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Contents

Review Article

Immune Aspects of Female Infertility

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Abstract

Immune infertility, in terms of reproductive failure, has become a serious health issue involving approximately 1 out of 5 couples at reproductive age. Semen that is defined as a complex fluid containing sperm, cellular vesicles and other cells and components, could sensitize the female genital tract. The immune rejection of male semen in the female reproductive tract is explained as the failure of natural tolerance leading to local and/or systemic immune response. Present active immune mechanism may induce high levels of anti-seminal/sperm antibodies. It has already been proven that iso-immunization is associated with infertility. Comprehensive studies with regards to the identification of antibody-targets and the determination of specific antibody class contribute to the development of effective immuno-therapy and, on the other hand, potential immuno-contraception, and then of course to complex patient diagnosis. This review summarizes the aspects of female immune infertility.

Keywords: Immunoglobulins, Infertility, Sperm

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Introduction

The World Health Organization declares infertility as a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse. Infertility has been reported to be one of the most prevalent chronic health disorders regardless of age (1, 2). The decreased fecundity is associated with other health issues (severe avitaminosis, severe renal impairment, cancer and cachexia due to malnutrition or tumor), age, lifestyle and environment. The male partner accounts for the infertility 40% of the time, 40% from the female partner as well and 20% shared by both the man and the woman. The factors involve congenital, hormonal, morphological and immunological disorders (3). The main disorders involved in infertility include pathologic spermiogram, ovulation problems/anovulation, tubal diseases, pelvic adhesion/endometriosis, cervical factors and idiopathic reason usually qualified as the so-called unexplained infertility (UI) (4-6).

UI is diagnosed in a couple when the standard investigations including the semen analyses, test of ovulation and tubal potency do not provide specific results or do not detect any abnormality. Several reports (4, 5, 7) suggested that the diagnosis of UI is subjective and often misdiagnosed for endometriosis, tubal infertility, premature ovarian ageing and immune infertility. The prevalence of UI reaches up to 30% of infertile couples with regards to standard investigation. Severe endometriosis affects the fecundity potential. Mild endometriosis is not, however, associated with infertility in the absence of secondary organic disruption. It has been reported that approximately 20% of infertile females suffer from tubal disease, either distal or peritubal (2, 4). Follicle number is genetically dependent. Female subfertility caused by poor ovarian reserve is declared when the remaining follicle amount represents a fraction of the original value (8). In some women, the so-called poor ovarian response has been noticed when the age-

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ing ovary produces fewer follicles, follicles grow poorly and follicular atresia occurs (5). Molecular and cellular endometrial deficiency resulting in an implantation failure can be related to UI since the natural immunosuppression does not prevent maternal immune rejection. T regulatory (Treg) cells are believed to protect the fetus from an immune attack. Treg cells function in immune tolerance exhibiting the immuno-suppressive activity. A factor of spontaneous abortion is displayed in the case of a lower number of $CD4^+CD25^+$ $Foxp3^+$ Treg cells that is, under normal conditions, elevated in the first trimester of physiological pregnancies (9, 10). UI is not necessarily linked to Treg differentiation, thus to immune suppression failure, but also to its recruitment into the implantation site. This fact is caused by the reduced expression and insufficient function of lymphocyte and chemotactic agents present in the uterus. Since Treg differentiation is regulated by transforming growth factor beta (TGFβ), idiopathic infertility may be related to a reduced availability of this factor. The lack of TGFβ results in insufficient Treg induction. Diminished $CD4^+CD25^+$ Treg population, the lower expression of Foxp3 and the failure of lymphocyte adherence and chemotaxis seem to play, however, a role in primary cause of UI (11).

Immune/immunological infertility is diagnosed when spontaneously produced antibodies bind to the antigens occurring on either the male or female gametocytes. In particular, antibodies bind to seminal proteins or structures present on the sperm or oocyte. So far, anti-sperm antibodies (ASA) have been observed more frequently than anti-oocyte antibodies (12).

Antibody formation

After an exposure to an antigenic agent, the level of immunoglobulin M (IgM) antibodies is supposed to be dominant at the early phase of a primary immune response. In response to some allergens, IgE antibodies may be prevalent in genetically predisposed individuals. The switch into IgG and IgA antibodies is induced at the late phase of primary immune response or after repeated exposure to the same antigen (13-15). When chronically exposed to the antigens, IgG_1 and IgG_4 become the predominantly produced subclasses of IgG isotype. IgG_4 is a unique antibody unable to activate the classical complement pathway and is then known as an anti-inflammatory Ig and a blocking antibody towards IgE antibodies, depending on the antigenic model. It remains unclear whether IgG_4 is a protective or pathogenic antibody (6, 16, 17). Schroeder and Cavacini speculated that IgG_1 and IgG_3 antibodies are generally induced in response to protein antigens whereas IgG_2 and IgG_4 to polysaccharide antigens (18). Other studies related to antibody distribution neither refute nor endorse this hypothesis (6, 19). We have reported IgG_1/IgG_4 predominance in anti-seminal antibodies and IgG_4/IgG_1 predominance in ASA. We have also proposed the distribution of seminal/sperm-specific antibody isotypes showing that immunoglobulins E, M, $A_{1,2}$, G_3 are not significantly involved in pathophysiological female sensitization. Specific IgG_4 appears to be mainly produced together with specific IgG_1 (20).

Anti-sperm antibodies

The sperm antigenicity concerning the animal kingdom was first described by Landsteiner, Metchnikov and Metalnikova in 1899 as sperm toxins. In 1932, Baskin observed circulating antibodies against sperm and in 1954, Rümke observed and described the first type of ASA. They have cytotoxic, immobilizing and agglutinating functions. ASA are detectable on the systemic (blood and lymph) as well as the local level [seminal fluid (SF), cervical-vaginal mucus]. In general, the IgG isotype of ASA is mainly related to the blood circulation and IgA isotype to mucosal immunity in women. In men, IgG and IgA fractions are the most prevalent in SF, while IgG and IgM isotypes in serum (6, 21).

Semen has a very heterogeneous antigenic content. Since sperm has auto-antigenic (auto-immunization) as well as iso-antigenic (iso-immunization) potential, it is able to induce the production of sperm-reactive T-cells in men as well as in women, thus is opsonized and then targeted by the leukocytes (sperm-cytotoxic effect) (22-24). It is not a single ASA that influences fertility but more likely multiple ASA causing infertility. Furthermore, it has been postulated that antibodies against a single sperm antigen cannot cause infertility. It has also been reported that not all ASA, either produced in women or men, affect the fertility potential since the cognate antigen is not necessarily involved in the fertilization process (6, 23-26).

A highly heterogeneous sperm antigenic content could be modified during maturation and ejaculation based on antigen sequestration. Newly expressed antigens could then be in contact with any immunocompetent cells, e.g., a sperm membrane-incorporated fibronectin exhibits changes in regional antigenic expression during sperm maturation, whereas secreted fibronectin is a product of male accessory sex glands and can be attached to sperm tail during ejaculation (12, 27). Considering gastrointestinal exposure, ASA formation may be operative (21).

In men, sperm germ cells are protected in the testis from an auto-immune attack by the blood-testis barrier. When the barrier is disrupted, auto-antibodies are produced and are then detectable in blood serum, seminal plasma or directly attached to the sperm surface membrane (28). An increased risk of ASA formation may follow the congenital absence of reproductive tract components. ASA are mostly associated with genital inflammation/infection (e.g., orchitis), epididymis trauma, genital surgery, cryptorchidism and varicocele (27). The theory of auto-immune disease was supported by proving that ASA formation is related to certain human leukocyte antigen classes (29).

In women, the failure of natural tolerance may lead to sensitivity resulting in sperm elimination. ASA affect fertility potential through various pre/post-fertilization processes, such as sperm agglutination and motility, cervix mucus penetration, capacitation, acrosome reaction, zona pellucida (ZP) binding and penetration, oolemma binding, sperm-oocyte fusion and embryo implantation (30, 31). The active local immuno-regulatory mechanism is based on vaginal and cervical tissues having an active and sensitive mucosal immune system, by which the fertility potential is affected. This explains the rather high percentage of infertile women with the local reactions leading to inflammation as well as with high levels of serum anti-semen antibodies. Furthermore, ASA-coated sperm may be more vulnerable to phagocytosis in the female reproductive tract (28). Serum ASA are related to the long-term exposure of female to sperm and then to seminal deficiency in immuno-suppressive factors (32, 33).

Nevertheless, there is the evidence of ASA occurrence in fertile women and men. Some fertile individuals are positive in serum sperm agglutinins. It has been suggested that these ASA are not clinically significant. It is a physiological effect without a pathologic background as they do not inhibit the fertilization process either *in vitro* or *in vivo* (12, 23). In this case, they may be considered as the so-called natural ASA (34). They are produced by auto-reactive B cells in men that were stimulated to grow. Furthermore, natural autoantibodies may be more poly-reactive antibodies, hypothetically help remove senescent molecules and cells, and participate in immune auto-treatment of cancer (35, 36). The poly-reactive character may play a part in cytotoxic reaction at early fertilization associated with infertility.

Role of seminal fluid in female immune infertility

SF represents a part of the semen containing a range of organic/inorganic substances (e.g., neutral α-glucosidase, hyaluronidase, carnitine, glycerolphosphocholine, fructose, prostaglandins (PGs), citrate, zinc, selenium) that are necessary for the physiological metabolism of sperm. The seminal complex mixture of secretions originates in the testis, epididymis and accessory glands including the prostate, seminal vesicles and Cowper's gland. It also acts as a nutritive, transport and buffering medium of pH=7.35-7.5 that defines the main SF functions: sperm protection from the acidic environment of the vagina, metabolic support, liquefaction and clot formation. SF composition is similar to blood plasma; however, it differs in saccharide content (37-39).

Prostate specific antigen (PSA), prostatic acid phosphatase (PAP) and prostate-specific protein-94 belonging to the prostate secretion are in direct contact with sperm and thus may be the first to confront the cervical tissues. PSA is a 33 kDa member of the glandular kallikrein subfamily of serine proteases participating in the liquefaction of the seminal coagulum. Its activity is strongly inhibited by zinc ions (40-42). Serum PSA is a commonly used marker of prostate cancer (43-45). PAP, a member of the histidine acid phosphatase family, is a non-specific tyrosine phosphatase that dephosphorylates macromolecules and inactivates lysophosphatidic acid in SF (46, 47). Seminal components, e.g., heparin (48) or zinc-2-glycoprotein (ZAG) (49), bind to the acrosomal sperm head region protect sperm and are then carried together

into the higher female genital tract. SF plays an important role in moving the sperm into the female reproductive tract due to its high content of TGFβ and PGE, both of which inhibit the function of natural killer (NK) cells and neutrophils that are recruited into the superficial epithelial layers of the cervical tissues. TGFβ is synthesized in the prostate and is testosterone-dependent. This glycoprotein belongs to cell-secreted molecules and occurs in 75% in the latent form in SF. It is further activated in the female reproductive tract by either the enzymes of male/female origin, acidic vaginal pH or through conformational change after an interaction with epithelial cells. The remaining proportion of TGFβ, 25%, exists in an active form (50, 51). TGFβ acting may result in the immune tolerance of seminal antigens. A divergent member of this family is growth/differentiation factor 15 (GDF 15), which is highly abundant in SF. GDF 15 has anti-tumorigenic activity, serves as a cancer marker and is likely to promote a pro-inflammatory immune response. High level of GDF 15 in female serum corresponds to spontaneous abortion as it is expressed in the placenta. It has been suggested that due to the presence of seminal antigens on a fetus, TGFβ facilitates the female immune tolerance to the fetus (52, 53).

Some seminal constituents, such as cathepsin D, are able to degrade proteins vaginally exposed that may be involved in antibody formation related to immune infertility (54). Seminal ZAG has been reported as a novel adipokine playing a significant role in fertilization, lipid mobilization, and peptide/antigen/ligand binding. ZAG may participate in the expression of female immune response since the fold is similar to major histocompatibility complex (MHC) molecules, in particular MHC I, on the antigen-presenting cells. ZAG has been proven to be an IgG-binding protein related to a pathophysiological iso-immunization. This protein belongs to immunoglobulin gene family and may have a protective role by blocking the elicited female anti-semen antibodies (6, 31, 49). SF includes a repertoire of signaling molecules interacting with the epithelium in the female reproductive tract. SF may modulate the chemotactic and phagocytic responses of the female reproductive tract. Phagocytes serve to filter out morphologically abnormal sperm. Sperm selection is based on morphological or antigenic structures. Mainly, the immune modulating properties are mediated by the PGs of the E series, complement inhibitors, cytokines and proteins capable of binding IgG antibodies (55, 56). Local reactions may lead to an inflammation. However, SF has a built-in mechanism preventing an immunological sensitization of the female against sperm as well as seminal structures. This protective system exists due to the presence of immune inhibitors originating in the male sex accessory glands (52, 57).

SF has been suggested to be the modulator of sperm-induced inflammation that leads to sperm elimination from the female genital tract. Antibody fraction interacting with seminal antigen targets most of the seminal proteins adsorbed on sperm. However, SF induces the recruitment of macrophages and dendritic cells into cervical and endometrial tissues (58). SF elicits endometrial changes by inducing pro-inflammatory cytokines and cyclooxygenase-2. Their presence leads to macrophage and dendritic cell recruitment into the female reproductive tract. Seminal components activate the income of neutrophils into the endometrial stroma (24, 52, 59). However, it has been reported that the influx of neutrophils is higher and faster when the washed sperm inseminated (60). This fact demonstrates the protective and signaling activity of SF. The immuno-suppressive activity prevents the iso-immunization of the female reproductive tract and suppresses cell-mediated cytotoxicity (61). Seminal prostaglandin D2 is known for its immuno-suppressive effect, by which ASA formation is prevented in the female genital tract. The immuno-modulating properties are mediated by PGE, complement inhibitors, cytokines and proteins capable of binding the Fc region of IgG. These IgG-binding proteins are Fcγ receptor-like soluble proteins.

In general, seminal antibody-binding proteins contribute to sperm protection against immune-mediated damage by enabling successful sperm passage in the female reproductive tract and by blocking an interaction with immune effectors such as prolactin-inducible protein, which is a secretory glycoprotein located in seminal vesicles, binds to immunoglobulin G via its Fc fragment. It may therefore be involved in immune regulation by trapping ASA and neutralizing them (62, 63). Particular deficiencies in seminal factors may lead to higher antibody production in infertile women (64).

SF has already been considered to be linked to the IgE-mediated rare reaction to semen (65). This rare phenomenon was first reported in 1945 (66). Human seminal plasma allergy (HSPA), the so-called hypersensitivity to semen, is defined by local and/or systemic symptoms after exposure to SF. The symptoms occur immediately after contact with semen or even within several hours after intercourse. The local symptoms include vulvar/vaginal itching, burning, redness and swelling. Local reaction can appear on any semen contact site and can be misdiagnosed as chronic vulvo-vaginitis caused by bacteria, yeasts, viruses and other parasites. Systemic symptoms include generalized urticaria, angioedema (face, tongue, lips, throat), dyspnea, wheezing, cough, chest tightness, rhinorrhea, nausea, vomiting, diarrhea. Generalized malaise may result in an anaphylactic shock, which is a life-threatening reaction. The symptoms can manifest after the first time intercourse in up to 50% of cases. Response mediated by IgE antibodies is then the most common mechanism. It has been suggested that female patients experiencing any allergic symptoms after/during the first time intercourse might be sensitive to other antigens/allergens that cross-react with SF. IgE cross-reactivity has already been proven among proteins from dog epithelium and PSA (67). Patients diagnosed with HSPA have difficulties conceiving but infertility has not been demonstrated, so far (65, 68, 69).

Auto-immune aspects in infertility

Auto-immune phenomena have already been associated with increased prevalence of female immune infertility. This fact concerns anti-phospholipid, anti-nuclear, anti-thyroid, anti-annexin V, anti-prothrombin, anti-laminin, anti-ZP antibody formation, the high level of NK cells as the risk factors but not as those pathognomonic (4).

ZP, as the protective layer, is composed of glycoproteins. It represents a broad antigenic content. Antibodies against ZP prevent sperm from penetrating it. Anti-ZP autoantibody concentration can be elevated if ZP shape is abnormal (deformed, thickened, thinned). These antibodies interfere with the implantation process since ZP protects a fertilized oocyte up to the 7th day after fertilization, up to embryo hatching. During this time the ZP is thickened (15-17 μm). ZP-specific antibodies are detectable in follicular and peritoneal fluid, and cervical mucus in IgG, IgA and IgM isotypes (21).

Anti-phospholipid antibodies (APA) have been associated with e.g. miscarriage, intrauterine fetal death, and placental thrombosis since the time of their discovery by Wasserman in 1906. These components of the female immune system are autoantibodies directed in particular against β2-glycoprotein, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, annexin V and cardiolipin. APA are mostly produced in IgG fractions accompanied by IgA and IgM. Phosphatidylserine-specific APA cause fetus hypotrophy as a consequence of placental vascular damage, against which the maternal immune system produces anti-coagulating factors (70). The risk of spontaneous abortion increases with the presence of anti-coagulating antibodies. Antibodies specific to annexin V and placental anti-coagulating protein are also related to reproductive failure and detectable in 5-6% of women diagnosed with pregnancy loss, 8-10% of women after unsuccessful *in vitro* fertilization, 1% of not pregnant and healthy women, and 0% of pregnant women without a pathophysiologic aspect. Complex complication is called anti-phospholipid syndrome also known as Hughes syndrome. It may cause hyper-coagulation leading to rapid organ failure (6, 21, 70).

Endometrium-specific antibodies are, inter alia, associated with polycystic ovary syndrome (PCOS) that is mainly classified as an endocrine genetic disorder. PCOS is known as Stein-Leventhal syndrome first described in 1935. It is characterized by enlarged ovaries caused by cysts, irregular ovulation, irregular or no menstruation, and increased androgen levels. With regards to androgen levels, PCOS is associated with hirsutism. On the other hand, it is associated with obesity, type 2 diabetes and high cholesterol levels. Women suffering from PCOS have usually problems with conceiving (21, 71).

Pregnancy is also complicated by endometriosis, a serious gynecological complication affecting up to 10% of women of reproductive age. Twenty-five % of women diagnosed with endometriosis are infertile. Peritoneal endometriosis is characterized by retrograde menstruation causing secondary inflammation. Factors typical for such a condition are high level of autoantibodies, presence

of T-lymphocytes in peritoneal fluid, and elevated level of NK cells (72, 73).

Mucosal immunity of the female genital tract

The mucosal immune system operates on a local level and is represented by lymphoid tissues in mucosae and external secretory glands. It limits the access of environmental antigens by which the fertility potential is significantly regulated as well. It restricts and/or prevents the penetration in the systemic compartment. The female genital tissues and secretion (vaginal washes and cervical mucus) provide the protection that differs from systemic reaction by the cell types involved and by their products, the antibodies. However, it is the initial antigen exposure to mucosae that leads to the systemic T cell hypo-responsiveness (74, 75).

Mucosal immunity in the female genital tract is influenced by the level of antibodies, cytokines and hormones. Humoral defense displayed in mucosal tissue surface provides the antibodies of the IgG, IgA and IgM isotypes. IgG, IgA and IgM levels are dependent on the menstrual cycle and are influenced by hormones. IgA and IgG reach their maximum concentrations before ovulation, which is linked to the increased level of interleukin 1 component β. In particular, estrogen causes a higher expression of secretory IgA (S-IgA), thus its selective transport is increased. This way of regulation is responsible for antibody-isotype distribution including their properties, the transport of immunoglobulin-containing cells, antigen-presenting cells, in addition to $CD4^+$ and $CD8^+$ cells in the vagina, uterus and fallopian tubes (76). In addition, it has been shown (77) that oral contraception influences IgA as well as IgG levels in the cervical mucus. It is almost one third higher than in the cervical mucus of naturally cycling women. The vaginal washes of women on oral contraception display an elevated level of IgG in comparison to IgA. Several observations showed (76, 78) that the concentration decreases in this manner: IgG>IgA>IgM. This ratio is related to the presence of IgG/A/M-producing cells. The uterine endocervix contains the highest amount of IgG- and IgA-secreting cells compared to the ectocervix, fallopian tubes and vagina (76, 79). Cervical mucus contains higher levels of IgG than IgA, both of which are locally produced. On the contrary, women on oral contraception have IgA as the predominant antibody present in cervical mucus. Among the three mentioned isotypes, IgM is the less efficiently transported antibody. The mucosal IgA antibodies are selectively transported to an external secretion based on a receptor-associated mechanism. The distribution of IgG subclasses in mucosal secretions displays a plasma proportion. IgD occurs rarely or in very low concentrations in external mucosae. The level of IgE depends on the genetic predisposition to develop allergies and then on the allergenic nature of the presented antigen (74). Despite the low IgA affinity, the avidity is high regarding the multibinding sites. Environmental antigens are usually degraded by proteolytic enzymes. IgA itself is resistant to the enzymes of proteolytic character. It has been suggested that a certain amount of not eliminated antigens circulates in the complex with IgA, which further activates the systemic immune response. It is less probable that antigens entering, at first, the mucosal tissue could circulate on itself (75, 78, 80). IgA is a multivalent antibody existing in two subclasses, IgA_1 and IgA_2. IgA has an anti-inflammatory activity proved by the inhibition of complement activation and by a diminishing effect on NK cells. These properties may avoid an early-precise diagnosis as an inflammatory marker and may not be detected. In cervical mucus as well as a vaginal wash, the IgA_1 concentration is equal to IgA_2. S-IgA is locally produced by sub-epithelial plasma cells. Most of the time, it is a polymeric molecule, which corresponds to IgA_2 since IgA_1 is rather monomeric. It has been suggested that cervical mucus contains approximately 80% of the polymeric form and the vaginal wash contains approximately 50% (74, 76). Eosinophils that cover mucosal surfaces can be degranulated by IgA antibodies. This pathology is observed when natural immune tolerance is disrupted. Further reactions may evoke an allergic reaction to the presented antigen, such as seminal and/or sperm component. Semen rejection at the level of mucosal immunity may not be reflected at the systemic level (74). The protective role depends on an antibody-dependent cell-mediated cytotoxicity, opsonization, the activation of innate humoral factors, removal and further elimination of already formed immune complexes within epithelial cells and lamina propria. IgA is able to diminish absorption of an entire antigen as well as a part of it on mucosal tissues. In comparison with IgG, which after antigen-recognition activates complement resulting in inflam-

mation, IgA acts as an inhibitor or directly avoids the adherence of antigen (81). S-IgA in a complex with an antigen is not able to efficiently activate the complement pathway. IgA antibodies have been reported to be a part of the natural antibody pool showing the characteristics of polyreactivity and hypothesized to act as the first barrier defense (82).

The uterine cervix participates in the local immune reaction by the presence of immunoglobulin-producing cells in a complex mixture known as cervical mucus/fluid/plasma. The fluid located in and around the cervix. Cervical mucus is composed mostly of water, up to 90%, depending on the menstrual cycle. Its composition is based on a glycoprotein web filled by mucus rich in immunocompetent proteins, electrolytes (calcium, sodium and potassium), simple sugars such as fructose and glucose, amino acids, C3 and C4 complement components, Th1 and Th2 cytokines, PGE, and trace elements (zinc, copper, iron, manganese, selenium). The misbalance in its content is frequently associated with immune infertility and spontaneous abortion (83-86). The value of pH is alkaline especially at ovulation in order to allow sperm survival by the elevated level of water and electrolytes. After menstruation, cervical mucus becomes rather acidic. Acidic pH is characteristic for vaginal mucus as well (87).

The basic role of cervical mucus consists in a barrier blocking uterus entrance. It is connected to "stick and thick" properties and acts as a natural lubricant because of its glycerol content. Its amount is not hormone-dependent. The mucus functions also as a transport and nourishing medium for sperm by being less concentrated, transparent with a lower amount of immuno-competent agents and high fructose level, which is essential for efficient sperm metabolism. The sugar level is progesterone dependent (21, 84, 85). On the other hand, the cervix always acts as a reservoir for sperm after sexual intercourse. Regarding the iso-immunization within the entire menstrual cycle, cervical mucus contains the antibodies directed to sperm. Their level is then crucial for sperm-cervical mucus penetration and following fertilization. ASA-positive female patients have been commonly diagnosed with immune infertility. An iso-immunization rate is observed by a local ASA level which determines the appropriate treatment (84, 85, 88). It has been shown (86) that ASA present in cervical mucus are of an agglutinating character. These locally produced ASA do not differ from those systemically produced, thus they affect sperm capacitation, acrosome reaction and may interfere with ZP penetration as well as embryo implantation. The peak of ASA in IgA and IgG fraction is reached at the luteal and follicular phases of the menstrual cycle. In contrast, their level is lowest at ovulation. The peak is related to the highest level of estradiol, usually one day before ovulation (77).

Conclusion

Infertility has been defined as reproductive failure and recognized as a disease. Idiopathic infertility correlates with certain immune aspects such as natural tolerance, in addition to the levels of immunoglobulins and specific antibodies in local and systemic secretions. Sperm displays a very heterogeneous antigenic content and has highly auto- as well as iso-antigenic potential. SF, a protective and nutritive sperm medium, is the first contact with the local immune system of the female genital tract, thus representing the potential antibody-targets. Mucosal immunity in the female genital tract is influenced by the level of natural and specific antibodies, cytokines and hormones. Seminal components also bind to the acrosomal sperm to protect it and are then carried together with it into the higher female genital tract. Iso-immunization has been associated with female immune infertility. The thorough comprehension of this pathophysiological process consists of the determination of antibody isotype mostly involved in antigen targeting; and on the other hand, consists of the characterization and identification of semen antibody-binding proteins. In particular, early determination of serum seminal/sperm-specific immunoglobulin G subclasses may make patient profiling more precise and complete the information for diagnosis. Furthermore, based on our studies, anti-seminal/sperm IgG_1 and IgG_4 could be of interest for further therapy targets. The identification of uniform auto- and iso-immunization markers would contribute to a comprehensive, detailed patient diagnosis.

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Original Article

Evaluation of Risk Factors Associated with Endometriosis in Infertile Women

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Abstract

Background: Endometriosis affects women's physical and mental wellbeing. Symptoms include dyspareunia, dysmenorrhea, pelvic pain, and infertility. The purpose of this study is to assess the correlation between some relevant factors and symptoms and risk of an endometriosis diagnosis in infertile women.

Materials and Methods: A retrospective study of 1282 surgical patients in an infertility Institute, Iran between 2011 and 2013 were evaluated by laparoscopy. Of these, there were 341 infertile women with endometriosis (cases) and 332 infertile women with a normal pelvis (comparison group). Chi-square and t tests were used to compare these two groups. Logistic regression was done to build a prediction model for an endometriosis diagnosis.

Results: Gravidity [odds ratio (OR): 0.8, confidence interval (CI): 0.6-0.9, P=0.01], parity (OR: 0.7, CI: 0.6-0.9, P=0.01), family history of endometriosis (OR: 4.9, CI: 2.1-11.3, P<0.001), history of galactorrhea (OR: 2.3, CI: 1.5-3.5, P=0.01), history of pelvic surgery (OR: 1.9, CI: 1.3-2.7, P<0.001), and shorter menstrual cycle length (OR: 0.9, CI: 0.9-0.9, P=0.04) were associated with endometriosis. Duration of natural menstruation and age of menarche were not correlated with subsequent risk of endometriosis (P>0.05). Fatigue, diarrhea, constipation, dysmenorrhea, dyspareunia, pelvic pain and premenstrual spotting were more significant among late-stage endometriosis patients than in those with early-stage endometriosis and more prevalent among patients with endometriosis than that of the comparison group. In the logistic regression model, gravidity, family history of endometriosis, history of galactorrhea, history of pelvic surgery, dysmenorrhoea, pelvic pain, dysparaunia, premenstrual spotting, fatigue, and diarrhea were significantly associated with endometriosis. However, the number of pregnancies was negatively related to endometriosis.

Conclusion: Endometriosis is a considerable public health issue because it affects many women and is associated with the significant morbidity. In this study, we built a prediction model which can be used to predict the risk of endometriosis in infertile women.

Keywords: Case-Comparison Study, Endometriosis, Infertility, Symptoms

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Introduction

Endometriosis is the benign proliferation of functioning endometrial glands and stroma, located outside of the uterine cavity. It is diagnosed by laparoscopic observation of lesions, nodules, or cysts on the pelvic peritoneum or the pelvic organs (1), and is one of the most common diseases in gynecology field (2), as well as a source of an exorbitant economic burden in public health field (3). Endometriosis could be considered as an epigenetic, hormonal regulated disease (4, 5) which is progesterone resistance, and estrogens promote perilesional angiogenesis and neo-innervation and allow endometriotic foci to growth. Moreover, estrogens may contribute to decreases in the local immune surveillance by Peritoneal Fluid Mononuclear Cells (6, 7) and enhance the pro-inflammatory microenvironment typical of the disease (8, 9). Endometriosis, as an enigmatic disease, is responsible for chronic pelvic pain, dysmenorrhea, menorrhagia, dyspareunia and infertility (2, 10-12). The range of the variable influence on the resulting pain syndrome in endometriosis is very wide, for example, classified according to the revised American Society for Reproductive Medicine (rASRM) classification, previous surgical procedures, Douglas obliteration, extent of the sub-peritoneal infiltration and pelvic wall implants (13). It has been observed that there is no relation between the intensity of the pain experienced and stages of disease. Regardless of disease stage, women with endometriosis seem to have similar menstrual patterns and ages at menarche (12). Some studies have revealed an increased risk of other diseases among the women with endometriosis (14, 15). Approximately, one half of the infertile women facing surgery are diagnosed with endometriosis (16). Despite this relatively high prevalence and morbidity, little information has been published about the risk factors for endometriosis in infertile women, who are more likely to have endometriosis as an underlying cause of their infertility.

The relationship between endometriosis in infertile women and clinical symptoms is a complex association, which is influenced by multiple factors including psychological, different cultural conditions, ethnic, and climatic conditions. Therefore, the aim of this study is to determine the demographic, personal characteristic, reproductive variables, contraception and menstruation pattern associated with the presence of endometriosis in infertile women. We also investigated the parameters that might predict the risk of an endometriosis diagnosis.

Materials and Methods

In retrospective study, the subjects were 1282 currently infertile [the failure to achieve a clinical pregnancy after 1 year or more of regular unprotected sexual intercourse (17) women aged 16-46 years who underwent laparoscopy between 2011 and 2013. The study was approved by the Institutional Review Board of the Royan Institute Research Center and the Royan Ethics Committee according to the Helsinki Declaration; signed informed written consent was obtained from all participants. The information was collected by using a self-administered questionnaire, which included questions about demographic characteristics, family history, health of reproductive and infertility, symptoms and physical characteristics. Characteristics of the menstrual cycle included age at menarche, average length of menstrual bleeding and cycle, previous use of oral contraceptives (OC, total number of years taken), previous usage of intrauterine device (IUD), age at first pregnancy and pelvic pain.

The most common indications for laparoscopy were as follows: symptoms of endometriosis, such as dyspareunia, dysmenorrhea and pelvic pain, unexplained factor in infertility, uterine abnormality and tubo-peritoneal disorder. After laparoscopy, we divided the 1282 participants into three groups, shown in the diagram. The first group of 341 patients had visual lesions of endometriosis (52 had stage I, 85 had stage II, 111 had stage III, and 84 had stage IV); the second group of 609 patients had adhesions, fibroids, leiomyomas, and/or uterine abnormalities; the third group of 332 subjects had no visual lesions of endometriosis, i.e., a normal pelvis without any complications (Fig.1). In analysis, the second group was excluded in order to evaluate the risk factors in women with endometriosis (pure endometriosis) compared with the group with a normal pelvis and no other complications.

All clinicians of the study were required to collect the following information from all participants: history of infertility, gravidity, parity, ectopic pregnancies, abortion, pathologies of the reproductive tract (e.g., sexually transmitted diseases, pelvic inflammatory disease (PID), and salpingitis), any observations during surgery (e.g., uterine anomalies,

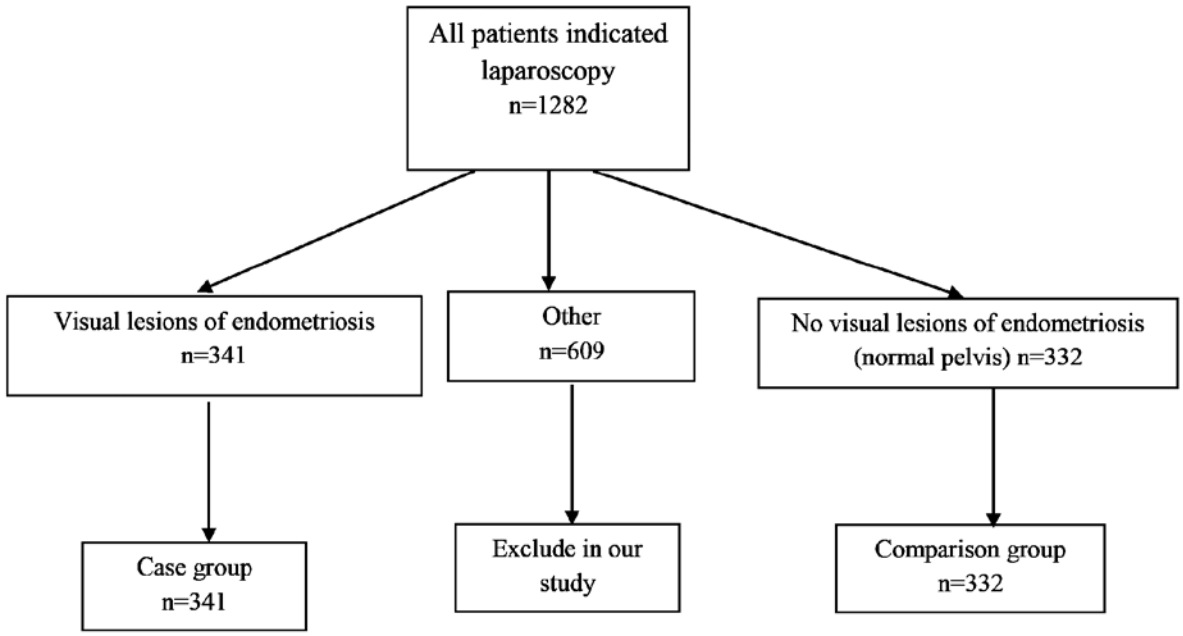

Fig.1: Flow chart showing longitudinal analysis of the population.

tubal obstruction, leiomyoma, and adhesions), and any medication taken on a regular basis. Weight and height of all female infertility referring to the Center are measured during the first visit. Body mass index (BMI) is defined as weight divided by height squared (kg/m^2).

Symptoms of endometriosis (in last 6 months) were collected, such as dysmenorrhoea, pelvic pain, dyspareunia, premenstrual spotting, fatigue, diarrhea and constipation. Other symptoms indicative of endometriosis are as follows: irregular bleeding, severe bleeding, bloat, nausea, vomiting, dyschezia and dysuria.

Following surgery, the stage of the disease was defined according to the rASRM as stage I (minimal), stage II (mild), stage III (moderate), and stage IV (severe) (18). In 80.2% of women with endometriosis, histologic confirmation was also made.

Statistical analysis was performed by using SPSS program (Version 18, Chicago, IL, USA), comparing those with a diagnosis of endometriosis and those with a normal pelvis using Chi-square or t tests, as appropriate. In order to predict a diagnosis of endometriosis, we used logistic regression. The data were expressed as means ± SD. Odds ratio (OR) and 95% confidence interval (95% CIs) were also calculated for each factor. In order to build a prediction model, we used stepwise logistic regression in a backward manner. In this model, a P value of 0.15 was used as entry criterion, whereas a P value of 0.1 was the threshold for a variable to stay in the model. The area under the receiver operating characteristic (ROC) curve (AUC) shows the discriminative performance of the fitted logistic model. An AUC equal to 0.5 shows no discriminative performance, whereas an AUC of 1.0 indicates perfect discrimination. In ordinal regression analysis, predictors which probably reflect symptoms of endometriosis (such as dyspareunia, dysmenorrhea and pelvic pain) were examined. P values less than 0.05 were considered to be statistically significant. Moreover, we assessed the calibration of the model by comparing the predicted probability in a category of patients and the observed percentage of endometriosis in that category. According, we categorized the predicted probabilities in 10 groups, based on percentile points with steps of 10% per step. In each category, we compared the mean predicted probability in that particular category with the observed probability, i.e., the number of women with endometriosis in that category divided by the total number of women in that category. The results were plotted graphically.

