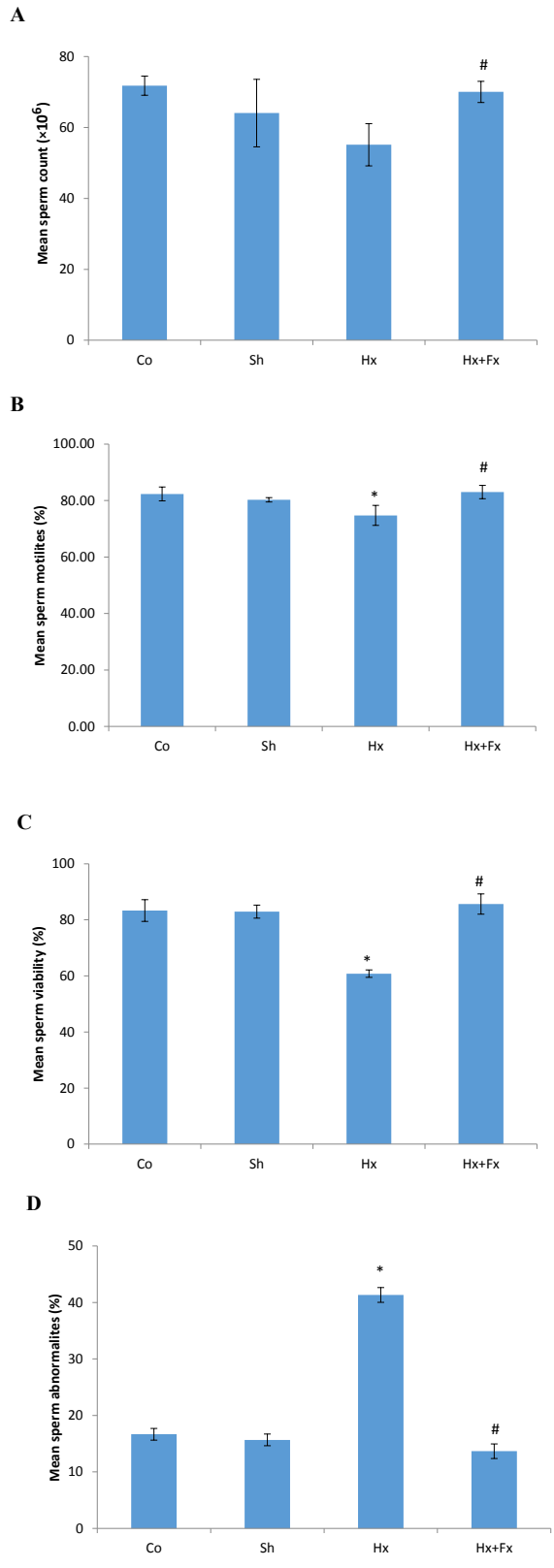


**Fig.3:** Effects of oral flaxseed on sperm parameters of rats following hypoxia. **A.** Sperm count, **B.** Sperm motility, **C.** Sperm viability, and **D.** Sperm abnormality.
*; P<0.05 compared to control and sham groups, #; P<0.05 compared to HX group, Co; Normal group that received normal oxygen levels and normal food, Sh; Sham group maintained in hypoxia chamber with normal oxygen levels and food, Hx; Animals were exposed to hypoxia and received normal food, and Hx+Fx; Animals were exposed to hypoxia and received normal food supplemented with 10% Fx food.

### Effects of flaxseed on diameter of seminiferous tubules and thickness of the germinal epithelium in rats exposed to hypoxia

The effects of oral Fx on the diameter of seminiferous tubules and thickness of the germinal epithelium were evaluated after hypoxia in rats. According to ANOVA test, the mean diameter of seminiferous tubules was significantly different in the studied groups compared to control and sham (P=0.0001, Fig.4). A significant difference was found in the diameter of seminiferous tubules of Hx group (10.58 ± 0.34 µm) in comparison to the control (11.77 ± 0.22 µm, P=0.031) and sham (12.28 ± 0.4 µm, P=0.001) groups (Fig.4) and a significant difference was observed in diameter of seminiferous tubules of Hx+Fx group (13.04 ± 0.2 µm) as compared to the Control (P=0.022), sham (P=0.048) and Hx (P=0.0001) groups (Fig.4). The thickness of the germinal epithelium was significantly different among the studied groups (P=0.008, Fig.4). A significant difference was observed in the thickness of the germinal epithelium of Hx+Fx [3.5 (IQR: 3.13-3.83) µm] group as compared to the Hx [2.28 (IQR:2-2.56) µm, P=0.005] group (Fig.4).

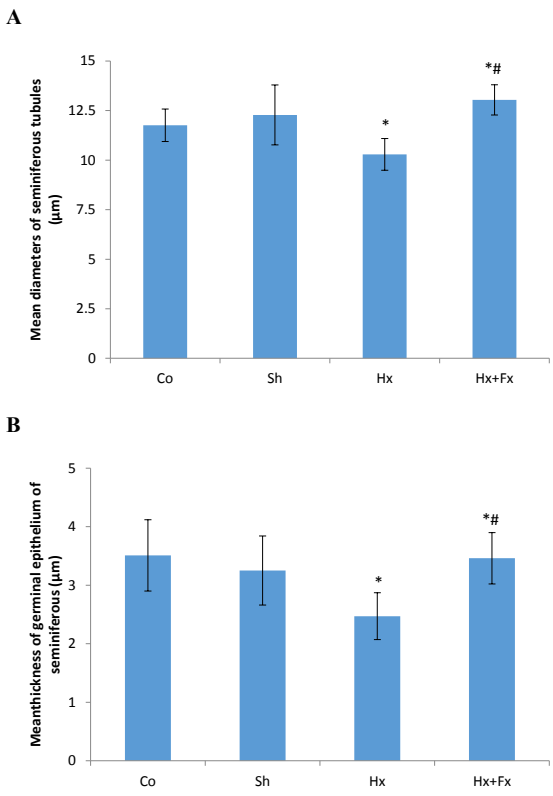


**Fig.4:** Effects of flaxseed on diameter of seminiferous tubules and thickness of the germinal epithelium in rats exposed to hypoxia. Comparing **A.** The diameter of seminiferous tubules and **B.** Thickness of the germinal epithelium in different groups.
*; P<0.05 compared to Control and Sham groups, #; P<0.05 compared to Hx group, Co; Normal group that received normal oxygen levels and normal food, Sh; Sham group maintained in hypoxia chamber with normal oxygen levels and food, Hx; Animals were exposed to hypoxia and received normal food, and Hx+Fx; Animals were exposed to hypoxia and received normal food supplementated with 10% Fx food.

### The effects of oral flaxseed on MDA and TAC concentrations were evaluated after hypoxia in rats exposed to after hypoxia

No significant difference was observed in the mean MDA among studied groups (control=7.78 ± 0.11 nmol/mg and sham=7.13 ± 0.09 nmol/mg, Hx=8.57 ± 0.28 nmol/mg and Hx+Fx=6.7 ± 0.81 nmol/mg) (P=0.075, Fig.5). The mean TAC was significantly different among the studied groups (P=0.01, Fig.5). A significant difference was observed in TAC of Hx+Fx (2.07 ± 0.12 nmol/mg) group compared to control (1.51 ± 0.13 nmol/mg, P=0.011), sham (1.53 ± 0.06 nmol/mg, P=0.014) and Hx (1.18 ± 0.02 nmol/mg, P=0.001) groups (Fig.5).

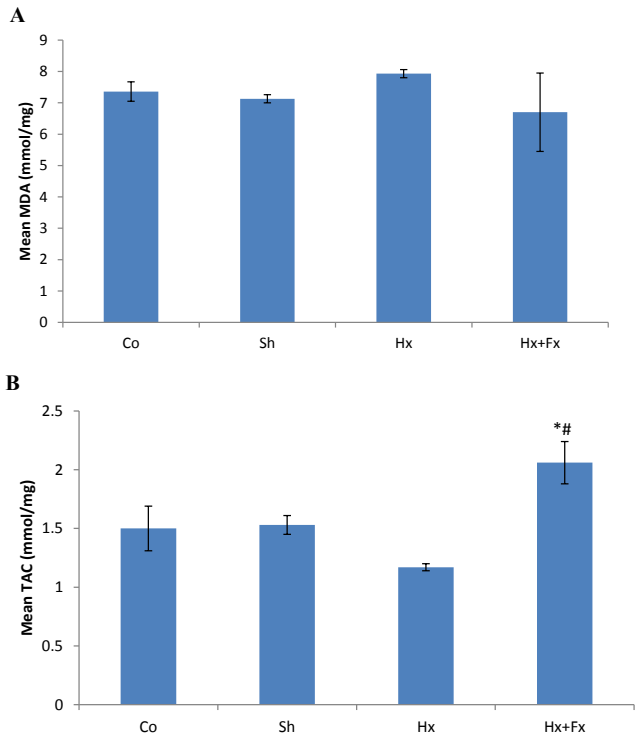


**Fig.5:** Effects of oral flaxseed on MDA and TAC concentrations in rats exposed to hypoxia. **A.** MDA and **B.** TAC concentrations of rats following hypoxia.
MDA; Malondialdehyde, TAC; Total antioxidant capacity, *; P<0.05 compared to Control and Sham groups, #; P<0.05 compared to Hx group, Co; Normal group normal oxygen and normal food, Sh; Sham group maintained in hypoxia chamber with normal oxygen and food, Hx; Animals were exposed to hypoxia and received normal food, and Hx+Fx; Animals were exposed to hypoxia and received normal food supplementated with 10% Fx food.

## Discussion

Hypoxia is a condition can result in overproduction of ROS which along with a decrease in the level of antioxidants, may give rise to oxidative stress. Oxidative stress as an imbalance between generation of ROS and ability of endogenous antioxidant systems to scavenge ROS has adverse influence on testes structure and sperm parameters.

In this study, we found that hypoxia leads to reduction in the germinal epithelial thickness and some changes in the serum, testes and sperm parameters in rats also hypoxia results in excessive formation of ROS. We also observed that hypoxia increases interstitial space of the testes, which extends the oxygen diffusion distance and impairs oxygen delivery to germ cells. It makes germ cells more susceptible to damage, which was confirmed by our observation concerning degeneration of germ cells in hypoxic rats. A similar outcome was reported by other researchers (26). In the present study, we observed that

flaxseed improves testicular structure as reflected by increased diameter of seminiferous tubules of Hx+Fx group as compared to the Hx group and increased thickness of the germinal epithelium of Hx+Fx group as compared to the Hx group.

Spermatogenesis is vulnerable to hypoxia because spermatogenesis has a high proliferation rate, damanding notable oxygen levels in the testes and it has been reported that breathing 10% $O_2$/90% $N_2$ results in a 24% decrease in testicular blood flow, but a 23% increase in cerebral blood flow. These characteristics may attribute to the morphological changes of spermatogenesis induced by hypoxia. Besides, a significant decrease in testicular mass followed by adverse effects on reproductive hormones such as testosterone was observed (27). In this study the sperm count, motility and viability significantly decreased in Hx, but increased in Hx+Fx group which might indicate that hypoxia affects sperm sperm differentiation process. We found that flaxseed can improve sperm parameters following exposure to hypoxia.

In our study there was significant reduction in body weight of Hx+Fx group in comparison to the control and sham groups. Researchers have observed that doses of 5 and 10 g of flaxseed fibers result in prolonged decrease in the levels of ghrelin a hunger-signaling gut peptide (29).

Dissimilar to many other cell types, sperm lipid membranes contain an exceptionally high percentage of polyunsaturated fatty acids (PUFAs) that provide the fluidity to the membrane contraction events associated with fertilization. However, PUFAs are readily oxidized and produce malondialdehyde.

We reported that lipid peroxidation assessed by MDA levels in all groups exposed to hypoxia was increase but the differences among different groups were not significant. The hypoxia-induced changes in lipid metabolism were mediated via hepatic stearoyl coenzyme A desaturase (25, 30). Lipid peroxidation in mice exposed to sever hypoxia is different from those exposed to moderate hypoxia and the degree of lipid peroxidation rate depend on hypoxia intensity (30). Therefore, probably due to this reason, our result is different from other those of reports. These adverse effects of hypoxia have also been reported to decreased the supplementation of antioxidants such as melatonin and ascorbate (31).

This study shows an increase in serum inflammatory markers (i.e.IL-18) only in group who expose to hypoxia and higher levels of lipid peroxidation and reduces antioxidant activity. In addition, we found flaxseed could effectively counteract peroxidation damage, mediated by the attenuation of systemic and tissue oxidative stress induced by Hypoxia. This is reflected by an increase in TAC values in Hx+Fx group as compared to the Hx group. This is in agreements with previous studies (26).

A high rate of death was observed among animals during the last time of hypoxia procedure.

To confirm the results of this study, we suggest to evaluate the testicular tissue superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRD), and glutathion-S-transferase (GST) activities to confirm the obtained findings.

## Conclusion

The conclusion the present study revealed that flaxseed as an antioxidant drug can reduce hypoxia-induced damages in the testes.

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## Author's Contributions

G.H., M.A.; Contributed to conception and design. M.P., F.N., S.M.; Contributed to all experimental work. K.M.; Contributes to data and statistical analysis, and interpretation of data. N.Y., N.D.; Were responsible for overall supervision, drafted the manuscript, which was revised by F.A., S.I. , K.M., M.B. and B.N. All authors read and approved the final manuscript.

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Original Article

# Protective Effects of *Matricaria chamomilla* Extract on Torsion/ Detorsion-Induced Tissue Damage and Oxidative Stress in Adult Rat Testis

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## Abstract

**Background:** There is some evidence indicating that *Matricaria chamomile* (MC) had protective effects on ischemia-reperfusion. In the present study, a rat model was used to investigate the effect of hydroalcoholic extract of MC on torsion/detorsion-induced testis tissue damage.

**Materials and Methods:** In this experimental study, 28 male Wistar rats were randomly divided into 4 groups as follows: G1, Sham operated; G2, testicular torsion/detorsion (T/D); G3, rats with testicular torsion/detorsion that received 300 mg/kg of MC extracts 30 minutes before detorsion (T/DMC); and G4, healthy rats that received 300 mg/kg of MC extracts (MC). Also, the reperfusion period was 24 hours. After blood sampling, the oxidative stress marker [e.g. superoxide dismutase (SOD) levels], blood levels of testosterone, and anti-oxidant enzyme levels [e.g. glutathione peroxidase (GPx)] were assessed by ELISA methods. Serum activity of malondialdehyde (MDA) was evaluated by spectrophotometry. Another assessment was carried out by histomorphometry, 24-hour post-procedure. The histological parameters investigated by Johnson's scores (JS), also the seminiferous tubule diameter (STD) and the height of the germinal epithelium (HE) measured using the linear eyepiece grids using light microscopy.

**Results:** Histological features significantly differed between sham and the other groups. The levels of SOD, GPx, and testosterone hormone were significantly decreased in T/D group as compared to sham group, while these parameters increased in T/DMC group as compared to T/D group. During ischemia, the MDA levels increased; however, treatment with MC extract decreased the MDA levels in G3 and G4 groups.

**Conclusion:** Results of the present study demonstrated that MC can protect the testis tissue against torsion/detorsion-induced damages by suppressing superoxide production.

***Keywords:*** Chamomile, Oxidative Stress, Testicle, Torsion/Detorsion

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## Introduction

Testicular torsion, as an abnormal twisting of the spermatic cord due to rotation of a testes or the mesorchium (i.e. a fold in the area between the testes and epididymis), is one of the dangerous pathologic conditions which leads to severe scrotal pain and further injuries of the testes which is regarded as an emergency condition. In males, it has been reported the incidence of testicular torsion peaks under the age of 25 years old; however, it may be seen in any age group and it is estimated to occur in 1 out of 4000 males (1).