Results

The prevalence of endometriosis in the total sample of women undergoing laparoscopy was 26.5%. The women's demographic and reproductive characteristics are listed in Table 1. The study group (cases) consisted of 341 infertile women, who were diagnosed to have endometriosis by laparoscopy. The severity of the disease was staged according to the rASRM classification of endometriosis. Endometriosis was staged as 15.2% minimal (rASRM stage I), 24.9% mild (rASRM stage II), 32.5% moderate (rASRM stage III) and 24.3% severe (rASRM stage IV). All these women were infertile [primary infertility in 296 (89.2% of women) and secondary infertility in 36 (10.8% of women)], with mean age of 32.4 ± 4.9 years, mean age at menarche of 13.1 ± 1.2 years, mean duration of infertility of 5.8 ± 1.6 years (Table 1).

As a comparison, the 332 infertile women who referred to the same center for infertility and were laparoscopically confirmed to be without endometriosis were included. All these women were infertile [primary infertility in 296 (87.4%) of women and secondary infertility in 34 (12.6%) of women], with mean age of 31.4 ± 5.2 years, mean age at menarche of 13.1 ± 1.3 years and mean duration of infertility of 6.0 ± 1.8 years (Table 1).

Independent t test analysis showed no significant difference in BMI between the case and the comparison groups (P>0.05). Those with endometriosis did not differ from the comparison group with regard to age at menarche, menstrual status, duration of menstrual bleeding, type of infertility, duration of infertility and cigarette smoking. In contrast, a significant difference was found concerning the length of menstrual cycles, age, gravidity and history of abortion (Table 1).

We also found no association between endometriosis and the use of IUD or previous exposure to OCs. No significant difference was found between the two groups in previous cervical trauma, genital tract abnormality, history of PID and sexually transmitted diseases (STD) (i.e., chlamydia, herpes, condylomas, and gonorrhea).

Table 1: Selected demographic, personal, and lifestyle characteristics of the case and the comparison groups

Parameters	Comparison group	Cases	OR (95% CI)	P value
Age (Y)				
<30	155 (45.5)	118 (35.5)	1†	
30-35	103 (30.2)	126 (38)	1.6 (1.1- 2.2)	0.02
>35	83 (24.3)	88 (26.5)	1.3 (0.9- 2.04)	
Age at menarche (Y)	13.1 ± 1.3	13.1 ± 1.2	0.9 (0.8-1.10)	0.7
Age at marriage (Y)	20.3 ± 3.8	22.1 ± 4.6	1.1 (0.9-1.2)	0.8
Night worker				
No	338 (99.1)	325 (97.9)	1†	
Yes	3 (0.9)	7 (2.1)	2.4 (0.6-9.4)	0.2
Menstruation status				
Irregular	47 (86.2)	42 (12.7)	1†	0.6
Regular	294 (13.8)	290 (87.3)	1.10 (0.7-1.7)	
Menstrual cycle length	31.1 ± 7.8	29.9 ± 7.5	0.9 (0.9-0.9)	0.04
Duration of bleeding menstrual (days)	6.0 ± 1.8	5.8 ± 1.6	0.9 (0.8-1.05)	0.4
History of live birth				
Yes	43 (12.6)	36 (10.8)	1†	
No	298 (87.4)	296 (89.2)	1.1 (0.7-1.9)	0.4
Type of infertility				

Table 1: Continued

Parameters	Comparison group	Cases	OR (95% CI)	P value
Secondary	43 (12.6)	36 (10.8)	1†	0.4
Primary	298 (87.4)	296 (89.2)	1.1 (0.7-1.9)	
Duration of infertility (Y)	6.90 ± 4.3	6.7 ± 4.5	0.9 (0.9-1.03)	0.8
Contraceptive				
None	189 (55.4)	167 (50.3)	1†	
OCP	26 (7.6)	27 (8.1)	1.1 (0.6-2.09)	0.2
IUD	8 (2.3)	3 (0.9)	0.4 (0.1-1.6)	
Other	118 (34.6)	135 (40.7)	1.2 (0.9-1.7)	
Duration of consume contraceptive (month)	30.9 ± 20.09	30.2 ± 20.1	0.9 (0.9-1.009)	0.7
Gravidity	0.5 ± 1.1	0.3 ± 0.8	0.8 (0.6-0.9)	0.01
Parity	0.4 ± 1.08	0.3 ± 0.6	0.7 (0.6-0.9)	0.01
BMI (kg/m^2)	25.6 ± 3.8	25.05 ± 4.01	0.9 (0.9-1.002)	0.06
No. of spontaneous abortion	0.4 ± 1.06	0.2 ± 0.5	0.7 (0.5-0.8)	0.02
Smoking				
No	337 (98.8)	326 (98.2)	1†	
Yes	4 (1.2)	6 (1.8)	1.5 (0.4-5.5)	0.5
Family history of endometriosis				
No	334 (97.4)	301 (90.7)	1†	<0.001
Yes	7 (2.1)	31 (9.3)	4.9 (2.1-11.3)	
History of galactoreahea				
No	302 (88.6)	255 (76.8)	1†	<0.001
Yes	39 (11.4)	77 (23.2)	2.3 (1.5-3.5)	
Abnormality genital tract				
No	259 (76)	272 (81.9)	1†	0.05
Yes	82 (24)	60 (18.1)	0.6 (0.4-1.01)	
History of STD				
No	340 (99.7)	331 (99.7)	1†	0.9
Yes	1 (0.3)	1 (0.3)	1.02 (0.06-16.4)	
History of PID				
No	337 (99.8)	325 (97.9)	1†	0.3
Yes	4 (1.2)	7 (2.1)	1.8 (0.5-6.2)	
History pelvic surgery				
No. surgery	126 (37)	77 (23.2)	1†	
Laparoscopy	180 (52.8)	189 (56.9)	1.7 (1.2-2.4)	
Laparoscopy and laparotomy	11 (3.2)	42 (12.7)	1.6 (0.8-3.08)	<0.001
Laparotomy	24 (7)	24 (7.2)	6.2 (3.03-12.8)	
Previous cervical trauma				
No. trauma	332 (97.7)	317 (95.5)	1†	0.1
Trauma	9 (2.6)	15 (4.5)	1.7 (0.7-4.04)	

Data are presented as n (%) or mean ± SD.
†; Reference category, OR; Odds ratio, CI; Confidence interval, OCP; Oral contraceptives, IUD; Intrauterine device, BMI; Body mass index, STD; Sexually transmitted disease and PID; Pelvic inflammatory disease.

However, patients with endometriosis were significantly more likely to have a family history of endometriosis, a history of galactorrhea, and a history of pelvic surgery (Table 1).

Symptom distribution among patients with early-stage (stage I or II disease) and late-stage (stage III or IV disease) endometriosis is summarized in Table 2, which shows that dysmenorrhea, dyspareunia, pelvic pain, premenstrual spotting, fatigue, diarrhea and constipation were more common among late-stage endometriosis patients than in those with early-stage endometriosis and more prevalent among patients with endometriosis than those with a normal pelvis (Table 2).

Finally, in order to build a prediction model and to find the most important factors affecting endometriosis, we used a logistics regression model in a backward manner. Table 3 shows the result of fitting logistic regression model to the data.

In the logistic regression model, gravidity, family history of endometriosis, history of galactorrhea, history of pelvic surgery, dysmenorrhoea, pelvic pain, dysparaunia, premenstrual spotting, fatigue, and diarrhea were significantly positively associated with endometriosis. However, number of pregnancies was negatively related to endometriosis (Table 3).

Table 2: Distribution of symptoms associated with endometriosis according to disease stage and comparison group

Parameters	Disease stage				Comparison group	P value	OR (95% CI)
	1st	2nd	3rd	4th			
	n (%)	n (%)	n (%)	n (%)	n (%)		
Dysmenorrhoea							
Yes	28 (53.8)	52 (61.2)	79 (71.2)	68 (81)	140 (41.1)		3.1 (2.2.-4.2)
No	24 (46.2)	33 (38.8)	32 (28.8)	16 (19)	201 (58.9)	<0.001	1†
Dysparunia							
Yes	15 (28.8)	32 (37.6)	39 (35.1)	46 (54.8)	68 (19.9)		2.6 (1.8-3.7)
No	37 (71.2)	53 (62.4)	72 (64.9)	38 (45.2)	273 (80.1)	<0.001	1†
Pelvic Pain							
Yes	16 (30.8)	56 (34.1)	52 (46.8)	44 (52.4)	65 (19.1)		3.1 (2.2-4.4)
No	36 (69.2)	29 (65.9)	59 (53.2)	40 (47.6)	276 (80.9)	0.02	1†
Premenstrual spotting							
Yes	7 (13.5)	27 (31.8)	31 (27.9)	41 (48.8)	42 (12.3)		3.3 (2.2-4.6)
No	45 (86.5)	58 (68.2)	80 (72.1)	43 (51.2)	299 (87.7)	<0.001	1†
Fatigue							
Yes	3 (5.8)	12 (14.1)	12 (10.8)	20 (23.8)	16 (4.7)		3.3 (1.8-6.0)
No	49 (94.2)	73 (85.9)	99 (89.2)	64 (76.2)	325 (95.3)	<0.001	1†
Diarrhea							
Yes	2 (3.8)	6 (7.11)	8 (7.2)	9 (10.7)	1 (0.3)		27.6 (3.7-205.5)
No	50 (96.2)	79 (92.9)	103 (92.8)	75 (89.3)	340 (99.7)	<0.001	1†
Constipation							
Yes	3 (5.8)	16 (22.4)	13 (11.7)	16 (19)	32 (9.4)		1.7 (1.09-2.8)
No	49 (94.2)	66 (77.6)	98 (88.3)	68 (81)	309 (90.6)	0.003	1†

†; Reference category, OR; Odds ratio and CI, Confidence interval.

The AUC shows the discriminative performance of the logistic model. The AUC value equal to 0.5 shows no discriminative performance, while AUC value of 1.0 indicates perfect discrimination. The AUC value for the fitted logistic model was 0.78 (95% CI 0.74-0.81), showing a good predictive performance for the fitted logistic regression model (Fig.2). We checked the goodness of fit of our model by the Hosmer-Lemeshow goodness of fit test. The P value for this test was 0.13 showing good predictive performance of our model. Also validity of the model was assessed by calibration plot; all predicted probabilities were almost similar to the observed rate of endometriosis in each group and show the good calibration of the model.

Table 3: Result of fitting multiple logistic regression

Parameters	OR (95% CI)	P value
Gravidity	0.8 (0.6-0.9)	0.04
Family history of endometriosis	2.7 (1.06-7.1)	0.03
History of galactoreahea	1.8 (1.1-3.05)	0.01
History of pelvic surgery		
No. surgery	1†	
Laparoscopy	3.4 (1.5-7.8)	<0.001
Laparotomy	4.1 (2.4-6.8)	
Laparoscopy and laparotomy	14.5 (6.1-34.2)	
Dysmenorrhoea	1.8 (1.1-2.8)	0.006
Pelvic pain	4.1 (2.4-6.8)	<0.001
Dysparunia	1.6 (1.09-2.4)	0.01
Premenstrual spotting	2.2 (1.3-3.6)	0.002
Fatigue	2.6 (1.3-5.1)	0.006
Diarrhea	19.06 (2.4-150.6)	0.005
Area under ROC curve (95% CI)=0.78 (0.74-0.81)		

†; Reference category, OR; Odds ratio, CI; Confidence interval and ROC; Receiver operating characteristic.

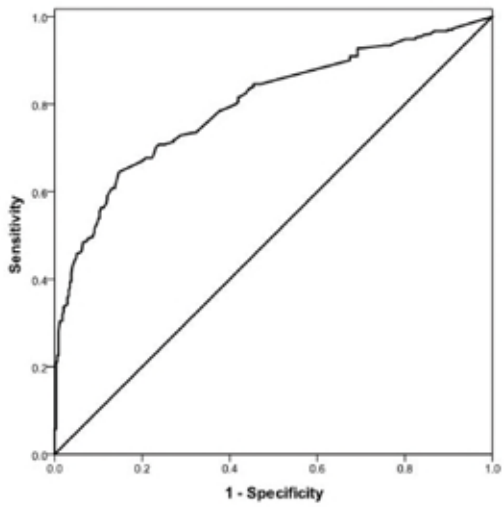

Fig.2: Receiver operating characteristic (ROC) curve for assessment discriminative preformance of logistic regression.

Discussion

Endometriosis is one of the most common gynecological diseases in the different countries. It is a confusing disease with little known about its distribution, true prevalence and risk factors in the population (19). In our study, prevalence of endometriosis in the total sample of women undergoing laparoscopy was 26.5%. Ozkan et al. (20) found endometriosis has a prevalence of 25-40% in infertile women. The goal of this study was to investigate the relation between some relevant factors and risk of an endometriosis diagnosis. Our study represents an analysis of 673 infertile women undergoing laparoscopy (cases and comparison group) for further understanding of the different risk factors and associated symptoms of endometriosis with infertility.

A significant difference was observed between the average age of infertile women with and without endometriosis (32.4 ± 4.9 vs. 31.4 ± 5.2). Increasing age, alcohol use, low body weight, family history of endometriosis, early menarche, prolonged menstrual flow, and short cycle interval, intercourse during menses, infertility are also alleged risk factors (21, 22). Our results show no significant difference association between BMI or smoking intake and endometriosis. This is consistent with the several studies showing no association with these parameters and endometriosis (22, 23). Some authors have reported inverse relation between BMI and endometriosis (24, 25), although in these latter studies the comparison group was not infertile women.

The present study indicated a higher rate of endometriosis among more educated women (data not shown), is consistent with the results of other studies (2, 26). The possible association of endometriosis with higher education level is due to a delay in childbearing. The association between education level and endometriosis probably reflects the socioeconomic issues, such as access to the medical care. The absence of gravidity in endometriosis group was associated with significantly increased odds of suffering from endometriosis; a finding that is consistent with several other reports (26, 27). However, spontaneous abortions and ectopic pregnancy were not linked to endometriosis (14). In the present study, we did not find any significant difference between duration of infertility and endometriosis. Akande et al. (28) reported that the effects of duration of infertility and primary infertility were not observed to be statistically significant for women with endometriosis. Several studies show that prolonged duration of infertility itself may be a precursor of endometriosis in the absence of other causes (29, 30). Duration and heaviness of flow and premenstrual spotting were also risk factors for endometriosis ($P<0.05$). The majority of studies to date have reported that early menarche (<11 years) increases the risk of endometriosis (23, 31, 32), but our result did not find any significant difference between age of menarche and endometriosis. Peterson et al. (33) reported that there was no relationship between endometriosis and menstrual cycle history, including average cycle length, number of menstrual cycles, and age at menarche.

Cramer et al. (32) found in their case-control study that women with infertility associated with endometriosis had a lower age at menarche, shorter menstrual cycles and longer duration of menstrual bleeding than those of the control group. Likewise, we found no significant difference between the heavy menstrual flow and the risk of endometriosis. Treloar et al. (34) have also reported the same result, even though heavy flow is associated with endometriosis in the other studies (1, 35). A recent study has reported that a shorter cycle length is associated with an increasing risk of endometriosis, but none of these studies has examined specifically this association before the onset of symptoms (1, 36). Overall, these findings support the Sampson's theory of retrograde menstruation, in which women with greater opportunity for menstrual contamination of the pelvis are at increased risk of endometriosis (34, 37).

We found significant correlation between length of cycle and the presence of endometriosis, but other studies reported no significant association between length of cycle, length of menses, as well as age at menarche with the presence of endometriosis (2, 34). Multiple lines of evidence have indicated that endometriosis is associated with increased exposure to menstruation, an assumption supporting the ret-

rograde menstruation theory (1, 36). Menstrual factors, previously shown to be associated with endometriosis, include early menarche, shorter cycle length, longer menses, late pregnancies and longtime delay between first pregnancy and menarche (2). However, the association between menstrual factors and endometriosis remains unclear because some studies fail to show a relationship between these factors and the disease (27, 38). Nevertheless, other studies found that women with endometriosis reported apparently either shorter or longer and heavier menstruation than normal cycle (39, 40). Stovall et al. (41) have found menstrual cycle ≤27 days as a risk factor that seems to be associated to endometriosis in infertile women.

The results of the present study showed that the stage of endometriosis, according to the rASRM classification, is related to the presence of pain. We found a significant difference between stage and symptoms of endometriosis.

Vercellini et al. (42) reported that endometriosis associated symptoms of endometriosis, such as dyspareunia, dysmenorrhea and pelvic pain and endometriosis stage is directly related to the persistence of that symptom. Arruda et al. (43) reported disease stage was significantly associated with severity of dysmenorrhoea and non-menstrual pain.

The Italian study (44) showed no relationship between the intensity of pain and the stage of the disease. In another study, on 469 women aged 18-45 years old revealed that no clear-cut association between stage, site or morphological characteristics of pelvic endometriosis and pain (45).

In our study, infertile women who experienced dysmenorrhea were more likely to have endometriosis rather than women reported no pain during their menstruation. Therefore, if more severe dysmenorrhea is associated with increased risk of contractility and expulsion menstrual debris into the pelvic, severe cramps may suggest susceptibility to the disease (1). Pelvic pain is often used as a diagnostic tool for endometriosis with dysmenorrhea being the most commonly reported symptom (14, 27). Pelvic pain might predispose women to endometriosis via retrograde menstruation (23). There was also a significant difference of increasing risk of endometriosis with the reported pelvic pain, dysmenorrheal and dyspareunia (12). In our study, we found significant difference between endometriosis and pelvic pain occurring during ovulation in contrast to what was found by Treloar et al. (34). The cul-de-sac and uterosacral ligaments are the most common sites of endometriosis (46). Dyspareunia may be common complaint among women with endometriosis because these areas are stretched during intercourse (9).

Use of contraception, as OCs and IUD, are also known to affect menstrual flow. If retrograde menstruation is involved in the etiology of endometriosis, usage of IUD (a common cause of menorrhagia) would be expected to increase the risk of the disease. Hughes et al. (47) have suggested that use of IUD not influence the development of endometriosis. In other study, OC exposure was associated with a lower risk of endometriosis (48). Our results indicate no significant difference between IUD and OCs exposure and endometriosis in infertile women.

In the present study, we observed a positive correlation between the previously operated pelvic and endometriosis. In addition, family history of endometriosis was prevalent among the patients with the disease compared with patients with a normal pelvis ($P<0.001$). This point was also confirmed by the other authors (49-51). These data appear to confirm that a familial tendency toward endometriosis, and also suggest that genetic risk factor in the pathogenesis of endometriosis exist (33).

Interestingly, in our study, the endometriosis group commonly reported constipation (61.4 vs. 38.6%) and diarrhea (96.2 vs. 3.8%), suggesting that irritable bowel syndrome be considered as a co-morbidity.

Finally, various factors may be useful in screening for endometriosis and predict risk of an endometriosis diagnosis. Patient characteristics, gravidity, family history of endometriosis, history of galactorrhea, history of pelvic surgery, dysmenorrhoea, pelvic pain, dysparaunia, premenstrual spotting, fatigue, diarrhea, and the number of pregnancies have been evaluated as predictors of endometriosis. Thus, infertility center should have enough information about the symptoms of endometriosis in order to pro-

vide more information for patients. The findings suggest that gravidity, family history of endometriosis, history of galactorrhea, history of pelvic surgery, dysmenorrhoea, pelvic pain, dysparaunia, premenstrual spotting, fatigue, and diarrhea were significantly positively associated with endometriosis. However, number of pregnancies was negatively related to endometriosis.

Our models can be used with the almost and highest reliability as a guide to screen for endometriosis, in patients comparable to the developing population. The effects of using these models in patient care have to be further investigated. In addition to high prevalence of endometriosis, consultation in relation to risk factors for endometriosis will be helpful for early detection and prevention of disease.

Only a prospective cohort study can specify to what extent any of these characteristics indicate risk-factors for different stages of endometriosis. Although our results show that there is a relationship between pain symptoms, disease severity and infertility, it may also help to focus the future of epidemiologic studies regarding prevention and treatment for the endometriosis.

Conclusion

There is a decreasing risk of endometriosis in currently infertile women with history of pregnancy and an increased risk in infertile women reporting a history of dysmenorrhea, family history of endometriosis, history of galactorrahea, history of pelvic surgery, dysmenorrhoea, pelvic pain, dysparunia, premenstrual spotting, fatigue and diarrhea. Due to the high prevalence of endometriosis, consultation in relation to risk factors for endometriosis, we will be helpful for early screening, detection and prevention of disease.

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Original Article

Metabolic and Endocrine Characteristics of Indian Women with Polycystic Ovary Syndrome

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Abstract

Background: Polycystic ovary syndrome (PCOS) is one of the most common endocrinological disorders among women of reproductive age and the leading cause of female infertility. This study intends to evaluate the lipid profile, hormonal levels [free T3 (fT3), free T4 (fT4), thyroid stimulating hormone (TSH), insulin, luteinizing hormone (LH), follicle stimulating hormone (FSH), and prolactin] in PCOS women from Nellore and its surrounding districts of Andhra Pradesh, India.

Materials and Methods: This cross-sectional study included 80 newly diagnosed PCOS women and an equal number of age and body mass index (BMI) matched healthy controls. We used the photometry methods to determine serum glucose levels and the lipid profile. An immunoturbidometry method was employed to measure high sensitive C-reactive protein (hsCRP). All hormonal parameters were measured using chemiluminescence immunoassays. Insulin resistance was evaluated using the homeostatic model assessment-insulin resistance (HOMA-IR) method. Statistical analysis was done using SPSS software version 20.0.

Results: The PCOS patients presented statistically higher levels of total cholesterol (TC), triglycerides (TG) and low density lipoprotein cholesterol (LDL-c, $P<0.0001$) when compared to those of controls. PCOS patients had elevated fasting glucose, hsCRP, fasting insulin, TSH, LH and prolactin levels ($P<0.001$). An increased LH/FSH ratio (>1.5) was seen in women with PCOS compared with control women. In addition, we observed a direct correlation between fasting insulin with fasting glucose and HOMA-IR. LH was inversely proportional to BMI.

Conclusion: The present study showed a higher prevalence of insulin resistance, dyslipidemia, and hypothyroidism in PCOS women. Furthermore this study showed increased LH concentrations, a higher LH/FSH ratio, and higher prolactin levels in PCOS women.

Keywords: Polycystic Ovary Syndrome, Gonadotropin Hormones, Insulin Resistance, Dyslipidemia, Hypothyroidism

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrinological disorders among adolescent girls and women of reproductive age. PCOS is the leading cause of female infertility (1). Menstrual irregularity, chronic anovulation, hyperandrogenism, and multiple small sub-capsular cystic follicles in the ovary on ultrasonography characterize the syndrome. PCOS is associated with insulin resistance, increased risk of type 2

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diabetes mellitus and cardiovascular disorders (2). Obesity, mainly central obesity, is present in varying degrees (30-70%) in women with PCOS (3, 4). Central obesity, being a prominent feature of the so-called metabolic syndrome, is directly linked to increased peripheral insulin resistance (5). It has been shown that insulin resistance is responsible for the development of polycystic ovaries in PCOS women although obesity seems to be the major cause (6). Hence pathogenic determinants of PCOS include insulin resistance and β-cell dysfunction. Therefore, women with PCOS have an increased risk for type 2 diabetes (7).

The majority of studies that evaluated the prevalence of glucose intolerance in PCOS primarily included obese women, which aggravated their risk for glucose intolerance. Likewise, a high prevalence of abnormal glucose intolerance has also been documented in women with PCOS (6). An elevated luteinizing hormone/follicle stimulating hormone (LH/FSH) ratio is typically seen in PCOS patients. This elevated ratio was considered as a gold standard for clinical diagnosis of the disease (8) before the proposal of the Rotterdam criteria. However LH/FSH levels, as a gold standard, became controversial after a number of studies have reported a variable prevalence of these ratios (30-90%) among PCOS women (9, 10). An ethnic variation of the metabolic and endocrine pattern in PCOS was also reported (11-13).

All features of this syndrome may not be present in an individual patient (2, 13). Depending on the interactions of different hormones in PCOS patients, the pathogenesis, clinical presentation, and biochemical profile varies in an individual. In general, the treatment of PCOS patients is targeted towards regularization of menses and recover of fertility. PCOS women are at high risk of developing type 2 diabetes, cardiovascular disorders, and endometrial carcinoma (2, 7, 9). Hence, in addition to symptomatic relief, correction of the underlying endocrinological pathology and biochemical abnormalities at the earliest is necessary. Hence biochemical parameters and the hormone profile become important in understanding the pathogenesis of PCOS. Assessment of the lipid profile, glycemic status and endocrine status in PCOS patients may help in making a decision regarding treatment, better outcome, differential diagnosis, and prognosis of the disease. With this background we have planned the present study to assess the metabolic profile and endocrine pattern of PCOS women from Nellore and its surrounding districts of Andhra Pradesh, India.

Materials and Methods

We conducted this cross-sectional study at Narayana Medical College and Hospital, Nellore, Andhra Pradesh, India during the period of October 2012 to January 2014. The study comprised 80 newly diagnosed PCOS women and an equal number of age and body mass index (BMI) matched healthy females as controls. All participants were in the age group of 19 to 35 years. Patients have been diagnosed with PCOS on the basis of the Rotterdam criteria (14). A total of two out of three of the following are required for diagnosis: oligo- and/or anovulation (defined by the presence of oligomenorrhea or amenorrhea); clinical and/or biochemical signs of hyperandrogenism [defined by presence of hirsutism (Ferriman-Gallwey score ≥6), acne or alopecia, and/or elevated androgen levels]; and polycystic ovaries by gynecological ultrasound. We excluded patients with congenital adrenal hyperplasia, Cushing's syndrome, androgen-secreting tumors, known hypothyroidism on treatment and intake of any medication that affected endocrinal parameters.

Height and weight were obtained from each subject. The BMI was calculated as the weight in kilograms divided by the square of height in meters. About 5 ml of blood was collected from the antecubital vein. Fasting blood samples were collected in plain and sodium fluoride tubes, and then centrifuged at 3500 rpm for 10 minutes to separate the serum. Analysis of total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-c) and glucose were performed using commercial kits available for a fully automated Humastar 600 biochemistry analyzer (Germany). We used Friedwald's formula to calculate low density lipoprotein cholesterol (LDL-c) and very low density lipoprotein (VLDL-c) levels. High sensitive C-reactive protein (hsCRP) levels were measured by immunoturbidometry (hsCRP Reagent Kit, CRP-ULTRA - turbilatex, Spinreact, Spain). Hormones free T3 (fT3), free T4 (fT4), thyroid stimulating hormone (TSH), LH, FSH, prolactin and insulin were measured by the chemilumines-

cence immunoassay (CLIA) method using a Beckman Coulter Access fully automated analyzer. The hormone kits used in the Beckman Coulter Access analyzer (USA) were from Beckman Coulter, Ireland. We estimated insulin resistance by the homeostatic model assessment-insulin resistance (HOMA- IR) method (15).

Statistical analysis

All the results were tabulated as mean and standard deviation. We used the SPSS 20.0 version for statistical analysis. The unpaired student t test was used to determine the statistical significance between the study groups. Pearson correlation was used for correlating different parameters. A P value of <0.05 was considered to be statistically significant.

Ethical considerations

The study was approved by the Narayana Medical College and Hospital Institutional Ethics Committee, Nellore, Andhra Pradesh, India. Written and informed consent was obtained from the individuals who have participated in the study.

Results

There were 80 clinically proved, confirmed PCOS patients in the age range of 19-35 years chosen for the study. The mean age of controls was 26.7 ± 3.4 years and for PCOS patients, it was 25.6 ± 3.9 years (P=0.06). The mean BMI for controls was 26.1 ± 4.2 kg/m^2 and 27.0 ± 5.6 kg/m^2 for PCOS patients (P=0.25). There was no significant statistical difference in age or BMI between PCOS women and controls. We assessed both PCOS women and controls for serum lipid profile, fasting blood sugar, fasting insulin, insulin resistance (HOMA-IR), thyroid profile, LH, FSH, LH/FSH ratio and prolactin levels. Comparisons were made between the two groups. The results of biochemical and hormonal profile findings of this study are shown in Table 1.

As seen in Table 1, PCOS patients had higher TC, TG, LDL-c and VLDL-c levels (P<0.001) compared to controls which indicated that PCOS women had dyslipidemia. On the other hand, HDL-c showed no significant statistical difference (P=0.481) between the groups. Fasting glucose, fasting insulin and insulin resistance showed a significant increase (P<0.001) in cases compared to controls. Figure 1 shows a graphical representation of the mean values of the thyroid and gonadotropin hormones. PCOS patients had increased TSH compared to controls (P<0.0001). LH and prolactin showed increase levels (P<0.0001) whereas FSH (P<0.0001) showed mildly decreased levels in PCOS women compared to healthy controls (Table 1).

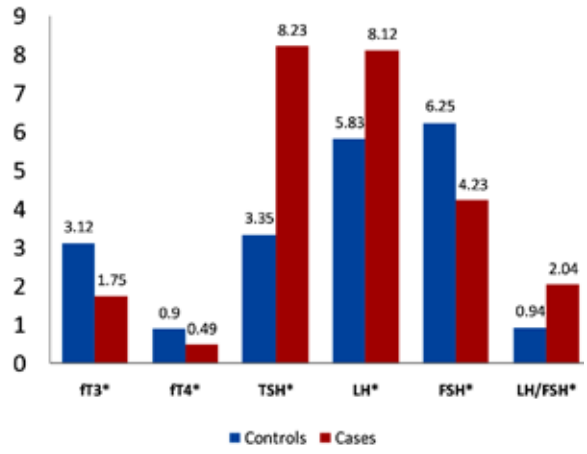

Fig.1: Mean values for gonadotropin hormones in control women and polycystic ovary syndrome (PCOS) women (cases). *; P<0.0001, fT3; Free T3, fT4; Free T4, TSH; Thyroid stimulating hormone, LH; Luteinizing hormone and FSH; Follicle stimulating hormone.

Significant positive correlations between BMI with fasting insulin (r=0.493) and insulin resistance (r=0.401, P<0.01) and a significant negative correlation with LH (r=-0.279, P<0.01) were found in PCOS patients (Table 2). hsCRP positively correlated with fasting glucose (r=0.816), fasting insulin (r=0.518), insulin resistance (r=0.609), LH/FSH ratio (r=0.631) and prolactin (r=0.688, P<0.01), and had a negative correlation with FSH (r=-0.514, P<0.001). Fasting glucose showed positive correlations with fasting insulin (r=0.703), insulin resistance (r=0.811), LH/FSH ratio (r=0.615), and prolactin (r=0.699, P<0.01), and a negative correlation with FSH (r=-0.533, P<0.01). Similarly, fasting insulin also showed significant positive correlations with insulin resistance (r=0.981) and prolactin (r=0.542, P<0.01). TSH showed positive correlations with BMI, hsCRP, fasting glucose, fasting insulin, insulin resistance, LH/FSH ratio and prolactin (r=0.173, P<0.05, r=0.757, r=0.772, r=0.499, r=0.583, r=0.492, r=0.693,

respectively and P<0.01). LH showed a positive correlation with LH/FSH ratio (r=0.556, P<0.01). FSH showed a negative correlation with LH/FSH ratio (r=-0.571, P<0.01). The LH/FSH ratio showed a positive correlation with prolactin (r=0.469, P<0.01).

Table 1: Serum concentrations of lipid profiles and hormones in normal controls and polycystic ovary syndrome (PCOS) women

Parameters and their normal ranges	Controls (Mean ± SD)	PCOS cases (Mean ± SD)	P value
TC (<200 mg/dl)	181 ± 32.9	214 ± 35.7	0.0001
TG (60-165 mg/dl)	105 ± 48.9	189 ± 42.9	0.0001
HDL-c (45-65 mg/dl)	47.5 ± 8.5	46.6 ± 7.6	0.481
LDL-c (<130 mg/dl)	114 ± 20.1	130 ± 30.2	0.001
VLDL-c (12-40 mg/dl)	21 ± 9.8	37.3 ± 8.8	0.001
Fasting glucose (70-110 mg/dl)	86.0 ± 9.1	127 ± 11.4	0.0001
Fasting insulin (0.7-9.0 µlU/ml)	7.4 ± 1.8	13.4 ± 5.3	0.001
HOMA-IR (<2.0)	1.6 ± 0.5	4.3 ± 2.0	0.001
fT3 (2.50-3.90 pg/ml)	3.1 ± 0.3	1.8 ± 0.7	0.0001
fT4 (0.34-5.60 mIU/L)	0.9 ± 0.2	0.5 ± 0.1	0.0001
TSH (0.34-5.60 mIU/L)	3.4 ± 1.3	8.2 ± 2.4	0.0001
hsCRP (<5 mg/L)	1.9 ± 1.2	8.5 ± 2.7	0.0001
LH (0.5-10.5 mIU/L)	5.8 ± 1.7	8.1 ± 3.0	0.0001
FSH (3.0-13.0 mIU/L)	6.3 ± 1.9	4.2 ± 1.6	0.0001
LH/FSH (<1.2)	0.9 ± 0.2	2.0 ± 0.8	0.0001
Prolactin (1.2-19.5 ng/ml)	13.5 ± 3.5	50.7 ± 27.1	0.0001

TC; Total cholesterol, TG; Triglycerides, HDL-c; High-density lipoprotein cholesterol, LDL-c; Low-density lipoprotein cholesterol, VLDL; Very low density lipoprotein, HOMA-IR; Homeostatic model assessment-insulin resistance, fT3; Free T3, fT4; Free T4, TSH; Thyroid stimulating hormone, hsCRP; High sensitive C-reactive protein, LH; Luteinizing hormone and FSH; Follicle stimulating hormone.

Table 2: Pearson correlation values for different parameters among polycystic ovary syndrome (PCOS) women

	BMI	hsCRP	Fasting glucose	Fasting insulin	HOMA-IR	TSH	LH	FSH	LH/FSH	Prolactin
BMI	1	0.158*	0.203*	0.439**	0.401**	0.173*	-0.279**	-0.207**	0.040	0.272**
hsCRP		1	0.816**	0.518**	0.609**	0.757**	0.279**	-0.514**	0.631**	0.688**
Fasting glucose			1	0.703**	0.811**	0.772**	0.256**	-0.533**	0.615**	0.699**
Fasting insulin				1	0.981**	0.499**	0.021	-0.410**	0.409**	0.542**
HOMA- IR					1	0.583**	0.049	-0.469**	0.466**	0.603**
TSH						1	0.222**	-0.440**	0.492**	0.693**
LH							1	0.244**	0.556**	0.089
FSH								1	-0.571**	-0.481**
LH/FSH									1	0.469**
Prolactin										1

*; Correlation is significant at the 0.05 level (2-tailed), **; Correlation is significant at the 0.01 level (2-tailed), BMI; Body mass index, hsCRP; High sensitive C-reactive protein, HOMA-IR; Homeostatic model assessment-insulin resistance, TSH; Thyroid stimulating hormone, LH; Luteinizing hormone and FSH; Follicle stimulating hormone.

Discussion

PCOS is multi-factorial endocrine disorder associated with derangement in the metabolic profile and endocrine pattern. In the present study we have attempted to explore the changes in metabolic and hormonal parameters in PCOS women from Nellore and its surrounding districts of Andhra Pradesh, India. Out of 80 PCOS women recruited for the study, 42 women were in the age range of 20 to 25 years, 29 women were 25 to 30 years of age, and 9 women were 30 to 35 years of age. There were 15 patients who presented with a BMI lower than 20 kg/m^2, 10 patients showed a normal BMI (20 to 25 kg/m^2), 29 patients were overweight (BMI 25 to 30 kg/m^2), and 26 patients were obese (BMI>30 kg/m^2). The most common abnormalities seen in PCOS are increased BMI, low HDL-c levels and elevated TG. In the present study an abnormal lipid profile was found in women with PCOS. The findings of elevated TC, TG, LDL-c and HDL-c agreed with those of Naidu et al. (2), Zhang et al. (16), Kim and Choi (17), Talbott et al. (18), and Saha et al. (19).

PCOS may represent (20) a major segment of the female population at a risk of cardiovascular disease, which may be related to increased VLDL-c levels. As shown by Wetterau et al. (21), this increase in VLDL-c is basically due to insulin resistance. Insulin normally inhibits the expression of microsomal triglyceride transfer protein that is responsible for apo-B and VLDL secretion. Hence, insulin resistance may be responsible for increased VLDL secretion in PCOS individuals (22). In the present study we have observed that 70% of PCOS patients exhibited an abnormal lipid profile and the mean values of cholesterol, TG, LDL-c and, VLDL-c were increased.