The degree and the duration of torsion are two important predictors of testicular damage (2). If detorsion occurs within 4 to 6 hours after torsion, testis can be saved in 90% of cases. On the other hand, the success rate decreases to 50% after 12 hours and it drops to 10% after 24 hours. Therefore, in order to maintain the testicular tissue and prevent orchiectomy, an immediate correct diagnosis along with essential interventions, are necessary (1, 3). The twist of spermatic cord leads to reduced testicular blood flow; therefore, for reperfusion of the affected testis, an immediate surgery is needed. However, further damage to the testis results from any attempt to reperfuse the ischemic tissue.

Several studies have been reported that disruption of the seminiferous epithelium and disappearance of germ cells may occur after ischemia/reperfusion (IR) injury in the testis (3-5). Reactive oxygen species (ROS) have

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been reported as a possible cause of IR-induced damage (3). An increase in the level of ROS leads to DNA damage and testicular germ cell apoptosis (3, 4). Thus, to prevent reperfusion injury, combinations of enzymes, chemical drugs, and herbal extracts have been used after testicular torsion/detorsion or ischemic/reperfusion, along with performing histopathological assessments (6-8). These protocols are intended for inhibition of oxidative stress. For example, several studies have been reported that using zinc aspartate reduces IR-induced injury and also increases the activity of antioxidant enzymes (2, 3). Medicinal herbs are cost-effective and less severe side effects than conventional pharmacological drugs. Therefore, nowadays, they have a special place in the treatment of infertility (9, 10).

One of the perennial plants that belongs to Asteraceae family is chamomile (*Matricaria chamomile* (MC) which grows in the West Europe and North Africa. It has been used as a tea to treat stomach disorders in traditional medicine. Moreover, the antispasmodic effects of chamomile can reduce the possibility of preterm delivery in women and also alleviate menstrual cramps. It is also used to stimulate menstruation. The stimulating effects of MC extract on leukocytes, such as macrophages and B lymphocytes, can be effective in the treatment of skin inflammation and eczema. The soothing effect of MC extract on the central nervous system is useful for the treatment of insomnia. Also, both lipophilic and hydrophilic components of chamomile extract have great therapeutic activities (9, 10).

Unstable oils and flavonoids, including apigenin, rutin, and luteolin, are the most main active compounds of hydroalcoholic extract of chamomile. Flavonoids, as phenyl benzopyrone chemicals, are observed in all vascular plants. Also, it has been reported that the benzopyranone ring system is a molecular scaffold of considerable interest, and this scaffold is found in certain flavonoid natural products and has aromatase inhibitory activity (9, 10). Several clinical and experimental studies which were performed on M. recutita reported that the majority of its pharmacological actions are dependent on its antioxidant activity that reduces the free radicals and inhibits lipid peroxidation (9-11). Therefore, we decided to investigate the hydroalcoholic extract of MC on oxidative stress and tissue damage caused by torsion/detorsion in the testes of rats.

## Materials and Methods

In this experimental study, all experimental procedures were approved by the animal Ethics Committee of Gonabad University of Medical Sciences, Gonabad, Iran. Twenty-eight male Wistar rats weighing 200-250 g were maintained for 2 weeks on a moderate fiber (MF) diet and had free access to food and water. They were kept in the animal room at a constant temperature (25 ± 2°C) at 30-70% humidity with 12 hour light/12 hour dark cycles. Rats were randomly divided into 4 groups as follows: sham group (G1) that underwent a surgery without induction of torsion; torsion/detorsion group (T/D or G2) in which testicular torsion was induced for 4 hours followed by detorsion for 24 hours; G3 or T/DMC group in which testicular torsion was induced for 4 hours and rats intraperitoneally received 300 mg/kg of hydroalcoholic extracts of MC, 30 minutes before detorsion then experienced detorsion for 24 hours; and G4 or MC group in which rats intraperitoneally received 300 mg/kg of hydroalcoholic extracts of MC for 24 hours without application of torsion (5-7).

### Preparation of the hydroalcoholic extract of *Matricaria chamomile*

In order to prepare chamomile whole-plant-extract, 500 g of chamomile flower was dried at 25°C and protected from direct sunlight. For extraction, the dried plants were grounded and treated with 2 L of alcohol 96% and distilled water and left for 48 hours at room temperature. Over this period, the mixture was frequently shaken and then filtered. Next, the mixture was centrifuged at 3000 rpm for 5 minutes. At the end of the process, the resulting solution was poured into an open-top container and the solvent was evaporated. About 90 g of a semi-solid extract was obtained from chamomile powder. In order to achieve appropriate concentrations, the extract was dissolved in normal saline.

### Surgical procedure

The surgical procedure was carried out based on previous experimental studies (6, 7). In brief, using ketamine (50 mg/kg) and xylazine (10 mg/kg), the rats were anaesthetized. Then, through a longitudinal scrotal incision, their left testis was exposed and dissected. Afterwards, torsion of the left testis was induced by 720° counterclockwise rotation and fixed to the scrotum in the torsion position using three 6/0 non-absorbable silk sutures. These procedures were described in our previous study.

Testicular torsion maintained for 4 hours in T/D groups and afterward, detorsion was performed and maintained for 24 hours. At the end of the treatment period, 24-hour post-procedure, rats were anaesthetized using ketamine-xylazine and their blood was drawn from the hearts in order to measure the levels of testosterone and antioxidant enzymes. Blood samples were centrifuged at 3000 rpm for 10 minutes and then the serum was removed and kept at -70°C until further analysis. Moreover, in order to examine tissue oxidative stress markers and perform histological study, the left testicular underwent orchiectomy.

### Tissue fixation and preparation of specimens

After the surgical procedure, the testicular specimens were immersed in the Bouin's solution for 48 hours. After fixation, testicles were dehydrated in a series of increasing concentrations of ethanol and embedded in paraffin. Then, sections were cut into 5-µm thickness,

deparaffinized, stained with hematoxylin-eosin (H&E), and studied under an optical microscope (NIKON) at a final magnification of ×400.

### Histological evaluation and maturation of seminiferous tubules

In order to evaluate the spermatogenesis in seminiferous tubules, the Johnson's score was used. For this propose, 50 seminiferous tubules were examined in each cross-section and a score of 1-10 was given to each tubule according to the following criteria (8).

### Morphometry of seminiferous tubules

The morphometry of the seminiferous tubules was randomly recorded by measuring 20 cross sections of seminiferous tubules that were prepared as circular as possible or nearly round cross sections. In the same sections, the height of the seminiferous epithelium (HE) was also measured from the basal membrane on one side of the tubule to the luminal edge. These measurements were done using the linear eyepiece grids on the light microscope at ×400 magnification (3).

### Evaluation of biochemical parameters (malondialdehyde, superoxide dismutase, and glutathione peroxidase levels) in the serum

Measurement of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx) levels were described in our previous study.

Briefly, the level of MDA was measured by placing 0.20 $cm^3$ of plasma into a test tube which contained 3.0 $cm^3$ of glacial acetic acid. Then, 1% thiobarbituric acid (TBA) in 2% NaOH was added to the tube which was placed into a boiling water bath for 15 minutes. The absorbance of the pink product was read at 532 nm after cooling, using a spectrophotometer device (Biospect Inc., USA). The calibration curve was constructed using malondialdehyde tetrabutylammonium salt obtained from Sigma (USA) (7). The levels of SOD and GSH peroxidase activity (GPx) were assayed in the serum using an ELISA reader (Antus) according to the protocols of the kits (Randox and Ransod, UK).

### Measurement the oxidative stress markers in the testis tissue

For measuring tissue oxidative stress markers, testis tissues were homogenized. Next, lipid peroxidation level was assessed as the amount of MDA. In order to prepare a solution of TBA-TCA-HCL, 375 mg of TBA was dissolved in 2 ml of HCl, then added to 100 ml of 15 % trichloroacetic acid (TCA). For dissolving the sediment, a water bath at 50ºC was used. The tissue was weighed and immediately homogenized using a solution of potassium chloride 5.1% to obtain a 10% homogenized mixture. Then, 1 ml of the homogenized tissue mixture was mixed with 2 ml of TBA-TCA-HCl solution and heated in boiling water for 45 minutes (a pink-orange solution). After cooling, it was centrifuged at 1000 rpm for 10 minutes. The absorption (A) at 532 nm was read using a spectrophotometer (Biospect). The levels of SOD and GPx were assessed in the testis tissue using an ELISA reader (Antus) according to the manufacturer's protocols (Randox and Ransod, UK).

### Measurement of testosterone level

The serum level of testosterone was determined by a testosterone ELISA kit (Demeditec Diagnostics, Germany) and absorbance was measured at 405 nm using an ELISA reader (Antus).

### Statistical analysis

Statistical analysis of data was carried out IBM SPSS Statistics Software (Version 20, IBM Corp., Armonk, NY, USA). All data were presented as mean ± SE and compared using One-way ANOVA and Tukey's post-hoc test. Differences with $P<0.05$ were considered statistically significant.

## Results

### Testicular histological parameters

In T/D and T/DMC groups, the mean Johnson's score (MJS) was significantly lower than that of sham group (P=0.001). On the other hand, MC extract significantly increased the MJS in T/DMC and MC groups compared to T/D group (P=0.001). However, the MC and sham groups did not show significant differences in terms of MJS (Fig.1, Table 1).

**Table 1:** A comparison of the testicular mean Johnson's score, seminiferous tubule diameter, and the height of epithelium among sham, T/D, T/DMC, and MC groups

| Groups | Mean Johnson's Score ± SD | STD ± SD | HE ± SD |
|---|---|---|---|
| Sham | 9.685 ± 0.11 | 264.42 ± 2.69 | 69.2 ± 3.21 |
| T/D | 4.458 ± 0.15$^{+}$ | 156.80 ± 0.34$^{+}$ | 34.42 ± 5.32$^{+}$ |
| T/DMC | 7.478 ± 0.41$^{*}$ | 195.65 ± 7.42$^{*}$ | 54.75 ± 3.6$^{*}$ |
| MC | 9.56 ± 0.10$^{*}$ | 264.62 ± 6.30$^{*}$ | 70.3 ± 4.25$^{*}$ |

**T/D; Group underwent testicular torsion/detorsion, T/DMC; Group underwent testicular torsion/detorsion and received hydroalcoholic extract of MC, 30 minutes before detorsion, MC; Goup received hydroalcoholic extract of MC, STD; Seminiferous tubule diameter, HE; The thickness or height of the seminiferous epithelium, *; Shows significant difference as compared to T/D, and $^{+}$; Means significant difference as compared to sham group (P≤0.05). All data are displayed as mean ± SD.**

Moreover, the seminiferous tubule diameter (STD) was significantly decreased in T/D group in comparison to sham group ($P<0.001$). Also, the STD was significantly increased in T/DMC and MC groups, which received the hydroalcoholic extract of MC, as compared to T/D group ($P<0.001$). In addition, there were no significant differences between MC and sham groups for STD ($P>0.05$). Furthermore, the HE was significantly decreased in T/D group compared to sham group ($P<0.001$) while treatment with MC extract significantly increased the HE in T/DMC and MC groups compared to T/D group ($P<0.001$).

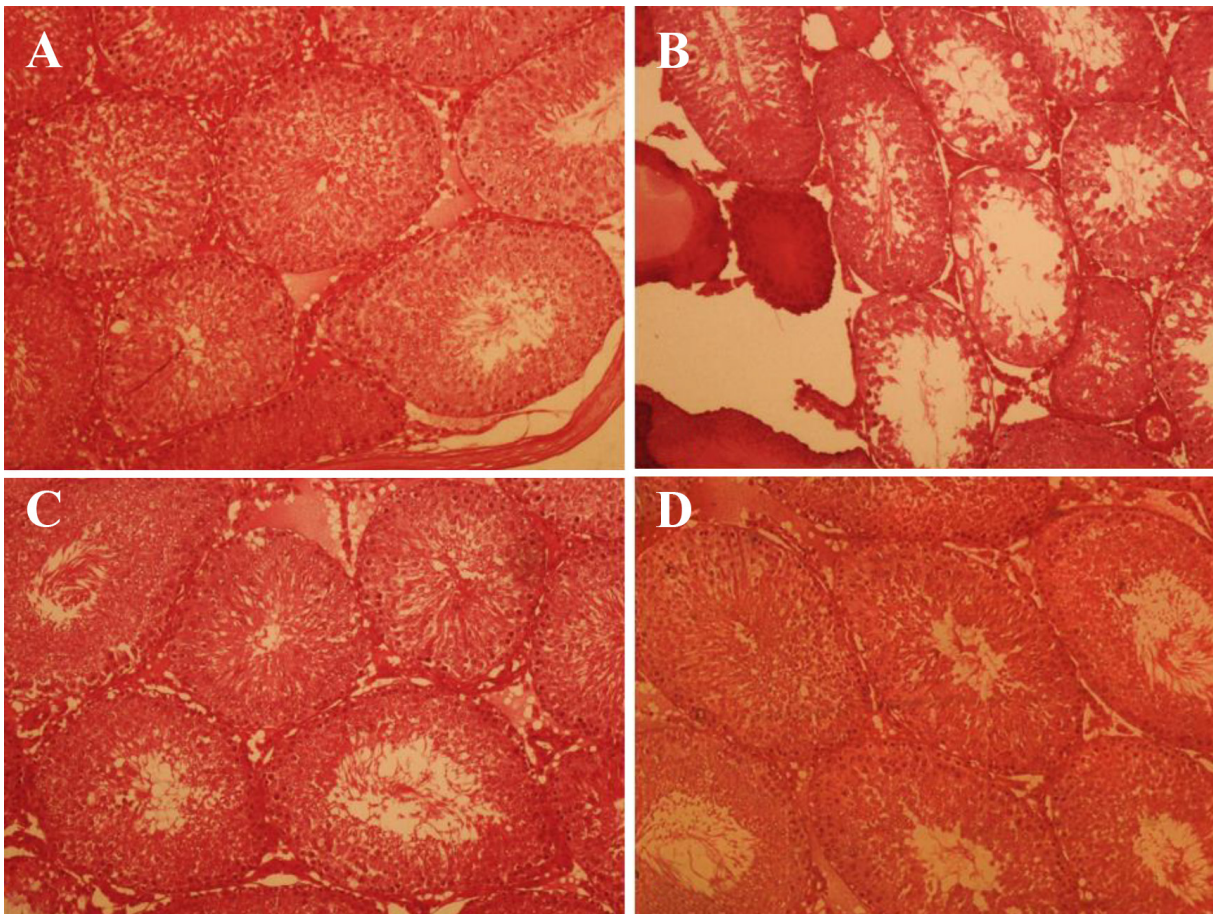


**Fig.1:** Histological findings in sham, T/D, T/DMC and MC groups, 24 hours after surgery. **A.** Sham, the lumen of tubules is quite regular and the thickness of the germinal epithelium is normal, also no congestion and edema were observed, **B.** Testicular torsion induced for 4 hours followed by detorsion. The thickness of germinal epithelium was substantially declined, **C.** Testicular torsion detorsion which received hydroalcoholic extract of MC, 30 minutes was before detorsion (T/DMC). Edema and congestion were substantially reduced and MC prevented reductions in the thickness of the germinal epithelium, and **D.** Received hydroalcoholic extracts of MC. The lumen of seminiferous tubules is quite regular and the thickness of the germinal epithelium is normal, and no congestion and edema were observed (H&E).

## Biochemical parameters

In all subgroups of T/D, T/DMC, and MC, the serum levels of testosterone were significantly decreased in comparison to sham group (P<0.001). Moreover, in the groups treated with MC extract, T/DMC and MC groups, testosterone level was significantly higher than that of T/D group (P<0.001, Fig.2). On the other hand, in all subgroups of T/D and T/DMC, the serum levels of GPx were significantly decreased in comparison to sham group (P<0.001). Also, it was significantly increased in T/DMC and MC groups compared to T/D group (P<0.001, Fig.3). The serum level of SOD was significantly lower in T/D group than sham group (P<0.001). Also, the comparison between T/D group to T/DMC and MC groups showed that the serum level of SOD was significantly increased in T/DMC and MC groups as compared to T/D group (P<0.001, Fig.4). Moreover, the serum level of MDA was significantly higher in T/D group than sham group (P<0.001). In this regard, the level of MDA was significantly decreased in T/DMC and MC groups in comparison with T/D group (P<0.001, Fig.5).

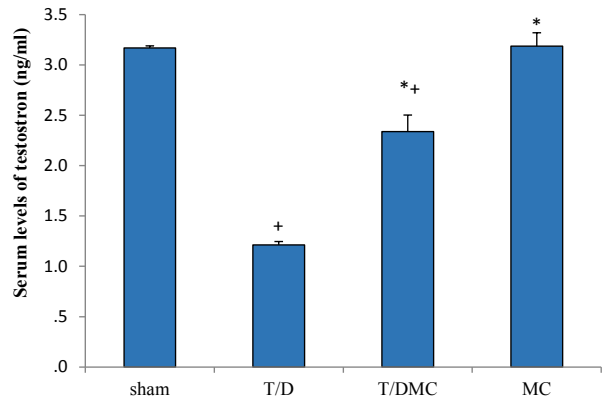


**Fig.2:** A comparison of testosterone levels in sham, T/D, T/DMC and MC groups. T/D; Group underwent testicular torsion/detorsion, T/DMC; Group underwent testicular torsion/detorsion and received hydroalcoholic extract of MC, 30 minutes before detorsion, MC; Group received hydroalcoholic extract of MC, *; Shows significant difference compared to T/D group, and +; Means significant difference compared to sham group (P≤0.05).

## The level of oxidative stress markers in testis tissue

The mean level of MDA in testis tissue was significantly higher in T/D group compared to sham group.

Also, it was significantly decreased in T/DMC and MC groups when compared to T/D group. The mean activity of SOD in the testis tissue was significantly decreased in T/D group as compared to sham group. In this regard, it was significantly increased in T/DMC and MC groups in comparison with T/D group. The mean activity of GPx in sham group was significantly higher than that of T/D group. Moreover, in T/DMC and MC groups, the level of GPx was significantly higher than that of T/D group (P<0.001, Table 2).

**Table 2:** The level of oxidative stress markers in testis tissue in sham, T/D, T/DMC, and MC groups

| Groups | MDA ± SD | SOD ± SD | GPx ± SD |
|---|---|---|---|
| Sham | 80 ± 9 | 1.52 ± 0.21 | 31 ± 3.21 |
| T/D | 140 ± 11† | 0.62 ± 0.11† | 13.25 ± 2.32† |
| T/DMC | 100 ± 13* | 0.96 ± 0.18* | 24.75 ± 4.6* |
| MC | 85 ± 10* | 1.47 ± 0.24* | 28.65 ± 3.25* |

**T/D; Group underwent testicular torsion/detorsion, T/DMC; Group underwent testicular torsion/detorsion and received hydroalcoholic extracts of MC, 30 minutes before detorsion, MC; Group received hydroalcoholic extracts of MC, MDA; Malondialdehyde, SOD; Superoxide dismutase, GPx; Glutathione peroxidase, *; Shows significant difference as compared to T/D, and †; Means significant difference as compared to sham group (P≤0.05). All data are displayed as mean ± SD.**

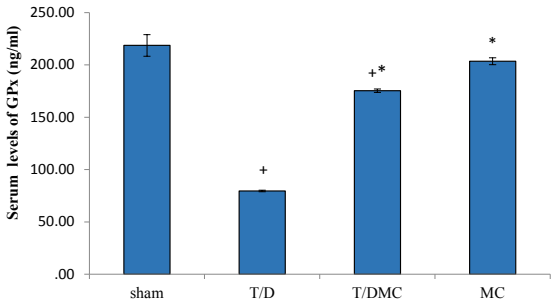


**Fig.3:** A comparison of the GPx in sham, T/D, T/DMC and MC groups. GPX; Glutathione peroxidase, T/D; Group underwent testicular torsion/detorsion, T/DMC; Group underwent testicular torsion/detorsion and received hydroalcoholic extracts of MC, 30 minutes before detorsion, MC; Group received hydroalcoholic extracts of MC, *; Shows significant difference compared to T/D group, and +; Means significant difference compared to sham group (P≤0.05).

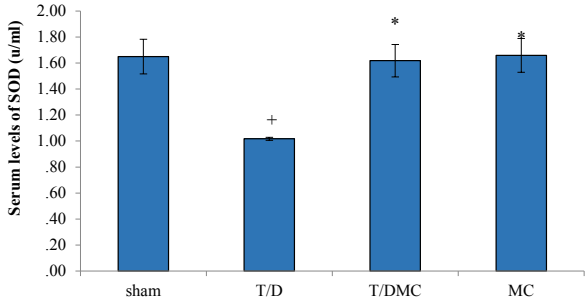


**Fig.4:** A comparison of SOD levels in sham, T/D, T/DMC and MC groups. SOD; Superoxide dismutase, T/D; Group underwent testicular torsion/detorsion, T/DMC; Group underwent testicular torsion/detorsion and received hydroalcoholic extract of MC, 30 minutes before detorsion, MC; Group received hydroalcoholic extract of MC, and *; Shows significant difference with T/D group (P≤0.05). Values are expressed as mean ± SD.

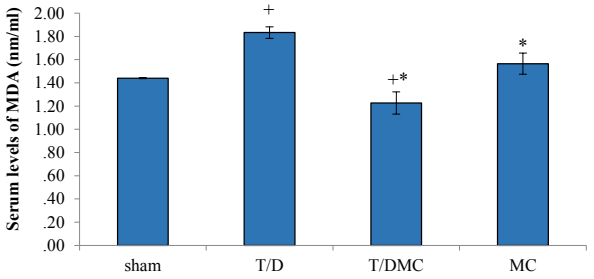


**Fig.5:** A comparison of the MDA in sham, T/D, T/DMC and MC groups. MDA; Malondialdehyde, T/D; Group underwent testicular torsion/detorsion, T/DMC; Group underwent testicular torsion/detorsion and received hydroalcoholic extracts of MC, 30 minutes before detorsion, MC; Group received hydroalcoholic extracts of MC, *; Shows significant difference compared to T/D group, and +; Means significant difference compared to sham group (P≤0.05).

## Discussion

Ischemia-reperfusion (IR) is the main phenomenon that occurs following testicular torsion and causes testicular damage, apoptosis, and even infertility. The histological damage caused by IR injury in testis has been shown in several studies with different time period and degree of torsion and different time period of detorsion. As in this study, 4-hour torsion and 24-hour reperfusion caused damage to the testicles (6, 12, 13). Ischemia and reperfusion can lead to tissue damage through several mechanisms including increasing ROS levels and production and secretion of inflammatory factors (6). Former research has shown that the severity of ischemic histological damage depends on two important factors namely, the duration and degree of torsion (14).

Yuluğ et al. (6) showed that 4-hour ischemia followed by 24-hour reperfusion could cause testicular tissue damage. Previous studies have also shown that torsion of 720 degrees is enough to stop the testicular blood flow in a rat model (6, 7, 12-16). In the present study, according to previous studies, we induced 4-hour ischemia following by 24-hour reperfusion.

Furthermore, our present study showed that torsion of 720 degrees for 4 hours and a consecutive reperfusion for 24 hours led to edema. Moreover, histological features such as degeneration of germ cells layer and decreases in the seminiferous tubule diameter, Johnson's score and the number of germ cells were observed. Spermatogenesis is an extremely regulated process which is mainly controlled by testosterone and gonadotropins (17). In a study, Moghimian et al. (1, 18) showed that 5-hour ischemia followed by 24-hour reperfusion reduced serum levels of testosterone.

As a fact, the half-life of testosterone in the blood is 24 hours. Also, IR in testicles results in damages in testis tissue such as Leydig cells, which act as the source of testosterone secretion. In the present study, a statistically significant difference in serum levels of testosterone was observed. It was significantly decreased in the T/D group. One study reported that 30 minutes of ischemia followed

by reperfusion leads to decreased levels of GPx but increased levels of SOD level, 24 hours after the procedure (19, 20). These findings show that the antioxidant defense against oxidative stress is activated after the testicular ischemia and reperfusion. On the other hand, Ozkan et al. (2) reported that 4-hour torsion followed by detorsion led to decreased levels of SOD but increased levels of MDA 4 hours after the procedure.

In the present study, the serum and tissue levels of SOD and GPx in the T/D group significantly decreased while the serum and tissue levels of MDA increased. In agreement with our results, Ozbek et al. (21) and Ozturk et al. (22) in separated studies showed that testicular torsion for 4 hours and detorsion increase tissue levels of MDA and reduce SOD and GPx levels. According to the previous studies, it can be concluded that the effects of chamomile on serum testosterone levels act in a dose-dependent manner so that low doses can reduce serum testosterone levels while high doses increase serum testosterone levels (23).

In an experimental study, Johari et al. (23) showed that an intraperitoneal injection (10, 20, and 40 mg/kg) of *M. chamomile* flower extract reduced the serum level of testosterone in male rats. Another study has reported that testosterone levels decrease in rats which received *M. chamomile* extract (400 mg/kg) for 8 weeks (24). Moreover, Hatami and Estakhr (25) reported that MC 100 mg/kg increases serum testosterone levels, the function of the hormonal pituitary-testis axis, and spermatogenesis. The present study showed that 300 mg/kg of MC extract can significantly increase the serum levels of testosterone in the T/DMC and MC groups as compared to the T/D group. Therefore, MC extract by preventing Leydig cells damage and its components, increases the serum levels of testosterone.

On the other hand, in the present research, we observed that the serum level of testosterone in MC group was higher than that of sham group. Possibly, chamomile extracts exert its effect via its flavonoids, phenolic compounds, and alpha-bisabolol content and also through its antioxidant potentials which result in neutralization free radicals (9, 10). One study has reported that hydroalcoholic extract of MC and its compounds such as flavonoids increase the serum levels of testosterone (26). Antioxidants are compounds that prevent the formation of free radicals and inhibit lipid peroxidation; therefore, they can be effective in treatment of infertility induced by oxidative stress (27, 28). The enzymatic antioxidants, such as SOD and catalase have an important role in the prevention of cells insults induced by oxidative conditions (29).

Several studies have reported that extract of MC reduced the lipid peroxidation (as reflected by MDA levels) and increased the serum level of SOD, catalase, and glutathione (10, 30). In addition, one study reported that MC extract decreased the level of MDA in the brain tissue and increased the tissue level of SOD and GPx (5). Finally, our study showed that the dose of 300 mg/kg of MC extract decreased the level of MDA while increased the levels of SOD and GPx.

## Conclusion

According to the results of the present study, the extract of Matricaria chamomile could change the level of testosterone and protect the tissue against damage and oxidative stress following testicular torsion/detorsion.

## Acknowledgements

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## Author's Contributions

M.Sh., M.M., S.H.A.-E.; Contributed to conception and design. H.Sh., A.Kh.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. M.Sh., M.M.; Were responsible for overall supervision. M.S.; Drafted the manuscript, which was revised by H.Sh. All authors read and approved the final manuscript.

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Original Article

# Histopathological Effects of Titanium Dioxide Nanoparticles and The Possible Protective Role of N-Acetylcysteine on The Testes of Male Albino Rats

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## Abstract

**Background:** Titanium dioxide ($TiO_2$) is a white pigment which is used in paints, plastics, etc. It is reported that $TiO_2$ induces oxidative stress and DNA damage. N-acetylcysteine (NAC) has been used to fight oxidative stress-induced damage in different tissues. The objective of this study was to evaluate the toxic effects of orally administered $TiO_2$ nanoparticles and the possible protective effect of NAC on the testes of adult male albino rats.

**Materials and Methods:** In this experimental study, 50 adult male albino rats were classified into five groups. Group I was the negative control, group II was treated with gum acacia solution, group III was treated with NAC, group IV was treated with $TiO_2$ nanoparticles, and group V was treated with 100 mg/kg of NAC and 1200 mg/kg $TiO_2$ nanoparticles. Total testosterone, glutathione (GSH), and serum malondialdehyde (MDA) levels were estimated. The testes were subjected to histopathological, electron microscopic examinations, and immunohistochemical detection for tumor necrosis factor (TNF)-α. Cells from the left testis were examined to detect the degree of DNA impairment by using the comet assay.

**Results:** $TiO_2$ nanoparticles induced histopathological and ultrastructure changes in the testes as well as positive TNF-α immunoreaction in the testicular tissue. Moreover, there was an increase in serum MDA while a decrease in testosterone and GSH levels in $TiO_2$ nanoparticles-treated group. $TiO_2$ resulted in DNA damage. Administration of NAC to $TiO_2$-treated rats led to improvement of the previous parameters with modest protective effects against DNA damage.

**Conclusion:** $TiO_2$-induced damage to the testes was mediated by oxidative stress. Notably, administration of NAC protected against $TiO_2$'s damaging effects.

***Keywords:*** N-acetylcysteine, Oxidative Stress, Testis, Titanium Dioxide, Toxicity

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## Introduction

Titanium dioxide ($TiO_2$), also recognized as titanium oxide or titania, is a naturally occurring oxide of titanium which is commonly used as a colouring pigments to provide a white colour in products such as dyes, plastic, paper, and foods (1). $TiO_2$ either alone or in mixtures, is broadly used for a wide range of medical procedures. However, in case of imbalanced biological conditions such as low pH, $TiO_2$ devices can release enormous amounts of particles at both micrometer and nanometer levels (2).

The lethal effects of nanoparticles can be accredited to their small size and hence outsized surface area which results in increased rates of chemical reaction and infiltration into the cells interfering with numerous subcellular physiological mechanisms (3). For instance, studies presented that nanoparticles may infiltrate into nuclei and later may interfere with the organization and functions of DNA (4). Furthermore, $TiO_2$ nanoparticles may produce reactive oxygen species (ROS) resulting in cell toxicity (5). Previous studies reported that $TiO_2$ nanoparticles elicited different antagonistic cellular properties including DNA injury (6).

Previous studies have established that $TiO_2$ nanoparticles may pass into the cells of the reproductive system and induce damage (7). Takeda et al. (8) declared that $TiO_2$ nanoparticles were found in the testes and brain, which indicated that $TiO_2$ nanoparticles may penetrate

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both blood-testis and blood-brain barriers. Komatsu et al. (9) confirmed that $TiO_2$ nanoparticles were absorbed in Leydig's cells leading to disruption of cellular proliferation and dysregulation of the expression of heme oxygenase-1 (HO-1), a steroidogenic regulatory protein, which regulates mitochondrial cholesterol transfer. This implies that long-period exposure to small doses of $TiO_2$ nanoparticles should not be ignored and the possible risks that these particles may impose to reproductive health should be considered, particularly in those who are occupationally exposed to $TiO_2$ nanoparticles.