Hypothyroidism is the disease state caused by insufficient production of thyroid hormone by the thyroid gland. Some authors have affirmed that insulin resistance and increased androgen production can cause hypothyroidism. Insulin resistance has also been considered to be the principal factor in the genesis of PCOS (23). In our study, we observed an increased level of serum TSH and decreased level of fT3 and fT4 hormones. There were 29 out of 80 PCOS patients with TSH levels <5.5 mIU/L, 37 patients reported TSH levels in the range of 5.5 to 10 mIU/L, and 14 patients presented with TSH levels >10 mIU/L. The minimum TSH reported value was 3.6 and the maximum value was 13.4 mIU/L. fT3 and fT4 levels decreased (P<0.0001) in PCOS women compared to controls. Our study results agreed with previous studies by Eldar-Geva et al. (24), Yasmin et al. (25), and Anwary et al. (26).

Insulin resistance and hyperinsulinemia are factors that play an important role in the pathogenesis of PCOS. In the present study we have shown predominant insulin resistance, hypothyroidism, dyslipidemia, and an increased LH/FSH ratio in women with POCS compared to control women (2, 5, 6). The direct effect of testosterone adipocytes has been investigated and induction of androgen receptor mediated insulin resistance via testosterone was established (27). Hyperandrogenism is due to increased LH and low-to-normal FSH levels. Due to the increase in LH and estrogen, FSH is negatively inhibited. Theca cell hyperplasia ensues, leading to hyperandrogenemia that clinically presents as hirsutism. In our study, we have used hirsutism as one of the clinical features for the diagnosis of hyperandrogenism. BMI has a negative association with the baseline levels of LH in PCOS patients. We observed this association in the present study, which supported results from previous studies (27, 28).

PCOS has been the subject of continuous studies on diagnosis, management, and therapy. During the 1980s-1990s, the LH/FSH ratio was perceived to be the gold standard for the diagnosis of PCOS. A higher LH/FSH ratio is no longer a characteristic attribute in PCOS as there is excess production of LH in PCOS patients, which is associated with the inconsistency in LH/FSH ratios in various studies. Of the 80 PCOS patients in this study, 18 patients presented with LH/FSH ratios lower than 1.5, 33 patients ranged from 1.5 to 2.0, and the remaining 29 had LH/FSH ratios higher than 2. Normal prolactin levels were reported in 15 patients, prolactin levels in the range of 19.5 to 50 ng/ml were shown in 26 patients, 36 patients had levels in the range of 51 to 100 ng/ml, and the remaining 3 patients presented with levels higher than 100 ng/ml.

In the current study, we observed a mild increase in the prolactin level (50.65 ± 27.11 ng/ml) in 68% cases, which was similar to previous studies conducted by Kalsum and Jalali (29) where

69.51% of subfertile women suffered from hyperprolactinemia. Nizam et al. (30) also showed that hyperprolactinemia was a major cause of subfertility. Treatment with drugs that lowered prolactin levels resulted in pregnancy for 24% of the infertile women. This finding was consistent with our study.

Conclusion

In the present study, we showed the biochemical and hormonal imbalances that underlie the complex endocrinological cascade of PCOS in Nellore and its surrounding districts of the Andhra Pradesh population. PCOS patients in this population presented with a higher prevalence of insulin resistance, dyslipidemia, and hypothyroidism. Metabolic and endocrine patterns indicated that PCOS patients were at higher risk of developing diabetes as well as cardiovascular disease. An increased concentration of LH, mild hyperprolactinaemia, higher LH/FSH ratio, and decreased FSH has suggested that there is a disturbance in the normal gonadotropin ovarian axis. Further correlation study has revealed that LH is inversely proportional to BMI. A LH/FSH ratio greater than 2.0 can be useful in the diagnosis of PCOS women in our population. Hence we recommend that screening of PCOS patients for metabolic and endocrine parameters will help in the management and treatment of PCOS for a better outcome.

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Original Article

Correlation of Serum Lipoprotein Ratios with Insulin Resistance in Infertile Women with Polycystic Ovarian Syndrome: A Case Control Study

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Abstract

Background: Dyslipidemia and insulin resistance (IR), occurring in most infertile women with polycystic ovarian syndrome (PCOS), increase the risk of cardiovascular disease (CVD) and type 2 diabetes. This study aimed to assess the relationships between lipoprotein ratios and IR in PCOS women.

Materials and Methods: Thirty six infertile women with PCOS selected based on Androgen Excess Society (AES) criteria and 29 healthy women matched for age were recruited to this case-control study. After physical measurements, fasting serum glucose (Glu), insulin and lipid profile levels [triglycerides (TGs), total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C)] were measured, while lipoprotein ratios (TC/HDL-C, LDL-C/HDL-C, TG/HDL-C) were calculated. IR was also calculated using homeostasis model assessment (HOMA)-IR. The optimal cut-offs of lipoprotein ratios in relation to HOMA-IR were calculated based on the Receiver Operating Characteristics (ROC) curve analysis using the area under curve (AUC).

Results: Waist circumference (WC), insulin levels, HOMA-IR, TG levels, and all lipoprotein ratios were significantly higher, while HDL-C was lower in PCOS group as compared to healthy controls. All lipoprotein ratios, TG levels, and WC are significantly correlated with insulin levels and HOMA-IR. Among lipoprotein ratios, the highest AUC of the ROC belonged to TG/HDL-C ratio with sensitivity of 63.6% and specificity of 84.4% (TG/HDL-C>3.19) as a marker of IR in infertile PCOS women.

Conclusion: Lipoprotein ratios, particularly TG/HDL-C, are directly correlated with insulin levels and can be used as a marker of IR (HOMA-IR) in infertile PCOS patients.

Keywords: Lipoprotein, Infertility, PCOS, Insulin Resistance

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Introduction

Polycystic ovary syndrome (PCOS) is the most common gynecological endocrinopathy disorder and the frequent cause of oligo-ovulatory infertility (1). Abnormalities with ovulation are the cause of infertility in about one third of couples attending infertility clinics that count for 90% of these cases (2). PCOS is generally characterized by chronic anovulation, hyperandrogenism and ovarian polycystic changes that are detected by an ultrasound scan in a clinic (3). The estimated prevalence of PCOS based on the criteria used for diagnosis and recruitment process of the study population has been reported between 2.2 and 26% in different countries (4). Although the etiology of PCOS is still unknown, it has been demonstrated that PCOS is a metabolic disorders rather than a reproductive endocrine disease (3). Insulin is a key component in the pathophysiology of PCOS (1). On average, PCOS patients have higher triglyceride (TG), lower high density lipoprotein-cholesterol (HDL-C) and higher low density lipoprotein-cholesterol (LDL-C) levels than their non-PCOS matched group (5). Insulin resistance (IR), hyperinsulinaemia and dyslipidemia are diagnosed among 50 to 70% of patients with PCOS (6). There is a drastic improvement in PCOS complication when is accompanied by modulation of IR (1). Therefore, PCOS is associated with increased risk of metabolic abnormalities, indicating that the patients are at the risk of developing type 2 diabetes and cardiovascular disease (CVD) (3).

Despite modern treatment options for infertilities and considering economic aspects, it is reasonable to give specific attention to cost effective and easily applied methods for predicting metabolic abnormalities at population level (7). Routine methods for measuring IR are hyperinsulinemic-euglycemic clamp technique (a gold standard to assess insulin sensitivity) (8), homeostasis model assessment (HOMA)-IR, Bennett index, Li Guangwei index, quantitative insulin sensitivity check index (QUICKI), and fasting serum glucose (Glu)/insulin ratio (G/I). Due to being complex, expensive and time-consuming, the latter methods are of limited use in clinical and epidemiological studies (9). Thus for daily clinical practice, it is necessary to use other methods for measuring IR, which are lower in costs and applicable to the general population.

In order to provide a new idea to evaluate IR in infertilities associated with PCOS, the possibility of establishing the values of total cholesterol (TC)/HDL-C, TG/HDL-C, and LDL-C/HDL-C ratios, waist circumference (WC) as surrogates, as well as LDL-C, TC, and TG levels to estimate insulin levels and IR was investigated. By using Receiver Operating Characteristic (ROC) curves in our subjects, the accuracy of the mentioned parameters was recived.

Materials and Methods

Subjects

In this case-control study, subjects were selected among women aged 19 to 35 years who visited a private reproductive medical center, Tabriz, Iran, during the period of February till April 2013, for infertility due to PCOS. Selection was done by the standardized protocol for the initial evaluation. A total of 35 patients were identified as PCOS cases according to the Androgen Excess Society (AES, 2006) criteria (1), while 29 age-matched healthy women (without any infertility and PCOS disorders) were recruited in the study as the control group. Inclusion criteria for case group were as follows: married, clinical and/or biochemical hyperandrogenism, and ovarian dysfunction (oligoanovulation and/or polycystic ovaries detected by ultrasound scans). Exclusion criteria were as follows: congenital adrenal hyperplasia, androgen-secreting tumors, taking androgenic/anabolic medications, Cushing syndrome, severe IR syndrome, thyroid dysfunction, hyperprolactinemia, diabetes, hypertension, CVD, taking vitamins and supplements during the 3 months prior to the study, evidence of recent or recurrent infection, and smoking or drinking alcohol.

Physical measurements

Body weight was measured without shoes with minimal amount of clothing using a digital scale (SECA, Germany) to the nearest 0.1 kg. Height was measured using a non-stretchable stadiometer (SECA, Germany) to the nearest 0.1 cm. Body mass index (BMI) was calculated as weight in kg divided to height in squared me-

ters. WC was measured at the midpoint between the lowest rib and the top of the lateral border of iliac crest during minimal respiration. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured using Spot Vital Signs Device (Welch Allyn, USA). Participants were asked to lie down and relax for approximately 8 to 10 minutes, after which three blood pressure measurements were recorded at five-minute intervals.

Blood analysis

After 12-hour overnight fast, blood samples were collected. Serum and plasma samples were separated using a centrifuge (Beckman Coulter Inc., USA) at 1500 rpm for 15 minutes. Fasting insulin levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (Monobind Inc., USA). Fasting plasma Glu was measured using enzymatic procedures by an automatic analyzer (Abbott, USA). IR was estimated by HOMA using the following formula: HOMA-IR=fasting insulin (μU/ml)×fasting Glu (mg/dl)/405 (10). The concentrations of TC and TG were measured using enzymatic procedure with commercial kits (Pars Azmon, IRI), while HDL-C was measured by a direct method using polyethylene-glycol-pretreated enzymes by an automatic analyzer (Abbott, USA). LDL-C was calculated using Friedewald's formula (11). Lipoprotein ratios (TC/HDL-C, TG/HDL-C and LDL-C/HDL) were then calculated.

Ethical considerations

This study was approved by the Medical Ethics Committee of Ahvaz Jundishapur University and all participants gave an informed consent before commencing the study. The code of Ethics Committee is ETH-702, and registered code of study is NRC-9110.

Statistical analysis

Results were expressed as mean ± SD. Levene's test for equality of variances was used. The differences between concerning continuous and categorical variables were analyzed using unpaired t test (or Mann-Whitney U test for non-normally distributed data) and λ^2 test, respectively. Correlations were determined by Spearman correlation coefficient method. ROC curves were used to estimate the sensitivity and specificity of serum lipoprotein ratios to diagnose IR. P values less than 0.05 were considered statistically significant. All statistical analyses were performed using Statistical Package for Social Sciences 20.0 (SPSS, SPCC Inc., USA) software.

Results

The control group was matched with the patient group for age. Although the values of BMI, BP, TC, LDL-C, TG and fasting serum Glu were found to be higher in the infertile PCOS group than in the control group, indicating that these differences were not statistically significant. A higher insulin level and HOMA-IR value were observed in patients group compared to the control group (P<0.001 and P=0.024, respectively). TG levels (P=0.009) as well as the values of TC/HDL (P=0.002), TG/HDL (P=0.047), LDL/HDL (P=0.002) and WC (P<0.001) were significantly higher, while HDL-C levels (P=0.003) were lower in the cases compared to those of their healthy counterparts. The results are shown in Table 1.

HOMA-IR value in the patients showed a positive correlation with TG levels (r=0.56, P<0.01) as well as the values of TC/HDL-C (r=0.34, P<0.05), TG/HDL-C (r=0.49, P<0.01), LDL-C/HDL-C (r=0.33, P<0.05), and WC (r=0.37, P<0.05). However, HOMA-IR value showed no significant correlation with TC, LDL and HDL concentrations. Serum insulin levels are positively correlated with TG level (r=0.46, P<0.01), TC level (r=0.33, P<0.05), and TG/HDL value (r=0.39, P<0.05). We found no significant correlation between serum insulin levels and LDL-C, HDL-C, TC/HDL, LDL/HDL and WC values in our patients. The results are shown in Table 2.

According to the ROC curve analysis, all lipid ratios (TG/HDL-C, TC/HDL-C, and LDL/HDL-C) showed an area under curve (AUC) greater than 0.5. Thus, as an effective diagnostic marker for IR in PCOS patients, the AUC of TG/HDL-C was the highest with sensitivity of 63.6% and specificity of 84.4% (TG/HDL-C>3.19). The results are shown in Table 3 and Figure 1.

Table 1: Baseline and clinical characteristics of two groups (age range 19-35 years)

Variables	Infertile PCOS (n=36)	Healthy control (n=29)	P value[a]
Age	26.36 ± 4.2	27.96 ± 2.47	0.107
BMI (kg/m^2)	26.72 ± 4.39	25.55 ± 4.3	0.286
BMI (%)[c] BMI≥25	72.2	48.3	0.049
WC (cm)	94.77 ± 10.36	85.06 ± 8.48	<0.001
SBP (mmHg)	118.66 ± 8.98	116.89 ± 6.03	0.209
DBP (mmHg)	78.19 ± 6.98	76.37 ± 5.15	0.274
Fasting serum Glu (mg/dL)	94.47 ± 11.88	89.86 ± 8.25	0.081
Insulin (µU/mL)[b]	21.41 ± 14.14	16.24 ± 11.55	0.029
HOMA-IR[b]	5.16 ± 3.72	3.41 ± 2.53	0.024
TC (mg/dL)	214.83 ± 43.97	202.68 ± 46.44	0.285
TG (mg/dL)	139.28 ± 66.98	98.17 ± 50.72	0.009
HDL-C (mg/dL)	42.88 ± 10.2	52.06 ± 13.71	0.003
LDL-C (mg/dL)	143.69 ± 36.25	129.51 ± 35.70	0.119
TC/HDL-C ratio	5.16 ± 1.22	4.11 ± 1.36	0.002
TG/HDL-C ratio	3.62 ± 2.17	2.44 ± 2.52	0.047
LDL-C/HDL-C ratio	3.44 ± 0.98	2.62 ± 1.02	0.002

PCOS; Polycystic ovarian syndrome, BMI; Body mass index, WC; Waist circumference, SBP; Systolic blood pressure, DBP; Diastolic blood pressure, Glu; Glucose, HOMA-IR; Homeostasis model assessment of insulin resistance, TC; Total cholesterol, TG; Triglyceride, HDL-C; High density lipoprotein-cholesterol, LDL-C; Low density lipoprotein-cholesterol, [a]; Statistical analyses performed by unpaired t test for comparison, [b]; Statistical analyses performed by Mann-Whitney U test and [c]; Statistical analyses performed by Chi-squared test. Data are the mean ± SD.

Table 2: Spearman's correlations of lipid profile, lipoprotein ratios and WC values with serum insulin level and IR in infertile women with PCOS

Variables	Serum insulin levels	IR
TGs	0.46[b]	0.56[b]
TC	0.33[a]	0.316
LDL-C	0.29	0.28
HDL-C	0.14	0.08
TC/HDL	0.3	0.34[a]
TG/HDL	0.39[a]	0.49[b]
LDL/HDL	0.28	0.33[a]
WC	0.32	0.37[a]

IR; Insulin resistance, PCOS; Polycystic ovary syndrome, WC; Waist circumference, TC; Total cholesterol, TG; Triglyceride, HDL-C; High density lipoprotein-cholesterol, LDL-C; Low density lipoprotein-cholesterol, [a]; P<0.05 and [b]; P<0.01.

Table 3: Serum lipoprotein ratios, AUC, cut-off points and sensitivity and specificity calculated from ROC curves for the detection of PCOS with IR

Serum lipoprotein ratios	AUC± SE	95% CI	Cut-off point	Infertile PCOS patients		P value
				Sensitivity (%)	Specificity (%)	
TG/HDL	0.743 ± 0.062	0.622-0.864	3.19	63.6	84.4	0.001
TC/HDL	0.651 ± 0.069	0.515-0.786	4.37	69.7	65.6	0.037
LDL/HDL	0.638 ± 0.070	0.502-0.775	2.84	69.7	63.5	0.055

CI; Confidence interval, IR; Insulin resistance, AUC; Area under curve area, ROC; Receiver operating characteristic, PCOS; Polycystic ovarian syndrome, TC; Total cholesterol, TG; Triglyceride, HDL; High density lipoprotein and LDL; Low density lipoprotein.

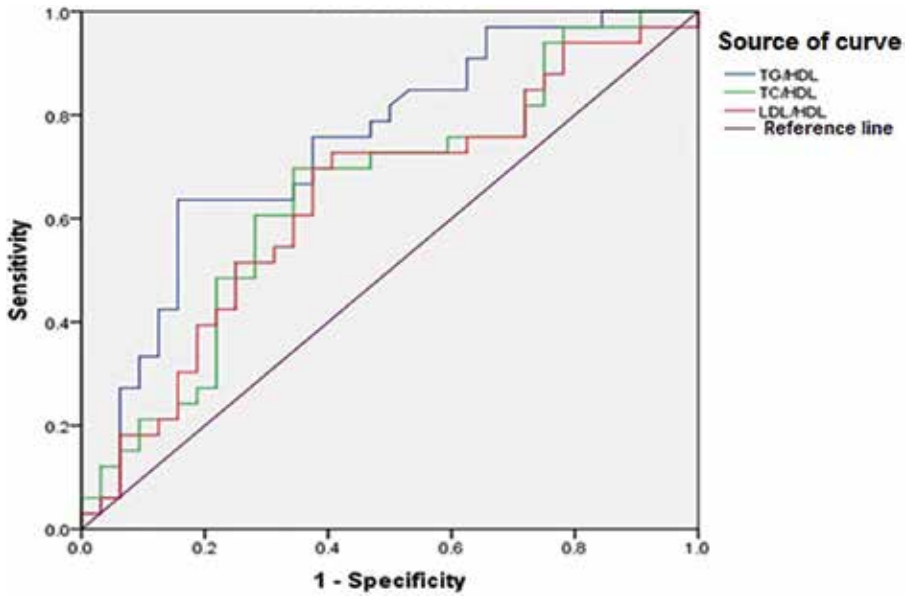

Fig.1: ROC curves of serum lipoprotein ratios for detection of insulin resistance in PCOS cases. Diagonal segments are produced by ties. PCOS; Polycystic overian syndrome and ROC; Receiver operating characteristic.

Discussion

Among the factors responsible for a reduction in fecundity and successful pregnancy, the hormonal changes associated with various factors are considered as an important cause for interrupting normal ovulatory menstrual cycles (12). Among these factors, visceral adiposity is a common finding in PCOS patient, even when the subjects are not classified as overweight (25<BMI<29.9) (13). According to our findings, we observed a significant different in WC between groups. Although the difference in BMI index between cases and controls was not significant, but in case group, percentage of overweight BMI was higher than that of controls. Also there was a significantly positive correlation between WC and IR. Our Findings are in agreement with those of some previous studies (13-15). However, the latter results differ from those of the study conducted by Iuhas et al. (16). In their study, visceral fat area showed no significant difference in PCOS and healthy subjects, which might be due to the difference in method of measuring visceral fat and larger sample size. Pathophysiology of PCOS is unknown. It is regarded as an endocrinal disorder due to IR, which presents in about 70% of PCOS patients (17, 18). In PCOS patients, IR is mostly associated with dyslipidemia. Methods used for measuring IR are mostly sophisticated and expensive that are not applicable for epidemiological studies. Hence, more reasonable methods for IR measurements have been investigated in several studies, of which lipoprotein ratios were proposed for the identification of IR as an alternative method. Our investigation was carried out in order to provide evidences for the application of lipoprotein ratios as an indicator of IR in infertile PCOS women. In this investigation, PCOS was diagnosed by AES criteria, while for the first time, subjects were selected among infertile PCOS women.

This study showed the case group had higher TG levels and lower HDL-C levels compared to control group. While no significant difference was detected in TC and LDL-C levels between groups. Most studies have shown low levels of HDL-C in women with PCOS, but composition of HDL in PCOS is still unknown. There is still a need for further studies in order to determination of the HDL-C composition in these patients. One of the mechanisms that could explain the observed difference is the activity of hepatic lipase (HL) enzyme induced by IR and hyperan-

drogenemia, which removes lipid from HDL and plays as a key role of the lipid-depleted HDL particles in PCOS patients. Also insulin-resistant states along with low HDL levels are frequently associated with hypertriglyceridemia. However, another possible mechanism of dyslipidemia in PCOS could be a reduction in clearance of triglyceride-rich proteins (19).

Result of this study demonstrated a significant association in TC/HDL-C, TG/HDL-C and LDL-C/HDL-C ratios and TG with IR (HOMA-IR) in PCOS patients. In a study on women with PCOS, Xiang et al. (20) also suggested that serum lipoprotein ratios could be used as a marker of IR due to the significant positive correlation of the indices with IR. However, in their study, Rotterdam criteria were used for diagnosing PCOS, so there was a significant difference in terms of BMI between case and control groups, which could be a confounding factor. Hence the present study was designed more specifically by use of updated criteria (AES) on infertile women for diagnosing PCOS. Moreover in our study BMI was not significantly different between case and control groups, which could justify the confounding impact of BMI on results. Serum lipoprotein ratios were also reported to be significantly correlated to IR in type 2 diabetes patients (21). Furthermore TG/HDL could be considered as a simple reliable indicator to determine IR in healthy (22) and severely obese non-diabetic individuals (23). Our results on women with ovulatory disorder infertility also confirmed these findings. ROC curve analysis showed that TG/HDL-C, TC/HDL-C, and LDL-C/HDL-C with an AUC greater than 0.5 were effective and useful diagnostic markers for IR in infertile PCOS women. AUC of TG/HDL-C was the highest with sensitivity of 63.6% and specificity of 84.4% (TG/HDL-C>3.19). Xiang et al. (20) have also shown that AUC of TC/HDL-C had the highest sensitivity and specificity (TC/HDL-C>3.6). This discrepancy could be partially due to lesser sample size in our study or possible racial differences.

Future studies with higher sample size and more specific markers are needed to show the correlation between lipid ratios and IR in an extended level.

Conclusion

Our investigation demonstrated that despite the routine methods used for measuring IR, TC/HDL-C, TG/HDL-C, and LDL/HDL ratios could be regarded as simple, reliable and economic indicators of IR in PCOS infertile women. Moreover the combination of higher serum lipoprotein ratios and TG levels with abdominal obesity may predispose a group of patients to more marked risks for IR.

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Original Article

Relationships between Serum Luteinizing Hormone Level, Endometrial Thickness and Body Mass Index in Polycystic Ovary Syndrome Patients with and without Endometrial Hyperplasia

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Abstract

Background: The endometrial hyperplasia measured by ultrasound in polycystic ovary syndrome (PCOS) women is strongly related to pathologic endometrial thickness, but there is no consensus on the relation between serum luteinizing hormone (LH) and either of these factors: pathologic endometrial hyperplasia and body mass index (BMI).

Materials and Methods: In this observational cross-sectional study, three hundred fifty infertile PCOS women were involved in this research. An endometrial biopsy was taken by using a pipelle instrument, regardless of menstrual cycle's day and all samples were reported by the same pathologist. Basal serum LH level was compared between two subgroups (hyperplasia and non-hyperplasia). The intended population was divided into three groups according to BMI and basal serum LH, later on the comparison was made in three groups. Chi-square test was applied to compare nominal variables between groups. Mann-Whitney U, and one way ANOVA tests were used to compare means on the basis of the result of normality test.

Results: The frequency of endometrial hyperplasia was 2.6%. Endometrial thickness in the patients with endometrial hyperplasia was significantly higher than that of a normal endometrium (10.78 ± 3.70 vs. 7.90 ± 2.86 respectively, P=0.020). There was no relation between endometrial hyperplasia and serum LH (P=0.600). The ANOVA test showed serum LH levels were not the same among three BMI groups (P=0.007). Post hoc test was also performed. It showed that the LH level in normal BMI group was significantly higher than those of other groups (P=0.005 and P=0.004), but there was no statistical difference between overweight and obese groups (P=0.8). We found no relationship between BMI and endometrial thickness in PCOS patients (P=0.6).

Conclusion: Sonographic endometrial stripe thickness is predictive for endometrial hyperplasia in PCOS women. We could not find out any relationship between serum LH level and BMI with endometrial thickness in PCOS patients. However, our study confirmed a diverse relationship between serum LH level and BMI in PCOS patients.

Keywords: Polycystic Ovary Syndrome, Endometrial Hyperplasia, Luteinizing Hormone, Body Mass Index

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Introduction

Polycystic ovary syndrome (PCOS), the most common cause of anovulatory infertility, affects 5-10% of women of fertile age (1). The definition of PCOS in compliance with the 2003 Rotterdam criteria was confirmed in ESHRE/ASRM consensus meeting, provided that at least two out of three following features exist: oligo-ovulation or anovulation, elevated levels of androgens (Hyperandrogenemia) or clinical manifestations of androgen excess (Hyperandrogenemism) and polycystic ovaries as observed by ultrasonography (2). The endometrium in PCOS women has a wider spectrum compared to that of normal endometrium and has a higher incidence of hyperplasia and carcinoma (3, 4).

The incidence rate of hyperplasia in PCOS women is higher than that of normal women (5, 6). High prevalence of endometrial hyperplasia in such women is attributed to persistently high levels of estrogen (mainly estrone) without progesterone (that inhibit proliferation). However, the endometrial function of women with PCOS completely differs from a normal endometrium and is consistent with a predisposition to hyperplasia and carcinoma (7-10).

Because of the increased gonadotropin-releasing hormone (GnRH) pulsatility, luteinizing hormone (LH) hyper secretion is one of the hallmarks of PCOS. Increasing levels observed in about 70% of PCOS patients with elevated LH pulse amplitude and increased LH pulse frequency leading to a two to three fold elevation in serum LH level versus follicle stimulating hormone (FSH) serum level (11).

An increased LH/FSH has been used as a diagnostic test for PCOS for many years, but recent consensus recommendations are against the ones which were used before (12). Some studies reported the basal serum LH levels correlated inversely with body mass index (BMI) in PCOS patients (13, 14), but it is not approved by Hendriks et al. (6), who found no relationship between BMI and LH level in PCOS patients. The aim of this study is to investigate the relationship between serum LH level and endometrial thickness with endometrial hyperplasia. Besides, we want to compare serum LH levels in PCOS women with different BMI.

Materials and Methods

In this cross-sectional study, three hundred fifty PCOS infertile women were enrolled between December 2009 and March 2011 in Royan Institute which is a referral-based fertility and endocrinology clinic. The present study was approved by the Institution Review Board and Ethics Committee of Royan Institute. The research was performed in accordance with Helsinki Declaration and acted in compliance with the committee of Publication Ethics (COPE) guidelines. All participants signed informed consent. The diagnosis of PCOS was based on the 2003 Rotterdam criteria (2). Cases with hyperprolactinemia, thyroid dysfunction, hypothalamic amenorrhea, Cushing's syndrome and ovarian failure were diagnosed by hormonal investigations and excluded from this study. Eligible PCOS patients were asked about menstrual retardation, if the patient had had a menstrual retardation, beta-human chorionic gonadotropin (β-hCG) would have been checked, then in the absence of pregnancy, endometrial thickness was measured by trans-vaginal ultrasound, and endometrial biopsy was taken on the same day. If the patient had not had a delay in menstruation, endometrial thickness would have been measured and endometrial biopsy had been taken on the same day as well. Serum LH level was measured during the next cycle's days 2 or 3 in patients with regular menstrual cycles and after administration of progesterone in patients with irregular menstrual cycles. Irregular menses defined as menstrual periods were shorter than 21 days or longer than 35 days. Intermenstrual interval was recorded and divided into two groups fewer than 3 months and 3 or more than 3 months. Endometrial thickness was measured by using trans-vaginal ultrasound by the same gynecologist for all patients. In the same way, an endometrial biopsy was taken by using a pipelle instrument (Endo cell, wallanch surgical devices Inc., orange, CT, USA) by the same gynecologist.

The endometrial tissues were sent for pathological diagnosis. All specimens were diagnosed by the same pathologist. The world health organization (WHO) criteria were used for the diagnosis of endometrial hyperplasia (15). Endometrial hyperplasia was reported as a morphologic classification into four classes of hyperplasia, composed of complex or simple architecture combined variously with the presence or absence of cytologic atypia (14).

Before the initiation of treatment cycle, height and weight were measured by well-trained nurse. BMI was calculated as body weight in Kg divided by the square of height in meters. To investigate the relationship between serum LH concentration and BMI, the patients were divided into three groups in accordance with their BMI: normal (20<BMI≤25), overweight (25<BMI<30) and obese (30≤BMI).

Statistical analysis

The data was statistically analyzed by using SPSS software version 20. P<0.05 was considered as statistically significant level. T test and Mann-Whitney U test were used to compare means on the basis of the result of normality test. With regard to the results of the Kolmogorov- Smirnov Normality-test for endometrial thickness (Z=2.57, P=0.0001), we used non parametric Mann-Whitney U test to compare endometrial thickness between the two groups and the result revealed a significant difference among groups (Z=2.32, P=0.020). One way analysis of variance (ANOVA) was used to compare LH means between three BMI groups. Chi-square test was used to compare nominal variables between groups. We used multivariate logistic regression by backward to determine predictive factors for endometrial hyperplasia. Female age, BMI, serum LH level and endometrial thickness were included in the regression model.

Results

Three hundred fifty infertile PCOS patients were involved in this study. The women's age, BMI and duration of infertility were 28.5 ± 4.4 year, 28.8 ± 5.1 kg/m^2 and 7.2 ± 4.4 year (mean ± SD) respectively. We found the frequency of endometrial hyperplasia was 2.6%. Basic characteristics of participants are summarized and illustrated in Table 1.

Table 2 shows participants' endometrial pathology reports. Endometrial hyperplasia (simple, complex with or without atypia) was reported in 9 cases and a normal pathology (proliferative, secretory and polyp) was reported in 313 cases. Twenty eight biopsies were reported inadequate.

The mean of endometrial thickness in the normal group was 7.90 ± 2.86 mm and in the hyperplasic group was 10.78 ± 3.70.

Although other characteristics of two groups were not similar, no statistical significant difference was found between normal and hyperplastic groups (Table 1).

Table 1: Comparison of two groups (hyperplastic vs. non-hyperplastic) of PCOS women

		Normal n=313 Mean (SD)	Hyperplasia n=9 Mean (SD)	Total n=350 Mean (SD)	P value
Age		28.45 (4.42)	29.67 (4.74)	28.54 (4.41)	0.416**
Age of menarche		13.30 (1.69)	13.00 (1.32)	13.27 (1.65)	0.756***
Duration of infertility		7.15 (4.45)	8.94 (5.18)	7.21 (4.45)	0.279***
LH level		8.41 (6.67)	9.47 (6.16)	8.42 (6.49)	0.600***
BMI		28.81 (5.11)	30.96 (6.08)	28.82 (5.14)	0.286***
		n (%)	n (%)	n (%)	
Type of infertility	Primary Secondary	260 (83.1%) 53 (16.9%)	8 (88.9%) 1 (11.1%)	294 (84.0%) 56 (16.0%)	0.537****
Menstrual Pattern	Regular Irregular	18 (5.3%) 323 (94.7%)	0 (0%) 9 (100%)	18 (5.1%) 332 (94.9%)	0.592****
IMI	<3 month ≥3 month	162 (47.8%) 177 (52.2%)	7 (77.8%) 2 (22.2%)	169 (48.6%) 179 (51.4%)	0.070****

*; 28 (8.0%) of pathology reports were inadequate, **; t test, ***; Mann-Withney test, ****; Fisher exact test, IMI; Inter menstrual interval, LH; Luteinizing hormone, BMI; Boy mass index and PCOS; Polycystic ovary syndrome.

Table 2: Pathology report of endometrial biopsy in PCOS women

Report	n	% (valid)	Classification
Proliferative	216	61.6 (67.1)	Normal
Secretory	94	26.9 (29.2)	n=313, 89.4%
Polyp	3	0.9 (0.9)	
Simple hyperplasia	5	1.4 (1.6)	Hyperplasia
Complex hyperplasia with atypia	3	0.9 (0.9)	n=9, 2.6%
Complex hyperplasia without atypia	1	0.3 (0.3)	
Total	322	92.0 (100)	
Inadequate (missing)	28	8.0	

PCOS; Polycystic ovary syndrome.

Table 3: Comparison of serum LH level and endometrial thickness in three groups (normal, overweight and obese) of PCOS women

BMI groups	Normal[a] n=82	Overweight[b] n=148	Obese[c] n=120	P value
Serum basal LH	10.39[**] ± 7.4	7.89 ± 6.9	7.73 ± 4.8	0.007[*]
Endometrial thickness	7.9 ± 3.1	7.7 ± 2.7	8.0 ± 2.9	0.68

[*]; One Way ANOVA (LSD post Hoc test), [a]; (20<BMI≤25), [b]; (25<BMI<30), [c]; (30≤BMI), [**]; Significantly higher than two other groups, PCOS; Polycystic ovary syndrome, BMI; Body mass index and LH; Luteinizing hormone. Data presented as mean ± SD.

We also compared serum LH level and endometrial thickness in three groups (normal, overweight and obese) of participants. Results are illustrated in Table 3. One way ANOVA was performed which showed serum LH levels were not equal between groups (F=5.05, P=0.007). Least significant difference (LSD) post hoc test was also conducted which showed that the LH level in normal BMI group was significantly higher than that of other groups (P=0.005 and P=0.004), but there was no statistical difference between overweight and obese groups (P=0.841). There were no significant differences among three BMI groups in terms of endometrial thickness (P=0.6).

Multivariate logistic regression test demonstrated that the endometrial thickness was predictive factor for endometrial hyperplasia in PCOS women (odds ratio: 1.26, 95% confidence interval, 1.05-1.53, P=0.01). Female's age, BMI and LH level weren't predictive for endometrial hyperplasia.

Discussion

Important risk factors for endometrial cancer in PCOS women were reported in previous studies including obesity, age≥50 years, nulliparity, hypertension, infertility and diabetes (7, 16-18). Therefore, PCOS women particularly those with chronic anovulation may be exposed to higher risk of endometrial hyperplasia and endometrial cancer. The mechanisms which cause endometrial hyperplasia and carcinoma are possibly hyperestrogenemia. Hyperandrogenism, hyperinsulinemia and obesity are also risk factors (19, 20). Hyperinsulinemia stimulates adrenal and ovarian androgen production, endogenous estrogen production from progesterone, and it also decreases hepatic sex hormone binding globulin production (18, 21). On the other hand, the combination of insulin resistance and hyperinsulinemia seems to increase the circulation of androgen levels (22, 23), and induce constant production of LH (24). Insulin, androgens and estrogens raise mitotic activity through insulin-like growth factor (7, 18). All These alterations motivate endometrial proliferation and mutagenic potential, which may elevate the risk of endometrial hyperplasia and cancer (18).

The prevalence of endometrial hyperplasia in our study was 2.6%. Holm et al. (18), in a large cohort of Danish premenopausal women (n=963) with PCOS found a low prevalence of endometrial

thickness (1%) and endometrial cancer (0.1%). In comparison to previous study, our population had higher risk of hyperplasia; this difference may be related to variation of PCOS phenotypes between two different races.

In our survey, only endometrial thickness was predictive of hyperplasia. It means that for every 1 mm increase in endometrial stripe thickness, the odds ratio of hyperplasia increased by 1.26. Higher endometrial thickness in hyperplastic group in our study is similar to Cheung (10) and McCormick et al. (25) studies. They reported the only endometrial stripe thickness was predictive of hyperplasia and for every 1 mm increase in endometrial stripe thickness the risk of hyperplasia increased by 1.48. On the other hand, some previous studies had contrast results about the usefulness of endometrial stripe thickness in PCOS patients. We could not find statistical significant difference in serum LH level between normal and hyperplastic groups either. We could not find any other studies conducted on the relationship between serum LH level and hyperplasia. In our study, serum LH level was higher in hyperplastic group, but the difference was not statistically significant. Similar to the previous studies, we could not find any relationship between age and endometrial hyperplasia (10, 26).