N-acetylcysteine (NAC) is an antioxidant and free-radical scrounger. It acts as a cysteine contributor and upholds or even upsurges the intracellular levels of glutathione (GSH) (a tripeptide which guards cells against toxins). NAC is characterized by its antioxidant ability through releasing sulfhydryl groups which in turn, reduce ROS levels and possess the ability to reduce oxidative stress directly, suppress the nuclear factor kappa b (NF-κB) inflammation pathway and inflammatory cytokines secretion, and enhance the GSH production (10). NAC was proven to fight oxidative stress-induced injury in several tissues. For instance, administration of NAC suppressed oxidative stress-induced cellular damage in different injury models including head injury, endotoxin-induced lung damage, liver injury, and heart disorders (11). In order to explore the reproductive toxicity of $TiO_2$ nanoparticles, the present study was conducted. The toxic effects of orally administered $TiO_2$ nanoparticles were evaluated in the testes of adult albino rats through histopathological, ultrastructural examinations, immunohistochemical detection of tumor necrosis factor (TNF)-α, measurement of total testosterone and oxidative stress levels, and comet assay. Moreover, the possible protective effects of co-administration of NAC and $TiO_2$ nanoparticles were assessed.

## Materials and Methods

The current experimental study was carried out in Animal Laboratory, Zagazig University. $TiO_2$ nanoparticles (Titanos, China) were nanopowder of 21 nm size with ≥99.5% purity dissolved in gum acacia solution. NAC was purchased from SEDICO, Egypt. Fifty male albino rats weighing 150-200 g were obtained from the Animal House, Faculty of Medicine, Zagazig University. The study was conducted in accordance with the guidelines of the Ethics Committee for Research of Zagazig University. The rats were divided into 5 groups. Group I was the control group that received no treatment. Group II was treated with 1 ml of 5% gum acacia solution (the solvent used for titanium dioxide) by oral gavage once daily for 12 weeks. Group III was orally treated with 100 mg/kg of NAC once daily for 12 weeks. Group IV was orally treated with 1200 mg/kg of $TiO_2$ nanoparticles once daily for 12 weeks. Group V was orally treated with a combination of 100 mg/kg of NAC and 1200 mg/kg of $TiO_2$ nanoparticles once daily for 12 weeks.

For histopathological analysis, the left testis was fixed in Bouin's solution and the tissue was processed and embedded in paraffin blocks for preparation of 5-µm thick sections. Sections were stained with Haematoxylin and Eosin and examined by light microscopy. Ultrastructural examination of the left testis was conducted using the Transmission Electron Microscope (TEM). The analysis was performed according to the method described by Glauret and Lewis (12). The stained sections were examined by TEM in Electron Microscope Center in the department of histology, Faculty of Medicine, Zagazig University. Immunohistochemistry of the left testis was performed using labeled streptavidin-biotin (LSAB) technique. The deparaffinized sections were incubated with hydrogen peroxide to block the endogenous peroxidase. Then, sections were incubated with primary antibodies for TNF-α (rabbit polyclonal TNF-α antibody). Then, sections were incubated with the secondary antibodies and peroxidase-labeled streptavidin. Staining was completed by incubation with substrate chromogen, which resulted in the brown-colored precipitates at the antigen sites.

Blood was collected from the tail vein then it was centrifuged to collect serum. Total testosterone level was measured by enzyme-linked immunosorbent assay (ELISA). Malondialdehyde (MDA) was estimated by the thiobarbituric acid assay. Estimation of reduced GSH level was done using 5, 5'-dithiobis nitro benzoic acid assay. The comet assay was performed according to the method of Singh et al. (13) to evaluate the in vivo genotoxic potential of $TiO_2$ nanoparticles in rats using the single-cell gel electrophoresis. Cells fixed in agarose were lysed to form nucleoids containing the DNA material. Electrophoresis at high pH results in comets which were detected by fluorescence microscopy. Based on the integrity of the comet tail and the head, we determined the number of DNA breaks.

### Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences software (SPSS version 22.0, IBM, USA). Differences between multiple means (quantitative variables) were evaluated by one way ANOVA test, followed by LSD. A $P<0.05$ was considered statistically significant.

## Results

Macroscopic examinations of the left testis in terms of color, testis to body weight ratio, and infarction of treated groups revealed no significant changes compared to the control group. Histopathological examination of groups I, II, and III showed the same histological features without any abnormal histopathological finding such as dark nuclei, hyaline fluids, and blood extravasation into the interstitial spaces (Fig.1A, B). Meanwhile, histological examination of group IV showed disorganized seminiferous tubules, spermatogenic cells with dark pyknotic nuclei, separation of basement membranes, hyaline fluids, vacuolation, and extravasation of blood in the interstitial tissue. Moreover, some tubules showed thin layers of spermatogonia and sperms (Fig.1C). In Group V, there was a minimal separation of basement membranes with hyaline exudates in the interstitium (Fig.1D).

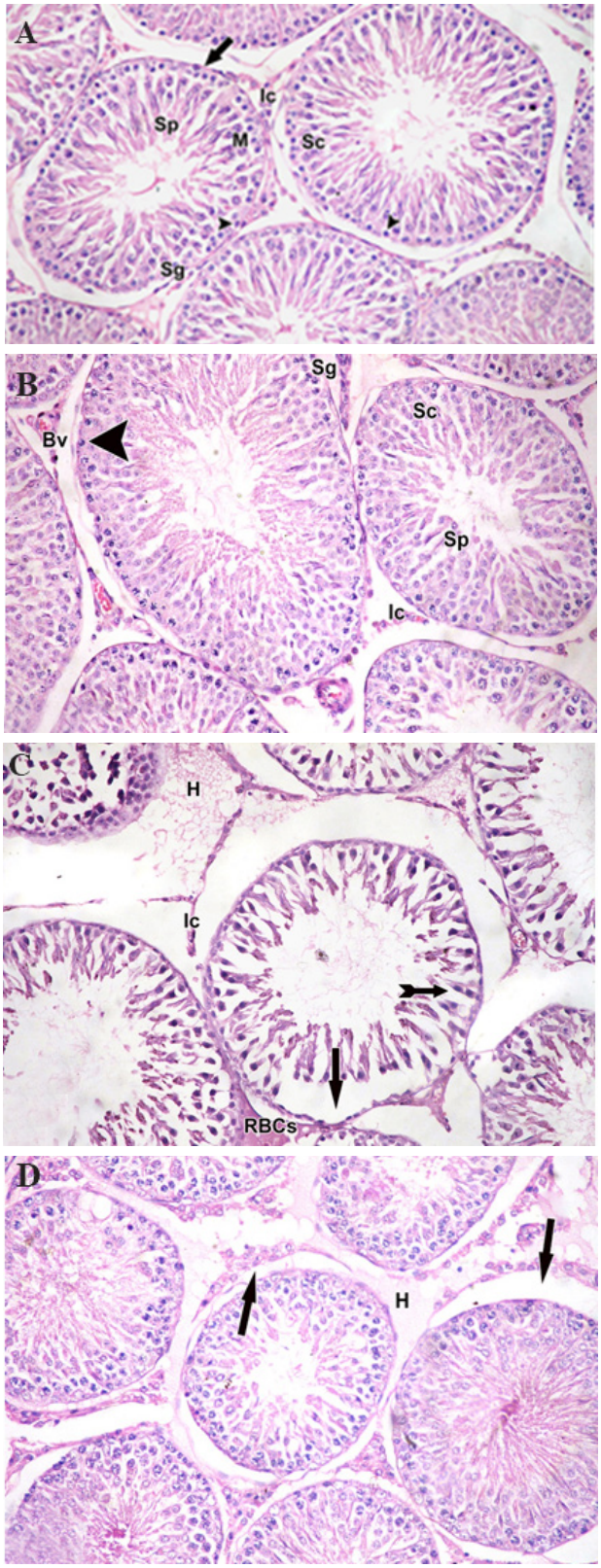


**Fig.1:** Photomicrograph showing histology of seminiferous tubules. **A.** Group I, II, and **B.** Group III showing normal seminiferous tubules lined by spermatogonia (Sg) close to the basal membrane (arrow), spermatogenic cells (Sc) with many mitotic figures (M) and Sertoli cells (arrow head). Seminiferous tubules lumen containing spermatid (Sp) with normal interstitial tissue (Ic) in between, **C.** Group IV showing marked disorganization, spermatogenic cells with dark pyknotic nuclei (tailed arrow), interstitial cells (Ic), basement membrane separation in many areas (arrow), extensive area between seminiferous tubules, hyaline exudate (H), and extravasation of blood (RBCs) in the interstitium, and **D.** Group V showing: separation of basement membrane of seminiferous tubules (arrow) and hyaline exudate (H) in the interstitium (H&E: ×200).

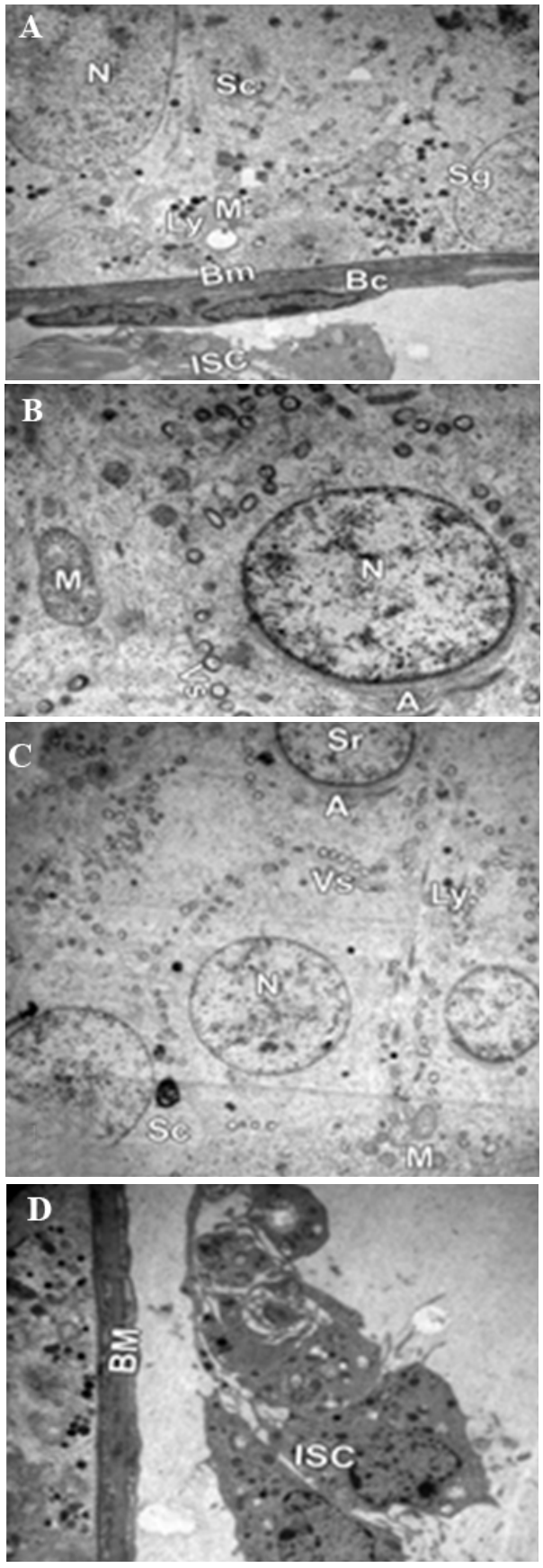


**Fig.2:** Photomicrograph showing electron microscopy of a seminiferous tubule. **A, B.** Group I, II ×4000, ×8000, **C,** and **D.** group III ×4000, showing spermatogenic cells (Sc) with its euchromatic nucleus (N). The cytoplasm contains mitochondria (M) and lysosomes (Ly); spermatogonia (Sg) resting on the basement membrane (Bm) with adjacent blood capillary (Bc) and interstitial cell (ISC); and a spermatid (Sr) with its acrosomal cap (A) and numerous vesicles (Vs).

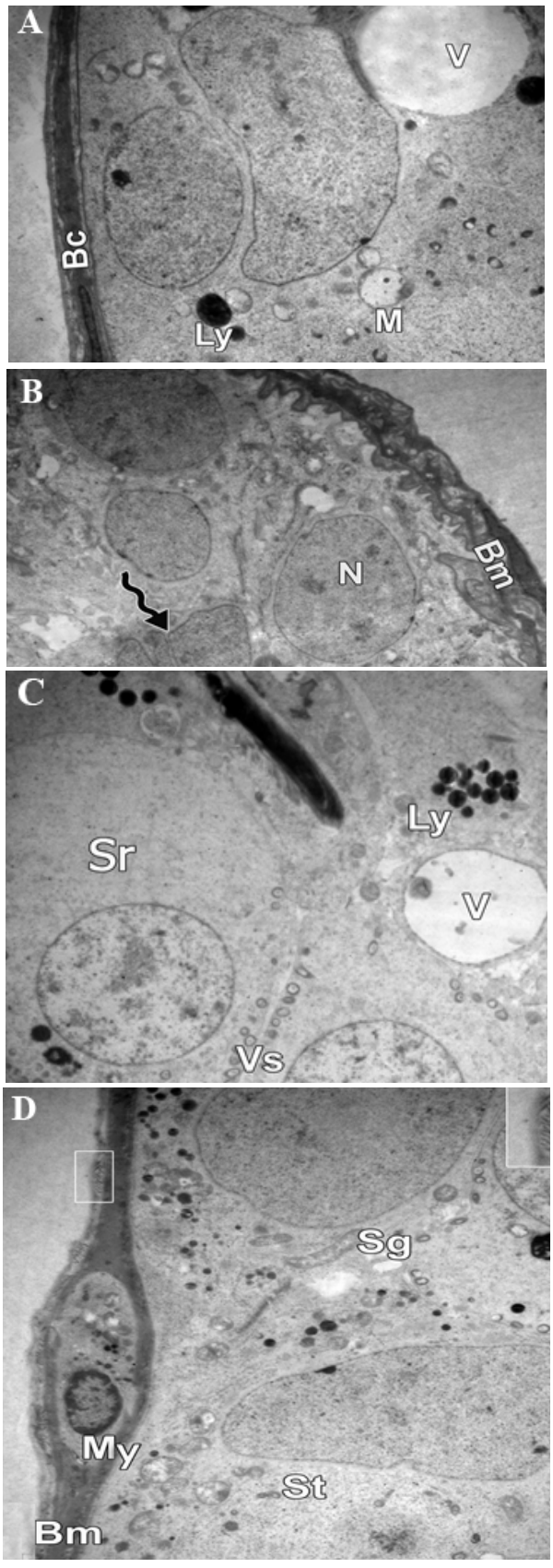


**Fig.3:** Photomicrograph showing electron microscopy of a seminiferous tubule. **A, B.** Group IV ×4000 showing irregular and thickened basement membrane (Bm), multi-folded euchromatic nuclei (N) with marked indentation (waved arrow), marked cytoplasmic vacuolation, mitochondria (M), lysosomes (Ly) and blood capillary (Bc), **C,** and **D.** Group V ×4000 showing vacuolation (V), spermatogonia (Sg) early spermatids (Sr) with their euchromatic nuclei and numerous vesicles (Vs), flattened myoid cell (My) and collagen fibers deposition (square) in the basement membrane (Bm).

Using TEM, groups I, II, and II showed normal ultrastructures including normal seminiferous tubules lined with spermatogonia close to the basal membrane, spermatogenic cells with many mitotic figures and sertoli cells (Fig.2A-D). Group IV revealed signs of inflammatory damage in the form of thickened irregular wavy basement membrane with collagen fiber deposition, many abnormal multi-folded euchromatic nuclei with marked indentation, marked cytoplasmic vacuolation, and swollen mitochondria (Fig.3A, B). Cytoplasmic vacuolations with mild deposition of collagen fibers in the basement membrane were observed in spermatid and spermatogonia cells in group V (Fig.3C, D). Groups I, II, and III showed a relatively low TNF-α immunoreactivity (Fig.4A, B). On the other hand, a strong positive TNF-α immunoreaction was detected in group IV (Fig.4C) compared to group V which showed weaker immunoreaction (Fig.4D).

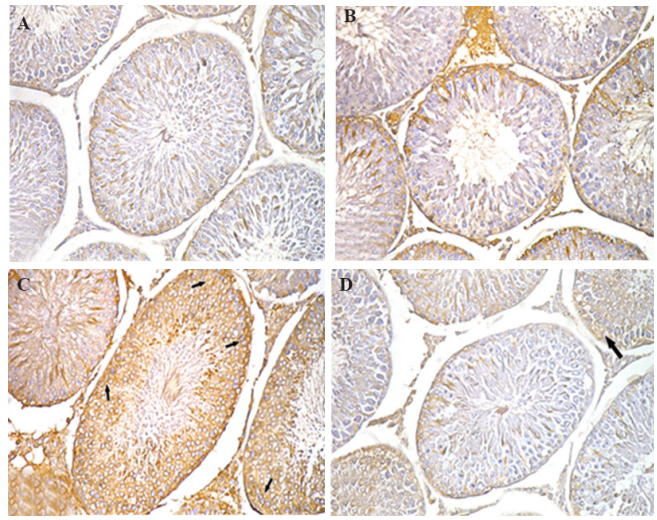


**Fig.4:** Photomicrograph showing immunohistochemistry of seminiferous tubules. **A.** Group I, II, **B.** group III showing weak TNF-α immuno-reactivity, **C.** Group IV showing strong positive TNF-α immunoreaction (arrow), and **D.** Group V showing decreased TNF-α immuno-reactivity (×200).

There was an insignificant difference in total testosterone level among groups I, II, and III. However, the total testosterone level of group IV displayed a significant decrease compared to groups I, II, and III. Total testosterone level was significantly decreased in group V compared to groups I, II, and III. Meanwhile, there was a significant increase in total testosterone level of group V compared to group IV (Table 1). GSH values were insignificantly different among groups I, II, and III. However, there was a significant decrease in GSH level of group IV when compared with groups I, II, and III. Also, there was a significant increase in GSH level of group V compared to groups I, II, and III. Furthermore, there was a significant increase in GSH level of group V compared to group IV (Table 1). Additionally, there was a significant increase in MDA level of group IV compared to groups I, II, and III in addition to a significant increase in MDA level of group V compared to groups I, II, and III. On the other hand, there was a significant decrease ($P<0.05$) in MDA level of group V compared to group IV (Table 1).

**Table 1:** Total testosterone, serum reduced glutathione (GSH), and serum malondialdehyde (MDA)

| Biochemical parameters | Group I | Group II | group III | Group IV | Group V |
|---|---|---|---|---|---|
| Total testosterone (nmol/L) | $23.8 \pm 8.8^{bc}$ | $21.2 \pm 8.6^{bc}$ | $26.2 \pm 8.8^{bc}$ | $0.38 \pm 0.0^{ac}$ | $15.4 \pm 6.0^{ab}$ |
| GSH (nmol/L) | $50.2 \pm 4.0^{bc}$ | $49.1 \pm 3.9^{bc}$ | $52.8 \pm 4.7^{bc}$ | $31.8 \pm 5.7^{ac}$ | $61.9 \pm 3.5^{ab}$ |
| MDA (nmol/L) | $74.9 \pm 3.5^{bc}$ | $75.0 \pm 3.9^{bc}$ | $73.9 \pm 3.4^{bc}$ | $136.3 \pm 21^{ac}$ | $85.7 \pm 6.6^{ab}$ |

**Values are referred as mean ± SD. P< 0.05 was considered statistically significant.**
**[a]; significance with group I ,II and III, [b]; significance with group IV, and [c]; Significance with the group V.**

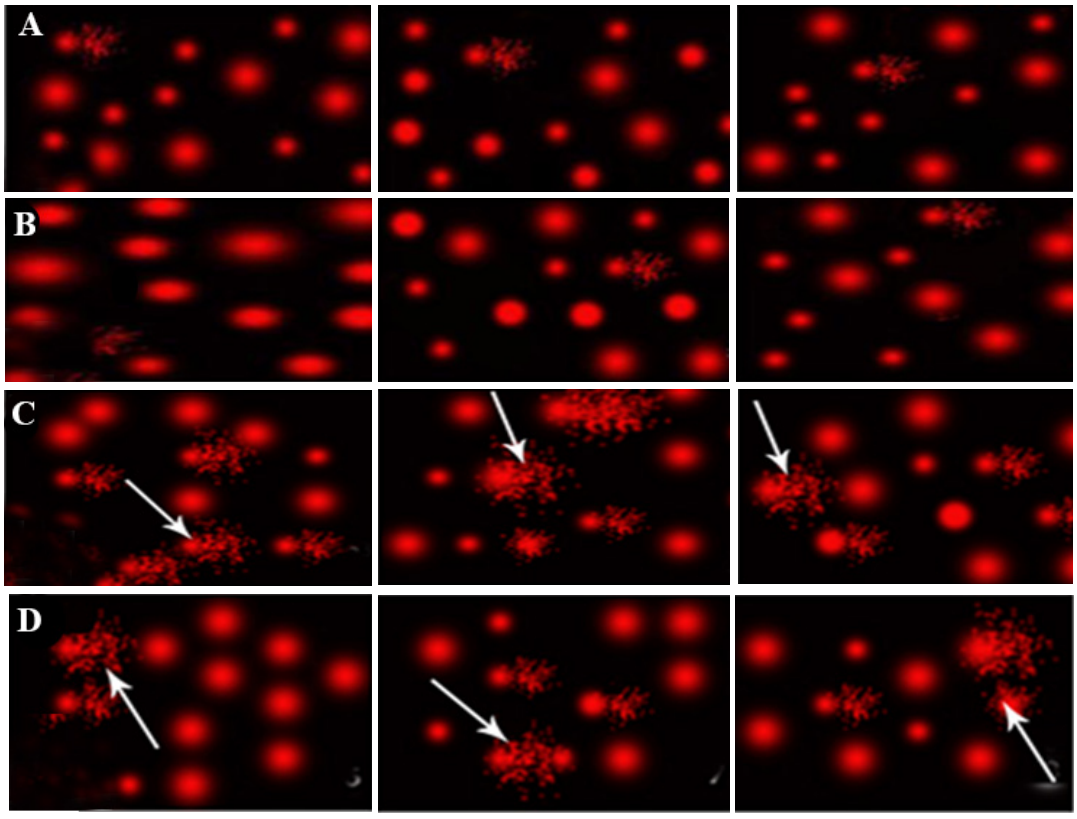


**Fig.5:** Photomicrograph of comet test showing nuclei of testes cells. **A.** Group I, II, **B.** group III showing almost normal condensed type nuclei and undamaged cells, **C.** Group IV showing abnormal tailed nuclei and damaged cells (white arrow), and **D.** Group V showing less number of abnormal tailed nuclei and damaged cells.

According to comet assay results, cells nuclei of group IV showed a significant increase in percentage (%) of tailed nuclei, tail length, tail DNA% and unit tail moment compared to groups I, II, and III. Co-administration of NAC and $TiO_2$ nanoparticles caused a significant decrease in DNA damage parameters in group V compared to group IV. However, there was a significant increase ($P<0.05$) in % of tailed nuclei, tail length, tail DNA % and unit tail moment of group V compared to groups I, II, and III (Fig.5).

## Discussion

Prevalent applications of nanomaterial cause a huge potential for human exposure to these compounds. However, many experts and establishments have upstretched the environmental and toxicological concerns regarding nanotechnology (14). $TiO_2$ nanoparticles have the ability to drift through diverse paths and accumulate in body tissues, which may lead to inflammation and apoptosis, resulting in various organ damages. The present study showed that $TiO_2$ nanoparticles induced several histopathological alterations in the testes compared to the control group. Administration of NAC along with $TiO_2$ nanoparticles showed improvements with minimal alterations in seminiferous tubules. Gao et al. (7) showed that nanoparticles-induced testicular injury and inhibition of spermatogenesis may attribute to changes in male sex hormone levels and testicular gene expression.

Our results are supported by data reported by Takeda et al. (8) which showed that exposure to $TiO_2$ nanoparticles resulted in disrupted seminiferous tubules and tubule lumens with few mature sperms. Moreover, They observed aggregates of nanoparticles in Leydig's cells, sertoli cells, and spermatids. Guo et al. (15) demonstrated a reduction in sperm density and motility in mice injected with $TiO_2$ nanoparticles. On the other hand, Wang et al. (16) reported no abnormal pathological changes in the testes following acute toxicity induced by nano-sized $TiO_2$ particles. The inconsistencies among these studies may be due to the differ-

ences in experimental conditions such as the animal model as well as administered dose, exposure duration, and the physicochemical characteristics of $TiO_2$.

In the present study, examination of the testis sections of $TiO_2$ nanoparticles-treated group revealed signs of inflammatory damage in the testicular tissue. For instance, administration of NAC along with $TiO_2$ nanoparticles showed partial improvement in testicular tissue, which was found by histological and immunohistochemical examination. However, cytoplasmic vacuolation was still observed with mild collagen fibers deposition in the basement membrane. From an ultrastructural point of view, variable sized intercellular spaces were observed. El Ghazzawy et al. (17) stated that intercellular spaces represented advanced degenerative alterations damaging the cell membrane integrity as a result of oxidative stress. ROS induce oxidative phosphorylation of cell membranes resulting in interruption of the integrity of the intercellular junctional complex. These results are in accordance with those reported by Fouad et al. (18) which were obtained based on electron microscopy investigation of testicular tissues exposed to ROS and inflammatory cytokines measurements.

Co-administration of NAC and $TiO_2$ nanoparticles led to a reduction in TNF-α immunoreactivity. Our results are in accordance with those indicated by Park et al. (19). Furthermore, there was a significant increase in testosterone level of the NAC+$TiO_2$-treated group. A previous study found that sex hormone balance in the male reproductive system was disrupted by $TiO_2$ nanoparticles exposure as the amount of testosterone was greatly reduced which led to suppressed spermatogenesis (7). Furthermore, EL-Sharkawy et al. (20) detected lowered testosterone levels in rats administered with $TiO_2$; authors stated that reduction in testosterone secretion may be due to the high level of NO, which led to hypospermatogenesis, testicular inflammation, and disturbance of gonadotropin-releasing hormone secretion.

Co-administration of NAC and $TiO_2$ resulted in a significant increase in GSH. These results showed a time-dependent reduction in GSH level in $TiO_2$ nanoparticles-treated rats. Similar findings were reported by Long et al. (21) who observed GSH exhaustion and an upsurge in the lipid peroxidation levels after exposure to $TiO_2$ nanoparticles. ROS generation was suggested as a probable mechanism involved in the toxicity of nanoparticles (5). Jeon et al. (22) speculated that a part of the ROS generation may be due to the catalytic properties of nanosized-$TiO_2$. GSH level was remarkably decreased in the $TiO_2$-treated group. However, it is worthy to say that GSH level was higher in NAC and $TiO_2$ treated-group compared to NAC-treated group which was supposed to be decreased by $TiO_2$. This may be explained by the ability of NAC to induce antioxidant effects in injury models rather than normal models (11, 23).

There was a significant rise in MDA level in $TiO_2$ nanoparticles-treated group compared to the group treated with NAC+$TiO_2$. However, MDA levels in NAC+$TiO_2$ nanoparticles-treated group were significantly higher than those of the control group. Significant changes in MDA levels suggest that induction of pathological lesions is probably mediated through the oxidative stress enhanced by the dumped nanoparticles. These results were consistent with those reported by Attia et al. that showed a time-dependent significant release of oxidative stress in the liver as evident by increased MDA and reduced GSH levels (24). Furthermore, Gurr et al. (5) revealed an exponential increase in the MDA production caused by $TiO_2$, and they attributed this increase in lipid peroxidation to excessive ROS generation.

The comet assay is a broadly used assay for investigation of DNA damage and repair, genotoxic properties of chemicals and pharmaceuticals, environmental biomonitoring, and also human monitoring. However, comet assay has been used for determination of the toxicity of highly reactive nanoparticles and several studies used it to test the potential toxicity of manufactured nanoparticles by assessing DNA strand breaks or oxidative DNA lesions (25). In the present study, results of *in vivo* comet assay showed that oral administration of $TiO_2$ resulted in an increase in DNA damage in the testes. These results are in accordance with those noted by Shukla et al. (26) indicating that $TiO_2$ nanoparticles generate ROS and cause DNA damage and genotoxicity in mammalian cells. The direct association between ROS generation and oxidative DNA damage further proposes that oxidative stress can act as a significant path through which, $TiO_2$ nanoparticles cause DNA damage. Previous studies showed that $TiO_2$ nanoparticles caused DNA injury indirectly through inflammation (27) and generation of ROS (5).

Furthermore, $TiO_2$ nanoparticles in aqueous suspension release free radicals which can result in DNA damage by oxidation, nitration, methylation or deamination reactions (28). Since $TiO_2$ nanoparticles prompt inflammatory reactions and DNA injury, it was suggested that $TiO_2$ nanoparticles act an indirect genotoxicity inducer as suggested by Dankovic et al. (29). Previous studies reported DNA damage caused by $TiO_2$ nanoparticles using *in vitro* (5, 16) and *in vivo* comet assays (30). On the other hand, negative results were reported concerning $TiO_2$ nanoparticles-induced DNA damage in studies using *in vitro* experiments (31) and *in vivo* comet assays (32). Tao and Kobzik (33) suggested that discrepancies among studies may be due to irregular $TiO_2$ release, particle size, the extent of accumulation, and incubation circumstances, suggesting that additional studies should be done to determine the situations in which $TiO_2$ nanoparticles genotoxicity arises.

NAC acts as an antioxidant through expanding the synthesis of endogenous GSH which is frequently exhausted as a result of augmented oxidative stress (23). Additionally, NAC performs as a direct scavenger of free radicals (34). Together, these antioxidant activities of NAC can attribute to guard against oxidative stresses. These results are consistent with those mentioned in El-Kirdasy et al. (35) study. The protective effects of NAC on testicular damage and dysfunction, were also demonstrated by oth-

er studies (36). NAC has been shown to have significant effects on testicular dysfunction. Consistent with the decrease in TNF-α immunoreactivity in the current study, Dick et al. (37) reported that NAC pretreatment stops TNF-α production in alveolar macrophages treated with nickel particles. Attia et al. (24) stated that co-treatment with NAC and $TiO_2$ restored MDA and liver cells GSH levels. Furthermore, Xue et al. (10) detailed that NAC powerfully repressed ROS production in $TiO_2$-treated cells and blocked nano-$TiO_2$ induced lipid peroxidation, and apoptosis. The diminished level of DNA damage in nuclei of the testes following treatment with NAC was in accordance with results reported by Shi et al. (38) which showed that NAC administration suppressed the level of $TiO_2$ nanoparticles-induced DNA injury in human lymphocytes. The suppressive effect of NAC on ROS formation in cells exposed to $TiO_2$ was also noted by Xue et al. (10). Moreover, NAC showed significant effects on the volume and motility of semen by increasing the antioxidant level and reducing peroxide and oxidative stress index when compared to the control group, in a clinical trial. This was explained by NAC ability to diminish ROS and reduce the viscosity of the semen (39).