As the menstrual cycle length increased and PCOS women extended menstrual cycles of more than 60 days, they were at risk of endometrial hyperplasia (27). In our study similar to McCormick et al. (25) inter menstrual interval was not associated with hyperplasia, but prior studies whose participants had longer durations of amenorrhea reported conflicting results (8, 26).

Similar to previous studies (11, 13), we observed serum LH Level to be significantly higher in some PCOS women with normal BMI, but Hendriks et al. (6) had found no correlation between LH concentration and age or BMI in PCOS patients. Pagán et al. (14) found the LH pulse frequency is elevated in PCOS, but no influence of BMI on either marker of hypothalamic function was detected. In PCOS, the pituitary response to a weight-based dose of GnRH is inversely related to BMI, these evidences suggested that in PCOS patients the effect of BMI on LH be interposed at a pituitary and not a hypothalamic level.

The present study reveals that there is no relationship between BMI and endometrial thickness in PCOS patients in compliance with to Iatrakis et al. (28) study. In contrast, McCormick et al. (25) reported that women with hyperplasia had significantly higher BMI in comparison with those without hyperplasia. Heller et al. (29) reported that higher BMI was associated with endometrial hyperplasia in comparison with lower BMI. Likewise, Zeng et al. (30) compared endometrial thickness and endometrial blood flow in three BMI groups in non-PCOS patients; they found no relationship between BMI and endometrial thickness in these patients. They also reported obesity ($BMI \geq 28\ kg/m^2$) seems to have a negative effect on endometrial and subendometrial blood flow. Due to the limitations, we did not evaluate endometrial pattern, endometrial spiral arterial resistance index (RI) and pulsatility index (PI) values and systolic/diastolic ratio (S/D) in PCOS patients in our study. We suggest comparing these variables among normal weight, overweight and obese PCOS women in future studies.

Conclusion

Sonographic endometrial stripe thickness is predictive for endometrial hyperplasia in PCOS women. We could not find any relationship between serum LH level and BMI with endometrial thickness in PCOS patients. However, our study confirmed a diverse relationship between serum LH level and BMI in PCOS patients.

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Original Article

Effect of Laparoscopic Ovarian Drilling on Outcomes of *In Vitro* Fertilization in Clomiphene-Resistant Women with Polycystic Ovary Syndrome

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Abstract

Background: Recently the laparoscopic ovarian drilling (LOD) has been used as a surgical treatment for ovulation in women with polycystic ovarian syndrome (PCOS), although its mechanism and outcomes are still unclear. This study was undertaken to evaluate the *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) outcomes in clomiphene-resistant women with PCOS who were treated with LOD.

Materials and Methods: In this retrospective study, we reviewed the medical records of 300 women between 20 to 35 years old with clomiphene-resistant PCOS who had an ovulatory infertility and who were nominated for IVF/ICSI. Based on their treatment history, they were located into the following two groups: group I (n=150) including PCOS women who had history of LOD at least 6 months to 3 years before IVF/ICSI, and group II (n=150) including PCOS patients without history of drilling. Both groups were treated with antagonist protocol in the assisted reproductive technology (ART) process. The duration of treatment cycles, number of oocytes and embryos obtained, chemical and clinical pregnancy rate, the number of embryos transferred, and presence of ovarian hyper stimulation syndrome (OHSS) were measured. To compare means and frequencies, Student's t test, Mann-whitney and chi-square tests were used.

Results: Our results showed that ovarian cauterization before IVF/ICSI in patients with PCOS reduced the risk of OHSS (P=0.025). Despite the same pregnancy rate in both groups (P=0.604), more obtained oocytes and embryos were seen on women without ovarian drilling than women with LOD (P<0.001 and P=0.033, respectively).

Conclusion: There is no difference between the pregnancy rate in both groups. Due to significant reduction in OHSS in women undergoing LOD, this surgical treatment may be considered as a useful technique in the management of patients who have previously developed OHSS. However, there are ongoing concerns about long-term effects of LOD on ovarian function.

Keywords: Ovary, Surgical Diathermy, Polycystic Ovary Syndrome, IVF/ICSI, Assisted Reproductive Technology

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Introduction

Polycystic ovary syndrome (PCOS) with 5-10% prevalence among the reproductive-age women involves reproductive and metabolic systems (1-3) that leads to ovulation dysfunction (4). Lifestyle modifications and administration of selective estrogen receptor modulators (SERMs), including clomiphene citrate (CC), are considered as the first-step approach in treatment for PCOS patients (2). But in 20% of cases, CC is not successful in ovulation induction (5). Gonadotropin therapy as the second option for these patients often causes overproduction of follicles that lead to risks of ovarian hyperstimulation syndrome (OHSS) and multiple pregnancies (6). Recently laparoscopic ovarian drilling (LOD) has been used widely by gynecologists as an alternative surgical method for ovulation induction using gonadotropins for PCOS patients unresponsive to clomiphene, but there is a lack of consensus on effectiveness of this method (7). In a study by Flyckt and Goldberg (8), they showed that serum luteinizing hormone (LH) and testosterone levels were normalized following LOD, while their levels remained unchanged over long-term follow-up. They also evaluated ovulation and pregnancy rates after gonadotropin therapy for ovulation induction and LOD. They concluded that although the mechanism of LOD is unknown, this method prevents the risks of multiple pregnancy and OHSS. Also several studies have reported the impact of LOD prior to assisted reproduction technology (ART) in decreasing the OHSS risk and improving the pregnancy rate in women with a history of cancellation of *in vitro* fertilization (IVF) treatment cycle due to risk of OHSS or even risk of OHSS in previous treatment cycle (9-11). Other study also showed that LOD can reduce the risk of cancellation of the ART treatment cycle, but there are no significant differences in pregnancy, miscarriage, or live birth rate (12).

The effect of LOD on ART outcomes in clomiphene-resistant PCOS patients is still unknown; therefore, we aimed to evaluate IVF/intracytoplasmic sperm injection (ICSI) outcomes in clomiphene-resistant women with PCOS who were treated with LOD.

Materials and Methods

After Institutional Review Board approval was received, a retrospective review of hospital records was performed at the Research and Clinical Center for Infertility, Yazd, Iran. In this study, about 1000 medical records of clomiphene-resistant PCOS women undergoing IVF/ICSI treatment from 2006 to 2010 were reviewed. The inclusion criteria were as follows: age between 20 and 35 years old, history of at least one year infertility, and no response to CC (dose up to 150 mg/day for at least three cycles) (13). Women with any other cause of oligomenorrhea and hyperandrogenism were excluded. Furthermore the patients with the following criteria were excluded: history of previous IVF/ICSI, chronic diseases such as thyroid disorders and diabetes mellitus, infertility due to severe male factor (azoospermia), severe endometriosis, and body mass index (BMI)>30. Therefore, 150 clomiphene-resistant PCOS women meeting our inclusion criteria with a history of LOD, (performed at least 6 months to 3 years before IVF/ICSI) were assigned to the group I, while 150 clomiphene-resistant PCOS women with no history of electro-cauterization who underwent IVF/ICSI were assighned to the group II (control group). Two groups were matched in terms of age, duration of infertility, and BMI. PCOS diagnosis was defined as having at least 2 signs of the following Rotterdam criteria: anovulation or oligomenorrhoea, clinical or biochemical signs of hyperandrogenism and the typical ultrasound (US) patterns (polycystic ovaries) (4).

This study was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran, for collecting the data from medical records.

Laparoscopy was performed under general anaesthesia, using 10 mm laparoscope and a unipolar needle electrode with a coagulating current set at 40 W power. In each ovary, four drilling points were made and duration of each diathermy application was about 3-5 seconds.

The patients of groups I and II were treated with gonadotropin-releasing hormone (GnRH) antagonist protocol. They received 150 IU/daily of recombinant human follicle stimulating hormone (r-hFSH, Gonalf, Serono, Switzerland) from second day of menstrual cycle that was assessed by serial vaginal sonography. When mean diameter of dominant follicles reached to 14 mm, 0.25 mg/day of GnRH antagonist (Cetrotide, Sereno) was started and continued until the day of human chorionic gonadotropin (hCG, Pregnyl, Organon, Netherland) injection. When at least two follicles with a mean diameter of 17 mm or one leading follicle larger than 18 mm was observed, 10000 IU hCG was injected. Oocyte retrieval was done using a 17-gauge needle under vaginal ultrasonography guidance, 34-36 hours after hCG injection. Subse-

quently conventional IVF or ICSI was performed.

In all patients, 2-3 embryos were transferred 2 days after oocyte retrieval using an embryo transfer catheter (Labotect Labor-Technik-Göttingen GmbH, Germany). The patients then inserted 800 mg daily Cyclogest suppository (Aburaihan, Iran) on the day of oocyte collection for luteal phase support, and it continued until the fetal heart activity was documented by ultrasonography. To determine chemical pregnancy, the serum hCG level on day 16 after the oocyte recovery was measured. Chemical pregnancy was defined by serum beta-hCG (β-hCG)>50 IU/L, and clinical pregnancy was defined by observation of fetal heart activity by transvaginal ultrasonography 2-3 weeks after positive β-hCG.

The patients were considered at risk of OHSS if more than 15 follicles over 14 mm were observed in each ovary and serum estradiol (E2) levels were more than 3000 pg/ml on the day of hCG administration. In these patients, cycle was canceled; embryos were frozen and not transferred.

The following outcome measures were compared between two groups: duration of treatment cycles, the number of oocytes obtained, chemical and clinical pregnancy rate, number of embryos obtained, the number of embryos transferred, and the risk of OHSS.

Statistical analysis

Data was analyzed using Statistical Package for the Social Sciences 16.0 (SPSS, SPSS Inc., USA). Normal quantitative variables were described as mean ± SD and 95% confidence interval (CI), qualitative data were presented as frequency, and categorical variables were expressed as a percentage. Student's t test and Mann-Whitney U test were used to ascertain the significance of differences between mean values of the variables such as demographic characteristics, number of oocytes and embryo obtained. Chi-squares analysis (χ^2 tests) was performed to meausr the proportions of categorical variables between two groups. P value<0.05 was cosidered as statiscally significant.

Results

From 1000 medical records of clomiphene-resistant PCOS patients who underwent IVF/ICSI treatment and who were reffered to our center from 2006 to 2010, 300 women were enrolled in the study and assigned to two groups (n=150/each).

The demographic, clinical and endocrinological characteristics of participants are showed in Table 1. There were no significant difference in mean age, BMI, duration and type of infertility, and duration of treatment between two groups, but basal FSH (day 3 FSH) levels in groups I and II showed a statisticaly significant difference (Table 1, P=0.019).

There was no significant difference between two groups regarding chemical and clinical pregnancy rate (P=0.604), but mean number of oocytes and embryos obtained were more in group II (P=0.001); however, this difference was not clinically significant (Table 2). Among 150 patients who were treated by electro-cauterization, 10 women (6.7%) were diagnosed with OHSS as compared with 22 (7.14%) patients in group II, indicating that there is a significant difference (P=0.025, Table 2).

Table 1: Demographic, clinical and endocrinological characteristics of participants in two groups

Characteristics	Group I	Group II	P value (Student t test)
Age (Y)*	27.96 ± 3.82	27.21 ± 4.13	0.106
BMI (kg/m^2)*	25.02 ± 2.71	24.86 ± 2.55	0.569
Duration of infertility (Y)*	7.01 ± 2.52	6.64 ± 2.75	0.222
Basal FSH level (day 3 FSH) (IU/L)*	6.64 ± 1.83	5.93 ± 1.89	0.019**
Duration of treatment cycle (IVF/ICSI) (day)*	12.06 ± 1.18	11.88 ± 1.13	0.197
	n (%)	n (%)	P value (Chi-square test)
Type of infertility			
Primary	136 (90.7%)	139 (92.7%)	0.531
Secondary	14 (9.3%)	11 (7.3%)	

*; All data are presented as mean ± SD. **; Significant at P<0.05, BMI; Body mass index, IVF; *In vitro* fertilization, ICSI; Intracytoplasmic sperm injection and FSH; Follicle-stimulating hormone.

Table 2: ART outcomes in two groups

	With LOD	Without LOD	P value (Mann-Whitney test)
Number of oocytes obtained*	12.44 ± 3.25	13.48 ± 3.02	<0.001**
Number of embryo obtained*	9.84 ± 2.65	10.50 ± 2.67	0.033**
	n (%)	n (%)	P value (Chi-squqre test)
Chemical pregnancy	61 (40.7%)	60 (40%)	0.906
Clinical pregnancy	53 (35.3%)	52 (34.7%)	0.604
Chemical pregnancy	61 (40.7%)	60 (40%)	0.906
OHSS	10 (6.7%)	22 (14.7%)	0.025**

*; All data are presented as mean ± SD. **; Significant at P<0.05, OHSS; Ovarian hyperstimulation syndrome, ART; Assisted reproductive technology and LOD; Laparoscopic ovarian drilling.

Discussion

As an alternative to treatment of clomiphene-resistant patients with PCOS, LOD has been proposed due to its quick and easy approach (8).

In this research, we evaluated the IVF/ICSI outcome in 150 clomiphene-resistant women with PCOS who were treated by ovarian electrosurgical drilling and then compared with 150 patients without history of ovarian drilling.

Based on our results, the ovarian drilling in patients with PCOS reduces the risk of OHSS, known as a potential life-threatening disorder. PCOS paients respond differently to controlled ovarian hyperstimulation compared with normal ovaries; therefore, they experience a higher cycle cancellation rate due to an exaggerated response to gonadotropin therapy that leading to the increased risk of OHSS (2, 6, 14).

Tozer et al. (15) retrospectively compared IVF outcomes between PCOS patients undergoing LOD and PCOS patients who did not undergo LOD. They found a trend toward increased ongoing pregnancy rates and decreased risk of developing severe OHSS despite the fact that all LOD-treated patients remained anovulatory after the procedure.

In another study by Greenblatt and Casper (16), they have showed that ovarian trauma disrupts local androgen synthesis that leads to a reduction in intraovarian androgen concentration that is followed by negative effects of androgen on follicular maturation. Subsequently it results in decreased peripheral conversion of androgen to estrogen that causes positive feedback on LH secretion (17). Although, other factors such as inhibin and other local ovarian substances may be involved (18).

Breborowicz et al. (19) in their study showed that prior to IVF, transvaginal ovarian drilling in patients with severe PCOS on metformin therapy leads to an increase in E2 level, meaning an increase in number of mature oocytes and embryos as well as available blastocyst. In our paper, the pregnancy rate in the two groups did not differ, but the number of oocytes and embryos obtained was less in patients with history of LOD, although these differences were not clinically significant. A decrease in number of retrieved oocytes and embryos in the current study suggests the possibility of increased risk of diminished ovarian reserve (DOR) or premature ovarian failure.

Weerakiet et al. (20) in a cross sectional study evaluated the effect of LOD on ovarian reserve. Anti-mullerian hormone (AMH), inhibin B, basal FSH, antral follicle count (AFC) and ovarian volume were measured and compared with related values in PCOS women underwent LOD, PCOS women who did not undergo LOD and normal women with regular menstrual cycles. Their findings revealed that AMH level was lower in LOD-PCOS group (4.6 ± 3.16 ng/mL) as compared to the non-LOD-PCOS group (5.99 ± 3.36 ng/mL), but the difference was not statistically significant. Furthermore AMH level was significantly lower in normal women with regular menstrual cycles, indicating the reduced risk factors for developing OHSS and good ovarian reserve. The serum FSH mean levels were significantly higher in LOD-

PCOS group. There was no significant differences in inhibin B mean levels between groups. Therefore, they concluded that the ovarian reserve was diminished in LOD-PCOS women as compared to non-LOD-PCOS women.

Mural in his literature review demonstrated that although the available data in the literature is limited, there was no concrete evidence of a diminished ovarian reserve or premature ovarian failure associated with LOD in women with PCOS. He indicated that LOD is considered as an effective method to enhace the overain function and nomalize ovarian morphologic and endocrinologic properties if it is performed properly (21).

This study has several limitations such as lack of access to early pregnancy outcomes and life birth rate information. However, further studies with bigger samples and a prospective follow-up for a long period of time on electrosurgical drilling effects on ovarian reserve are recommended. Furthermore review of the possibility of premature menopause in LOD-PCOS and its related complication may benefit from further studies.

Conclusion

There was no difference in the pregnancy rate in women with clomiphene-resistant PCOS undergoing LOD as compared to patients without history of LOD. Due to significant reduction in OHSS in women undergoing LOD, this surgical treatment may be considered as a useful technique in the management of patients who have previously developed OHSS. Though there are ongoing concerns about long-term effects of LOD on ovarian function.

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Original Article

A Comparison of Success Rates of Embryo Transfer on Weekdays and Weekends

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Abstract

Background: The aim of this study is to examine the effect of the embryo transfer (ET) day on clinical pregnancy success rates in *in vitro* fertilization-ET (IVF-ET) cycles.

Materials and Methods: In this retrospective study, we divided patients with infertility who underwent IVF-ET with fresh embryos into two groups depending on whether the ET was performed on weekdays or weekends. The main outcome measure was to compare the clinical pregnancy rates of patients with similar demographic and clinical characteristics who underwent ET on weekdays or weekends.

Results: A total of 188 patients underwent IVF-ET on weekdays (n=156) or weekends (n=32). Both groups had similar demographic and cycle characteristics. The overall pregnancy rate was 42.8%. Among the study groups, the weekday group had a 40.2% ET success rate and the weekend group had a 54.8% success rate (P=0.517). Although no statistically significant difference existed between the two groups, we observed an absolute 14.6% increase in pregnancy rate for ETs performed during weekends compared to those performed on weekdays, with a 35% statistical power.

Conclusion: ETs performed during weekends were more successful than ETs performed during weekdays with an absolute 14.6% increase in clinical pregnancy rate. This finding should be confirmed by conducting further studies with larger groups of patients.

Keywords: Embryo Transfer, *In Vitro* Fertilization, Pregnancy

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Introduction

Infertility is a common health care problem with a prevalence of 4 to 14% worldwide (1). Improvements in the success rates of the *in vitro* fertilization-embryo transfer (IVF-ET) cycles can benefit couples from the physical, psychiological and financial points of view. Thus, every possible factor which can potentially improve the success of an ET should be considered.

IVF-ET cycles were started at the beginning of the natural cycle in the first years of assisted reproductive technologies. Conventional ovarian hyperstimulation was later replaced by controlled ovarian stimulation to avoid the need for clinical and laboratory staff to be on duty during weekends (2). Since then, no studies have compared pregnancy success rates between weekdays and weekends. To the best of our knowledge, this is the first study

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that compares the effect of the ET day on clinical pregnancy success rates in IVF-ET cycles.

Materials and Methods

This was a retrospective observational study based on a review of medical records from Celal Bayar University Infertility Research and In vitro Fertilization Center. We included patients who referred to our clinic because of primary or secondary infertility and underwent assisted reproduction (IVF-ET) with a gonadotropin-releasing hormone (GnRH) antagonist protocol between August 2013 and January 2015.

A total of 188 patients were divided into two groups, depending on whether the ET performed on weekdays (n=156) or weekends (n=32). Clinical characteristics that included age, duration of infertility, and body-mass index (BMI) were collected for each patient. The results of sperm analysis were collected from each patient. We defined abnormal sperm analysis as any abnormality in one of the following parameters: total count, morphology and/or motility. Serum follicle-stimulating hormone (FSH), estradiol (E_2) and luteinizing hormone (LH) levels were measured at the beginning of the ovulation induction and at the day of human chorionic gonadotropin (hCG) administration (E_2 and LH). Ovulation induction protocol, oocyte retrieval days, ET days, endometrial thickness at the beginning of ovulation induction and day of oocyte retrieval, and clinical pregnancy rates were collected for each patient. Only first ovulation induction cycle of each patient was included in the study.

Exclusion criteria for IVF-ET were high serum FSH (>13 mIU/mL), serum LH (>13 mIU/mL) or serum E2 (>80 pg/mL) levels, use of long or short ovulation induction protocols, patients with unavailable or arrested embryos, chemical pregnancies and pregnancies with gestational sac without fetal heart beat. Also excluded were patients with IVF-ET cycles with cryopreserved embryos and those with a history of >4 IVF cycle failures.

Ovulation induction protocol

We used the antagonist protocol in each patient with the same ovulation induction agents. Ovulation induction was started with recombinant FSH (r-FSH, Puregon®, Australia) or human menopausal gonadotropin (HMG, Menogon®, Switzerland). GnRH antagonist (Orgalutran®, Australia, 0.25 mg/day) was added to the cycle on 5th to 7th day depending on the clinical characteristics of the patient. Ovulation was triggered by intramuscular hCG (Pregnyl®, Germany, 10000 IU) when at least two follicles larger than 17 mm in diameter were detected on ultrasound. Oocyte retrieval was performed 36 hours after the injection by the same operator. ETs were performed between the 2nd and 5th days after oocyte retrieval depending on the embryo characteristics; all transfers were performed with the same technique, equipment, and by the same operator. One embryo was transferred in one cycle in patients younger than 35 years of age and two embryos (in case of available two good quality embryos) in patients equal or older than 35 years of age based on legal arrangements in Turkey. Patients received luteal phase support by micronized vaginal progesterone (Progestan, 800 mg/day) from the day of oocyte retrieval which continued until a pregnancy test was performed.

Main outcome measure

This study compared the pregnancy rates of patients with similar clinical and cycle characteristics who underwent ET on weekdays versus weekends. We considered the primary outcome measure to be the clinical pregnancy rate. A blood pregnancy test was performed 9 to 12 days after ET and ultrasound was performed 2 weeks later if the pregnancy test was positive. Clinical pregnancy was defined as presence of a gestational sac with a fetal heart beat.

Ethical consent

The research protocol was approved by the Institutional Review Board of Celal Bayar University Medical School (no. 20478486-302) on August 27, 2014.

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics 15.0 (SPSS Inc., USA). The Shapiro-Wilk test was used to calculate whether the numeric variables had normal distribution. For normally distributed variables, we used the student's t test to analyze any differences in the distributions of the patients' characteristics. The Mann-Whitney U test assessed abnormally dis-

tributed variables. Cross-tables and chi square analysis were employed in the evaluation of the categorical data. P value<0.05 was considered statistically significant. A power analysis was made and the effect size measured to determine the timing (weekdays versus weekends) of ET on the pregnancy success rate.

Results

Overall, there was a 42.8% pregnancy rate. The rate of clinical pregnancy was 40.2% in the weekdays group and 54.8% in the weekend group (P=0.517) with a power of 35% and an effect size of 15%. Although there was no statistically significant difference between the two groups, an absolute 14.6% increase in success rate existed for ETs performed during weekends compared to those performed on weekdays.

Table 1 summarizes the baseline and cycle characteristics of the study population. Abnormal sperm analysis results existed in 69.5% of those in the ET weekdays group versus 72.4% of those in the ET weekend group (P=0.755). Both groups had a history of 0-5 intra-uterine insemination (IUI) treatments and/or 0-3 IVF programs. The percentage of IUI treatments and/or IVF programs were similar in both groups, with 54.4% of patients who underwent IUI in the weekdays group compared to 43.8% in the weekend group (P=0.442) and 29.9% who under went IVF in the weekdays group compared 31.2% who underwent IVF in the weekend group (P=0.885). The type of transferred embryos were cleaved-staged embryos in 83.9% and blastocysts in 16.1% in the weekdays group and cleaved-staged embryos in 87.5% and blastocysts in 12.5% in the weekend group. A single embryo was transferred in 78.2% of weekday patients and in 93.7% of the weekends group. Two embryos were transferred in 21.8% of weekday patients and in 6.3% of patients in the weekends group.

Table 1: Baseline and cycle characteristics of the patients that underwent embryo transfer on weekdays and weekends

	ET weekdays n=156	ET weekends n=32	P value
Age of women (Y)	31.17 ± 4.34*	29.87 ± 3.91*	0.119
Age of man (Y)	33.90 ± 4.39*	32.48 ± 4.19*	0.125
Duration of infertility (Y)	5.50 (1-20)**	6 (1-22)**	0.963
BMI	25.72 ± 4.41*	24.31 ± 3.79*	0.095
Baseline FSH level (mIU/mL)	7.36 ± 3.19*	7.79 ± 2.19*	0.565
Baseline E_2 level (pg/mL)	51.57 ± 33.61*	46.89 ± 22.06*	0.555
Baseline LH level (mIU/mL)	5.15 ± 2.83*	4.99 ± 1.92*	0.805
Baseline endometrial thickness (mm)	5.38 ± 2.51*	5.58 ± 2.06*	0.737
Dosage of induction agent (IU/day)	187.50 (100-300)**	175 (100-300)**	0.150
Retrieved follicle (n)	7.40 ± 4.75*	7 ± 3.38*	0.723
M2 oocytes (n)	6.05 ± 3.68*	5.26 ± 2.66*	0.376
Grade 1 embryos (n)	2.9 ± 3.38*	3 ± 2.69*	0.909
Fertilization rate	4.18 ± 2.61*	3.84 ± 1.77*	0.590
E_2 level at hCG day (pg/mL)	1335.75 ± 910.33*	1321.22 ± 884.97*	0.935
LH level at hCG day (mIU/mL)	3.22 ± 3.11*	6.64 ± 9.44*	0.146
Endometrial thickness (mm)	9.87 ± 2.56*	9.44 ± 2.61*	0.388
Mean transferred embryo (n)	1.29 ± 0.45*	1.15 ± 0.37*	0.174
ET day after OPU	3 (2-6)**	3 (2-5)**	0.344

*; Mean ± SD, **; Median (minimum-maximum), BMI; Body-mass index, M2; Metaphase II, ET; Embryo transfer, OPU; Ovum pick-up, P<0.05 was considered to be statistically significant, FSH; Follicle-stimulating hormone, LH; Luteinizing hormone, E_2; Estradiol and hCG; Human chorionic gonadotropin.

Discussion

IVF involves a complex series of steps, including controlled ovarian hyperstimulation, oocyte retrieval, fertilization, embryo culture, and uterine transfer. Many studies have described factors that influence the success rate of implantation and clinical pregnancy rates in IVF-ET cycles (3-16), including endometrial receptivity (4-6), treatments targeted to improve implantation (5, 7, 8), the ET technique (9, 10), transfer day of the conception material (11, 12), serum E_2 level during the IVF cycle (4^{th} day) (13), decrease in serum E_2 level after hCG administration (14), the relationship between E_2 and LH levels (15), and type of the agent used in ovulation induction (16).

Craig et al. (17) recently showed that acupuncture on the day of ET had a detrimental effect on clinical pregnancy rates, although a Cochrane analysis did not confirm this finding (18). Manheimer et al. (19) have reported a small positive effect of acupuncture on ET rates. Acupuncture is known to increased nitric oxide (NO) production and vasodilatator effect via relaxation of the smooth muscles of the vessels (20). It is clear from the current literature that many factors which affect the success of IVF-ET procedures remain to be clarified.

Uterine receptivity and implantation is one of the most enigmatic parts of the fertilization process. The junctional zone (JZ), or subendometrial layer of the myometrium, is the most popular part of the uterus related to this subject. The degree of JZ contractility has been shown to change through the ovarian cycle. Increased contractility just before ET significantly reduces the success rate of ET (12, 21). It is known that uterine manipulation during ET has a negative effect on pregnancy rates and is possibly related to increased uterine JZ contractility with mechanical effect (10, 22). JZ contractility may be regulated by local synthesis of NO within the uterus and high progesterone levels. It is believed to be related to uterine receptivity although the mechanism is unclear (23). A higher implantation success rate with frozen-thawed ETs has been shown in recent studies. This is thought to be related to the potential detrimental effect of endometrial receptivity induced by the high hormonal environment in fresh ET cycles (5, 24).

We believe that every possible factor which can potentially improve the success of ET should be considered. To the best of our knowledge, there is no data that compares the success rate of ET performed on weekdays versus weekends. We have found an absolute 14.6% increase in success rate for ETs performed during weekends compared to those performed on weekdays. There is no known possible factor for increasing the implantation rate during weekends. The factors that underline the difference in the current study should be investigated. This difference may be due to patient and/or IVF team related factors. Saturdays are more relaxing days in terms of crowdedness and work load. This may help to perform easier ETs with less JZ contractions. A relaxing clinic environment as well as patient mood during the weekends may decrease patient anxiety and positively effect hormonal status related to the implantation process.

There are several limitations in this study. Miscarriage after fetal heart beats and live birth rates were not determined. The number of patients in the study population was relatively low. According to the statistical power analysis, a 35% power was reached in this study. In order to reach a statistical significance with a 14.6% difference between the two groups, 485 patients in the weekdays group and 97 patients in weekend group were required. A nearly threefold higher patient number would be needed to reach statistical significance. In order to reach this number of patients, approximately a 5-year study would be needed at our center. Therefore, the current study findings should be confirmed with studies powered with larger numbers of patients.

Conclusion

ETs performed during the weekends compared to weekdays can be more successful. We strongly believe that large prospective studies are required to demonstrate whether ETs performed during the weekends are more successful in terms of clinical pregnancy rates compared to those performed on weekdays and, if so, to identify the potential reasons that improve the success rate of IVF-ET cycles.

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Original Article

Effects of Crocin Supplementation during *In Vitro* Maturation of Mouse Oocytes on Glutathione Synthesis and Cytoplasmic Maturation

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Abstract

Background: Crocin is an active ingredient of saffron (*Crocus sativus L.*) and its antioxidant properties have been previously investigated. This carotenoid scavenges free radicals and stimulates glutathione (GSH) synthesis; consequently, it may protect cells against oxidative stress. The aim of this research is to protect oocytes from oxidative stress by the addition of a natural source antioxidant.

Materials and Methods: In the present *in vitro* experimental study, we collected cumulus oocyte complexes (COCs) from mouse ovaries of euthanized, 6-8 week-old female Naval Medical Research Institute (NMRI) mice. Oocytes were subjected to *in vitro* maturation (IVM) in the presence of either crocin (5 or 10 µg/ml), 5 mM buthionine-[S-R]-sulfoximine (BSO), or the combination of crocin plus BSO. Oocytes that matured *in vitro* in a medium without crocin or BSO supplements were considered as controls. Following 16-18 hours of IVM, matured oocytes (n=631) were fertilized by capacitated sperm from NMRI male mice, and cultured *in vitro* for up to 96 hours to assess preimplantation embryonic development. The levels of GSH in metaphase II (MII) oocytes after IVM (n=240) were also assessed by the 5, 5-dithio-bis (2-nitrobenzoic acid) (DTNB)-GSH reductase recycling assay.

Results: Supplementation of IVM media with 10 µg/ml crocin significantly ($P<0.05$) increased nuclear maturation, preimplantation development and GSH concentrations compared with the control group. Maturation of oocytes in IVM medium supplemented with BSO alone or the combination of 5 µg/ml crocin and BSO drastically decreased GSH concentrations and subsequently resulted in low rates of maturation, fertilization and blastocyst development. However, the combination of 10 µg/ml crocin with 5 mM BSO increased the level of nuclear maturation which was comparable to the control group.

Conclusion: Supplementation of IVM media with crocin can improve nuclear maturation rates and subsequent developmental potential of mouse oocytes. This may occur by its beneficial effect in increasing GSH concentrations in MII oocytes.

Keywords: *In Vitro* Maturation, Crocin, Glutathione, Mouse, Oocyte

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Introduction

Oocyte maturation has two phases: nuclear (visualized by the extrusion of the second polar body) and cytoplasmic (1). Successful maturation, fertilization and development prior to implantation depend on proper growth and differentiation of immature oocytes and the surrounding cumulus cells. *In vitro* culturing conditions have higher concentrations of O_2 than *in vivo* conditions. Oxygen tension in the oviduct is approximately one-quarter to one-third of atmospheric tension (2). This high concentration of O_2 may cause oxidative stress. Herein, oxidative stress is mediated by reactive oxygen species (ROS) and results in an imbalance of the intracellular redox potential. During in *vitro* maturation (IVM), the oocyte is much more vulnerable to oxidative stress due to ROS overproduction and lack of an innate antioxidant defense system (3, 4). IVM of oocytes and subsequent early embryo development have been shown to be low in the absence of certain supplements such as amino acids and/or antioxidants (5, 6). Recently, numerous attempts have been made to improve the quality and subsequent development of *in vitro* matured oocytes in several species. Modifications to the oocyte culture conditions are considered potential approaches that can help to achieve this improvement. Several studies have shown that addition of antioxidants such as β-mercaptoethanol and vitamins C and E to the culture media could protect the oocytes against oxidative stress that results from IVM (3).

Throughout history, saffron (*Crocus sativus L.*) has been used as a medicinal plant and a culinary spice (7, 8). Among the constituents of saffron stigmas, crocins and crocetin are the most pivotal carotenoids. Investigations into their specific pharmacological properties have led to the discoveries of their antioxidant and antitumor effects (8-11). The crocins are a group of hydrophilic carotenoids that are either mono- or di-glycosyl polyene esters of crocetin inside which D-glucose and/or D-gentiobiose occur as carbohydrate residues (12). These carotenoids scavenge free radicals, particularly superoxide anions and, as a result, they may protect cells against oxidative stress (13). According to reports, the methanolic solution of crocin extracted from *Crocus sativus L.* possesses high radical scavenging activity (14, 15). Numerous studies have reported anti-apoptotic (16), anti-inflammatory (17), and anti-hypertensive (18) therapeutic effects of crocin. *In vitro* studies show that crocin prevents cell death attributed to oxidative stress (19).

The aim of the present study was to evaluate the effects of crocin supplementation during IVM of mouse oocytes on nuclear maturation rates, fertilization events, and subsequent preimplantation development after *in vitro* fertilization (IVF). In addition, we assessed glutathione (GSH) concentrations in MII oocytes after IVM in relation to crocin treatment.

Materials and Methods

In the present *in vitro* experimental study, all chemicals were purchased from Sigma-Aldrich (Germany) except GSH assay chemicals that were prepared from Wako-Japan.

Animals

All procedures performed on animals received the prior approval of the Ethics Board at Royan Institute. Female Naval Medical Research Institute (NMRI) mice, 6 to 8 weeks old and males, 6 to 8 weeks old (Pasteur Institute, Iran), were used in the current study. The animals were kept on a 12 hour light/12 hour dark schedule at an adjusted temperature (20-25°C) and humidity of 50% with ad libitum access to both food and water. Male and female mice were euthanized by cervical dislocation to collect sperm and oocytes.

Collection of cumulus oocyte complexes and *in vitro* maturation

Ovaries (from 70 intact mice) were dissected and transferred into dissecting medium that contained minimum essential medium (α-MEM, Gibco, UK) supplemented with 5% fetal bovine serum (FBS, Gibco, UK), 100 IU penicillin and 100 IU streptomycin for collection of cu-

mulus oocyte complexes (COCs). COCs were retrieved by puncturing antral follicles under a stereomicroscope (Olympus, America) using a pair of 27 gauge needles. IVM was carried out according to Behbahanian et al. (20). Groups of 10-15 COCs were transferred to 30 µl droplets of maturation medium (covered with mineral oil) that consisted of α-MEM supplemented with 100 IU penicillin, 100 IU streptomycin, 5% FBS, 100 mIU/ml recombinant human follicular stimulating hormone (rhFSH, Organon, Holland) and 7.5 IU/ml human chorionic gonadotrophin (hCG, Organon, Holland), then covered with mineral oil. After the incubation of oocytes for 16-18 hours at 37ºC and 5% CO_2, some of oocytes were denuded in α-MEM medium supplemented with 40 IU hyaluronidase for the GSH assay. We recorded the percentages of oocytes at the germinal vesicle (GV) stage, the GV breakdown (GVBD) stage (when the GV was absent), and the metaphase II (MII) stage (when the first polar body was extruded) by using an inverted microscope (Nikon, Japan). Other MII oocytes surrounded by cumulus cells were used for IVF.

Treatment of oocytes with crocin and/or 5 mM buthionine-[S-R]-sulfoximine

We performed the following treatments to evaluate the effect of crocin and 5 mM buthionine-[S-R]-sulfoximine (BSO) supplementation during IVM on the quality and subsequent development of mouse oocytes. Crocin was dissolved in α-MEM medium and added to the IVM medium at final concentrations of 5 µg/ml or 10 µg/ml. BSO was dissolved in α-MEM medium and added to the maturation medium either alone at a final concentration of 5 mM or in combination with crocin.