## Conclusion

Oral administration of $TiO_2$ nanoparticles induced toxic effects and DNA damage in the testes and these adverse effects may be attributed to induction of oxidative stress. Administration of NAC along with $TiO_2$ nanoparticles, protected against $TiO_2$ damaging effect.

## Acknowledgements

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## Author's Contributions

A.M.B.E.; Designed experiments, analyzed data and co-wrote the paper. A.I.; Performed experiments, analyzed data and co-wrote the paper. A.M.S.; Analysed data and co-wrote the paper. All authors read and approved the final manuscript.

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Original Article

# Anti-Oxidative and Anti-Apoptotic Effects of Apigenin on Number of Viable and Apoptotic Blastomeres, Zona Pellucida Thickness and Hatching Rate of Mouse Embryos

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## Abstract

**Background:** Apigenin is a plant-derived compound belonging to the flavonoids category and bears protective effects on different cells. The aim of this study was to evaluate the effect of apigenin on the number of viable and apoptotic blastomeres, the zona pellucida (ZP) thickness and hatching rate of pre-implantation mouse embryos exposed to $H_2O_2$ and actinomycin D.

**Materials and Methods:** In this experimental study, 420 two-cell embryos were randomly divided into six groups: i. Control, ii. Apigenin, iii. $H_2O_2$, iv. Apigenin+$H_2O_2$, v. Actinomycin D, and vi. Apigenin+Actinomycin D. The percentage of blastocysts and hatched blastocysts was calculated. Blastocyst ZP thickness was also measured. In addition, viable blastomeres quantity was counted by Hoechst and propidium iodide staining and the number of apoptotic blastomeres was counted by TUNEL assay.

**Results:** The results of viable and apoptotic blastomeres quantity, the ZP thickness, and the percentage of blastocysts and hatched blastocysts were significantly more favorable in the apigenin group, rather than the control group ($P<0.05$). The results of the apigenin+$H_2O_2$ group were significantly more favorable than the $H_2O_2$ group ($P<0.05$); and the results of apigenin+actinomycin D group were significantly more favorable than actinomycin D group ($P<0.05$).

**Conclusion:** The results suggest that apigenin may protect mouse embryos against $H_2O_2$ and actinomycin D. So that it increases the number of viable blastomeres and decreases the number of apoptotic blastomeres, which may cause expanding the blastocysts, thinning of the ZP thickness and increasing the rate of hatching in mouse embryos.

***Keywords:*** Apigenin, Apoptosis, Blastomeres, Embryonic Development, Zona Pellucida

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## Introduction

Embryonic development in culture medium may be affected by several stressors such as high oxygen concentration and high level of reactive oxygen species (ROS) (1). It is important that embryos in culture media to be protected from oxidative stress. For this purpose, antioxidants are valuable candidates (2).

Apigenin is a plant-derived compound belonging to the flavonoids category presented in various fruits and vegetables, such as parsley, onion, celery, chamomile and orange (3). Apigenin has different biological activities such as anti-oxidative, anti-inflammatory, anti-cancer and anti-tumorigenic properties (4, 5). Apigenin protects DNA from oxidative stress by binding to nucleic acids. Moreover, apigenin prevents cell apoptosis by suppressing ROS compounds (6) and decreasing the expression of caspase-3, caspase-9 and TNF-α (7).

By studying the embryo morphology, prediction of embryo fate is largely possible. The most morphological indicators to select the best embryos for transferring are zona pellucida (ZP) thickness and blastomere quantity (8). ZP thickness is a reliable indicator of *in vitro* fertilization (IVF) success rate which can be applied as a criterion for embryo selection. Actually, ZP thickness is inversely correlated with embryo viability and hatching rate (9). Moreover, cleavage rate and development to blastocyst are applied as two quality parameters of mammal embryos (8).

Although the beneficial effects of apigenin on different cells and tissues have been investigated (10, 11), there is no report yet concerning the effect of apigenin on growth and quality of embryos. So, in this study, we evaluated for the

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first time the impact of apigenin on some morphological indicators of pre-implantation mouse embryos including ZP thickness, viable and apoptotic blastomere quantity and hatching rate. To evaluate the anti-oxidant and anti-apoptotic effects of apigenin, we used $H_2O_2$ and actinomycin D in the culture medium to create ROS and apoptosis.

## Materials and Methods

In this experimental study, female C57BL/6 mice (6-8 weeks) were kept under controlled temperature (25 ± 2˚C) and light (12 hours light/12 hours dark), with free access to food and water. All animal protocols were approved by the Research Council of Semnan University of Medical Sciences (Semnan, Iran).

### Superovulation and embryo collection

For superovulation, the mice received 10 IU pregnant mare's serum gonadotropin (PMSG, Sigma, China) intraperitoneally. 48 hours later, they received 10 IU human chorionic gonadotropin (hCG, Sigma, China) intraperitoneally (12). They were subsequently mated overnight with males and the mating was assessed by the presence of vaginal plug on the morning after hCG injection. Two-cell embryos were flushed from the oviduct at about 48 hours after hCG injection and washed in human tubal fluid (HTF) medium containing HEPES (Sigma, USA). A total of 420 two-cell embryos were used in this study.

### Embryo culture

The embryos were transferred into the HTF medium, supplemented with 10% human serum albumin (Sigma, USA). Two-cell embryos were randomly divided into six groups (70 embryos in each group): i. Control group, without any treatment, ii. Apigenin (Sigma, China) group, 10 µM apigenin was added into the medium, iii. $H_2O_2$ group, 500 µM $H_2O_2$ was added into the medium, iv. Apigenin+$H_2O_2$ group, 10 µM apigenin and 500 µM $H_2O_2$ were added into the medium, v. Actinomycin D (Sigma, USA) group, 0.005 µg/ml actinomycin D was added into the medium, vi. Apigenin+actinomycin D group, 10 µM apigenin and 0.005 µg/ml actinomycin D were added into the medium. In all groups, 10 embryos were placed in a drop (20 µl) of HTF medium under mineral oil (Sigma, USA) in a 35 mm Petri dish (Jet Biofil, Canada). Next, they were incubated at 37˚C with 95% humidity and 5% $CO_2$. To evaluate the antioxidant effect of apigenin, two- to four-cell embryos were exposed to 500 µM $H_2O_2$ in the culture medium for 72 hours. To evaluate the anti-apoptotic effect of apigenin, as soon as reaching two-cell embryos to eight-cell stage, they were incubated with 0.005 µg/ml actinomycin D in the medium for 4 hours (13). Eventually, on the fourth and fifth days of embryonic period, the percentage of embryos reaching the stages of blastocyst and hatched blastocyst was assessed (14).

### Measurement of zona pellucida thickness

To measure ZP thickness, the blastocysts were randomly selected. Measurement was taken from the images using an inverted microscope (Nikon, Eclipse Ti-U, Japan) and motic images plus 2.0 software. The thickness of each ZP was measured at three points (8, 15).

### Differential staining and TUNEL assay

The blastocysts were randomly selected for blastomere counting analysis. Differential staining of blastocysts and apoptotic nuclei detection were performed according to the method described by Fouladi-Nashta et al. (16, 17). The blastocysts were treated with 30 µg/ml propidium iodide (PI, Sigma, China) and 1% Triton X-100 (Sigma, China) at 37˚C for 5 minutes. Immediately after, the blastocysts were washed twice and fixed in 4% paraformaldehyde containing 10 µg/ml bisbenzimide (Hoechst 33342, Sigma, USA) for 20 minutes at room temperature leading to fixation of blastocysts and staining total cell nuclei. Next, embryos were washed and incubated in droplets of in situ cell death detection (TUNEL) kit solution (Roche, Germany) for 45 minutes according to the manufacturer's instructions. Then the embryos were mounted on glass slides in glycerol droplets and were observed under a fluorescent microscope (Motic, AE31, Spain). Trophectoderm (TE) nuclei labeled with PI were appeared red, total cells including inner cell mass (ICM) labeled with Hoechst were appeared blue and apoptotic cells labeled with TUNEL were appeared green. The number of ICM, TE, and apoptotic cells was counted.

### Statistical analysis

Statistical analysis was performed using SPSS software version 16.0 software (version 16.0 for windows, Chicago, IL, USA). Comparison of the percentage of embryos from two-cell to hatched blastocyst was analyzed by $x^2$ test. The results of embryo percentage in apigenin group were compared to control group, the results of apigenin+$H_2O_2$ group were compared to $H_2O_2$ group, and the results of apigenin+actinomycin D group were compared to actinomycin D group. The results of ZP thickness and number of viable and apoptotic blastomeres were analyzed by one-way ANOVA followed by the Tukey test. The results are presented as mean ± SEM. $P<0.05$ is considered statistically significant.

## Results

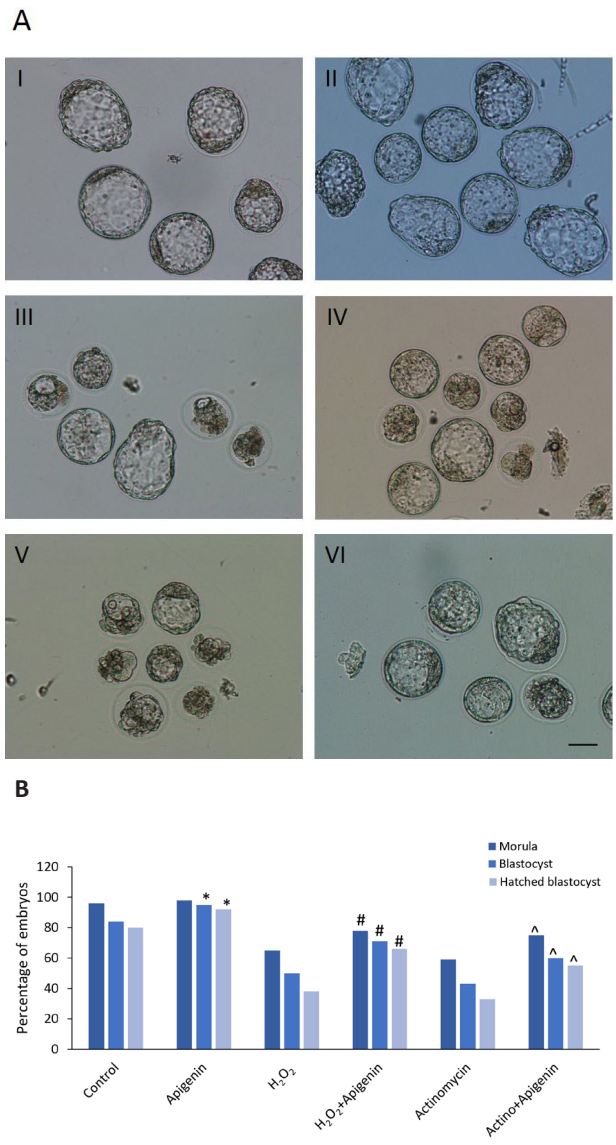
### Developmental rate of embryos

There was no statistically significant difference in the percentage of two-cell embryo development to eight-cell between the control and apigenin groups (P=0.404). There was no statistically significant difference between apigenin+$H_2O_2$ group and $H_2O_2$ (P=0.382). In addition, no statistically significant difference was determined between the apigenin+actinomycin D group and the ac-

tinomycin D (P=0.466, Table 1). Percentage of embryos reached to morula stage in the apigenin+$H_2O_2$ group was significantly higher than the $H_2O_2$ group (P=0.037), and in the apigenin+actinomycin D group was significantly higher than the actinomycin D group (P=0.016). There was no statistically significant difference in morula stage between the apigenin and control groups (P=0.087). Percentage of embryos reached to blastocyst and hatched blastocyst stages in the apigenin group was significantly higher than the control group (P=0.022). Additionally, this was significantly higher in the apigenin+$H_2O_2$ compared to the $H_2O_2$ group (P<0.001), and in apigenin+actinomycin D group compared to actinomycin D group (P<0.001, Fig.1A, B).

## The zona pellucida thickness of blastocysts

The results showed that ZP thickness of blastocysts in the apigenin group was significantly thinner than the control group (P=0.034). It was significantly thinner in the apigenin+$H_2O_2$ group compared to the $H_2O_2$ group (P=0.023), and in the apigenin+actinomycin D group rather than the actinomycin D group (P=0.003, Fig.1A, C).



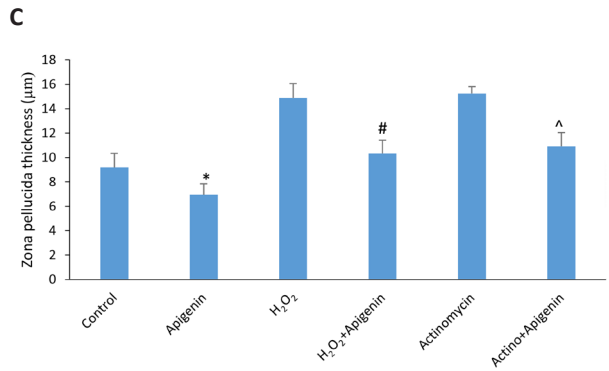




**Fig.1:** Apigenin protected the embryos against $H_2O_2$ and actinomycin D. **A.** The blastocysts of I. Control group, II. Apigenin group, III. $H_2O_2$ group, IV.Apigenin+$H_2O_2$ group, V. Actinomycin D group, VI. Apigenin+actinomycin D group, **B.** The results of the percentage of embryos that have reached to the stages of morula, blastocyst and hatched blastocyst, and **C.** The results of zona pellucida thickness of blastocysts (scale bar: 50 µm). Values are presented as mean ± SEM. *; P<0.05 apigenin versus the control group, #; P<0.05 apigenin+$H_2O_2$ versus the $H_2O_2$ group, and ^; P<0.05 apigenin+actinomycin D versus the actinomycin D group.

## Viable blastomeres quantity

The blastocysts were stained with Hoechst and PI followed by quantifying ICM and TE (Fig.2). The results showed the number of ICM and TE in the apigenin group was significantly higher than the control group (P=0.037). In addition, it was significantly higher in the apigenin+$H_2O_2$ group compared to the $H_2O_2$ group (P<0.001) and in the apigenin+actinomycin D group rather than the actinomycin D group (P<0.001, Fig.3).

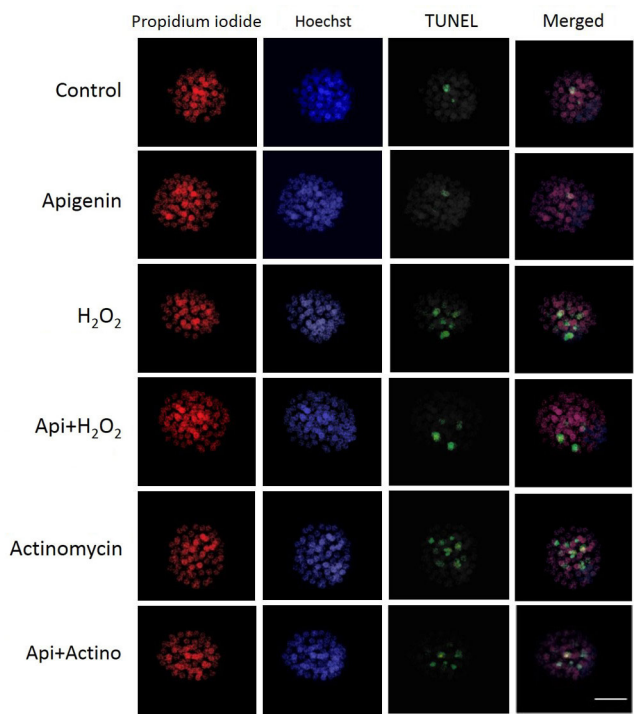



**Fig.2:** Differential staining and TUNEL labeling of the blastomeres. Staining with propidium iodide for trophectoderm cells (red), Hoechst for total cells (blue), and TUNEL for apoptotic cells (green) (scale bar: 50 µm).