Glutathione assay

We evaluated the total intra-cellular GSH concentration in the oocytes by the 5,5-dithiobis(2-nitrobenzoic acid) (DTNB)-GSH reductase recycling assay according to the method described by Viet Linh et al. (21). After IVM and removal of cumulus cells, oocytes were washed four times in phosphate-buffered saline (PBS) that consisted of 0.2 M sodium phosphate buffer and 10 mM EDTA, at pH=7.2. We transferred 10 MII oocytes in to 5 µL PBS in to the bottom of an Eppendorf tube after which 5 µL of 1.25 M phosphoric acid was added to each tube. The tubes were kept frozen at -80°C until the assay was performed. For the assay, oocytes were thawed at room temperature. Then, 175 µl of 0.33 mg/ml nicotinamide adenine dinucleotide phosphate (NADPH) and 25 µl of 6 mM DTNB were added to the tubes. Subsequently, 40 µl of distilled water was mixed within a microfuge tube. Up to 5 µl of 125 U/ml GSH reductase was prepared on ice and added to initiate the reaction. Absorbance was monitored at 412 nm using a spectrophotometer (Ziess, Japan) for 7 times at 30 second intervals (from 0 to 3 minutes). The quantity was determined from a constructed standard curve. For each group and replicate, the experiments were repeated 4 times with 10 MII oocytes.

***In vitro* fertilization**

Here and for IVF, we collected the epididymal sperm from the epididymides of male NMRI mice (6-8 weeks old). Spermatozoa were incubated in T6 medium for capacitation. IVF and capacitating medium consisted of T6 medium supplemented with 15 mg/ml bovine serum albumin (BSA, combination of them were equilibrated at 37ºC in 5% CO_2) (22) Following IVM, MII oocytes surrounded by cumulus cells was washed in IVF media. We transferred 3 or 4 oocytes to 50 µl droplets that were formerly covered by mineral oil. For IVF, 2×10^6 spermatozoa/ml were added to the droplets that contained the oocytes and the combination of sperm and oocytes were incubated at 37ºC and 5% CO_2 for 4-6 hours. Then, we recorded the numbers of two-pronuclear (2PN) formations observed with an inverted microscope (Nikon, Japan).

***In vitro* embryo culture**

Inseminated oocytes were respectively collected, washed, and transferred to 20 µl *in vitro* culture droplets (KSOM with 4 mg/ml BSA) (22). At 72 and 96 hours after IVF, we recorded the numbers

of morula and blastocyst embryos with an inverted microscope (Nikon, Japan).

Statistical analysis

Both ANOVA and Duncan's multiple range tests were applied to analyze maturation, 2PN and early development rate by using SPSS 16.0 software. All percentage values were subjected to log transformation prior to analysis. One-way ANOVA and Tukey HSD test were used for the GSH assay. All data were expressed as mean ± SEM. $P<0.05$ was therefore considered to be statistically significant.

Results

Experiment 1: effects of crocin on *in vitro* maturation, fertilization, and early embryo development

In this experimental study, we cultured the oocytes for 16-18 hours in IVM medium supplemented with either 5 µg/ml or 10 µg/ml of crocin. As depicted in Table 1, compared to the control group, supplementation of the maturation medium with 10 µg/ml crocin significantly increased the percentage of MII oocytes ($P<0.05$). There were no significant differences in the percentages of GVBD oocytes between treated groups and the control group ($P>0.05$, Table 1). Addition of 10 µg/ml crocin to maturation medium significantly increased 2PN formation (fertilization rate) compared to the control group ($P<0.05$, Table 1). Compared to the control group, the oocytes treated with 10 µg/ml crocin had the highest rate of embryo development and significantly increased blastocyst formation percentages ($P<0.05$). Significant differences did not exist for the 5 µg/ml dose of crocin in different stages of development ($P>0.05$).

Experiment 2: effects of crocin plus 5 mM buthionine-[S-R]-sulfoximine on *in vitro* maturation, fertilization, and early embryo development

Oocytes treated with BSO were cultured for 16-18 hours in IVM medium or in medium supplemented with either 5 µg/ml or 10 µg/ml crocin and 5 mM BSO. There was no significant difference between all groups in terms of GVBD percentage. As depicted in Table 2, the percentage of MII oocytes significantly decreased in the BSO treated groups compared to the control group. Compared to BSO alone ($P<0.05$), the addition of 10 µg/ml crocin to maturation medium with BSO increased the maturation rate to the same level as the control group ($P>0.05$, Table 2).

Oocytes matured in the presence of BSO had a significantly lower proportion of 2PN zygotes compared to the control group. Oocytes matured in the presence of 10 µg/ml crocin and BSO exhibited a no significantly higher proportion of 2PN zygotes compared to the groups treated with BSO ($P>0.05$, Table 2).

2PN zygotes from each treatment group were cultured further to assess the process of embryo development. The development rate and morula and blastocyst formation in both the BSO treatment and combined treatments of crocin plus BSO greatly declined ($P<0.05$, Table 2).

Table 1: Percentage of germinal vesicle breakdown (GVBD), metaphase II (MII), two-pronuclear (2PN), morula and blastocysts in crocin treatments

		After 4 hours	After 16-18 hours		After 4-6 hours (IVF)			72 hours after IVF	96 hours after IVF	
Crocin	Total COC	GVBD (%)	MII	P value (%)	Total MII inseminated	2PN formation	P value (%)	8-cell-morula (%)	Blastocyst	P value (%)
0	97	90 (92 ± 3)[a]	51 (52 ± 7)[a]		51	38 (70 ± 7)[a]		14 (40 ± 12)[a]	9 (20 ± 7)[a]	
5 µg/ml	98	92 (94 ± 2)[a]	65 (65 ± 7)[a]	P=0.18	65	57 (84 ± 4)[a]	P=0.061	20 (43 ± 8)[a]	14 (24 ± 10)[a]	P=0.19
10 µg/ml	104	97 (93 ± 2)[a]	79 (75± 3)[b]	P=0.02	75	67 (90 ± 3)[b]	P=0.013	32 (47 ± 7)[a]	29 (43 ± 3)[b]	P=0.03

Percentage of 2PN, 8-cell or morula, and blastocyst embryos in relation to 2PN cells. Data are expressed as mean ± SEM. All experiments have been repeated seven times.
Different superscripts indicate significant differences ($P<0.05$) and similar superscripts show no significant differences in a column ($P>0.05$). IVF; *In vitro* fertilization, COC; Cumulus oocyte complex and P value; Comparison between the experimental and control groups.

Table 2: Percentage of germinal vesicle breakdown (GVBD), metaphase II (MII), two-pronuclear (2PN), morula and blastocysts in crocin and 5 mM buthionine-[S-R]-sulfoximine (BSO) treatments

			After 4 hours	After 16-18 hours		After 4-6 hours (IVF)		72 hours after IVF		96 hours after IVF	
Crocin	BSO	Total COC	GVBD (%)	MII	P value (%)	2PN formation	P value (%)	8-cell-morula	P value (%)	Blastocyst	P value (%)
0	0	95	89 (93 ± 5)^{a}	52 (55 ± 5)		39 (74 ± 2)^{a}		15 (45 ± 7)^{a}		7 (20 ± 3)^{a}	
0	5 mM	80	65 (85 ± 9)^{a}	33 (42 ± 2)^{b}	P=0.024	15 (47 ± 6)^{b}	P=0.003	0^{b}	P=0.000	0^{b}	P=0.000
5 µg/ml	5 mM	77	72 (93 ± 3)^{a}	33 (43 ± 3)^{b}	P=0.034	17 (52 ± 6)^{b}	P=0.022	2 (7 ± 7)^{bc}	P=0.000	0^{b}	P=0.000
10 µg/ml	5 mM	80	75 (93 ± 2)^{a}	51(64 ± 4)^{a}	P=0.021	29 (59 ± 5)^{b}	P=0.042	4 (11 ± 5)^{c} P=0.002		2 (5 ± 3)^{b}	P=0.001

Percentage of 2PN, 8-cell or morula, and blastocyst embryos in relation to 2PN cells. Data are expressed as mean ± SEM. All experiments have been repeated seven times.
Different superscripts indicate significant differences (P<0.05) and similar superscripts show no significant differences in a column (P>0.05). IVF; *In vitro* maturation, BSO; Buthionine-[S-R]-sulfoximine, GVBD; Percentage of germinal vesicle breakdown, MII; Metaphase II oocytes, 2PN; Two-pronuclear, COC; Cumulus oocyte complex and P value; Comparison between the experimental and control groups.

Experiment 3: glutathione content of *in vitro* matured oocytes following maturation in crocin supplemented medium

Intracellular concentration of GSH among oocytes later to their maturation in culture media supplemented with 5 µg/ml or 10 µg/ml crocin was measured. We assayed four samples (40 oocytes per treatment) from four replications for the purpose of each treatment. Compared to the control group, the GSH concentration increased significantly in the group treated with 10 µg/ml crocin (P<0.05). Addition of 5 µg/ml crocin to the medium did not generate any significant difference compared to the control group (P>0.05, Table 3).

Experiment 4: glutathione content of *in vitro* matured oocytes in the presence of crocin and 5 mM buthionine-[S-R]-sulfoximine

The GSH content of the oocytes matured in the presence of BSO significantly decreased compared to the control group. Compared to the group treated with BSO alone, addition of 5 µg/ml or 10 µg/ml crocin to BSO treated oocytes did not significantly affect GSH content of MII oocytes after maturation (P>0.05, Table 3).

Table 3: Intracellular glutathione (GSH) levels on *in vitro* matured metaphase (MII) oocytes following supplementation of the maturation medium with crocin and 5 mM buthionine-[S-R]-sulfoximine (BSO)

		After oocyte maturation (16-18 hours)	
Crocin	BSO	Total GSH (pmol/oocyte)	P value
0	0	2.24 ± 0.43^{b}	
5 µg/ml	0	2.71 ± 0.58^{ab}	P=0.92
10 µg/ml	0	3.83 ± 0.29^{a}	P=0.046
0	5 mM	0.27 ± 0.09^{c}	P=0.045
5 µg/ml	5 mM	0.42 ± 0.15^{c}	P=0.018
10 µg/ml	5 mM	0.64 ± 0.28^{c}	P=0.010

Data are expressed as mean ± SEM. All experiments were repeated four times.
Different superscripts indicate significant differences (P<0.05) and similar superscripts show no significant differences in a column (P>0.05). P value; Comparisons between each experimental and the control group.

Discussion

The presence of high quality oocytes prior to IVF is an important factor that affects developmental competence of subsequent embryos (23). One of the most crucial and harmful factors which can affect *in vitro* oocytes and embryo development are free radicals. Free radicals have deteriorating effects on DNA repair, oocyte maturation, and meiotic spindle assembly (24). This research has investigated the improving effect of crocin (10 µg/ml) added to maturation medium on oocyte maturation, 2PN formation, and developmental competence of oocytes. However the lower dosage of crocin (5 µg/ml) did not affect either the maturation rate or the 2PN and blastocyst formation rates. Former *in vivo* investigations of different organs indicated positive effects of saffron extract and crocin against adverse effects of free radicals and oxidative stress (25-27). *In vitro* researches also demonstrated and emphasized the antioxidant properties of crocin (16, 28-30).

Assimopoulou et al. (14) reported that a metabolic solution of crocin extracted from *Crocus sativus L.* possessed a high level of radical scavenging activity. *In vitro* studies determined the direct linkage between total crocin concentration and antioxidant properties. Antioxidant function has seemed to be strongly influenced by the attached sugar moieties in crocin structures (31, 32). This natural antioxidant can impact cells by different mechanisms including nitrite scavenging ability, 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical cation inhibition, SOD-like activity, and elongation of lipid peroxidation (6). The radical scavenging potential of crocin is the source of its neuro-protective, anti-aging, anti-inflammatory, and anti-tumor activities (33). An *in vitro* experiment has shown that crocin can modulate cellular proteins and alter their functions. It can particularly affect several cell processes through the interaction with tubulin proteins or microtubules as an important cytoplasmic protein which affects cell division (34). We have observed in the current study, that the higher maturation rate caused by crocin indicated its positive effects during the *in vitro* culture. As observed, higher dosages of crocin (10 µg/ml) increased the oocyte maturation percentage. The effect of crocin appeared to be dose-dependent. By the time the 10 µg/ml dose of crocin was added to the maturation medium, the higher quality of *in vitro* matured oocytes resulted in a higher capacity for 2PN formation and embryonic development efficiency. Although the lower dosage of crocin (5 µg/ml) increased IVM, 2PN and blastocyst formation rate compared to the control group, this finding was not significant. The increased effects of the higher crocin dosage (10 µg/ml) on IVM, 2PN and early embryonic development rate were probably due to its dose-dependent antioxidant effects.

The current study results of the GSH assay have revealed that the addition of crocin (10 µg/ml) to the maturation medium increased the GSH content of MII oocytes after IVM. GSH is one of the fundamental non-enzymatic defensive structures against ROS in the mammalian oocyte and embryo. GSH is considered an indicator of cytoplasmic maturation in oocytes (35, 36). GSH content increases during development and oocyte maturation in the ovary, as the oocyte approaches the time of ovulation, and protects oocytes at later stages of fertilization (37). In oocytes, GSH stabilizes the meiotic spindle against oxidizing agents and is involved in the enhancement of MII, normal formation of the egg, male pronucleus formation, and inhibition of two-cell stage arrest (38). After fertilization, GSH participates in sperm decondensation parallel to oocyte activation and transformation of the fertilizing sperm head into the male pronucleus (39). *In vitro* culture conditions exacerbate the formation of ROS, which exert oxidative stress and deplete intracellular GSH content in the oocyte and embryo (40). A decline in oocyte GSH levels results in failure of 2PN formation and reduced embryo development (41). Previous studies have found that crocin can enhance the activities of GSH reductase and a rate-limiting enzyme, γ-glutamyl cysteine synthetase (γ-GCS), which is an enzyme involved in the synthesis of GSH. Thus it can contribute to a stable GSH supply in PC12 cells *in vitro* (42, 43). A series of studies by Soeda et al. (44) have indicated that a GSH dependent mechanism is involved in the inhibitory effects of crocin on oxidative stress-induced cell death. There are *in vivo* studies which indicate the effects of crocin on recovering levels of GSH and antioxidant enzymes against oxidative stress (45, 46).

The increased rate of GSH contents in the cyto-

plasm during IVM with the addition of 10 µg/ml crocin suggested that aside from the different antioxidant functions of crocin, increased GSH was one of the main causes of increased pronucleus production and early developmental competence. The effect of crocin increased the rate of IVM and subsequent *in vitro* development of oocytes. Addition of BSO caused depletion of GSH in the two groups of lower and higher crocin dosages. Depletion in the concentration of GSH caused by BSO supplementation affected oocyte maturation rate. However, the combination of crocin 10 µg/ml and BSO had no significant effect on maturation compared to the control group. The lower rate of subsequent developmental competence in all BSO treated groups appeared to be related to the lower preservation of GSH which was probably due to the impact of BSO during IVM. BSO decreased GSH production and it appeared that crocin addition did not compensate for this depletion.

Tavana et al. (47) have reported that higher dosages such as 40 µg/ml of saffron extract in maturation medium increases maturation rate. However, in order to have better IVF and embryo development, addition of lower saffron dosages such as 5 µg/ml is more effective. Our research has indicated that crocin (5 µg/ml) as a component of saffron did not affect any aspects of oocyte and embryo culture. Crocin, at the lower dosage, did not induce GSH synthesis and therefore it had lower antioxidant abilities. Saffron extract has been depicted to have higher antioxidant activities due to the presence of other components with antioxidant properties and synergic effects (48). We have reported that higher dosages of crocin (50, 100, 400 µg/ml) did not have any positive effect on IVF and embryo development. It seems that crocin affects cultured oocytes on a dose-dependent manner; hence 10 µg/ml of crocin would be the best dosage for supplementation.

Conclusion

Supplementation by crocin, an active ingredient of *Crocus sativus L.*, during IVM of oocytes can improve maturation, fertilization and early embryo development outcomes. Crocin can dose-dependently increase ooplasmic GSH concentration and cytoplasmic maturation during the maturation process. By the inhibition of GSH synthesis, BSO appears to have an inhibitory effect on crocin efficiency in maturation medium.

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Original Article

Fetal *RHD* Genotyping Using Real-Time Polymerase Chain Reaction Analysis of Cell-Free Fetal DNA in Pregnancy of RhD Negative Women in South of Iran

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Abstract

Background: Maternal-fetal RhD antigen incompatibility causes approximately 50% of clinically significant alloimmunization cases. The routine use of prophylactic anti-D immunoglobulin has dramatically reduced hemolytic disease of the fetus and newborn. Recently, fetal *RHD* genotyping in RhD negative pregnant women has been suggested for appropriate use of anti-D immunoglobulin antenatal prophylaxis and decrease unnecessary prenatal interventions.

Materials and Methods: In this prospective cohort study, in order to develop a reliable and non-invasive method for fetal *RHD* genotyping, cell free fetal DNA (cffDNA) was extracted from maternal plasma. Real-time quantitative polymerase chain reaction (qPCR) for detection of *RHD* exons 7, 5, 10 and intron 4 was performed and the results were compared to the serological results of cord blood cells as the gold standard method. *SRY* gene and hypermethylated Ras-association domain family member 1 (*RASSF1A*) gene were used to confirm the presence of fetal DNA in male and female fetuses, respectively.

Results: Out of 48 fetuses between 8 and 32 weeks (wks) of gestational age (GA), we correctly diagnosed 45 cases (93.75%) of *RHD* positive fetuses and 2 cases (4.16%) of the *RHD* negative one. Exon 7 was amplified in one sample, while three other *RHD* gene sequences were not detected; the sample was classified as inconclusive, and the RhD serology result after birth showed that the fetus was RhD-negative.
Conclusion: Our results showed high accuracy of the qPCR method using cffDNA for fetal *RHD* genotyping and implicate on the efficiency of this technique to predict the competence of anti-D immunoglobulin administration.

Keywords: Prenatal Diagnosis, Real-Time Polymerase Chain Reaction, Cell-Free Fetal DNA

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Introduction

Rh is systematically the most polymorphic blood group and clinically the most important group after ABO. RH complex is formed by two highly homologous *RHD* and *RHCE* genes, both of which are located on chromosome 1, and consist of 10 exons (1). Although complete deletion of *RHD* gene is found to be dominant in Caucasian D-negatives, there is a large diversity in other populations especially in Japanese and African blacks (2). About 66% of the RhD-negative African population carry a non-functional *RHD* gene named *RHD* pseudogene (*RHD* Ψ), and 15% of RhD-negative Africans have a special rearrangement of *RHD* and *RHCE* genes, named the hybrid allele *RHD-CE-Ds* (3, 4).

The D-negative phenotype has a wide range frequency in different ethnic populations. With regards to literatures, the frequency of RhD-negative is 3-7% in Africans,15-20% in Caucasians and less than 1% (0.3-0.5) in the far east (5). In a study conducted in Fars province of Iran, the frequencies of RhD negative phenotype were 13.05 and 9.62% in 1982 and 2001, respectively (6). Maternal-fetal RhD antigen incompatibility causes approximately 50% of clinically significant maternal alloimmunization cases. Since1960s, the routine use of prophylactic anti-D immunoglobulin has dramatically decreased the hemolytic disease of the fetus and newborn (7). Fetal *RHD* genotyping in RhD negative antenatal women can be effective for the appropriate use of anti-D antenatal prophylaxis, facilitating to reduce unnecessary prenatal interventions. In an immunized pregnant woman, the prediction of fetal RhD blood group is helpful for the appropriate management of the pregnancy and avoiding unnecessary invasive tests. At the same time, this reduces the concerns about the pregnancy outcome (8-11). For many years, prenatal diagnosis has been performed by chorionic villus sampling (CVS) and amniocentesis. These invasive tests increase the risk of feto-maternal hemorrhage and enhance the severity of alloimunization. In addition, performing these tests before 11 weeks (wks) of pregnancy is not recommended. Although CVS provides the result in the first trimester, it is associated with higher risk of miscarriage than amniocentesis: 1in 100-200 vs. 1 in 200-400, respectively (12-14).

Lo et al. (15) suggested the existence of cell-free fetal DNA (cffDNA) in the maternal plasma. Their hypothesis provided a new possibility for non-invasive prenatal diagnosis. Bianchi proposed three possibilities for cffDNA origin: hematopoietic cells, direct feto-maternal transfer of DNA molecules, and trophoblastic cells (16). Detection of cffDNA in anembryonic pregnancies demonstrated that the placental tissue is the main source of cffDNA in maternal circulation (17).

Detection of low fetal DNA concentration in maternal plasma (3% in early to 6% in late pregnancy) and distinguishing cffDNA from maternal DNA are the two major challenges that limit the use of cffDNA for non-invasive prenatal tests (NIPT) (18, 19).

Different methods have been used to confirm the presence of fetal DNA in maternal plasma, in previous studies. The most common system is to trace *SRY* sequence in maternal plasma; it also provides the possibility of determining the sex of the fetus, but this strategy is not applicable for female fetuses (4). Another possible method is an evaluation of polymorphic microsatellites and insertion/deletion markers in maternal plasma and buffy coat. Failure to detect a specific allele in maternal buffy coat together with its presence in maternal plasma is the basis for diagnosis. Such methods are not able to provide sufficient information and also have low sensitivity (20, 21). In a recent method, introduced as a universal marker, tracking is performed based on different methylation of the *RASSF1A* gene in maternal and fetal DNA (8, 22). The aim of our study was to set up a novel reliable protocol for non-invasive determination of fetal RhD status using cffDNA extracted from maternal plasma.

Materials and Methods

In this prospective cohort study, the plasma samples were collected from 50 RhD-negative women with singleton pregnancy at Hafez Hospital, Shiraz, Iran. Gestational age was between 8 and 32 wks, based on the last menstrual period (LMP). 10 blood samples were taken at 8-16 wks of gestation age (GA), 35 samples at 17-28 wks and 5 samples at ≥28 wks of GA. The participants were healthy women without any serious pregnancy complications, and their husbands were serologically RhD-positive.

Sample preparation

Peripheral blood samples were collected in a 6

ml tube containing Ethylenediaminetetraacetic acid (EDTA, INTERLAB Laboratory Products, Turkey) and processed within 6 hours. The samples were centrifuged at 2000 ×g for 10 minutes to separate the plasma, which were subsequently centrifuged at 3000 ×g for 10 minutes. The supernatants were then separated and stored at -80°C for further processing.

DNA extraction

QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) was used to extract cffDNA from plasma with minor modification. DNA was isolated from 200 µl of plasma according to manufacturer's instruction, but eluted in a final volume of 30 µl Buffer AE (INTERLAB Laboratory Products, Turkey). To minimize the risk of contamination, DNA was isolated under laminar airflow and aerosol-resistant tips were used.

Real-time polymerase chain reaction

Real-time PCR was performed on Rotor-Gene Q (Qiagen, USA) using SYBR Green Master Mix (2x Maxima SYBR Green/ROX qPCR Master Mix, Thermo Scientific, Lithuania). To determine the fetal RhD status, the presence of *RHD* exons 5, 7, 10 and intron 4 were evaluated. The AlleleID 7.5 primer software (PREMIER Biosoft, USA) was employed to design *SRY* primers using the *SRY* gene sequence obtained from GenBank nucleotide database (accession number: L08063). All other primers were selected according to previous studies presented in the Table 1 (23-28).

All quantitative polymerase chain reaction (qPCR) reactions were performed in a final volume of 25 µl containing 5 µl of DNA. The final concentration of primers in each qPCR reaction was 300 $nmol.L^{-1}$. The qPCR cycling condition was two-step holding temperatures: 50°C for 2 minutes, 95°C for 10 minutes followed by 50 cycles of 94°C for 60 seconds, 55°C for 60 seconds, and 72°C for 60 seconds. Two replicates were performed for the tested gene.

The fetuses were labeled either as a D-positive, when all *RHD* target sequences (exons 5, 10, 7 and intron 4) were properly amplified, or D-negative, when no amplification signal was detected. Fetuses were predicted to be inconclusive when one, two or three specific *RHD* sequences were amplified. The cycle threshold (Ct) values of 30-42 were considered positive.

Quality control

10-fold serial dilutions were prepared to determine the sensitivity of the test, the quality of primers, and qPCR reagents using DNA extracted from plasma of a male human. To rule out the possible contamination, positive controls, negative controls and no-template controls (NTCs) were also included in each PCR run, using sterile H_2O. The *β-globin* gene, as a reference gene, was tested to confirm the presence of cell free DNA (cfDNA).

Table 1: Sequences of PCR primers for real time PCR assays

Target genes	Sequence 5' to 3'
RHD (intron 4)	F: GATGACCAAGTTTTCTGGAAA
	R: CATAAACAGCAAGTCAACATATATACT
RHD (exon 5)	F: CGCCCTCTTCTTGTGGATG
	R: GAACACGGCATTCTTCCTTTC
RHD (exon 7)	F: CTCCATCATGGGCTACAA
	R: CCGGCTCCGACGGTATC
RHD (exon 10)	F: CCTCTCACTGTTGCCTGCATT
	R: AGTGCCTGCGCGAACATT
SRY	F: AATTGGCGATTAAGTCAA
	R: TGTATTCATTCTCAAGCAA
RASSF1A	F: AGCCTGAGCTCATTGAGCT
	R: ACCAGCTGCCGTGTG
β-globin	F: GTGCACCTGACTCCTGAGGAGA
	R: CCTTGATACCAACCTGCCCAG

PCR; Polymerase chain reaction.

Validating presence of the cell-free fetal DNA in RhD-negative female fetuses

SRY gene was used for all samples to confirm the presence of cffDNA. In the predicated samples as RhD negative female, the presence of hypermethylated *RASSF1A* gene was also tested. Investigations show that the *RASSF1* gene promoter is hypermethylated in DNA with placenta origin, but hypomethylated in maternal DNA (29). The cfDNA samples were initially treated with BstUI, a methylation-sensitive restriction enzyme. At this experiment, digestion reactions contained 0.5 µg DNA and 5 U BstUI restriction enzyme (New England Biolabs, England) were incubated at 60ºC for 2 hours followed subsequently by adding to qPCR reactions. Each run included three different controls: undigested non-pregnant control (DNA from a non-pregnant woman), digested non-pregnant control (DNA from a non-pregnant woman), and undigested pregnant control (DNA obtained from a pregnant woman).

RhD phenotype of newborns

Blood samples were collected at birth from cord blood. The direct agglutination test was carried out with anti-RhD reagents (CinnaGene, Iran). The concordance of test was determined by comparing the data from the prenatal genetic tests with serological results obtained from cord blood.

Statistical analysis

The this study, simple random sampling (SRS) method was used to collect clinical samples. As analytical values, limit of detection in qPCR test in clinical samples was defined. Using serology and neonate sex, as two gold standard test to respectively confirm *RHD* and *SRY* gene results, the diagnostic sensitivity, specificity and concordance were reported. Roc curve analysis was employed and P value>0.05 was reported as statistically significant level. All the statistical analyses were performed by SPSS, version 16.0.(Ltd, Hong Kong)

Ethical considerations

All procedures for this study were approved by the Ethics Committee (ec-p-90-3311) of Shiraz University of Medical Sciences (Shiraz, Iran). Informed consent was obtained from pregnant women who participated in this research project.

Results

Non-invasive prenatal determination of fetal RhD status, as well as gender analysis, was performed in 48 cases of RhD-negative pregnant women, while their husbands were RhD-positive. The mean gestational age was 26 wks at the time of blood sampling (ranging from 8 to 32 wks). Serological tests were performed on the cord blood sample, and the fetal gender was confirmed after delivery.

The minimum detection level of DNA in clinical samples was 4.2 (pg/µl). qPCR was performed on the samples in duplicates and the results were interpreted as positive, provided detection of the specific amplicons in both replicates.

Analysis of the standard curves of qPCR demonstrated a wide dynamic range and high efficiency for the investigated genes (Fig.1). The Ct value ranges in maternal plasma of clinical samples are presented in the Table 2. Figure 2 represents the qPCR results of *RHD* exon 7 in the controls and clinical samples.

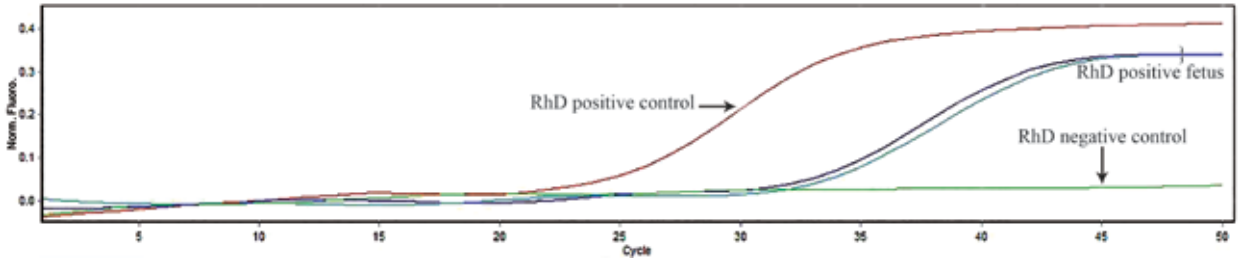

Fig.1: Real-time quantitative polymerase chain reaction (PCR). Amplification plots using real-time quantitative PCR for the *RHD* (exon 7) gene. Positive control; DNA from a RhD positive woman, Samples; Result observed from *RHD* negative women holding RhD positive fetuses, Negative control; Result observed from RhD negative women.

Analysis of two fetuses were not terminated and they were excluded from our samples due to abortion and hydrops fetalis. Following the amplification of all *RHD* gene target sequences, the fetuses were classified in different RhD positive groups. Out of 48 samples, the results of 45 cases (93.75%) were determined as RhD-positive, and 2 cases (4.16%) were detected as RhD-negative. Exon 7 was amplified in one sample (2.08%) while no signal was determined for the other three *RHD* gene fragments. The results obtained from this sample were considered to be inconclusive (Table 3) while serologic finding distinguished the fetus as Rh negative.

Serology of the cord blood indicated 45 RhD positive neonates (93.75%) and 3 RhD negative ones (6.25%). Based on a prenatal test for *SRY* gene, 5 cases (10.41%) were predicted to be male and 43 cases (89.58%) female (Table 3). There was complete concordance between *SRY* qPCR results and neonate gender after delivery. The Diagnostic concordance of the test was 100% for the *SRY* gene and 97.91% for the *RHD* gene (Table 4).

Three samples out of the 48 showed negative qPCR result for *RHD* and *SRY* genes. In order to confirm the presence of fetal DNA, *RASSF1A* qPCR was performed after methylation-sensitive restriction enzyme digestion. The obtained result confirmed the presence of cffDNA in all three samples.

Table 2: qPCR efficiencies, linear correlations (R^2) of standard dilutions, and ranges of Ct value for the tested genes

Target genes	qPCR efficiency (%)	R^2	Ct value ranges in clinical samples
β-globin	0.95	0.99	30-36.2
RHD intron 4	0.91	0.99	33.64-41.83
RHD exon 5	0.91	0.99	33.20-41.77
RHD exon 7	0.95	0.99	35.99-41.32
RHD exon 10	0.91	0.99	32.78-41.49
SRY	0.92	0.99	35.83-41.60

qPCR; Quantitative polymerase chain reaction, R^2; Linear correlations and Ct; Cycle threshold.

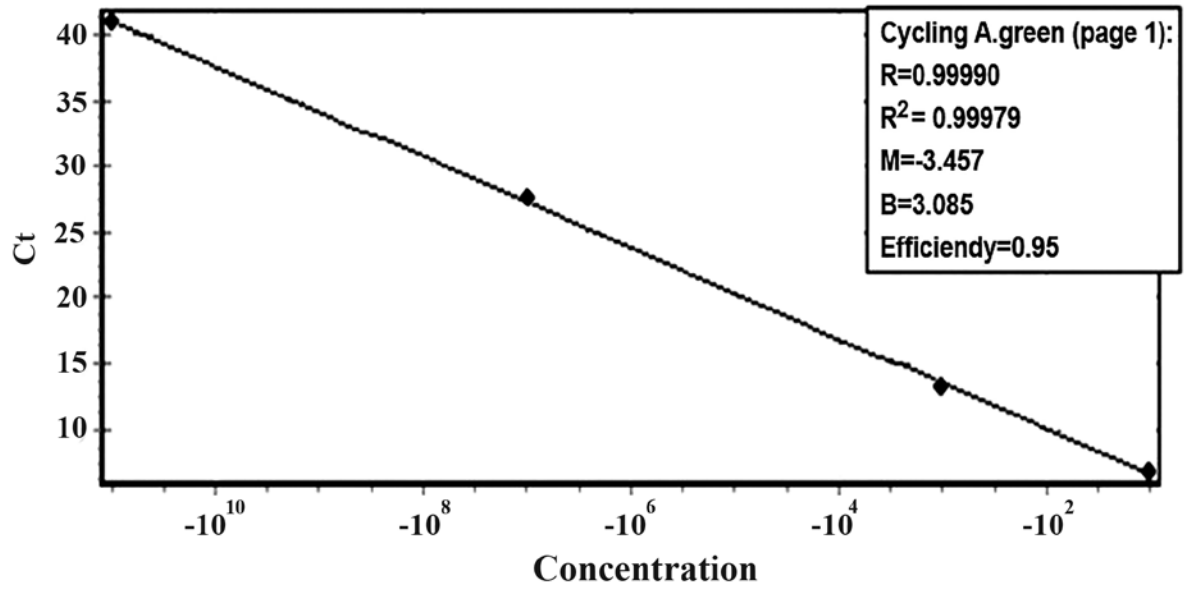

Fig.2: Real time standard curve of *RHD* exon 7 gene using 10-fold serially diluted samples. The plot shows the relationship between Ct value and DNA concentration. R; Correlation coefficient, R^2; Coefficient of determination, M; M-estimation, B; Beta coefficient and Ct; Cycle threshold.

Table 3: Fetal *RHD* and *SRY* genotyping results by qPCR and neonatal RhD phenotype and sex

Sample no.	Maternal RhD phenotype	Fetal genotyping in maternal plasma					Neonate RhD phenotype	Neonate sex
		RHD exon 5	*RHD* exon 7	*RHD* exon 10	*RHD* intron 4	*SRY*		
1	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
2	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
3	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
4	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
5	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
6	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
7	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
8	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
9	Neg	Pos	Pos	Pos	Pos	Pos	Pos	♂
10	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
11	Neg	Neg	Pos	Neg	Neg	Neg	Neg	♀
12	Neg	Pos	Pos	Pos	Pos	Pos	Pos	♂
13	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
14	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
15	Neg	Pos	Pos	Pos	Pos	Pos	Pos	♂
16	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
17	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
18	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
19	Neg	Neg	Neg	Neg	Neg	Neg	Neg	♀
20	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
21	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
22	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
23	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
24	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
25	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
26	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
27	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
28	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
29	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
30	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
31	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
32	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
33	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
34	Neg	Neg	Neg	Neg	Neg	Neg	Neg	♀
35	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
36	Neg	Pos	Pos	Pos	Pos	Pos	Pos	♂
37	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
38	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
39	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
40	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
41	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀

Table 3: Continued

Sampleno.	Maternal RhD phenotype	Fetal genotyping in maternal plasma					Neonate RhD phenotype	Neonate sex
		RHD exon 5	*RHD* exon 7	*RHD* exon 10	*RHD* intron 4	*SRY*		
43	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
44	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
45	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
46	Neg	Pos	Pos	Pos	Pos	Pos	Pos	♂
47	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀
48	Neg	Pos	Pos	Pos	Pos	Neg	Pos	♀

qPCR; Qantitative polymerase chain reaction.

Table 4: Diagnostic measures between genotyping and phenotyping

	RHD gene	*SRY* gene
Concordance	97.91% (47/48)	100%
Sensitivity	100%	100%
False-negatives	-	-
Specificity	100%	100%
False-positives	-	-

Discussion

Our findings confirmed the reliability of non-invasive prenatal testing to predict the fetal RhD status. This prediction can be helpful to determine the necessity of close fetal monitoring and the need of more invasive procedures in isoimmunized mothers. Another positive outcome of fetal *RHD* prediction is preventing unnecessary anti-D immunoglobulin injection in non- isoimmunized mothers with RhD negative fetuses. A study, performed in UK, showed that 38% of RhD-negative pregnant women bear RhD-negative fetus. Therefore, employing non-invasive prenatal test can reduce the cost of the health care system and risks of viral infection pertaining to anti-D administration (30).