## Apoptotic blastomeres quantity

The apoptotic blastomeres were detected by TUNEL

assay (Fig.2). The results showed that apoptotic blastomeres quantity in the apigenin group was significantly lower than the control group (P=0.011). Similarly this number was significantly lower in the apigenin+$H_2O_2$ group compared to the $H_2O_2$ group (P=0.003), and in the apigenin+actinomycin D group rather than actinomycin D group (P<0.001, Fig.3).

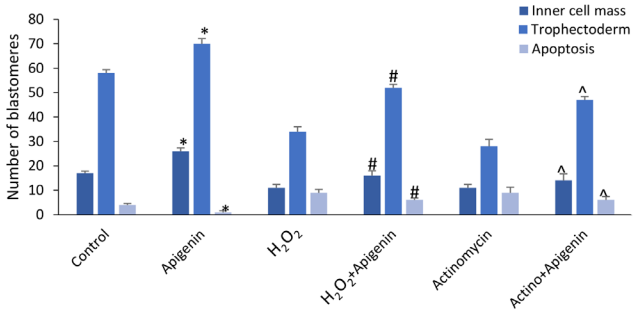



**Fig.3:** The results of viable blastomeres with propidium iodide and Hoechst staining and the apoptotic blastomeres with TUNEL assay. Values are presented as mean ± SEM. *; P<0.05 apigenin versus the control group, #; P<0.05 apigenin+$H_2O_2$ versus the $H_2O_2$ group, and ^; P<0.05 apigenin+actinomycin D versus the actinomycin D group.

**Table 1:** The results of number and percentage of two-cell embryos to eight-cell embryos in all groups

| Group | 2-Cell (%) | 4-Cell (%) | 8-Cell (%) |
|---|---|---|---|
| Control | 70 (100) | 68 (97.1) | 66 (94.2) |
| Apigenin | 70 (100) | 69 (98.6) | 68 (97.1) |
| $H_2O_2$ | 70 (100) | 66 (94.2) | 62 (88.5) |
| Apigenin+$H_2O_2$ | 70 (100) | 68 (97.1) | 65 (92.8) |
| Actinomycin D | 70 (100) | 67 (95.7) | 65 (92.8) |
| Apigenin+Actinomycin D | 70 (100) | 68 (97.1) | 67 (95.7) |

## Discussion

During development of embryos in vitro, various harmful factors can affect embryo quality and fertilization, although it seems that anti-oxidants can reduce the amount of damage (2, 18). In this study, for the first time, we evaluated the effect of apigenin on some morphological indicators of pre-implantation mouse embryos including ZP thickness, number of viable and apoptotic blastomeres and hatching rate. Moreover, to evaluate the anti-oxidant and anti-apoptotic effects of apigenin, we used $H_2O_2$ and actinomycin D in the culture medium to induce oxidative stress and apoptosis. Overall, the results showed that 10 µM apigenin, by protecting the embryos against $H_2O_2$ and actinomycin D, was able to enhance the quality and development of embryos and reduce apoptosis in the blastomeres.

Anti-oxidant capacity of apigenin has been shown in different cell types. So that Zhang et al. (19) reported that apigenin has neuro-protective effect on rats after contusive spinal cord injury. Zhao et al. (20) reported that apigenin has neuro-protective, anti-amyloidogenic and neuro-trophic effects on an Alzheimer disease mouse model. Liu et al. (21) reported that apigenin expresses Oct-4, Sox2, and c-Myc in dental pulp cells which helps maintain the dental pulp cells in an undifferentiated stage. However, no report concerns the effect of apigenin on growth and quality of embryos.

In the present study, to evaluate the anti-oxidant effect of apigenin, $H_2O_2$ was used, which similar to ROS easily penetrates from the cell membrane, causing damage and apoptosis (22). Sharma et al. (6) reported that apigenin attaches to nucleic acid bases and decreases oxidative DNA damage in epithelial cells of prostate. Lagoa et al. (23) showed that flavonoids including apigenin inhibit $H_2O_2$ production by increasing mitochondrial activity. The purpose of exposing embryos to $H_2O_2$ was to exacerbate the conditions of ROS in the culture medium (24) and evaluate the anti-oxidant effect of apigenin on protection of the embryos. The results of present study showed that apigenin by reducing the effects of $H_2O_2$ could protect the embryos and improve embryonic development. These results were in agreement with the other related study (25).

Moreover, to evaluate the anti-apoptotic effect of apigenin, actinomycin D was used as an inducer of apoptosis on different cell types by connecting to guanine-cytosine base pairs and inhibiting DNA transcription (26). Niknafs et al. (27) showed that melatonin improved development of the early mouse embryos exposed to actinomycin D. Abdelrazik et al. (13) reported that l-carnitine reduces apoptosis rate in blastomeres of mouse embryos exposed to actinomycin D. The results of present study showed that apigenin could protect the embryos exposed to actinomycin D and decrease the rate of apoptosis. These results were in agreement with other related studies (13, 27, 28).

Embryo quality is evaluated with morphological parameters. Viable and apoptotic blastomeres quantity, ZP thickness and ability to hatch of blastocyst are some of the most important morphological parameters of embryo (29, 30). Various studies have reported that reducing number of blastomeres could decrease chance of survival of embryos (31, 32). While the number of blastomeres increase, ZP thickness is decreased; in contrast the probability of blastocyst hatching and successful implantation are increased (15, 33).

Regarding the anti-oxidant and anti-apoptotic properties of apigenin, protective effect of this agent on improvement embryo growth is probably due to reduction of the ROS level (34), maintaining the mitochondrial activity (11, 35) and upregulating the gene expression of anti-oxidant enzymes like glutathione peroxidase (25). Glutathione peroxidase is an enzymatic anti-oxidant expressing in many cells and tissues during embryo formation and protecting the embryo against oxidative stress (18, 36). Since the glutathione peroxidase removes $H_2O_2$, apoptosis in embryonic cells reduces (18). Han et al. (37) reported that apigenin reduces oxidative stress and neuronal apoptosis in early brain injury following subarachnoid hemorrhage.

ZP thickness is a marker to select the best frozen-thawed embryos for transfer (38), because thin ZP increases the

probability of hatching rate and implantation. ZP thickness depends on inherent features of embryos to generate the lytic factors needed for ZP thinning (9, 39). There are many ways to thin or remove ZP such as partial zona dissection, using proteolytic enzymes, laser and Tyrode's solution (2). But those methods are invasive regarding that adding anti-oxidant into the embryo culture medium is probably less invasive and may cause thinning ZP thickness (15, 40). The present study showed apigenin could decrease ZP thickness of blastocysts. These results are in agreement with the results of Khanmohammadi et al. (15) indicating that l-carnitine, as an antioxidant, has the ability to reduce ZP thickness.

Despite obtaining these results, there are some limitations in this study. More research is required to clarify the molecular mechanisms underlying apigenin function on development and qualifying embryos. In addition, the number of samples was low. Hence, more samples would be needed in different conditions and with different doses of apigenin.

## Conclusion

The results of this study suggest that apigenin with anti-oxidant and anti-apoptotic properties may protect the embryos against $H_2O_2$ and actinomycin D. Apigenin can probably increase the number of viable blastomeres and decrease the number of apoptotic blastomeres, which may cause expanding blastocysts, thinning ZP thickness and increasing the rate of hatching in mouse embryos.

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## Author's Contributions

M.S.; Contributed substantially to the conception and design of the study and the acquisition of data. H.P., H.R.S., M.R.A.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. S.Z.; Gave the idea of the project, helped in the embryo staining and wrote the manuscript. All authors read and approved the final manuscript.

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Case Report

# Endometriosis of Diaphragm: A Case Report

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Abstract

Endometriosis affects about 10% of women of reproductive age. Its main feature is the presence of stroma and endometrial glands in sites other than the uterus, mainly in pelvis. Pelvic peritoneum, ovaries, uterine ligaments, bladder, intestines, andcul-de-sac are among the affected areas. Sometimes endometriosis can be found outside of the pelvis and even above abdominal cavity, like indiaphragm.Herein, we present a case of an asymptomatic diaphragmatic endometriosis that was discovered incidentally during laparoscopy of pelvic endometriosis, as well as our appropriately proposed treatment protocol.

***Keywords:*** Diaphragm, Endometriosis, Laparascopy, Shoulder Pain

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## Introduction

Endometriosis, which is characterized by the incidence of stroma and endometrial glands outside of the uterine cavity, is common in approximately 10% of women during their child bearing age (1). Most common site of endometriosis is pelvic peritoneum has been reported in extra-pelvic locations, like upper abdominal cavity and diaphragm, as well (2). In fact, diaphragmatic endometriosis involving the full thickness of the diaphragm includes 1-1.5% of patients diagnosed with endometriosis (1). This rare condition can be asymptomatic and has been discovered accidentally. A patient with diaphragmatic endometriosis experiences the following symptoms: upper abdominal pain on the right side, pain under the lower ribs, painful breathing, and sometimes nausea or vomiting (3, 4).

Here in, we present a case of diaphragmatic endometriosis associated with pelvic endometriosis in a 20-year-old female patient with chronic pelvic pain and dysmenorrhea with a high score. In a preliminary investigation, she was diagnosed with deep pelvic endometriosis. However, during laparoscopic surgery of the entire abdominal and pelvic cavity, diaphragmatic endometriosis was discovered incidentally, which had spread through the center and right parts of diaphragm. In this case report, we introduce a rare case of diaphragmatic endometriosis along with pelvic endometriosis and discuss its symptoms and therapeutic methods.

## Case Report

In March of 2017, a 20-year-old virgin female with achronic pelvic pain was referred to our center. The patient complained of severe pelvic pain with verbal numerical rating scale (VNRS) of 9 during the menstrual cycle. This chronic pain had lasted for almost one year. The patient did not mention dyschezia, pain during or afterurination, orother symptoms associated with diaphragmatic endometriosis, such as chest pain, shoulder pain, or right upper abdominal pain. Furthermore, she had used no hormone replacement therapy.

In abdominal examination, there was fullness on the left side, while in both rectal examination and abdominal examination, there was fullness in the posterior cul-de-sac. An immobile 10-cm mass wasfelt on the left side, whereas another immobile 5-6-cm mass was on the right side that was fixed to the uterus.

Pelvic ultrasonography results indicated a cyst with an approximate size of 12×7 cm consisting of thick contents in the left ovary with internal septae, raising suspicion regarding formation of the tubo ovarian complex in endometrial cavity. Furthermore, the ultrasound findings showed an endometrium a cyst with an approximate dimension of 4 cm on the right side with adhesion and endometrial nodule of the posterior fundus with moderate adhesion to the rectosigmoid. Therefore, magnetic resonance imaging (MRI) was performed to exclude the left mass from adenocarcinoma, while the results showed normal upper abdominal organs, including liver, spleen, pancreas, kidneys, adrenal, as well asthe lungs. In pelvic MRI findings, there was endometrium in both adnexae along with hydrosalpinx on the left side, whereas enhancement

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was not reported in the left adnexal masses.

In addition, the blood test showed an anti mullerian hormone (AMH) of 1.82 and CA-125 of 125.1, while other tumor markers, including risk of ovarian malignancy algorithm (ROMA) and HE4 were normal.

During laparoscopy, we noticed extensive endometriosis that involved the anterior and posteriorcul-de-sac, both pelvic side walls, both ovaries, and sigmoid colon. The left ovary contained a cyst measured 10-12 cm with severe adhesion to the rectum, while the right ovary contained a cyst measured approximately 6 cm with moderate adhesion to the tube and the right ovary. There was also no evidence of endometriosis in ureters. Anatomy of pelvis restored, pelvic Die corrected and a 2-cm endometriotic nodule attached to the rectovaginal septum (RVS) was shaved.

On exploring the upper abdomen, 5 to 6 areas of superficial endometriosis were discovered in the, anterior and center of the right hemi-diaphragm (Figs.1, 2), but the left hemidiaphragm was intact. The tota Redwine l surface area of the diaphragm (left side, center, and right side) was thoroughly investigated when the patient was put into reverse Trendelenburg position. The fulguration was performed using bipolar energy for endometriotic lesions of the diaphragm. The endoscopic exploration of thoracic cavity was not performed because the patient had no symptoms of shoulder or chest pain, no history of catamenial hemothorax or pneumothorax and diaphragmatic involvement was superficial.

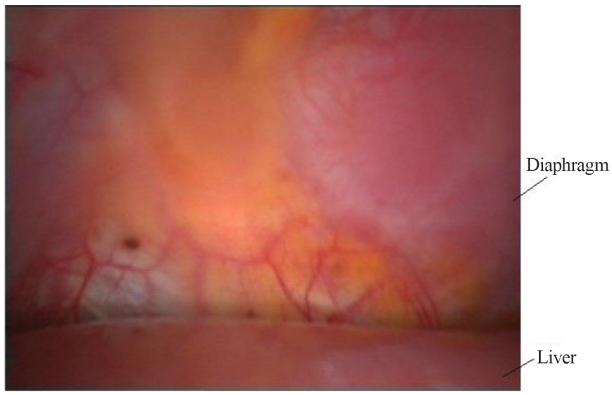


**Fig.1:** Lesions of endometriosis on the rightside and the center of the diaphragm.

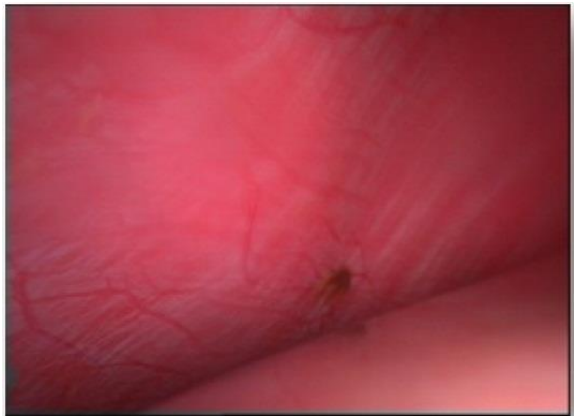

**Fig.2:** Lesion of endometriosis on the surface on right hemi diaphragm.

## Discussion

It has been reported that the incidence of endometriosis among the women of child bearing age is about 10% (5). Peritoneal cavity, especially the pelvic peritoneum, is the most common site of involvement, but endometriosis has been shown in almost all parts of the body (1). Endometriosis mainly occurs at the age of 30 to 45 years (6). The mean age for extra-pelvic endometriosis has been reported between 35 and 40 years with the prevalence of 12% (2, 3). In addition, the average age for pelvic endometriosis is 25 to 30 years (3).

Diaphragmatic endometriosis is a rare serious disorder, which has been reported for the first time as a separate term by Brews (7). Most diaphragmatic lesions occur on the right side. The pathogenesis of a higher prevalence of endometriosis in the subphrenic region is sampsons retrograde menstruation theory, which indicates that refluxed endometrium may be caught by falciform ligament in the right side of the diaphragm (1). Classical symptoms of diaphragmatic endometriosis are chest pain (pleuritic pain, especially on the right side), dyspnea, epigastric pain, shoulder pain, and upper abdomen painthat is sudden onset in many patients (2). It is noteworthy that although symptoms are usually periodic, some patients with diaphragmatic endometriosis experience continued symptoms, which are not associated with the menstrual cycle. Therefore, despite of atypical clinical symptoms, especially in patients with pelvic endometriosis, there should be a strong clinical suspicion to different types of thoracic endometriosis.

The pain in diaphragmatic endometriosis is due to stimulation of a sensory branch of the C5 nerve root. The severity of the symptoms varies depending on the location and depth of the lesions.It has been reported that diaphragmatic endometriosis can be asymptomatic, while some women may experience no clinical symptoms or an obscure pain (8). Some serious and life-threatening conditions associated with diaphragmatic endometriosis are the results of the expansion of the fenestrations or holes in the diaphragm due to necrosis of endometriosis lesions (9). These conditions are as follows: i. Catamenial pneumothorax (CPT) is a rare condition causing the lungs to collapse during menses and responsible for about one third of spontaneous pneumothorax in women (10-12). It occurs alone or with different manifestations of thoracic endometriosis syndrome (TES), including hemopneumothorax and catamenial hemoptysis, ii. Hemopneumothorax is known as presence of blood and air in the chest cavity (13), as well as iii. Intrathoracic endometriosis nodules.

Diaphragmatic endometriosis isdiagnostic error due to the similarity of clinical symptoms with other benign or malignant disorders. About 95% of diaphragmatic lesions occur in the right side of the diaphragm, although it has been previously seen in the left side alone or both sides of the diaphragm, even in some vital structures, like the phrenic nerve. Furthermore, in most of the reported cases, the lesions occur in the anterior or posterior portion of the

diaphragm and behind the liver. Therefore, due to the diversity of an organ site involvement, diaphragms and their surrounding areas should be thoroughly examined (3).

In terms of macroscopic appearance, lesions may appear in different colours and shapes that are mostly reported as bruised, purple and purple red. Computerized tomography (CT) scan or MRI may play an important role in diagnosis. Thoracic endometriosis may appear as small cystic lesions in chest radiography or CT scan (2). However, it has been shown that MRI may provide better details to diagnose endometriosis (6). In our case, MRI report showed no pulmonary endometriosis lesions.

Therapeutic measures for diaphragmatic endometriosis or suspicious thoracic endometriosis may be mainly based on the patient's medical history. It has been strongly indicated that the best treatment choice is the expectant approach as compared to the other interventions for those patients with asymptomatic diaphragmatic endometriosis (14).

However, for symptomatic patients, surgery will be beneficial, if the medicationis deemed to have failed (8, 15). Given a possibility of damage to the diaphragm, phrenic nerve, lungs, vessels or heart, it is crucial to choose a surgical plan after an informed consent is obtained from the patient. Also, alternative therapeutic options should be explained to the patient.

The patient's age, the type of treatment andthe medication as well as the surgeon's expertise should be also considered in this regard. Although there is still uncertainty about the efficiency of laparoscopic surgery in diagnosis and treatment of diaphragmatic endometriosis (3), this concern is being resolved in consultation with an expert laparoscopic surgeon regarding the use of different techniques such as proper patient positioning for an optimum view of the diaphragm and associated structures. The involvement of hidden area including the junction of the diaphragm and the posterior edge of the liver is common in the invasive conditions. In addition, the application of right sub-conundrum port or flexible laparoscope (16) may provide a precise view of the diaphragm. Simultaneous application of laparoscopy and thoracoscopic surgery (VATS) is also considered as an effective therapeutic plan in diagnosis and treatment of women with diaphragmatic endometriosis, suffering intolerable pain in the right upper abdomen and chest (due to hemopneumothorax).

The use of hormonal medications, such as danazol (oral contraceptives), has been suggested to the patients who are not interested in VATS or believe the thoracoscopy is not safe enough. The segmental resection is needed during VATS for the following disorders: tension pneumothorax, hemopneumothorax, lesions of pulmonary endometriosis, chemical pleurodesis, as well as pleurectomy (2). VATS as a procedure also provides the following abilities: resection of diaphragm implants, restoration of diaphragmatic fenestration, resection of apical blebs, and implants of lung parenchyma. This invasive procedure is known as an excisional technique dueto the complete removal of endometriosis lesions. In a number of control-randomized studies, it has been shown that complete disease eradication is the only definitive way to relieve pain, while the recurrence is negligible (17, 18). There are several different and effective methods for excision of the lesions, like vaporization, ablation, hydrodissection, and surgical scissor excision.

In our case, diaphragmatic endometriosis was discovered after inspection of the upper abdomen. In addition, the patient had nosymptoms, such as shortness of breath, shoulder pain, and right upper quadrant (RUQ) pain. Therefore, due to laparoscopic examination of the diaphragm, and appearance of lesions, the endometriosis lesions ablate. We decided to apply no other interventions for the patient.

It has been reported that the asymptomatic diaphragmatic endometriosis can be safely treated with use of laparoscopic surgery instead of laparotomy, diaphragmatic resection, or other interventions (3, 8, 9). Furthermore, VATS is known as a diagnostic and therapeutic method in selected symptomatic patients as compared to the laparotomy and thoracotomy. Medical treatment after surgery is different depending on the patient's decision for future pregnancies. In those patients who tend to getpregnant, there is no need for further medications after surgery, but assisted reproductive technology (ART) is recommended In contrast, for those who do not want to get pregnant, suppressive hormonal treatment is used.

After discharge, considering the virginity, we prescribed suppressive hormonal medicationsfor the patient. Furthermore, we advised her to go to a hospital immediately if she experience shoulder or RUQ pain, shortness of breath and other catamenial symptoms.

## Conclusion

Endometriosis is considered as a clinical puzzle for both physicians and patients. Although many efforts have been made for both diagnosis and treatment of this disease, it is still controversial in terms of clinical symptoms, pathophysiology, disease progression as well as management. The surgeon should be fully aware of the clinical symptoms, patient's medical history, and endometriosis lesions during a laparoscopic surgery. It is noted that if chest pain, shoulder pain, hemothorax, pneumothorax, and hemoptysis occur during the reproductive age, especially with acyclic pattern, then diaphragmatic endometriosis should be considered. Therefore, a close inspection of both anterior and posterior parts of hemi-diaphragm and applying a combined VATS/laparoscopy procedure are needed. This is especially true if there are lesions present. In order to achieve better outcomes with prevention of recurrence and re-occurring clinical symptoms, resection of any suspicious lesion is also recommended.

It is noteworthy that in contrast to conservative treatment which will be applied when endometriosis is detected during laparoscopic surgery in asymptomatic patients,

however, an interventional approach is needed in symptomatic patients with post-surgical complications.

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## Author's Contributions

A.M.K., M.K., K.T., B.T., G.M., K.S.; Contributed to conception and design. M.K.; A.M.K., Contributed to all experimental work. A.M.K., K.T.; Were responsible for overall supervision. M.K.; Drafted the manuscript, which was revised by A.M.K. All authors read and approved the final manuscript.

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# International Journal of Fertility and Sterility (Int J Fertil Steril)
# Guide for Authors

**Aims and Scope: International Journal of Fertility & Sterility** is a quarterly English publication of Royan Institute of Iran. The aim of this journal is to disseminate information through publishing the most recent scientific research studies on Fertility and Sterility and other related topics. **Int J Fertil Steril** has been certified by Ministry of Culture and Islamic Guidance since 2007. It has also been accredited as a scientific and research journal by HBI (Health and Biomedical Information) Journal Accreditation Commission since 2008. **This open access journal holds the membership of the Committee on Publication Ethics (COPE).**

**1. Types of articles**

The articles in the field of Fertility and Sterility can be considered for publications in Int J Fertil Steril. These articles are as below:

**A. Original articles** are scientific reports of the original research studies. The article consists of English Abstract (structured), Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgements, Author's Contributions, and References **(Up to 40).**

**B. Review articles** are the articles written by well experienced authors and those who have excellence in the related fields. The corresponding author of the review article must be one of the authors of at least three published articles appearing in the references. The review article consists of English Abstract (unstructured), Introduction, Conclusion, Author's Contributions, and References **(Up to 70).**

**C. Systematic Reviews**

Systematic reviews are a type of literature review that collect and critically analyzes multiple research studies or papers. The Systematic reviews consist of English Abstract (unstructured), Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgements, Author's Contributions, and References **(Up to 70).**

**D. Short communications** are the articles containing new findings. Submissions should be brief reports of ongoing researches. The short communication consists of English Abstract (unstructured), the body of the manuscript (should not hold heading or subheading), Acknowledgements, Author's Contributions, and References **(Up to 30).**

**E. Case reports** are short discussions of a case or case series with unique features not previously described which make an important teaching point or scientific observation. They may describe novel techniques or use equipment, or new information on diseases of importance. It consists of English Abstracts (Unstructured), Introduction, Case Report, Discussion, Acknowledgements, Author's Contributions, and References **(Up to 30).**

**F. Editorial** should be written by either the editor in chief or the editorial board.

**G. Imaging in reproductive medicine** should focus on a single case with an interesting illustration such as a photograph, histological specimen or investigation. Color images are welcomed. The text should be brief and informative.

**H. Letter to the editors** are welcome in response to previously published **Int J Fertil Steril** articles, and may also include interesting cases that do not meet the requirement of being truly exceptional, as well as other brief technical or clinical notes of general interest.

**I. Debate.**

**2. Submission Process**

It is recommended to see the guidelines for reporting different kinds of manuscripts here. This guide explains how to prepare the manuscript for submission. Before submitting, we suggest authors familiarize themselves with **Int J Fertil Steril** format and content by reading the journal via the website (www.ijfs.ir). The corresponding author ensures that all authors are included in the author list and agree with its order, and they must be aware of the manuscript submission.

**A. Author contributions statements**

It is essential for authors to include a statement of responsibility in the manuscript that specifies the contribution of every one of them. This participation must include conception and design of the manuscript, data acquisition or data analysis and interpretation, drafting of the manuscript and/or revising it for critically important intellectual content, revision and final approval of the manuscript and statistical analysis, obtaining funding, administrative, technical, or material support, or supervision. Authors who do not meet the above criteria should be acknowledged in the **Acknowledgments Section**.

**B. Cover letter**

Each article should be accompanied by a cover letter, signed by all authors specifying the following statement: "The manuscript has been seen and approved by all authors and is not under active consideration for publication. It has neither been accepted for publication nor published in another journal fully or partially (except in abstract form). I hereby assign the copyright of the enclosed manuscript to **Int J Fertil Steril**. The corresponding author must confirm the proof of the manuscript before online publishing. Also, is it needed to suggest three peer reviewers in the field of their manuscript.

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Authors whose first language is not English encouraged to consult a native English speaker in order to confirm his manuscripts to US or British (not a mixture) English usage and grammar. The manuscript should be prepared in accordance with the "International Committee of Medical Journal Editors (ICMJE)". Please send your manuscript in two formats (word and Pdf). The abstract and

text pages should have consecutive line numbers in the left margin beginning with title page and continuing through the last page of the written text. Each abbreviation must be defined in the abstract and text when they are mentioned for the first time. Avoid using abbreviation in title. Please use the international and standard abbreviations and symbols.

It should be added that an essential step toward the integration and linking of scientific information reported in published literature is using standardized nomenclature in all fields of science and medicine. Species names must be italicized (e.g., Homo sapiens) and also the full genus and species written out in full, both in the title of the manuscript and at the first mention of an organism in a paper.

It is necessary to mention that genes, mutations, genotypes, and alleles must be indicated in italics. Please use the recommended name by consulting the appropriate genetic nomenclature database, e.g., HUGO for human genes. In another word; if it is a human gene, you must write all the letters in capital and italic (e.g., OCT4, c-MYC). If not, only write the first letter in capital and italic (e.g., Oct4, c-Myc). **In addition, protein designations are the same as the gene symbol but are not italicized.**

**Of note, Int J Fertil Steril** will only consider publishing genetic association study papers that are novel and statistically robust. Authors are advised to adhere to the recommendations outlined in the STREGA statement (http://www.strega-statement.org). The following criteria must be met for all submissions:

**1.** Hardy-Weinberg Equilibrium (HWE) calculations must be carried out and reported along with the P-values if applicable [see Namipashaki et al. 2015 (Cell J, Vol 17, N 2, Pages: 187-192) for a discussion].

**2.** Linkage disequilibrium (LD) structure between SNPs (if multiple SNPs are reported) must be presented.

**3.** Appropriate multiple testing correction (if multiple independent SNPs are reported) must be included.

Submissions that fail to meet the above criteria will be rejected before being sent out for review.

Each of the following manuscript components should begin in the following sequence:

**Authors'** names and order of them must be carefully considered (full name(s), highest awarded academic degree(s), email(s), and institutional affiliation(s) of all the authors in English. Also, you must send mobile number and full postal address of the corresponding author).

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**Title** is providing the full title of the research (do not use abbreviations in title).

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**Abstract** must include Background, Materials and Methods, Results, and Conclusion (no more than 300 words).

**Keywords,** three to five, must be supplied by the authors at the foot of the abstract chosen from the Medical Subject Heading (MeSH). Therefore; they must be specific and relevant to the paper.

The following components should be identified in the abstract:

**Introduction:** The Introduction should provide a brief background to the subject of the paper, explain the importance of the study, and state a precise study question or purpose.

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**Supplementary materials** would be published on the online version of the journal. This material is important to the understanding and interpretation of the report and should not repeat material within the print article. The amount of supplementary material should be limited. Supplementary material should be original and not previously published and will undergo editorial and peer review with the main manuscript. Also, they must be cited in the manuscript text in parentheses, in a similar way as when citing a figure or a table. Provide a legend for each supplementary material submitted.

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**Conclusion:** It emphasizes the new and important aspects of the study. All conclusions are justified by the results of the study.

**Acknowledgements:** This part includes a statement thanking those who contributed substantially with work relevant to the study but does not have authorship criteria. It includes those who provided technical help, writing assistance and name of departments that provided only general support. You must mention financial support in the study. Otherwise; write this sentence "There is no financial support in this study".

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Example: Manicardi GC, Bianchi PG, Pantano S, Azzoni P, Bizzaro D, Bianchi U, et al. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. Biol Reprod. 1995; 52(4): 864-867.

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**Abstract book:**

Example: Nabavi SM. Stem cell therapy for multiple sclerosis. Cell J. 2013; 5 Suppl 1: Os-13.

**Thesis:**

Name of author. Thesis title. Degree. City name. University. Publication date (year).

Example: Eftekhari Yazdi P. Comparison of fragment removal and co-culture with Vero cell monolayer's on development of human fragmented embryos. Presented for the Ph.D., Tehran. Tarbiyat Modarres University. 2004.

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Example: Harnden P, Joffe JK, Jones WG, editors. Germ cell tumors V.Proceedings of the 5th Germ Cell Tumors Conference; 2001 Sep 13-15; Leeds,UK. New York: Springer; 2002.

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Surname(s) and first letter of name & middle name(s) of author(s). Manuscript title. Journal title (abbr). Publication date (year);

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Example: Jahanshahi A, Mirnajafi-Zadeh J, Javan M, Mohammad-Zadeh M, Rohani M. Effect of low-frequency stimulation on adenosineA1 and A2A receptors gene expression in dentate gyrus of perforant path kindled rats. Cell J. 2008; 10 (2): 87-92. Available from: http://www.celljournal.org. (20 Oct 2008).

**Book:**

Example: Anderson SC, Poulsen KB. Anderson's electronic atlas of hematology.[CD-ROM]. Philadelphia: Lippincott Williams & Wilkins; 2002.

**Law:**

Example: Embryo donation law. Iran Judicature, Official Gazette of the Islamic Republic of Iran. Available from: http://www.dastour.ir/Brows/?lid=245069.(20 Jul 2013).

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The authors must ensure that before submitting the manuscript for publication, they have to consider the following parts:

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**2.** Text of manuscript and References prepared as stated in the "guide for authors" section.

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