Based on previous experiences, there are several important steps in developing NIPT including: blood sample preparation (31), cffDNA extraction (32) and confirming presence of cffDNA (33). Additionally, regarding the reported genetic diversity at RH system within different ethnic groups, selection of *RHD* gene sequences for qPCR test and defining specific rules for interpretation of genotype are inevitable (34). Therefore, we developed a novel non-invasive prenatal diagnostic test using cffDNA in our laboratory, to evaluate the fetal RhD status within pregnant populations obtained from south of Iran. Previous studies have recommended the use of at least 2 *RHD* specific regions to avoid false positive results, although using multi-sequences to trace *RHD* diversity have recently become more widespread. In this study, all samples were tested for the presence of *RHD* exon 10 and intron 4 to distinguish between two homologous *RHD* and *RHCE* genes. In addition, exon 5 analysis was applied to identify the point mutations leading to *RHDΨ*. Moreover, in order to cover different types of partial D categories, especially DVI partial D as the most common hybrid *RHD-CE-Ds*, selected areas of *RHD* gene (intron 4, exons 5, 7 and 10) were included (35-38).

In this study, the false negative and false positive results were not observed, except in one sample that *RHD* exon 7 was amplified, while intron 4, exon 5 and exon 10 did not identify. This case was classified in the inconclusive group, and serology results showed the fetus as RhD negative. The possible cause of these findings was an *RHD* variant gene in the mother or fetus, but there was no access to maternal or newborn DNA for subsequent analysis. Comparison of three previously published studies (39, 35, 13) showed similar findings to our results.

Although the presence of fetal DNA was not confirmed in most of previously published studies (40, 41), our strategy was using *SRY* gene for all the samples and in cases that were negative for *SRY* and *RHD* genes, hypermethylation of *RASSF1* gene by BstUI restriction enzyme was evaluated. In order to avoid false-negative results followed by mismanagement of the pregnancy, analyzing *RASSF1A* gene is essential for the cases with *RHD* negative female fetuses.

Conclusion

In this study, diagnostic concordance of the predicted fetal gender (100%) and RhD status (97.91%) from free fetal DNA in the maternal plasma of 48 *RHD* negative women were obtained. With regards to observing no different Rh variants in this experiment, a large study from different region of our country- Iran- is suggested. Thus, this study can be helpful to find possible *RHD* variants as well as the cause of inconclusive cases. Conducting larger-scale studies will be the first step in establishing a guideline for running non-invasive *RHD* genotype testing on all *RHD* negative mothers in Iran.

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Original Article

Crocin Improves Damage Induced by Nicotine on A Number of Reproductive Parameters in Male Mice

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Abstract

Background: Crocin, a carotenoid isolated from Crocus sativus L. (saffron), is a pharmacologically active component of saffron. Nicotine consumption can decrease fertility in males through induction of oxidative stress and DNA damage. The aim of this study is to determine the effects of crocin on reproductive parameter damages in male mice exposed to nicotine.

Materials and Methods: In this experimental study, we divided 48 mice into 8 groups (n=6 per group): control (normal saline), nicotine (2.5 mg/kg), crocin (12.5, 25 and 50 mg/kg) and crocin (12.5, 25 and 50 mg/kg)+nicotine (2.5 mg/kg). Mice received once daily intraperitoneal injections of crocin, nicotine and crocin+nicotine for 4 weeks. Sperm parameters (count, motility, and viability), testis weight, seminiferous tube diameters, testosterone, and serum nitric oxide levels were analyzed and compared.

Results: Nicotine administration significantly decreased testosterone level; sperm count, viability, and motility; testis weight and seminiferous tubule diameters compared to the control group ($P<0.05$). However, increasing the dose of crocin in the crocin and crocin+nicotine groups significantly boosted sperm motility and viability; seminiferous tubule diameters; testis weight; and testosterone levels in all groups compared to the nicotine group ($P<0.05$).

Conclusion: Crocin improves nicotine-induced adverse effects on reproductive parameters in male mice.

Keywords: Crocin, Nicotine, Damage, Reproductive

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Introduction

A large, increasing number of patients worldwide use medicinal plants and herbs for health purposes (1). Crocin is a carotenoid obtained commercially from the dried trifid stigma of the culinary spice Crocus sativus L. (saffron) and is responsible for the red color of saffron (2, 3). It is the diester formed from the disaccharide gentiobiose and dicarboxylic acid crocetin (4). Saffron has been used traditionally as a coloring or flavoring agent, as well as an herbal remedy (5). In traditional medicine, throughout history, saffron has been used to treat infertility, impotence, and as a sexual potential stimulant (6). It contains four major bioactive constituents: crocin (color), crocetin (color), picrocrocin (taste), and safranal (aroma) (7).

Crocin can be purely isolated from the saffron extract and directly crystallized (8). The saffron spice contains numerous chemical substances such as carbohydrates, minerals, mucilage, vitamins (especially riboflavin and thiamin), and pigments that include crocin, anthocyanin, carotene, lycopene, and zigzantin (9, 10). Crocin has also shown various pharmacological activities - antioxidant,

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anti-tumor, radical scavenging, and genoprotective (11-13). The anti-tumor functions of crocin have a special place in pharmaceutics (14). According to research at pharmacological and high doses, crocin did not exhibit marked damages to all major organs of the body and no mortality was seen by crocin in mice (15).

Infertility is a health problem that causes adverse effects in personal, social, and economic domains and is observed in 10 to 15% of couples (16). Approximately 40% of infertility problems are associated with males (17). Infertility in males has been associated with sperm dysfunctions such as low sperm count, immaturity, abnormality, and lack of motility (18). Various studies have shown that consumption of nicotine-containing compounds decreases sperm count and motility (19). Nicotine is a highly toxic organic compound that contains nitrogen and alkaloids which are mostly found in tobacco (20).

Nicotine can easily pass through the cell membrane and react with tubulin protein present in the cytoplasm of multiplying cells, causing disorders to cell division (21). Nicotine can damage sperm membrane and DNA, and induce apoptosis in interstitial cells in the testis (22). We have taken into consideration the effect of saffron on hormone synthetics such as testosterone, the role of this hormone on spermatogenesis (23), the importance of the male reproductive system and lack of any report of a protective effect of crocin against nicotine in designing this study. Hence, the current study was conducted to analyze the protective effect of crocin on the damage induced by nicotine in a number of reproductive parameters in male mice.

Materials and Methods

Chemicals

In this experimental study, digentiobiosyl-8, 8'-diapocarotene-8, 8'-oate ($C_{44}H_{64}O_{24}$, crocin) powder (Merck, Germany) was diluted with normal saline (0.9%) to prepare the different doses. S)-3-[1-Methylpyrrolidin-2-yl]pyridine ($C_{10}H_{14}N_2$, nicotine) solution (Merck, Germany) was diluted with normal saline (0.9%) prior to administration (24).

Animals

A total of 48 healthy adult Balb/c male mice that weighed 27-30 g were purchased from Tehran Razi Institute. Animals were kept at 22 ± 2ºC under controlled environmental conditions, 12/12 hour light/dark cycle and free access to water and food. Animals were maintained in compliance with National Institutes of Health guidelines (25). This study was conducted in accordance with the approval granted by the Ethical Committee for Research on Laboratory Animals at Kermanshah University.

Experimental design

The mice were randomly divided into 8 groups (n=6): i. Control (normal saline; 1 ml distilled water (DW)/daily), ii. Nicotine (2.5 mg/kg) (26), iii. Nicotine+crocin (12.5 mg/kg), iv. Nicotine+crocin (25 mg/kg), v. Nicotine+crocin (50 mg/kg), vi. Crocin (12.5 mg/kg), vii. Crocin (25 mg/kg), and viii. Crocin (50 mg/kg). Mice received intraperitoneal (IP) injections of nicotine once per day for 4 weeks. Crocin and nicotine+crocin were administered in the same way to the animals (24).

Testis weight and seminiferous tubule diameter

The testes were carefully removed, washed in normal saline solution (0.9%), blotted, and weighed separately. The average weights were used. After testes fixation by formalin, the histological process that included dehydration, clearing, and embedding was carried out. The microscopic sections (5 μm) were prepared for hematoxylin and eosin (H&E) staining. The seminiferous tubule diameters were measured by a Motic camera and software (Moticam 2000, Spain). Seminiferous tubule average diameter (μm) was determined for each testis (27).

Sperm collection

The cauda epididymis was excised, minced and incubated in a pre-warmed petri dish that contained 10 ml Hank's balanced salt solution at 37°C. The spermatozoa were al¬lowed to disperse into the buffer. After 20 minutes, the cauda of the epididymides were removed and the suspension was gently shaken to homogenize. The solution was analyzed under light microscope at a magnification of ×400 (22, 28).

Sperm parameters

In order to count the sperm, we diluted 500 µL of the sperm suspension with formaldehyde fixative [10% formalin in phosphate buffered saline (PBS)] (Sigma, USA). Approximately 10 µL from the diluted solution was transferred into a hemocytometer using a Pasteur pipette (Thoma, Assistant Sondheim/Rhön, Germany) and the solution was allowed to remain for 7 minutes. Then, the sperm that settled were counted and evaluated per 250 small squares of a hemocytometer (27). Viability was assessed by eosin Y staining (5% in saline). We placed 40 µL samples of the freshly prepared sperm suspension on a glass slide. The suspension was mixed with 10 µL eosin (Sigma, USA) and subsequently observed under a light microscope at ×400 magnification. Live sperm remained unstained whereas sperm that showed any pink or red coloration were classified as dead. At least 200 sperm were counted from each sample in 10 random fields of vision and we recorded the percentages of live sperm (29). In order to assess the percentage of motile sperm, the suspension was prepared by pipetting. A small aliquot (40 µL) of freshly liquefied semen was placed on a glass slide at 37˚C for film recording with a video microscope (Olympus, BX51, Germany). Randomly, we recorded 10 fields from each slide with a camera for sperm motility assessment via analysis of the recorded films. Sperm motility was divided into four levels according to certain criteria: i. Quick progressive motility in direct line, ii. Slow progressive motility in direct or indirect line, iii. No progressive motility, and iv. No motility (18).

Testosterone and nitric oxide levels

The animals were anesthetized 24 hours after the last injection. Blood was taken from the hearts of the animals and preserved at 37ºC for 30 minutes, then centrifuged (1000 g) for 15 minutes. The collected blood was centrifuged at 25°C and 4000 rpm for 10 minutes in order to obtain the serum. The serum samples were kept frozen at -18°C. The blood testosterone level was analyzed by enzyme linked immunosorbent assay (ELISA, Abcam 108666, USA). Nitric oxide was measured based on Griess colorimetric assay. Accordingly, N-(1- naphthyl) ethylenediamine dihydrochoride (NEED), sulfonamide solutions and nitrite standards were prepared. To measure nitrite concentration in serum, the serum samples were thawed and 100 µl of the serum sample was deproteinized by zinc sulfate, then transferred to the wells. Subsequently, we added 100 µl chloride vanadium, 50 µl sulfonamide, and 50 µl NEED. The cells were incubated at 30°C in the dark. Optical densities (OD) of the samples were measured by an ELISA reader at a wavelength of 540 nm (30).

Statistical analysis

Data were presented as mean ± SEM and analyzed by one-way ANOVA followed by Tukey tests using SPSS package (version 18, SPSS Inc, USA). The Kruskal Wallis test was used to examine data normality and homogeneity of variances, considering a significance level of 0.05.

Results

Testis weight and seminiferous tubule diameter

The effective doses of nicotine (2.5 mg/kg) and crocin+nicotine (12.5 mg/kg) caused a significant decrease in testis weight and seminiferous tubule diameters compared to the control (saline) group (P=0.00). Crocin improved testis weight and seminiferous tubule diameters in treated animals of all doses compared with the nicotine group (P=0.00). Crocin+nicotine caused a significant increase in testis weight and seminiferous tubule diameters in all treated groups compared with the nicotine group. Crocin prevented the damage by nicotine on testis weight (P=0.00, Table 1, Fig.1).

Sperm parameters

Mean sperm count, progressive motility, and viability significantly decreased in the nicotine (2.5 mg/kg) and crocin+nicotine groups of all doses compared to the control (saline) group (P=0.00). However, crocin and crocin+nicotine significantly improved high motility and sperm viability in all treated groups compared with the nicotine group (P=0.01). Crocin significantly improved sperm counts in all treated groups compared with the nicotine administered group (P=0.01). Increasing crocin+nicotine doses revealed no significant increase in the sperm count in the treated groups compared to the nicotine group (P=0.35). Crocin prevented the damage caused by nicotine on sperm parameters (Table 2).

Table 1: Effects of nicotine, crocin and crocin+nicotine on mean testis weight and diameter of seminiferous tubules in male mice (n=6 for each group)

Groups	Mean testis weight (g)	Diameter of seminiferous tubules (µm)
Control	0.12 ± 0.007^{a}	46.12 ± 1.4^{a}
Nicotine	0.065 ± 0.01^{b}	25.39 ± 0.7^{b}
Crocin 12.5 mg/kg	0.12 ± 0.003^{ac}	44.25 ± 2.84^{ac}
Crocin 25 mg/kg	0.13 ± 0.003^{d}	46.99 ± 1.4^{ac}
Crocin 50 mg/kg	0.13 ± 0.003^{d}	50.87 ± 3.5^{ad}
Crocin+nicotine (12.5 mg/kg)	0.083 ± 0.01^{e}	34.79 ± 3.9^{f}
Crocin+nicotine (25 mg/kg)	0.1 ± 0.003^{f}	36.5 ± 0.8^{f}
Crocin+nicotine (50 mg/kg)	0.1 ± 0.003^{f}	37.88 ± 2.8^{f}

Data are presented as mean±SEM. Values with different letters indicating significant differences among groups at P<0.05.

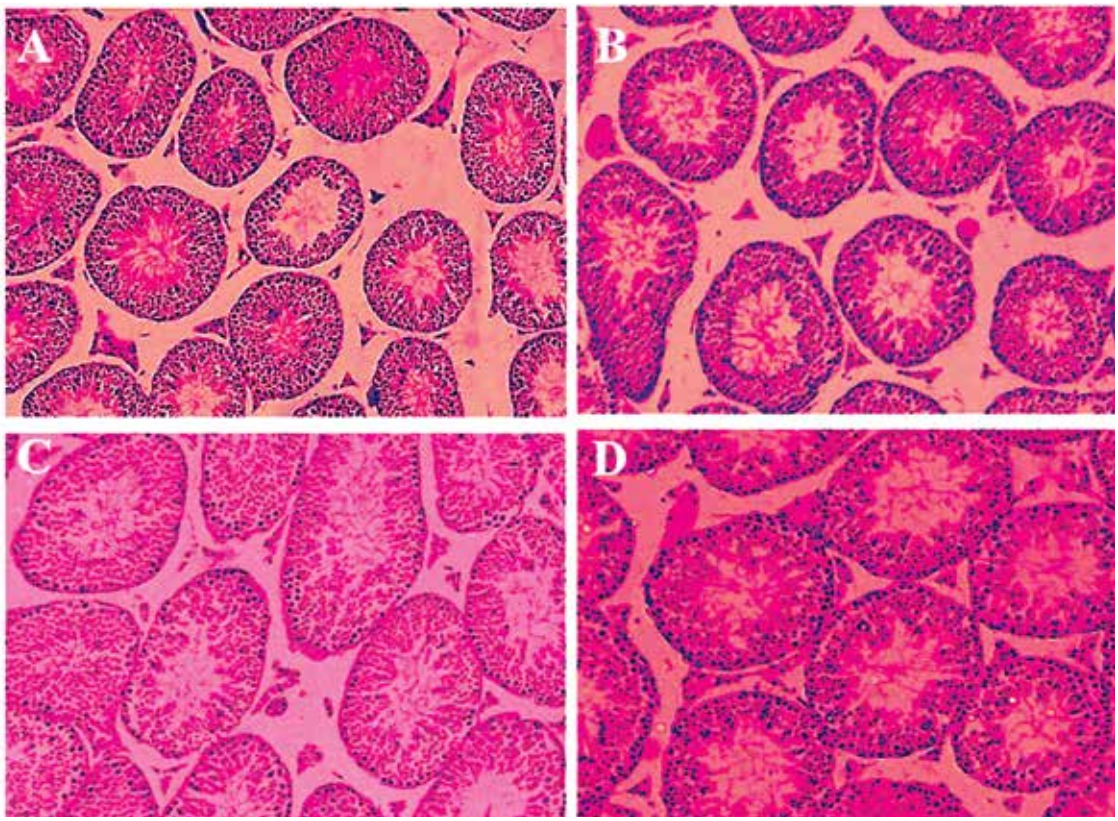

Fig.1: Effects of different concentrations of crocin on the diameters of seminiferous tubules according to hematoxylin and eosin (H&E) staining. **A.** Cross-section from the testis of mice from the control group with normal seminiferous tubules. Cross-sections from the testes of rats that received, **B.** 12.5 mg/kg of crocin, **C.** 25 mg/kg of crocin and **D.** 50 mg/kg of crocin (magnification: ×40).

Testosterone hormone and nitric oxide

Nicotine (2.5 mg/kg) and crocin+nicotine (12.5, 25 and 50 mg/kg) caused a significant decrease in testosterone compared to the control group (P=0.00). Increasing doses of crocin and crocin+nicotine showed significantly increased testosterone in all groups compared to the nicotine group. Crocin prevented the damage caused by nicotine on testosterone level (P=0.00, Fig.2). The mean level of nitric oxide in blood serum increased significantly in the nicotine (2.5 mg/kg) and crocin+nicotine (12.5 mg/kg) groups compared to the control group (P=0.00, Fig.3).

Table 2: Effects of nicotine, crocin and crocin+nicotine on sperm parameters in male mice (n=6 for each group)

Groups	Mean sperm count (10^6)	Sperm progressive motility (%)	Sperm viability (%)
Control	4.53 ± 0.06^{a}	6.6 ± 0.16^{a}	77.83 ± 0.16^{a}
Nicotine	2.16 ± 0.5^{b}	0.02 ± 0.05^{b}	30.03 ± 0.05^{b}
Crocin 12.5 mg/kg	4.5 ± 0.43^{ac}	6.83 ± 0.9^{a}	85.06 ± 0.9^{c}
Crocin 25 mg/kg	4.52 ± 0.7^{ac}	8.83 ± 1.04^{ac}	85.75 ± 1.04^{c}
Crocin 50 mg/kg	4.69 ± 1^{ac}	11.83 ± 3.07^{d}	89.45 ± 3.07^{c}
Crocin+nicotine (12.5 mg/kg)	2.16 ± 0.5^{bd}	0.3 ± 0.08^{e}	59.25 ± 0.08^{d}
Crocin+nicotine (25 mg/kg)	2.6 ± 0.7^{e}	1.3 ± 0.9^{f}	63.95 ± 0.9^{e}
Crocin+nicotine (50 mg/kg)	2.9 ± 0.2^{f}	1.5 ± 0.5^{f}	70.61 ± 0.5^{f}

Data are presented as mean ± SEM. Values with different letters indicating significant differences among groups at P<0.05.

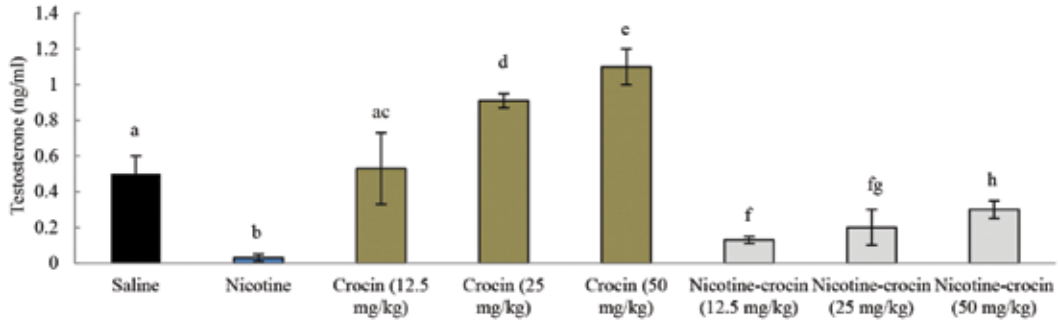

Fig.2: Effects of nicotine, crocin, and crocin+nicotine on testosterone levels in male mice (n=6 for each group). Different letters indicate significant differences among groups at P=0.00.

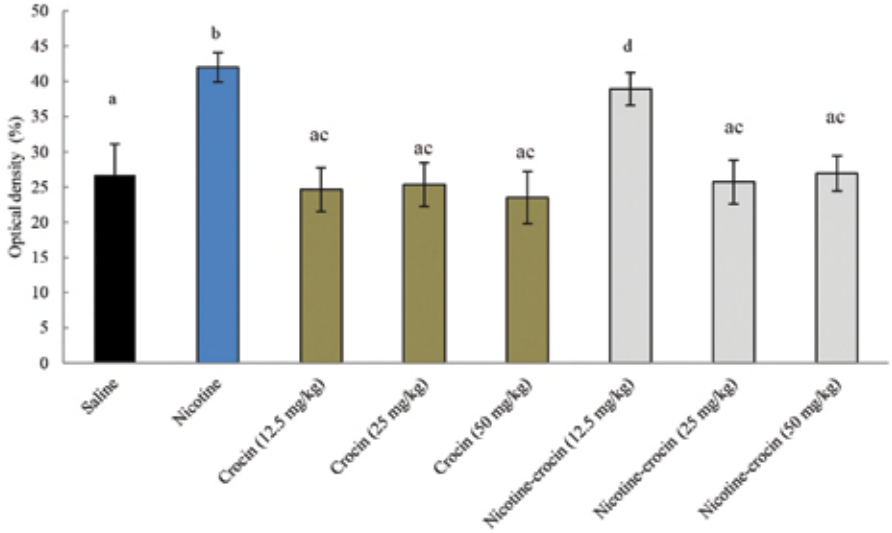

Fig.3: Effects of nicotine, crocin, and crocin+nicotine on mean nitric oxide levels in blood serum in male mice (n=6 for each group). Different letters indicate significant differences among groups at P=0.00.

Discussion

Currently, medicinal plants have numerous applications. One of the target tissues for plant extracts is reproductive organs such as the testis and reproductive parameters. The results of our experimental study have revealed that nicotine promoted male reproductive toxicity in mice. This agreed with results by Sankako et al. (31) who reported residual damage on sperm concentration, motility, and morphology after cigarette smoke exposure.

In fertile individuals, sperm motility levels have a direct relation to fertilization ability (32). Crocin has possibly increased the count and motility of normal sperm in treated groups through enhancing the antioxidant defense of the body (3). On the other hand, crocin caused a significant change in reproductive indices and inhibited the harmful effects induced by nicotine in the reproductive hormone. Crocin could act as an antioxidant and improve the sperm quality by increasing the expression of antioxidant genes in comparison with the nicotine group (11).

The findings of this study were in line with the results of a study conducted by Kalpana et al. (33) that investigated relative peroxidative and antioxidant effects of curcumin on nicotine-induced toxic fatty tissue. They reported that curcumin (as an antioxidant) decreased the toxicity induced by nicotine in fatty tissue. Researchers have stated that increasing free radicals causes the loss of epithelial cells, which can destroy cytoplasmic bridges and consequently decrease sperm count and motility levels, increasing sperm malformation (34).

Antioxidant properties of crocin can improve sperm quality by increasing expression of antioxidant genes (13). Nicotine can directly inhibit primary Leydig cell testosterone levels, but the mechanism of this effect is not known. Nicotine leads to lower testosterone hormone production, which may be a secondary reason for reduction of sperm number in seminiferous tubules (35). Changes in sperm vitality and motility after nicotine injection may be due to an increase in reactive oxygen species (ROS) levels in mice semen. Several lines of evidence indicate that ROS is involved in nicotine-induced testicular damage (36).

The results showed that sperm count, motility, and viability in the presence of crocin significantly improved compared to nicotine-only-treated animals. Therefore, positive changes in the sperm quality might be due to the hydroxyl radical scavenging activity of crocin which has been shown to inhibit lipid peroxidation (11). Increased sperm counts might possibility be caused by the anti-apoptotic effects of crocin (9). Crocin has been shown to act like an anti-oxidant in vivo, preventing the formation of free radicals and lipid peroxidation, hence, preventing oxidant-in¬duced apoptosis (12). The results of the present study have confirmed findings reported by Asadi et al. (6) which indicated that saffron improved epididymal sperm parameters in rats that were exposed to cadmium.

Crocin may reduce hypophyseal-hypothalamus sensitivity to testosterone feedback control on luteinizing hormone (LH) secretion. In light of crocin antioxidants' effects in biosynthesis of steroid hormones, it seems that crocin can affect male sexual hormone concentrations (37). Crocin administration improves sperm parameters and most changes that occur on testicle tis-sue in mice probably are the result of increasing testosterone levels. The findings of the present study have confirmed the results by Khayatnouri et al. (23) where saffron administration improved the spermatogenesis index in rats. However, the results of this study contrasted the findings of Safarinejad et al. (38) who reported that saffron administration for 26 weeks to infertile men with idiopathic oligoasthenoteratozoospermia (OAT) had no effects on semen parameters.

In the current study, the mean nitric oxide in blood serum has increased significantly in the nicotine group compared to the control group. Nitric oxide and the signal pathways of 3', 5'-cyclic guanosine monophosphate (cGMP), an important cascade signal, are found in many mammalian cells such as Sertoli cells and germinal cells in the testis tissue (39). Nitric oxide plays a pivotal role in blood circulation regulation in the reproductive system and previous studies have reported an increase in nitric oxide expression along with apoptosis in germinal cells (40).

Conclusion

The findings of this study showed that crocin improved some of the reproductive parameters in mice treated with nicotine. The antioxidant effects of crocin might have been a major reason for its

positive impact on reproductive parameters. However, further studies are required to define its exact mechanism of action.

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Original Article

Ethyl Pyruvate Ameliorates The Damage Induced by Cyclophosphamide on Adult Mice Testes

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Abstract

Background: Cyclophosphamide (CP) is a chemotherapy drug which causes deleterious effects on testicular tissue and increases free radicals in the body. The aim of this study is to investigate the protective effects of ethyl pyruvate (EP) on testicular improvement in CP treated animals.

Materials and Methods: In this experimental study, 15 male mice (6-8 weeks) were divided into 3 groups. The control group received normal saline (0.1 ml/day), intraperitoneal (IP), CP group received CP (15 mg/kg/week, IP), and the CP+EP group received EP (40 mg/kg/day, IP) plus CP. After 35 days, we assessed serum total antioxidant capacity (TAC) along with histomorphometric and histochemical analyses of the testicles.

Results: The mean thickness of the germinal epithelium, diameter of seminiferous tubules, and the number of Leydig cells in the CP+EP group were higher than those of the CP group ($P<0.05$). The number of the mast cells in the CP+EP group significantly reduced compared with the CP group ($P<0.05$). Alkaline phosphatase (ALP), periodic acid-schiff (PAS) positive reactions and lipid granules in cytoplasm of the Leydig cells in the CP group increased compared with the other groups ($P<0.05$). TAC in the CP group significantly reduced compared with the other groups ($P<0.05$).

Conclusion: This study showed the ability of EP to reduce the destructive side effects of CP in the adult mice reproductive system.

Keywords: Testis, Cyclophosphamide, Ethyl Pyruvate

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Introduction

A number of chemotherapeutic drugs such as cyclophosphamide (CP) that are used for neoplastic patients leave toxic side effects in various systems of the body, including the male reproductive system. Chemotherapy with CP disrupts reductive reactions in tissues and creates oxidative stress (1-3) as an alkylating agent, finally reducing fertilization in patients under treatment (4, 5). CP is converted into its active metabolites with the action of oxidase enzymes in the liver (6). Phosphoramide mustard and acrolein are active metabolites of CP (7). All anticancer effects of CP related to phosphoramide mustard and its toxic effects are related to acrolein (8). Acrolein, as a toxic metabolite of CP, interferes with the antioxidant system of tissues (9), producing a high level of reactive oxygen species (ROS) (2, 10). The cytotoxic effects of CP particularly target rapidly proliferating cells; hence the testicles are a target for the destructive effects of this drug (11). According to the importance of reproduction in humans and the use of antioxidants to decrease or eliminate free radicals produced by CP, we have chosen ethyl pyruvate (EP), a synthetic antioxidant with different therapeutic

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properties, for this study. EP is a primary anti-inflammatory, anti-oxidant molecule which improves local inflammation in the liver and, as a result, reduces secondary hepatic injury caused by acute pancreatitis (12). In addition, EP has a protective effect on nerves against paraquat toxicity (13). The effects of EP on oxidative stress caused by CP have not been studied in testicular tissue. Hence, this study evaluated the protective effects of EP on improvement of the testicles and serum antioxidants in CP treated animals.

Materials and Methods

Drugs and chemicals

CP (500 mg) was obtained from Baxter, Germany. EP was purchased from Sigma Aldrich (MO, USA).

Animals

In this experimental study, 15 adult male mice Naval Medical Research Institute (NMRI) mice (6-8 weeks) that weighed 20-25 g were used. The animals were randomly divided into three groups and maintained under standard conditions at 22 ± 2°C, 30-60% humidity, with 14 hours daylight and 10 hours darkness. All performed experiments in this study were in accordance with the guidance of the Ethical Committee for Research on Laboratory Animals at Urmia University.

Experimental design

Animals were divided into three groups, as follows: i. Control group (C) received normal saline [0.2 ml/day, intraperitoneal (IP)], ii. CP group received (15 mg/kg/week, IP) of CP, and iii. CP+EP group received EP (40 mg/kg/day, IP) plus CP (15 mg/kg/week, IP).
After 35 days, all mice were anesthetized and euthanized with ketamine (25 mg/kg, IP) after which serum and testicular samples were taken for further analyses.

Biochemical analysis

After the serum samples centrifuged at 3000 for 5 minutes twice, total antioxidant capacity (TAC) measured according to the Benzian method (14).

Histological analyses

The right testicles were fixed in 10% formal saline for 72 hours, after which the samples were dehydrated, cleared, and embedded in paraffin. Paraffin sections were prepared (6-7 µm in thickness) and stained with hematoxylin and eosin (H&E) for histomorphometry analyses with an Olympus light microscope (BH-2 model) and calibrated, graded objective lens. We measured the germinal epithelium thickness, diameter of the seminiferous tubules, and the number of Leydig cells in 1 mm^2 by using a latticed objective lens. We investigated the interstitial tissue in terms of edema and hyperemia, and seminiferous tubules in terms of morphological features such as the germinal epithelium. Toluidine blue staining was used to assess the mean number of mast cells (15).

Histochemical analyses

Oil red-O staining was performed on formalin buffer fixed specimens and frozen sections to evaluate the rate of lipid foci (brilliant red) supplement in Leydig cells and germinal epithelium (15). Other sections were stained with alkaline phosphatase (ALP) (16). ALP staining of testis tissue causes a dark brownish color reaction. Granules that contain carbohydrate compounds were stained with periodic acid-schiff (PAS) (17). PAS positive granules stained a brilliant red color.

Statistical analysis

The data were analyzed by SPSS software (version 20, SPSS Inc., USA); one-way ANOVA and the Bonferroni test were used. A $P<0.05$ was considered significant.

Results

Ethyl pyruvate ameliorates the germinal epithelium disarrangement induced by cyclophosphamide in the EP+CP group

Histological studies showed the presence of edema in the interstitial tissue, disruption of spermatogenic cells, and reduction of germinal epithelium height in most seminiferous tubules in the CP group compared to the control group. These conditions clearly improved in the CP+EP group (Fig.1).

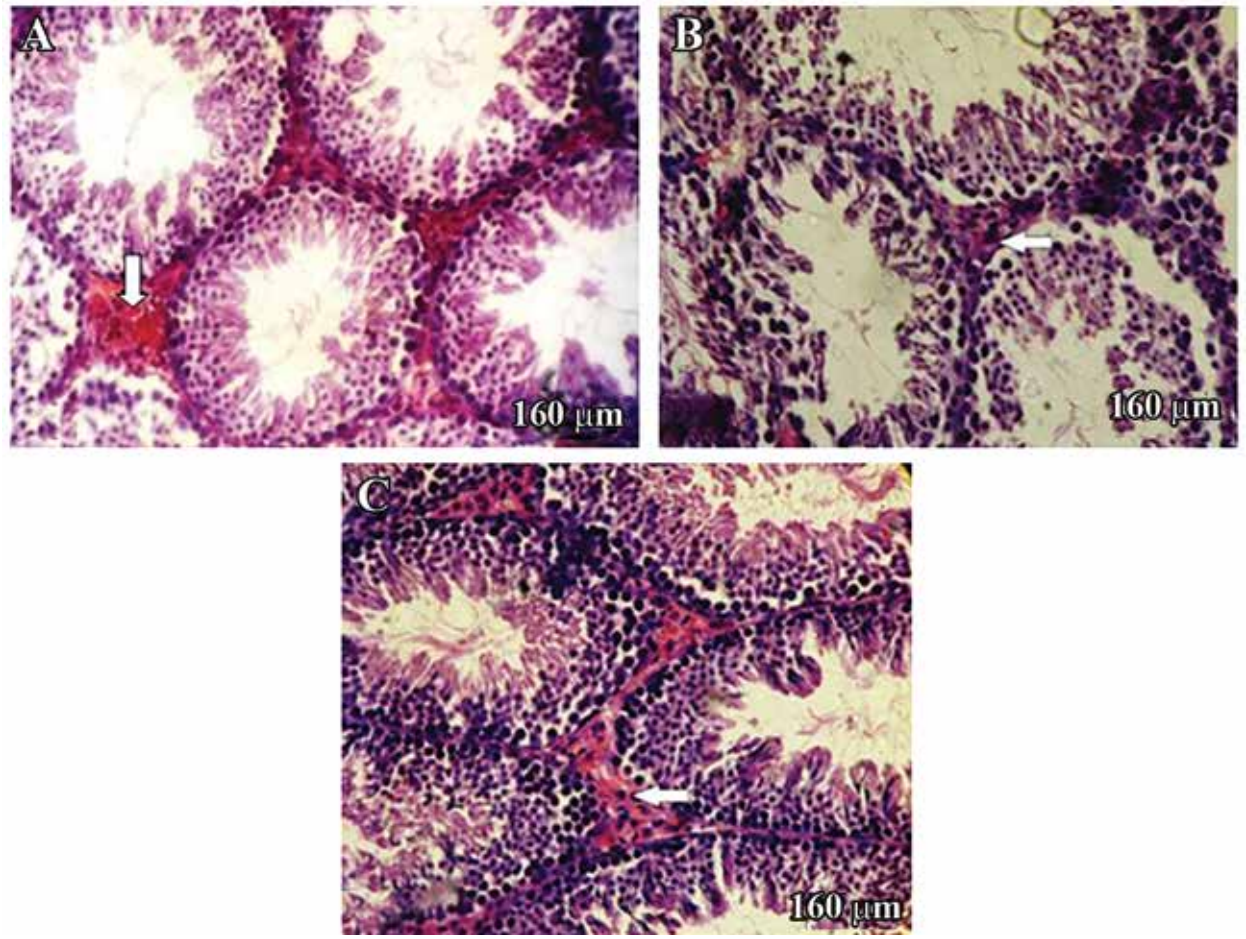

Fig.1: Histological changes in the: **A.** Control (C) group, **B.** Cyclophosphamide (CP) and **C.** CP+ethyl pyruvate (EP) groups. Leydig cells present in interstitial tissue (thick arrows), which was prominent in the C and CP+EP groups. The cytoplasm stained intensely with eosin (A) compared with the CP (B). Notice the germinal epithelium that is integrated in the CP+EP group (C), whereas it was disorganized in the CP group (B) (H&E; ×400).

Ethyl pyruvate ameliorates the thickness of germinal epithelium and seminiferous tubules in the EP+cyclophosphamide group

Morphometric studies showed that the germinal epithelium in the CP group was disarranged and disrupted. Its thickness significantly reduced compared to the control and EP+CP groups ($P<0.05$, Fig.2). There were significantly decreased seminiferous tubule diameters in the CP group compared with the other groups ($P<0.05$). However the control and EP+CP groups did not significantly differ (Fig.3).

Ethyl pyruvate increased the number of Leydig cells in the cyclophosphamide+EP group

This study showed that Leydig cells were in the interstitial tissue, almost accumulating around the blood vessels. They had an extensive acidophilic cytoplasm visualized by H&E staining, with spherical, euchromatic nuclei in the middle of the cells (Fig.1). There were a significantly reduced mean number of Leydig cells in the CP group compared with the other groups ($P<0.05$, Fig.4).

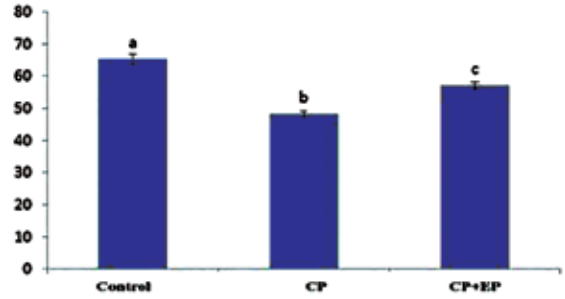

Fig.2: Germinal epithelium thicknessin testis (mean ± SE, µm). Non-similar letters (a, b, c) indicate significant differences (P<0.05). CP; Cyclophosphamide and EP; Ethyl pyruvate.

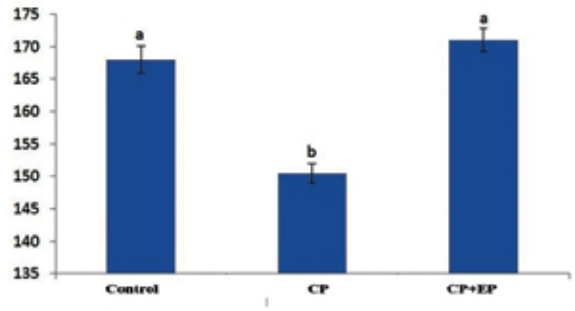

Fig.3: Diameter of seminiferous tubules (mean ± SE, µm). Non-similar letters (a, b, c) indicate significant difference (P<0.05). CP; Cyclophosphamide and EP; Ethyl pyruvate.

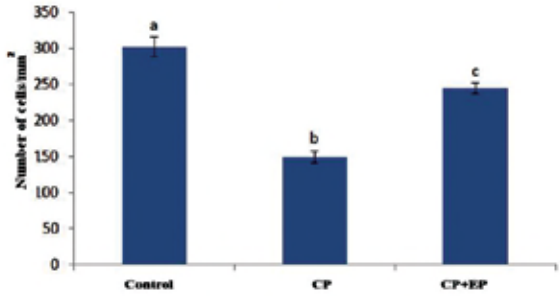

Fig.4: Number of Leydig cells in testis (mean ± SE). Non-similar letters (a, b, c) indicate significant difference (P<0.05). CP; Cyclophosphamide and EP; Ethyl pyruvate.

Ethyl pyruvate ameliorates the histochemical feature of the testis in cyclophosphamide treated mice

According to oil red-O staining, lipid granules in the cytoplasm of Leydig cells in the CP group increased compared to the other groups. Accumulation of lipid in the cytoplasm of spermatogenic cells adjacent to the basal lamina of the seminiferous tubules was observed in the CP group (Fig.5). PAS staining showed an increased PAS positive reaction in cells adjacent to the lumen of seminiferous tubules and Leydig cells in the CP group compared with the control and CP+EP groups (Fig.6). Reaction of ALP as dark brown fine granules in the interstitial tissue of the testicle was observed in the CP group; this reaction considerably reduced in the CP+EP group (Fig.7).

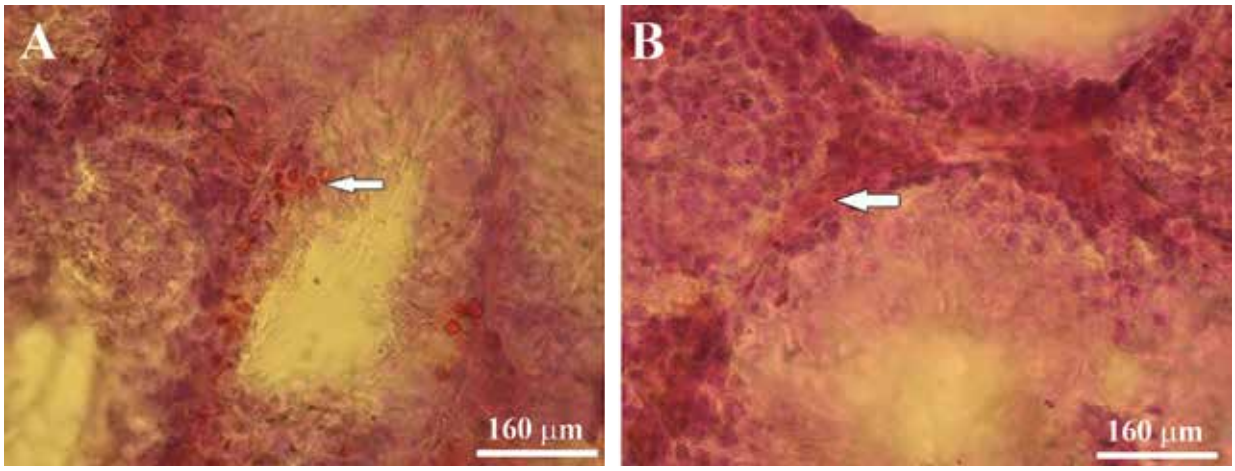

Fig.5: Lipid accumulation shown as red granules in the cyclophosphamide (CP), CP+ethyl pyruvate (EP) groups is detected in the **A.** Cytoplasm of spermatogenic and Sertoli cells in testes of the CP group (arrow) and **B.** Cytoplasm of Leydig or interstitial endocrine cells (arrow) in the CP+EP group (Oil red-O staining, ×400).

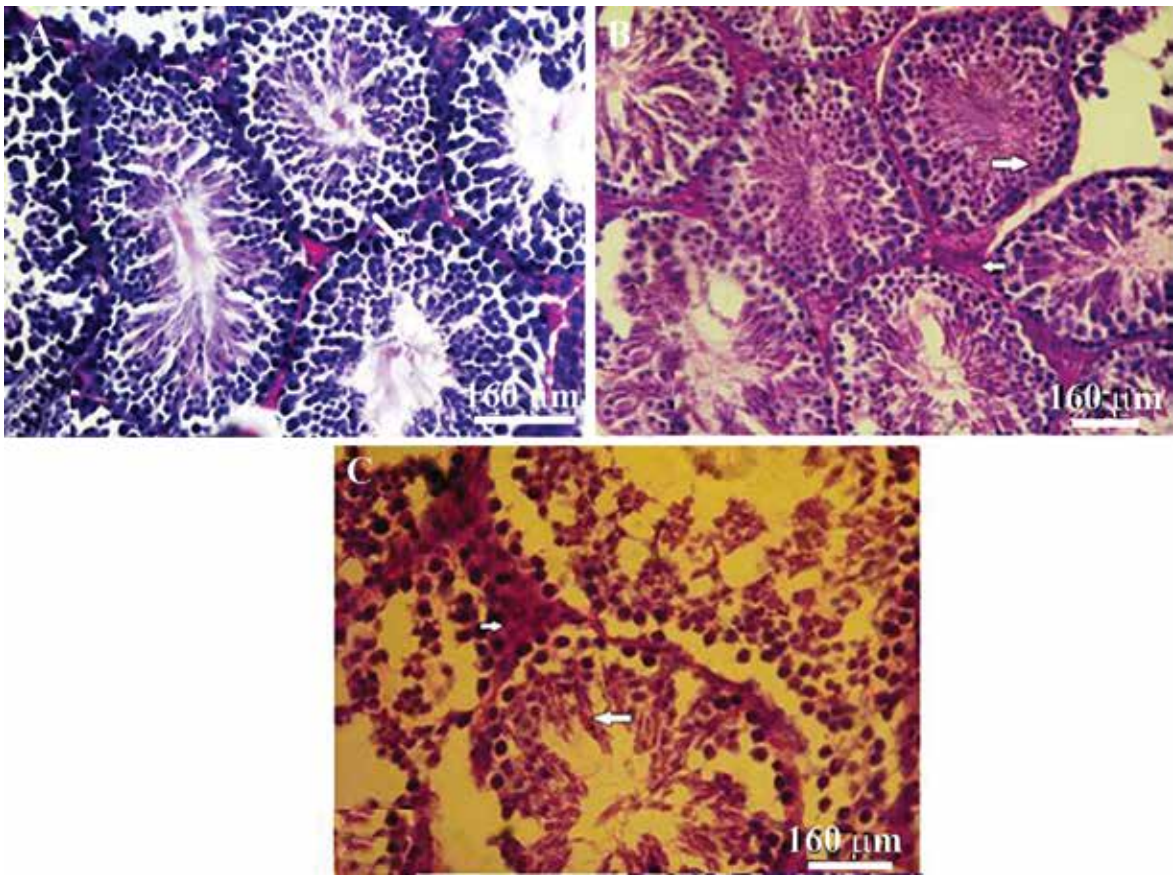

Fig.6: Periodic acid-schiff (PAS) reaction in the **A.** Control (C) group, **B.** Cyclophosphamide (CP) and **C.** CP+ethyl pyruvate (EP) groups. B. Accumulation of carbohydrate as red granules in the cytoplasm of Leydig cells (small arrows) shown in the CP group. PAS reaction was faintly observed in spermiogenic cells of the B. CP group (thick arrow) and in the cytoplasm of spermatogenic cells in the control, CP and CP+EP groups (thick arrows) (magnification: ×400).

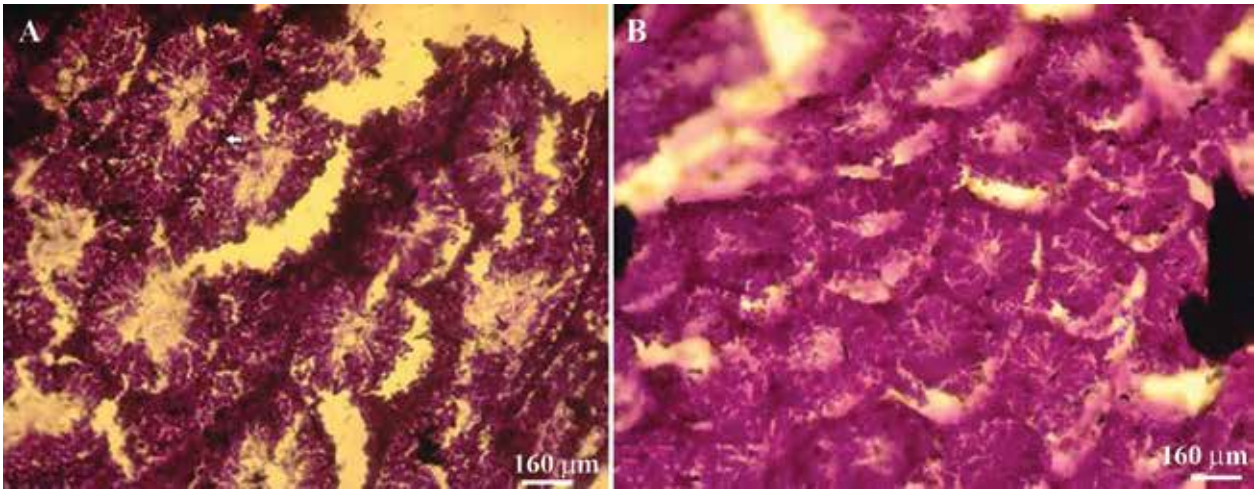

Fig.7: Alkaline phosphatase (ALP) reaction in the cyclophosphamide (CP) and CP+ethyl pyruvate (EP) groups shown as dark granules in Leydig cells located in the interstitial tissue of mice testis in the **A.** CP group (arrow), while this reaction was scant in the **B.** CP+EP group (magnification: ×400).

Ethyl pyruvate reduces the number of mast cells during oxidative stress

The numbers of mast cells in the testicular capsule were determined by toluidine blue staining. We observed that the cytoplasm of the mast cells were full of dark reddish violet granules (metachromatic) in the testicular capsule (Fig.8). There was a significantly higher mean number of mast cells in the CP group compared to the control group (P<0.05), while EP in the CP+EP group reduced the mean number of these cells to a level comparable to the control group (P<0.05, Fig.9).

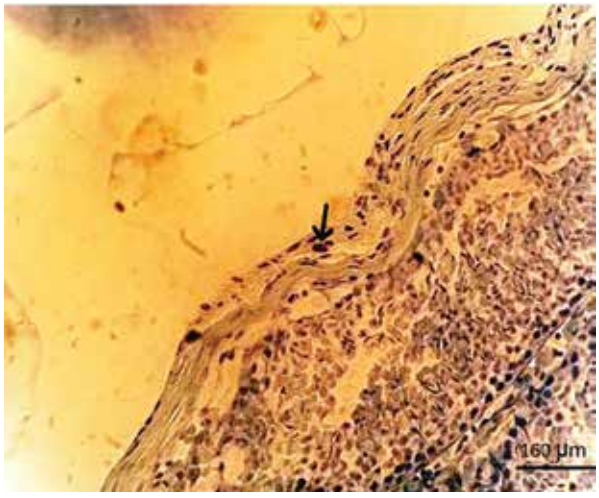

Fig.8: Mast cell localization in testicular capsule. Mast cell with dark purple granule that occupied the cytoplasm in the testicular capsule of the cyclophosphamide (CP) group (arrow). (Toluidine blue staining, magnification: ×400).

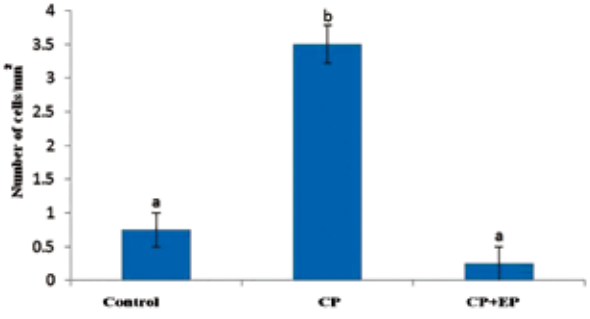

Fig.9: Number of mast cells in the testicular capsules (mean ± SE). Non-similar letters (a, b, c) indicate significant difference (P<0.05). CP; Cyclophosphamide and EP; Ethyl pyruvate.

Ethyl pyruvate elevates total antioxidant capacity against cyclophosphamide induced oxidative stress

There was reduced serum TAC in the CP group compared with the control and CP+EP groups. We observed moderate reduction in the CP+EP group. This reduction was significant only with the control group (P<0.05, Fig.10).

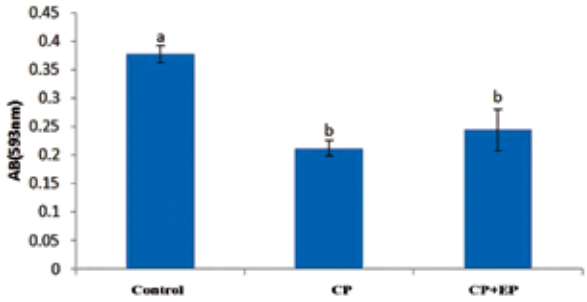

Fig.10: Total antioxidant capacity (TAC) levels in different groups (mean ± SE). Non-similar letters (a, b, c) indicate significant difference (P<0.05).

Discussion

According to previous studies, the toxic side effects of CP cause histological, histochemical and serological changes (2, 18, 19). Chemotherapy causes long-term or permanent azoospermiadue to destruction and damage of the testicular germ cells (20). We have observed that damage to germinal cells with CP was a main reason for the reduction in diameter of seminiferous tubules and height of germinal epithelium in the CP group. EP, with its antioxidant effects, caused a significant increase in these two parameters in the CP+EP group.

The toxic effect of chemotherapy on Leydig cells and indirect effect of damaging spermatogenic cells on negative performance of Leydig cells (21) justified the significant reduction in numbers of these cells in the CP group compared to the other groups. Accumulation of neutral lipids in the cytoplasm of spermatogenic cells adjacent to the basal lamina of seminiferous tubules in the CP group compared with other groups could be related to destruction of spermatogenic cells and accumulation of unconsumed lipid for biosynthesis of steroid hormones (22). Accumulation of lipid in this region might be related to increased phagocytosis of the apoptotic spermatogenic cells by Sertoli cells (23).

On the other hand, it has been shown that oxidative mechanisms increase active species of oxygen and lipid peroxidation by inactivating microsomal enzymes (24). The role of CP in production of free radicals and reduction of antioxidants (19, 25) was the logical reason for reduction of serum TAC in the CP group compared to the other groups.

Allergic and immunologic stimulations caused by prescription of CP increases the mean number of mast cells in the testicle capsule and consequently increase production of free radicals with degranulated mast cells, leading to reproductive disorders (26). On the other hand, degranulation of mast cells following acute physical and chemical stresses lead to secretion of histamine which increases permeability of the blood vessels (27). Increases in permeability of blood vessels and tissue edema by stimulation of apoptosis in endothelial cells (28, 29) and smooth muscular cells of the blood vessel wall (30, 31) also occur. With respect to results of the above studies, we have observed an increased number of mast cells in the testicle capsule, edema, and hyperemia in the interstitial tissue of the CP group. These would be expected side effects of CP on the testicles, which considerably reduced in the CP+EP group.

ALP enzyme activity in the testicles of rats with varicocele increased with degeneration of the reproductive cells (32). Therefore, the increased ALP reaction observed in the CP group was affected by the destructive effects of CP on reproductive cells of the testicle. Reduction of this reaction in the CP+EP group has supported results of previous studies. These degenerative changes in testicular tissue reduce glucose transmitters (33). CP disrupts transmission of glucose to the seminiferous tubules and spermatogenic cells, which have high a mitotic activity and a negative reaction against PAS staining due to the damage of these transmitters.

Conclusion

This study showed the protective effects of EP in the testis of CP treated mice.

Acknowledgements

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Original Article

The Effects of Testosterone on Oxidative Stress Markers in Mice with Spinal Cord Injuries

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Abstract

Background: Spinal cord injury (SCI) causes infertility in male patients through erectile dysfunction, ejaculatory dysfunction, semen and hormone abnormalities. Oxidative stress (OS) is involved in poor semen quality and subsequent infertility in males with SCI. The aim of this study is to examine the effects of SCI on the level of testosterone hormone.

Materials and Methods: In this experimental study, we evaluated the effects of exogenous testosterone on the activity of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) as well as the levels of malondialdehyde (MDA) and protein carbonylation (PCO), as markers of OS, in 10 groups of SCI mice. Total antioxidant capacity (TAC) was determined using the 2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation assay.

Results: Exogenous testosterone administration in mice with SCI significantly reduced SOD and GPx enzyme activities and MDA level. There was no significant decrease in PCO content. In addition, TAC remarkably increased in the sham and SCI groups not treated with testosterone but remained unchanged in all other experimental groups. Exogenous testosterone also reduced serum testosterone levels in all groups except the positive control group.

Conclusion: Our cumulative data indicated that SCI could cause sterility by disturbing the plasmatic testosterone balance. The normal level of endogenous testosterone was not completely restored by exogenous testosterone administration.

Keywords: Spinal Cord Injury, Infertility, Testosterone, Oxidative Stress, Reactive Oxygen Species

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Introduction

Spinal cord injury (SCI) is a traumatic or non-traumatic injury which occurs most often in 16-45 year-old males at the peak of their reproductive lives, permanently affecting quality of life (1). The majority of male patients with SCI have a weak reproductive function and distinct sperm profile with normal sperm count, but the sperm motility is abnormally low (1-4). However, the mechanisms responsible for poor sperm quality in men with SCI have not been clearly defined. It has been reported that abnormalities of hormones and hypothalamic-pituitary-gonadal axis dysfunction can be involved as a consequence of SCI in males (5). On the other hand, there is in-

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creasing evidence for the impact of oxidative stress (OS) on the sperm quality in this group of patients (2). Several studies have demonstrated a significant increase in the generation of reactive oxygen species (ROS) in males with SCI. It is well established that physiological level of ROS is necessary for normal sperm function and reactions that include oocyte fusion, capacitation and acrosome reaction (AR) while the excess amounts of ROS in semen can induce OS which negatively affect spermatozoa (2, 6). Seminal ROS strike a wide range of essential biomolecules such as proteins, lipids, carbohydrates and nucleic acids, and affect their functions. This impact may consequently be involved in DNA damage, decreased sperm motility, reduced sperm viability, sperm dysfunction, and semen hyperviscosity (7). Lipid peroxidation of sperm plasma membranes by ROS causes reduced membrane fluidity (1, 7).

Seminal fluid contains several defense mechanisms which are focused on oxidant scavenging to protect spermatozoa from detrimental oxidative injury. These include important antioxidant enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) which quench hydrogen peroxide and the excess free superoxide radicals. The seminal fluid also contains non-enzymatic antioxidants such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), carnitine and pyruvate. In this regard, elevated OS and reduced antioxidant activity in the seminal plasma lead to damaged sperm function and subsequent male infertility. It has been reported that infertile patients with and/or without SCI have discrepancies in seminal levels of ROS (7-9). Imbalanced hormonal levels, especially testosterone and follicle-stimulating hormone (FSH), are observed in SCI patients (2, 7). There are conflicting reports that demonstrate the direct roles of testosterone and FSH hormones in reproductive dysfunction in men with SCI.

Therefore, the present study aimed to examine the effects of SCI on the level of testosterone hormone. We determined whether a testosterone imbalance was involved in increased OS and resultant reproductive dysfunction.

Materials and Methods

Animals

Adult male mice, 4 to 6 months of age, that weighed 15 to 25 g were obtained from the School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. Mice were kept in temperature-controlled quarters on a 12:12 hour light:dark schedule, with standard mouse pellets and drinking water ad libitum.

Experimental design

In this experimental, we randomly divided the mice into 10 groups each, with 6 animals per group according to Table 1.

Table 1: Experimental design

	Laminectomy	SCI	Testosterone injection	Sampling day after SCI
P. Control	-	-	0.1 mg/kg BW-7 days	After completion of the period of injection
N. Control	-	-	-	Coincident with P. Control
SCI-0.0-7	+	+	-	1 week
SCI-0.0-35	+	+	-	35 days
SCI-0.1-7	+	+	0.1 mg/kg BW-7 days	2 weeks
SCI-0.1-14	+	-	0.1 mg/kg BW-7 days	3 weeks
SCI-0.1-35	+	+	0.1 mg/kg BW-35 days	35 days
SCI-0.1-42	+	+	0.1 mg/kg BW-35 days	42 days
P. Laminectomy (Sham group 1)	+	-	0.1 mg/kg BW-7 days	2 weeks after laminectomy
N. Laminectomy (Sham group 2)	+	-	-	Coincident with P. Laminectomy group

SCI; Spinal cord injury and BW; Body weight.

SCI-0.1-7 and SCI-0.1-42 received injections of testosterone one week after infliction of the SCI. SCI-0.1-14 received an injection of testosterone two weeks after infliction of the SCI. SCI-0.1-35 received an injection of testosterone at the same time as infliction of the SCI. N. Laminectomy was a sham group that did not receive any testosterone and P. Laminectomy was a sham group that received a testosterone injection.The N. Laminectomy and P. Laminectomy groups each underwent a sham operation. The N. Laminectomy did not receive testosterone replacement whereas the P. Laminectomy group received testosterone replacement.

Experimental protocol

We performed the SCI according to procedures by Yu et al. (10). We used a model that provided extradural compression of the spinal cord from the dorsal side. Briefly, the animals were anesthetized by xylazine [5 mg/kg body weight (BW)] and ketamine (50 mg/kg BW) injections; the laminae of the T9 and T11 vertebrae were removed, leaving the dura intact. The animals were placed in the prone position in a stereotaxic apparatus to perform the laminectomy and stabilize the spinal cord. A weight of 35 g was applied onto the intact dura for 5 minutes, using a curved rectangular plate (2.2×5 mm). According to evidence, serum testosterone levels have been shown to significantly reduce 3-7 days after SCI. The serum level of testosterone might return to normal levels by 14 days due to cellular compensatory mechanisms (11). The duration of spermatogenesis in mice is 32-35 days. Accordingly, we have divided the mice into six SCI groups, without and with testosterone administration, according to different intervals of administration and times for blood sampling (7, 14, 35 and 42 days).

Plasma levels of testosterone were estimated by a commercial enzyme linked immunosorbent assay (ELISA) kit (Cayman Chemical, USA) and expressed as ng/ml. The malondialdehyde (MDA, Hitachi, Japan) component of the blood samples was expressed as nanomoles of MDA created per mg protein and measured by the spectrophotometric method. We measured total antioxidant capacity (TAC) of plasma by the ABTS radical cation assay (12). SOD was examined by a Biovision kit (Biovision, USA). GPx was measured with a Randox kit (Randox, UK). Protein carbonylation content of mouse serum was assayed using a BioCell Protein Carbonyl Assay Kit (BioCell, New Zealand).

Statistical analysis

All data were expressed as mean ± SEM of three independent experiments. The data were analyzed by one-way ANOVA followed by the Tukey test. The statistical analyses were performed using Graphpad Prism statistical software (9). $P<0.05$ was regarded as statistically significant.

Results

During the 6 weeks of testosterone treatment, all of the mice groups remained healthy and grew at an ordinary rate. There was no significant difference between the body weight and weights of the testes, epididymis, seminal vesicles or ventral prostate of mice treated with testosterone or sham (data not shown).

The results presented in Figure 1 revealed that administration of exogenous testosterone led to a significant ($P<0.001$) reduction in total plasma testosterone level in all groups except for the positive control. However, the sham groups exhibited relatively less decrease compared with the other groups.

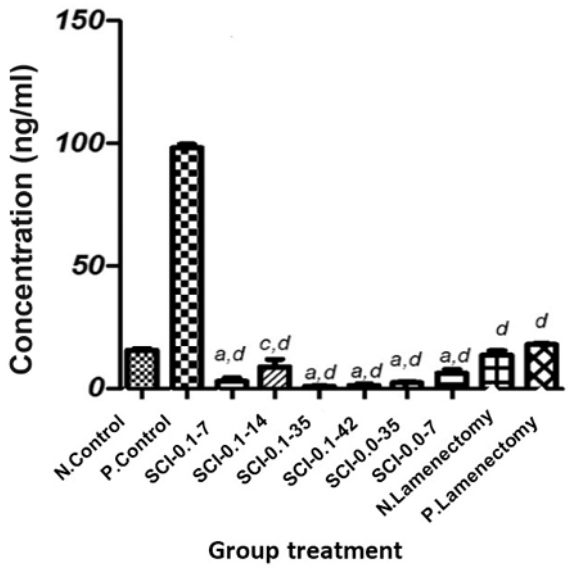

Fig.1: Effect of exogenous testosterone on plasma testosterone levels. The details of groups is in material and method section. Results are expressed as mean ± SD. a; $P<0.001$ compared with N. Control, c; $P<0.05$ compared with N. Control and d; $P<0.001$ compared with P. Control.

As shown in Figure 2, MDA levels decreased (significantly and insignificantly) in mice plasma as a consequence of testosterone administration

while the negative sham group had a significantly increased MDA level (P<0.01).

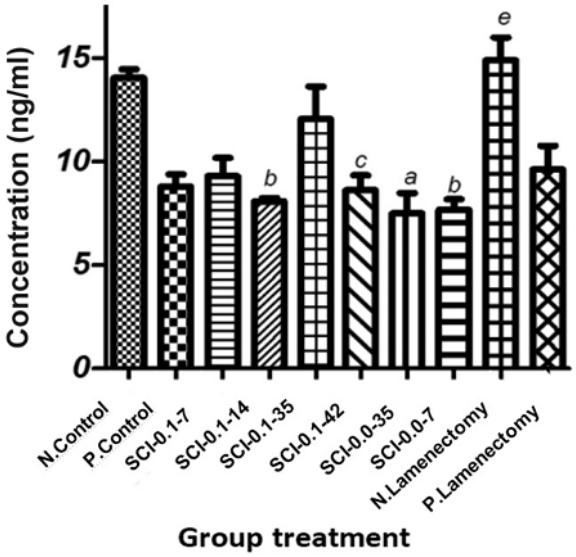

Fig.2: Effect of exogenous testosterone on malondialdehyde (MDA) level. The details of groups is in material and method section.Results are expressed as mean ± SD. a; P<0.001 compared with N. Control, b; P<0.01 compared with N. Control, c; P<0.05 compared with N. Control and e; P<0.01 compared with P. Control.

The influence of testosterone on other antioxidant biomarkers such as GPx, SOD, protein carbonyl and TAC are presented in Table 2. The findings depicted that testosterone administration had various effects on GPx and SOD in the tested groups. The results showed that testosterone administration significantly reduced GPx activity in the SCI-0.0-7 group relative to the control group. There was significantly diminished GPx activity in the sham groups with testosterone administration. On the other hand, the testosterone administration did not significantly affect GPx. In contrast, the testosterone treatment showed a considerable increase in GPx activity in the SCI group with administration of testosterone (0.1 mg/kg of body weight) after one week compared to the positive control group (P<0.001). There was a similar trend observed for SOD activity. According to Table 2, the testosterone administration showed fluctuations on SOD activity, which in some cases caused significantly increased (P<0.001 compared with both negative and positive groups) SOD activity in the positive sham group. However, in some tested groups, the administration of testosterone led to a nonsignificant reduction of SOD activity. In contrast, the effect of testosterone administration on TAC did not show considerable differences in the tested groups. We observed a significant increase in the sham (P<0.001) and SCI groups without testosterone treatment (P<0.05) in TAC plasma content in comparison with both positive and negative control groups. In addition, all tested groups showed a significant reduction (P<0.001) in protein carbonyl levels relative to the positive control, but not the negative control. The SCI groups without testosterone treatment showed considerably elevated (P<0.001 and P<0.01) protein carbonyl levels in comparison to the negative control. Generally, testosterone treatment failed to reduce protein carbonyl levels in all tested subjects.

Table 2: Effect of exogenous testosterone on testicular antioxidant enzymes (GPx and SOD)

	N. Control	P. Control	SCI-0.1-7	SCI-0.1-14	SCI-0.1-35	SCI-0.1-42	SCI-0.0-35	SCI-0.0-7	N. Lamenectomy	P. Lamenectomy
GPX	27.98 ± 1.64	31 ± 2.89	36.43 ± 8.41[d]	27 ± 2.60	33.5 ± 1.87	27.6 ± 3.13	24.02 ± 0.52[f]	22.5 ± 1.20[d]	28.1 ± 3.32	18.3 ± 2.40[a, d]
SOD	326.5 ± 183.20	276.7 ± 52.03	310.8 ± 101.90	319.7 ± 26.76	147.2 ± 21.14	292.2 ± 78.87	449.3 ± 27.51	308 ± 103.70	392.8 ± 46.86	619.2 ± 124.20[a, d]
Protein Carbonyl	0.28 ± 0.013	0.38 ± 0.051	0.30 ± 0.031[d]	0.27 ± 0.013[d]	0.29 ± 0.010[d]	0.28 ± 0.029[d]	0.36 ± 0.009[a]	0.35 ± 0.030[b]	0.26 ± 0.004[d]	0.28 ± 0.006[d]
TAC	2.11 ± 0.0	2.11 ± 0.0	2.11 ± 0.0	2.11 ± 0.0	2.11 ± 0.0	2.027 ± 0.16	2.955 ± 0.12	3.19 ± 0.31	2.792 ± 0.05	2.858 ± 0.12

SOD; Superoxide dismutase, GPx; Glutathione peroxidase, SCI; Spinal cord injury, TAC; Total antioxidant capacity, [a]; P<0.001 compared with N. Control, [b]; P<0.01 compared with N. Control, [d]; P<0.001 compared with P. Control, and [f]; P<0.05 compared with P. Control.

Discussion

Recently, attention has been paid to male sterility after SCI. Most male patients with SCI have poor semen quality, as evidenced by leukocytospermia and sperm motility (3, 4, 7, 13, 14). The presence of activated T cells in men with SCI leads to cytokine synthesis, which in turn affects human sperm motility and increases the production of ROS (15). It has been reported that SCI temporary and intensely influences the pituitary-testicular hormone axis. These alterations may be involved in Sertoli cell dysfunctions and consequent abnormalities in regular spermatogenesis (11). However, the cause of infertility in men with SCI is not clearly recognized. It has been reported that infertility might be related to sexual hormone imbalance and/or ROS production (2, 6). Therefore, elucidation of some of the differences in the literature might be essential.

In this regard, we evaluated whether SCI caused sexual hormone imbalances and ROS production. We also examined whether exogenous testosterone could make tribulation. Our results showed that the normal level of plasma testosterone was not restored by exogenous testosterone administration in all SCI mice except for the SCI 0.1 (2 W) groups. In agreement with a number of studies, our observations indicated that the administration of exogenous testosterone led to downregulation of natural testosterone production by the testes (16). Since such a decrease occurred only in the spinal cord-operated mice, and not in sham-operated ones that endured similar surgery-related stress.

It could be concluded that SCI might at least partially be involved in testosterone downregulation. However, the trivial immobilization of the mice following SCI might have resulted in more stress, which could have influenced the pituitary-testicular hormone axis and consequently suppressed natural testosterone production (11). It has also been reported that the denervation of testis in immature rats resulted in impairment of Leydig cell androgen production, which was comparable to the subjects with SCI damage (17).

The acute suppression of testosterone production in testes after SCI, with or without an attendant decline in serum FSH levels, could definitely compromise Sertoli cell functions and cause some of the abnormalities in spermatogenesis (11). These abnormalities might be attributed to increased OS that has resulted from an imbalance between the production of ROS and antioxidant agents (18). ROS, unstable and extremely reactive by-products of normal metabolism, mediate oxidative damages to cellular macromolecules (9). Since testosterone typically improves the metabolic rate (19, 20), it can be expected that a high dose of testosterone level may be involved in the imbalance between ROS production and antioxidant defenses causing an increased risk of OS. In this regard, several researchers have reported that testosterone plays a pro-oxidant role and induces OS in mammalian tissues (20). On the other hand, it has also been reported that testosterone has an antioxidant effect in the human prostate (21) and rat nervous system (9). These outcomes consequently indicate that the pro-oxidant property of testosterone is tissue and sex-dependent. In this regard, testes are principally susceptible to ROS-induced injury by virtue of testosterone's pro-oxidant activity.

Inconsistently, Chainy et al. (6) have reported that elevated MDA levels in response to testosterone treatment, whereas Peltola et al. (22) reported that testosterone decreased the level of MDA. In an *in vitro* study, Mooradian showed that administration of exogenous testosterone did not have significant pro-oxidant activity. Our results indicated that the MDA level did not increase, but reduced in groups that received testosterone, which suggested that testosterone suppressed H_2O_2 production and decreased production of MDA (23).

Protein carbonyl, as a marker of OS, is one of the OS by-products which form during the interaction between ROS and proteins. This interaction modifies biomolecules and changes their functions eventually leading to irretrievable cellular damage (24). In agreement with some studies, our results (SCI-operated without testosterone administration) have demonstrated that protein carbonyl increases while the level of plasma testosterone decreases (25). However, administration of exogenous testosterone showed no significant effect on protein carbonyl production in the SCI groups relative to the negative control. Therefore, this suggested that exogenous testosterone did not have a protective effect on this type of oxidative damage.

Plasmatic TAC is the other antioxidant defense

system against OS. The functional sum of antioxidants in plasma is used as measure of extracellular antioxidant barrier (26). Thus, we have measured TAC after testosterone administration in SCI-operated groups. Our findings indicated that administration of exogenous testosterone did not increase plasma TAC. However, TAC levels increased in the sham group and groups that did not receive testosterone. Therefore, our results reinforced the pro-oxidative properties of testosterone. Contrary to our results, Mancini et al. (26) reported that TAC had a significant association with total testosterone in male subjects. Such a discrepancy might be attributed to several factors such as gender-specific gene expression, vascular factors, distribution of body fat, and adaptation to aging. In addition, studies on exogenous testosterone administration were affected by dose, duration and route of administration. Thus, a study of antioxidant regulation by steroids could help to elucidate molecular mechanisms of testosterone function.

Injections of testosterone into adult mice manipulated the level of testosterone in the testes. Our results revealed that the testosterone injection caused antioxidant enzymes (GPx and SOD) reduction in testes in most groups, which agreed with findings reported by Chainy et al. (6). The precise mechanism of testosterone-induced reduction in the levels of GPx and SOD enzymes in the testis was not well reported. It has been reported that administration of 10 mg testosterone to intact rats caused a profound reduction (82%) in the serum luteinizing hormone (LH) level with no alteration in the FSH level (6) which regulated testosterone production in the testis (27). In addition, these antioxidant enzymes have synergistic functions. An abnormality of one of the antioxidant enzymes can affect the activities of the other enzymes. A reduction in SOD activity causes an elevation in the level of O_2-, which in turn causes inactivation of catalase (CAT) activity. Equally, when GPx or CAT fails to eradicate H_2O_2, the content of H_2O_2 may be upregulated by inactivation of SOD and vice versa (6). In general, administration of exogenous testosterone causes antioxidant enzyme reduction followed by OS induction.

Conclusion

Collectively, SCI, a neurogenic impairment, causes infertility through disturbing the plasma testosterone balance which could not be retrieved by administration of exogenous testosterone. In this regard, SCI led to a slight change in oxidative markers, with the exception of MDA which decreased. There was reduced free radical scavenging activities of SOD and GPx. Such an effect reinforced the pro-oxidant property of testosterone. Therefore, administration of exogenous testosterone would not compensate sexual hormone disturbance along with anti-oxidative protective effects. However, the exact causal mechanism leading to sexual hormone disturbances in males with SCI remains to be elucidated.

Acknowledgements

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Original Article

Investigation of Personality Traits between Infertile Women Submitted to Assisted Reproductive Technology or Surrogacy

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Abstract

Background: Personality traits affect human relationships, social interactions, treatment procedures, and essentially all human activities. The purpose of this study is to investigate the personality traits -including sensation seeking, flexibility, and happiness - among a variety of infertile women who were apt to choose assisted reproductive technology (ART) or surrogacy.

Materials and Methods: This is a cross-sectional study that was performed on 251 infertile women who visited Isfahan and Tehran Reproductive Medicine Center. These fertility clinics are located in Isfahan and Tehran, Iran. In this study, 201 infertile women who underwent treatment using ART and 50 infertile women who tended to have surrogacy were chosen by convenience sampling. Zuckerman's Sensation Seeking Scale Form V (SSS-V), Psychological Flexibility Questionnaire (adapted from NEO Personality Inventory-Revised) and Oxford Happiness Questionnaire (OHQ) were used as research instruments. All participants had to complete the research instruments in order to be included in this study. Data were analyzed by descriptive-analytical statistics and statistical tests including multivariate analysis of variance (MANOVA) and Z Fisher. Statistically significant effects were accepted for $P<0.05$.

Results: In the sensation-seeking variable, there was a meaningful difference between under-study groups. However, the flexibility and happiness variables did not have a significant difference between under-study groups ($P<0.001$). Interaction between education, employment, and financial status was effective in happiness of infertile women underwent ART ($P<0.05$), while age, education and financial status were also effective in happiness of infertile women sought surrogacy ($P<0.05$). A positive meaningful relationship was seen between sensation seeking and flexibility variables in both groups ($P<0.05$). And a negative meaningful relationship was seen between sensation seeking and happiness in infertile women who sought surrogacy ($P<0.05$). The difference in rate of relationship between sensation seeking and flexibility was meaningful in infertile women who sought either ART or surrogacy ($P<0.05$).

Conclusion: Sensations seeking as a personality trait is lower in infertile women who underwent treatment using ART compared women who tended to have surrogacy. This study shows that demographic variables are effective in happiness of infertile women. Also, there is a significant relation among sensation seeking, flexibility and happiness in infertile women.

Keywords: Flexibility, Happiness, Assisted Reproductive Technology

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Introduction

Infertility is a biological, mental, and social phenomenon; in other words, there are some mental, physiological, environmental, and interactional aspects affecting infertility (1). Infertility is defined as the inability to conceive, despite sexual intercourse, during a year without using any contraceptive devices. It is important to know that this period of time decreases to 6 months for women over 35 as this group of women naturally suffers a decrease in their conception ability (2).

Receiving treatments has increased the conception chance among infertile couples who have lost their ability to conceive (3). However, a research has indicated that a myriad of factors cause tension in these groups, including several medical tests, length of infertility treatment, low level of treatment success, and financial hardship from infertility treatment costs. These factors are so important that they cause infertile individuals to discontinue the treatment (4). Personality traits are among effective factors on human relation, social interaction, treatment procedure, and generally all human activities in the community (5). Sensation-seeking, flexibility, and happiness are three of the most important components of personality traits that can influence an individual's response to stressful situations.

Sensation seeking is a personality trait that puts emphasis on human behavior and interpersonal relations. As a result, many individual differences can be justified according to this trait. As Zuckerman explained, Sensation seeking is a personality trait which is experienced by seeking feelings and extensive experiences, and by accepting physical, social, legal and financial dangers (6). According to the Zuckerman, sensation seeking is a known trait that occurs with risk-taking (7). Risk-taking behaviors are those behaviors that make physical, psychological, and social results more likely to be negative and destructive (8).

A research has indicated that women who underwent assisted reproductive technology (ART) are at the increased risk of endometrial (9) and ovarian cancer (10-12). Children whose mothers gave birth with the help of ART are also more likely to suffer from physical problems. The study carried out by Ceelen et al. (13) has indicated that the risk of cardiovascular disease is more common in children born with *in vitro* fertilization (IVF). Furthermore in Iran, insurance companies refuse to cover the costs of fertility treatment, so patients have to accept the financial risks.

Surrogacy also has its own dangers; patients undergoing this treatment have to accept the probable risks (14, 15), including: increased risk of infertility, regret after facing surrogacy difficulties (16), refusing to give the baby to the couple, having a relationship with the baby's father (17), and not caring for the fetal health (18). Thus both ART and surrogacy have a number of risk factors that may be related with sensation seeking of women undergoing these procedures.

Another important personality trait that can affect infertility treatment procedures is flexibility. Psychological flexibility is defined as the ability to "recognize and adapt to various situational demands; shift mindsets or behavioral repertoires when these strategies compromise personal or social functioning; maintain balance among important life domains; and be aware, open, and committed to behaviors that are congruent with deeply held values" (19).

Some studies have demonstrated that flexibility in infertile couples could be an unknown protective factor against regression, resulting from infertility and reduction in life quality (20). In the research carried out by Repokari et al. (21), they have shown that some couples undergoing ART are more flexible to the negative effect of stressful psychological factors and consequently experience a feeling of bonding during infertility treatment.

In the case of surrogacy, flexibility again is a positive aspect of treatment. The American Society for Reproductive Medicine (ASRM) has pointed out that sympathy, compatibility, and flexibility in the couple seeking surrogacy guarantee the treatment success (22).

Happiness appears to be another personality variable. The following is the definition given by Argyle on happiness:

Happiness is the state of being happy or delightful (positive excitements), being satisfied with life being far from any anxiety and depression (negative sentiment) (23). Happiness is within the realm of health psychology and under the effect of different factors such as mental (24), physical (25), eco-

nomical (26) and religious (27) factors. Therefore, many personal differences can be justified based on these factors.

Research studies have revealed that the quality of marital relationship is considered as a meaningful predictive factor in happiness and desirable life, while low marital quality can lead to a myriad of social and family problems (28). Results of some other research studies, on the other hand, have demonstrated that infertility is associated with marital problems, causing a lot of mental and social problems (29). Therefore, it is hypothesized that infertility leads to low level of happiness in such individuals.

There is a number of research studies on depression and anxiety of infertile women, indicating that these women suffer from a high level of depression and anxiety (30-32). Depression could influence infertility treatment, follow-up programs, and future hope in these patients (33, 30). According to results from Ferreira's research, happiness increases the probability of continuing follow-up treatment programs in these women (34).

The purpose of this study is to investigate the personality traits including sensation seeking, flexibility, and happiness among a variety of infertile women who were apt to choose ART or surrogacy.

Materials and Methods

Approval for this cross-sectional study was obtained from the Isfahan University, Isfahan, Iran, in 2013. This study was performed on 251 infertile women who visited Isfahan Reproductive Center and Tehran Royan research Center. These fertility centers are located in Isfahan and Tehran, Iran. In this study, 201 infertile women who underwent ART and 50 infertile women who underwent surrogacy were selected by convenience sampling. Researchers attended these centers 4 days per week from April 13, 2013 to July 7, 2013. All participants were asked to sign an informed consent before entering the research. Data was collected using Zuckerman's Sensation Seeking Scale Form V (SSS-V), Psychological Flexibility Questionnaire (adapted from NEO Personality Inventory - Revised) and Oxford Happiness Questionnaire (OHQ). All participants also had to complete the research instruments in order to be included in this study.

Zuckerman's sensation seeking scale form V

This questionnaire was developed by Marvin Zuckerman in 1978. Different studies have estimated the reliability of this questionnaire higher than 0.85. In the study performed by Corulla (35), internal validity of this questionnaire was estimated 0.86 for females and 0.83 for males. In a research carried on university students, Mahvi Shirazi (36) reported the validity and reliability values of sensation seeking questionnaire were 0.78 and 0.80, respectively.

Psychological flexibility questionnaire

This scale is a collection of questions on three traits of imagination (O1), beliefs (O5) and familiarity factor values (O6), which was adapted from five main factors of personality. Different studies have estimated the reliability of this questionnaire higher than 0.85. In a study by Costa and McCrae (37), the validity of flexibility in the quintuple standard personality questionnaire was reported 0.87. In Iran, reliability coefficient for flexibility questionnaire was estimated 0.80 (38). Other studies in Iran by Keshavarz et al. (39) achieved Cronbach alpha of this questionnaire for 0.70.

Oxford happiness questionnaire

Argyle et al. (40) developed this scale in 1989. Different studies estimate reliability of this questionnaire about 0.9. A number of studies have been conducted on the validity and reliability of the OHQ by Liaghatdar et al. (41) as well as Alipur and Nurbala (42). They reported a satisfactory internal consistence, indicating that total score of all 29 items yielded high correlation coefficients. The reliability values achieved by Cronbach's alpha and split-half were 0.93 and 0.92, respectively.

Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Sciences 19.0 (SPSS, SPCC Inc., USA) software. Data were analyzed by descriptive-analytical statistics and

statistical tests including multi-variable analysis of variance (MANOVA) and Z Fisher. Statistically significant effects were accepted for $P<0.05$.

Results

In this study, 88.4% of samples were over 40 years of age and 11.6% were below 40. As far as education is concerned, 12.7% of participants did not have their high school diploma, 44.6% had received their high school diploma, 12% had associate degree, 25.5% had a bachelor's science (B.Sc.), and 5.2% had a master's degree or higher. Regarding employment status, 74.5% of participants were housewives and 25.5% were employees.

Indexes of descriptive statistics including mean and SD are available in Table 1. In order to analysis of the differences between under-study groups in the field of sensation seeking, flexibility, and happiness, we employed MANOVA, shown in Table 2. The assumption of homogeneity of variances was carried out using Levene's.

According to Table 2, there are meaningful differences regarding sensation seeking between groups. So the rate of sensation seeking is significantly higher in surrogacy group compared to ART treatment group. However, there are no meaningful differences regarding happiness and flexibility between two groups.

Tables 3 and 4 show the results of MANOVA, indicating the differences created in sensation seeking, happiness, and flexibility variables by participants' age, education level, employment status, and financial status.

Table 3 depicts the interaction of education, employment, and financial status affects the happiness in infertile women who sought treatment. Moreover, interaction between education and financial status as well as the interaction between employment and financial status affect happiness. However, interaction between education and employment affects the sensation seeking. Table 3 also demonstrates that in ART treatment group, education affects the sensation seeking, employment status affects the flexibility, and financial status affects the happiness.

Table 4 indicates that only age, education, and financial status affects happiness in infertile women who underwent surrogacy.

Table 1: Descriptive statistics of groups

Group variable	Sensation seeking			Flexibility			Happiness		
	n	Mean	SD	n	Mean	SD	n	Mean	SD
Seeking treatment	201	20.627	3.749	201	26.91	5.498	201	42.22	12.94
Surrogacy	50	23.24	3.879	50	26.98	5.192	50	39.98	11.54

Table 2: Summary of MANOVA

Difference source	Statistical index	SS	df	MS	F	Meaningful level
Intergroup	Sensation seeking	273.411	1	273.411	19.187	0.001*
	Flexibility	0.194	1	0.194	0.007	0.936
	Happiness	201.601	1	201.601	1.254	0.264
Intragroup	Sensation seeking	3548.135	249	14.25		
	Flexibility	7367.368	249	29.588		
	Happiness	40021.905	249	160.731		
Sum	Sensation seeking	116072	251			
	Flexibility	189322	251			
	Happiness	478296	251			

MANOVA; Multi-variable analysis of variance, SS; Sum of squares, df; Degree of freedom, MS; Mean squares, F; Function and *; P<0.001.

Table 3: The summary of MANOVA of infertile women underwent ART

Difference source	Statistical index	SS	df	MS	F	Meaningful level
Age	Sensation seeking	16.012	1	16.012	1.4	0.239
	Flexibility	2.437	1	2.437	0.08	0.774
	Happiness	34.27	1	34.27	0.25	0.618
Education level	Sensation seeking	300.15	5	60.031	5.24	0.000*
	Flexibility	58.417	5	11.683	0.4	0.851
	Happiness	1133.1	5	226.61	1.65	0.15
Employment status	Sensation seeking	13.514	1	13.514	1.18	0.279
	Flexibility	224.35	1	224.35	7.62	0.006*
	Happiness	29.385	1	29.385	0.21	0.645
Financial status	Sensation seeking	5.908	2	2.954	0.26	0.773
	Flexibility	9.125	2	4.563	0.16	0.857
	Happiness	4180.9	2	5.831	15.2	0.000*
Interaction between age and education	Sensation seeking	45.379	2	22.69	1.98	0.141
	Flexibility	1.704	2	0.852	0.03	0.971
	Happiness	147.19	2	73.594	0.54	0.587
Interaction between age and employment status	Sensation seeking	0.00	0	_	_	_
	Flexibility	0.00	0	_	_	_
	Happiness	0.00	0	_	_	_
Interaction between age and financial status	Sensation seeking	0.00	0	_	_	_
	Flexibility	0.00	0	_	_	_
	Happiness	0.00	0	_	_	_
Interaction between education and employment status	Sensation seeking	144.27	2	72.135	6.3	0.002*
	Flexibility	55.251	2	27.626	0.94	0.393
	Happiness	42.026	2	21.013	0.15	0.859
Interaction between education and financial status	Sensation seeking	94.956	6	15.826	1.38	0.224
	Flexibility	41.286	6	6.881	0.23	0.965
	Happiness	3096.5	6	516.08	3.75	0.002*
Interaction between employment status and financial	Sensation seeking	0.047	1	0.047	0.00	0.949
	Flexibility	28.763	1	28.763	0.98	0.324
	Happiness	758.39	1	758.39	5.51	0.02*
Interaction between age, education and employment status	Sensation seeking	0.00	0	_	_	_
	Flexibility	0.00	0	_	_	_
	Happiness	0.00	0	_	_	_
Interaction between age, education and financial status	Sensation seeking	0.00	0	_	_	_
	Flexibility	0.00	0	_	_	_
	Happiness	0.00	0	_	_	_
Interaction between age, employment status and financial status	Sensation seeking	0.00	0	_	_	_
	Flexibility	0.00	0	_	_	_
	Happiness	0.00	0	_	_	_

Table 3: Continued

Difference source	Statistical index	SS	df	MS	F	Meaningful level
Interaction between education, employment status and financial status	Sensation seeking	11.797	1	11.797	1.03	0.311
	Flexibility	20.076	1	20.076	0.68	0.41
	Happiness	885.92	1	885.92	6.44	0.012*
Interaction between age, education, employment status and financial status	Sensation seeking	0.00	0	_	_	_
	Flexibility	0.00	0	_	_	_
	Happiness	0.00	0	_	_	_
Error	Sensation seeking	2038.4	178	11.452		
	Flexibility	5243.4	178	29.458		
	Happiness	24505	178	137.67		
Sum	Sensation seeking	88330	201			
	Flexibility	151605	201			
	Happiness	391849	201			

MANOVA; Multi-variable analysis of variance, ART; Assisted reproductive technology, SS; Sum of squares, df; Degree of freedom, MS; Mean squares, F; Function and *; P<0.05.

Table 4: The summary of the MANOVA of infertile women sought surrogacy

Difference source	Statistical index	SS	df	MS	F	Meaningful level
Age	Sensation seeking	4.316	1	4.316	0.33	0.572
	Flexibility	4.967	1	4.967	0.23	0.636
	Happiness	38.165	1	38.165	0.48	0.494
Education level	Sensation seeking	40.051	5	8.01	0.61	0.695
	Flexibility	88.769	5	17.754	0.82	0.547
	Happiness	167.18	5	33.436	0.42	0.831
Employment status	Sensation seeking	0.018	1	0.018	0.00	0.971
	Flexibility	10.081	1	10.081	0.46	0.501
	Happiness	116.67	1	116.67	1.47	0.236
Financial status	Sensation seeking	34.458	2	17.229	0.26	0.286
	Flexibility	8.812	2	4.406	0.2	0.817
	Happiness	96.165	2	48.083	0.61	0.553
Interaction between age and education	Sensation seeking	11.836	3	3.945	0.3	0.826
	Flexibility	1.704	3	1.584	0.07	0.974
	Happiness	147.19	3	52.255	0.66	0.585
Interaction between age and employment status	Sensation seeking	0.00	0	_	_	_
	Flexibility	0.00	0	_	_	_
	Happiness	0.00	0	_	_	_
Interaction between age and financial status	Sensation seeking	0.06	1	0.06	0.01	0.947
	Flexibility	1.294	1	1.294	0.06	0.809
	Happiness	108.35	1	108.35	1.36	0.253

Table 4: Continued

Difference source	Statistical index	SS	df	MS	F	Meaningful level
Interaction between education and employment status	Sensation seeking	0.159	1	0.159	0.3	0.913
	Flexibility	37.398	1	37.398	1.72	0.2
	Happiness	128.14	1	128.14	1.62	0.214
Interaction between education and financial status	Sensation seeking	101.09	2	50.543	3.83	0.033
	Flexibility	46.476	2	23.238	1.07	0.356
	Happiness	224.14	2	112.07	1.41	0.261
Interaction between employment status and financial	Sensation seeking	0.00	0	_	_	_
	Flexibility	0.00	0	_	_	_
	Happiness	0.00	0	_	_	_
Interaction between age, education and employment status	Sensation seeking	0.00	0	_	_	_
	Flexibility	0.00	0	_	_	_
	Happiness	0.00	0	_	_	_
Interaction between age, education and financial status	Sensation seeking	26.501	1	26.501	2.01	0.167
	Flexibility	1.726	1	1.726	0.08	0.78
	Happiness	449.23	1	449.23	5.65	0.024*
Interaction between age, employment status and financial status	Sensation seeking	0.00	0	_	_	_
	Flexibility	0.00	0	_	_	_
	Happiness	0.00	0	_	_	_
Interaction between education, employment status and financial status	Sensation seeking	0.00	0	_	_	_
	Flexibility	0.00	0	_	_	_
	Happiness	0.00	0	_	_	_
Interaction between age, education, employment status and financial status	Sensation seeking	0.00	0	_	_	_
	Flexibility	0.00	0	_	_	_
	Happiness	0.00	0	_	_	_
Error	Sensation seeking	382.5	29	13.19		
	Flexibility	629.69	29	21.714		
	Happiness	2306.3	29	79.528		
Sum	Sensation seeking	737.12	50			
	Flexibility	1321	50			
	Happiness	6527	50			

MANOVA; Multi-variable analysis of variance, SS; Sum of squares, df; Degree of freedom, MS; Mean squares, F; Function and *; P<0.05.

Fisher's z was used to study the meaningful difference between rates of the correlations in ART treatment and surrogacy groups. In this statistical method, r coefficients are changed to z scores and then differences between z scores are computed. Achieved results are available in Tables 5 and 6. In Table 5, the transformation of Fisher's r into z-scores is shown.

Table 6 shows the meaningful differences between correlations of two groups and also it shows only the difference in correlation between sensation seeking and flexibility is significant. Based on Table 5, this correlation is significantly higher in surrogacy group compared to ART treatment group.

Table 5: Transformation of Fisher's r into Z-scores

Group	The correlation between sensation seeking and flexibility	The correlation between sensation seeking and happiness	The correlation between flexibility and happiness	Changing r into Z-scores for sensation seeking and flexibility	Changing r into Z-scores for sensation seeking and happiness	Changing r into Z-scores for flexibility and happiness
Group 1	0.206*	0.00	0.039	0.011	0.00	0.333
Group 2	0.394*	-0.321*	-0.258	0.375	0.039	0.264

*; P<0.05.

Table 6: The meaningful differences between correlations of two groups

Sensation seeking Z and flexibility in both groups	Sensation seeking Z and happiness in both groups	Flexibility Z and happiness in both groups
-2.25*	-0.24	0.94

*; P<0.05.

Discussion

The results of this research study provide families, therapists and mental health counselors, researchers, and infertile women with many practical applications. Understanding personality type of infertile woman can help her to choose a proper treatment method. Infertility is a phenomenon that therapists need to be aware of and skillful enough to prevent the tensions treating physical and mental health of infertile women.

According to the achieved results, there was a meaningful difference in terms of sensation seeking between groups. However, the same story was not true for flexibility and happiness. The researchers couldn't find a similar research that had considered these variables with the same groups. Although there are a number of studies pointing out the following risks that a person underwent surrogacy may experience (43): social risks; psychological risks including remorse (16), not withholding the baby, having a relationship with the baby's father (17) and not caring for the fetal health (18, 44); legal risks (45); financial risks (46, 47, 14, 15) as well as the potential risks of ART (43). However, our finding indicated high level of sensation seeking in a large number of individuals who underwent surrogacy.

According to Zuckerman (1979 and 1991), education, employment, and financial status affect the sensation seeking of infertile women underwent treatment. He showed that participants with university degrees achieved a higher score in sensation seeking. Moreover sensation seeking, adventure seeking, and experience seeking were seen among individuals appertaining to middle or high class of society (48, 49).

According to other findings, employment status can affect flexibility in infertile women who sought treatment. As Bradbury et al. (50) suggested a variety of factors can affect the marital compatibility and individuals' flexibility. Similar to their findings, we pointed out the sociocultural variables such as age, education, employment status, and financial status as effective factors.

Concerning the effect of demographic features on infertile women's happiness, our results indicated that financial status and education affected the happiness in infertile women who underwent treatment. Interaction between age, education and financial status also affected the happiness in infertile women who sought surrogacy. These findings were also in agreement with Ramezanzadeh et al. (51) and De Ree and Alessie (52). According to the results achieved by Ramezanzadeh et al. (51), education level causes a decrease in infertile women's depression, while it leads to an increase in happiness in them. Thus, education plays an important role in generating interests and developing self-satisfaction, resulting in happiness. Additionally, the relationship between education level and happiness is mostly resulted from high education level, successful career and high income (52).

According to achieved results, there was a positive meaningful relation between sensation

seeking and flexibility in both groups of infertile women, indicating the meaningful difference regarding rates of relationships in the two groups. These findings were also in agreement with Lauriola and Levin (53), Nicholson et al. (54), Soane and Chmiel (55), Anic (56) and Vries et al. (57). Sensation-seeking individuals are the most willing to take risks. They often seek new experiences and are willing to challenge possibilities. Sensation-seekers do not hesitate to go in different directions, especially when it comes to new ideas and innovations. They also have a high tendency to adapt quickly to changing circumstances because it feeds their desire for novel experiences. Therefore, flexibility is expected to have positive relationship with sensation seeking (54).

There were no meaningful differences between groups concerning the relationship between flexibility and happiness. Thus, flexible individuals increase their happiness because they own more choices (58). These findings are in agreement with those of Hayes and Joseph (59). However, since women who sought treatment failed many times in their programs, they may experience negative sensational feelings, suggesting no meaningful relation between flexibility and happiness.

Concerning the relationship between sensation seeking and happiness, a significant negative relationship is observed among infertile women seeking surrogacy. To shed light on these results, it should be noted that women seeking surrogacy, due to their specific conditions, must undergo a hazardous and costly treatment. The difficulties of these treatments lead to a reduction of overall happiness. However, this relationship is not observed in women undergoing ART. It is recommended this issue to be considered in future studies where personality traits can have a meaningful relation with infertile women's preferences over the treatment method. Another recommendation is to develop interference programs for recognizing personality traits and increasing flexibility and happiness in the group of infertile women, so women can respond to the pressures resulting from infertility and treatment more easily and make better decisions on treatment methods. One of the limitations of this study was the low number of women seeking surrogacy. Furthermore we only investigated three traits of personality traits, while other personality traits may be important to the subject of this study. Also a cross sectional study does not allow cause-effect conclusions, so the results of the study had no control over the confounding variables.

Conclusion

Based on the results of this study, sensations seeking as a personality trait is lower in infertile women who underwent ART as compared women who tended to have surrogacy. This study showed that demographic variables were effective in happiness of infertile women. Also there is a significant relation among sensation seeking, flexibility and happiness in infertile women. Thus in infertile women, those with higher rates of sensation seeking were more likely to choose surrogacy rather than ART treatment, and these individuals needed further psychological intervention to improve their happiness.

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Original Article

Effect of Group Positive Psychotherapy on Improvement of Life Satisfaction and The Quality of Life in Infertile Woman

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Abstract

Background: Positive psychotherapy is one of the new approaches in psychology which is innovated for treating psychological disorders and enhancing positive emotions. The aim of this study is to investigate the effectiveness of the group positive psychotherapy on elevation of life satisfaction and quality of life in infertile women.

Materials and Methods: In a randomized trial study, Beck Depression Inventory II (BDI-II) and clinical interview were used in a pre-test post-test control group design. After analyzing the result of the questionnaire, 36 infertile women who showed signs of mild to moderate depression were randomly placed into two following groups: control (n=18) and intervention (n=18). Before the treatment, the members of both groups answered BDI-II, Satisfaction With Life Scale (SWLS) and 12 item Short Form Health Survey (SF-12). The intervention group received six sessions of group positive psychotherapy, but the treatment of the control group began six weeks after the intervention group.

Results: The results showed that the life satisfaction scores of the intervention group were significantly elevated from 22.66 in pre-test to 26.13 in post-test ($P<0.001$), while this improvement was not significant in the control group ($P=0.405$). The difference between life satisfaction scores of the intervention and the control groups was also significant ($F=8.92$, $P=0.006$). However, no significant change in the quality of life level of the intervention and control groups was observed ($P=0.136$).

Conclusion: Thus it can be deduced from the findings that this treatment method could be introduced as solution to increase the life satisfaction in infertile women, but not as a treatment for elevating their quality of life (Registration Number: IRCT2013042810063N3).

Keywords: Psychotherapy, Female Infertility, Quality of Life, Depression

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Introduction

As the science has advanced, many of the once untreatable illnesses have now become treatable. However, there are certain problems which could still inflict a great deal of stress on people. Infertility is a reproductive system disease which is defined as inability to achieve clinical pregnancy 12 months after having sexual intercourse without any prophylactic device (1). It has been shown that the prevalence of lifetime infertility is between 6.6 to 26.4% worldwide, of which 9% showed infertility duration of 12 months. In 2007, 72 million women suffered from infertility worldwide (2). Also in another study (3), it has been shown that 11.2 to 14.1% of Iranian couples experienced one or another form of subfertility. Infertility is also considered as one of those diseases leading to mental health disorders (4). The high prevalence of infertility, therefore, necessitates the treatment for mental health stress of these couples. Approximately, one third of the infertility is associated with factors which are related to women, one third is related to men and one third is related to both men and women (3). Despite all this, infertility is still considered as woman's problem, especially in the context of developing countries (4). For example, in Iranian culture, the social implications of divorce, remarriage and separation causes severe mental stress for infertile women (5). The infertility is suggested to be associated with bio-psychosocial crisis (4), meaning stress contributing to many of the psychological disorders are the side-effects of infertility (6). Therefore, along with treatment of infertility, the individuals' mental health and social problems must be paid attention as well. Several studies have reported that sexual dysfunction (7), eating disorders (8), depression disorder (9-11), and psychiatric disorders (12-14) are more common in infertile women as competed to the women with a healthy reproductive system. Furthermore sexual desire and arousal are lower in infertile women (15).

Another common problem of the infertile women is the decreased levels of life satisfaction and quality of life. Life satisfaction is a cognitive and judgmental process that is based on comparison of the individual's conditions with what is considered as a proper standard (16). In a study by Callan and Hennessey (17), they found that the infertile women were less satisfied with their lives than the fertile ones. In addition quality of life as a multidimensional factor includes cognitive, behavioral capacities, emotional well-being and capabilities which are necessary for performing family, social and vocational roles (18). Another research showed that health-related quality of life and sexual function are significantly lower in the women with primary infertility (19). Also in a research, it has found that quality of life is lower in the infertile women than the men (20).

Many different methods have been developed for treating the psychological problems of the infertile individuals. For example, interpersonal psychotherapy (IPT) (21), cognitive behavioral therapy (CBT), supportive psychotherapy (9, 22), as well as emotion-focused and problem-focused coping strategies (23) have been known as a few of the useful methods. However, positive psychology is a new approach which emphasizes on the individuals' strengths that includes the scientific study of positive emotions, positive individual traits and positive institutions (24). Positive psychology as an intervention technique is applied to promote positive experiences, positive behaviors or positive cognitions (25). This treatment method has been shown to be effective due to the following factors: helping those who do not respond positively to drug therapy, cost effective, taking a short period of time to improve positive mood, no stigmatization and no negative side-effects (26). Some researchers have shown that since positive psychotherapy and interventions were effective on happiness and depression, it is likely to influence quality of life and life satisfaction (24). Since no research has been conducted with regard to this issue in the infertile women, the aim of this research is, therefore, to study the effectiveness of the group positive psychotherapy on elevation of the life satisfaction and the quality of life in infertile women.

Materials and Methods

Participants and procedure

This randomized trial study was approved by the Ethics Committee of the Kermanshah University of Medical Sciences, Kermanshah, Iran. During April and June 2013, the women paying a visit to Motazedi Infertility Treatment Center, Kermanshah, Iran, were selected using the convenience sampling method and asked to answer the Beck Depression Inventory II (BDI-II). All the participants were signed an informed consent before entering the study. A total of 121 individuals answered the questionnaire, of whom 115 fully completed the form. Then, a clinical psycholo-

gist conducted a diagnostic interview with those who showed mild to moderate symptoms of depression using the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR). Ultimately 49 people were found to meet the criteria to be included in the groups, of whom only 40 people were accepted to participate in the experiment. Out of these 40 individuals (four patients due to infertility treatment were dropped from the study), 36 people were randomly placed into two groups of intervention (n=18) and control (n=18) in a pre-test post-test control group design. The intervention group was divided into three subgroups of six individuals who received positive psychotherapy. However, the treatment of those in the control group was delayed until the end of the experiment. Before the treatment began, the participants in both groups were asked to answer the questions in the questionnaire regarding the life satisfaction and the quality of life.

The inclusion criteria in this study were as follows: i. Filling out the BDI-II, ii. Diagnosis of major depressive disorder (MDD) based on the major depression criteria mentioned in DSM-IV-TR criteria, iii. Not receiving psychiatric medication or any other form of psychotherapy treatment, and iv. Not having any other form of psychiatric disorder. The exclusion criteria in this study were as follows: i. Presence of physical problems interrupting the treatment process and ii. Presence of depression becoming more severe during the treatment period (in the event of intensified depression, the individuals from both groups were referred to a colleague who was a trained psychiatrist). Ultimately three people from the intervention group and two people from the control group were dropped out of the treatment process (Fig.1).

Instruments

Beck Depression Inventory II

The BDI-II measuring the symptoms for a two week-period contains 21 items. Each item is scored from 0 to 3 and total scores range from 0 to 63 that is interpreted as 0 to 13 for lowest depression, 14 to 19 for mild depression, 20 to 28 for moderate depression and 29 to 63 for severe depression (27). This questionnaire has copies in countries such as Japan (28) and Brazil (29). The Persian version of the questionnaire was evaluated with alpha value of 0.87 and test-retest reliability of 0.74 (30).

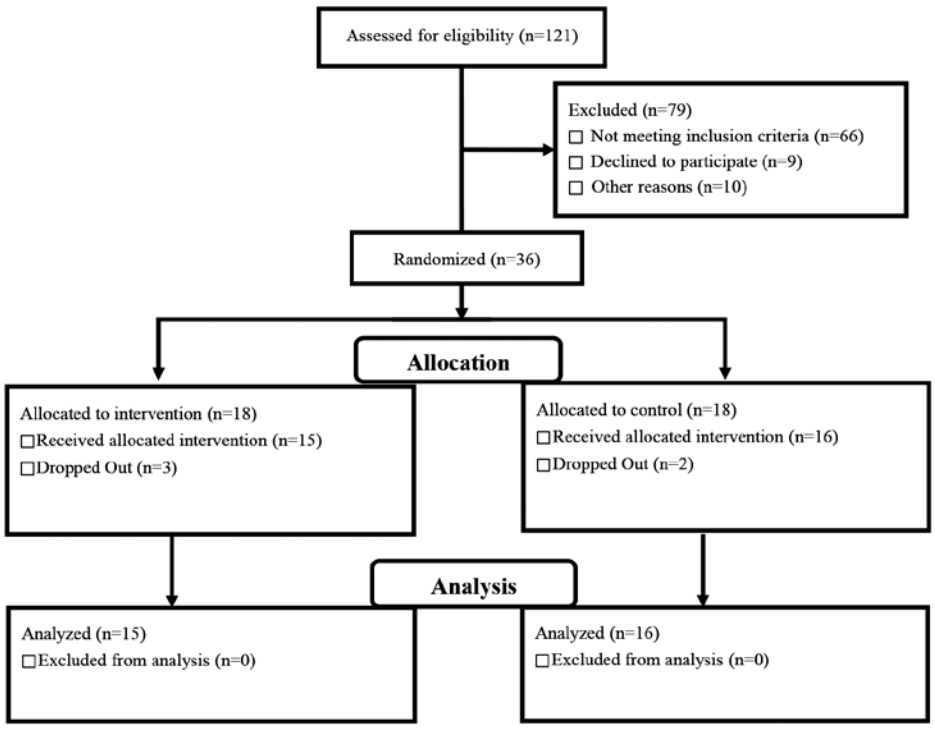

Fig.1: Graphic representation of participant flow.

Satisfaction With Life Scale

This scale was designed by Diener et al. (16) that is a very commonly used tool in researches on subjective-wellbeing (SWB) issues consisting of 5 items. Each item uses a Likert scale from 0 to 7 (31). In Iranian version, the reliability of Satisfaction With Life Scale (SWLS) is 0.83 when using the Cronbach Alpha and 0.69 when using the test-retest method. The structure reliability of this test is reported suitable using two other questionnaires (32).

12-item Short Form Health Survey

The 12-item quality of life questionnaire is the shortened version of the 36-item Short Form Health Survey (SF-36) (33). This questionnaire consists of eight sub-scales. Since there are a few questions, only the overall score of the person was used in this study. A research conducted on 5586 people in Tehran determined the validity (Cronbach's alpha= 0.72) and the reliability (with factor analysis) of this questionnaire, suggesting the questionnaire is suitable for the Iranian people both in terms of reliability and validity (34).

Intervention

The participants in this study were randomly placed into two groups of intervention and control. The treatment of the control group was delayed by six weeks, but the intervention group participated in a six-week group positive psychotherapy. This intervention was formulated and studied at the University of Pennsylvania in 2009 by Parks-Sheiner (35). Interventions were performed in a hospital room. Each session lasting an hour and half was held in a group therapy format. In each meeting, there were tasks that were completed by the participants for the following meeting. The meetings included the following six positive exercises: i. Using your strengths, ii. Gratitude visit, iii. A active-constructive response, iv. Counting blessings, v. Savoring, and vi. Biography (Table 1). The treatment sessions were carried out by a master degree student in clinical psychology program who had received training on positive psychotherapy at the Psychology and Counseling Organization of I.R. Iran. Moreover the intervention sessions were supervised by a tenure-track professor of clinical psychology at Kermanshah University of Medical Sciences.

Table 1: The definition of positive therapy and its sessions

Session	Content
Session one	Opening and positive introductions Preview next session and describe homework: value in action (VIA)/using your strengths Homework: take VIA strengths assessment to find out how to use one of your strengths every day
Session two	Discuss homework: using your strengths Preview next session and describe homework: gratitude Homework: write and deliver a gratitude letter
Session three	Discuss homework: gratitude letter Preview next session and describe homework: active-constructive responses Homework: make an active-constructive responses in social interactions
Session four	Discuss homework: active-constructive responses Preview next session and describe homework: blessings Homework: each night before bed, write down three good things that happened.
Session five	Discuss homework: blessings Preview next session and describe homework: savoring/biography Homework: pick one thing you usually rush through and take the time to savor it. Write a short essay (~1 page) detailing the characteristics and accomplishments that you hope to be remembered for and consider how much time you dedicated to pursue these Goals.
Session six	Discuss homework: biography and savoring Closing/maintenance Homework: pick at least one exercise and try to integrate it into your everyday life

Statistical analysis

The independent-samples t test and Chi-square were used to compare the demographic information and the pre-test depression scores between two groups. In addition the paired-samples t test and analysis of covariance (ANCOVA) were applied to study the differences between the scores of life satisfaction and quality of life, pre-tests and post-tests, of both groups.

Results

The demographic characteristics and pre-test depression scores of both groups are shown in Table 2, indicating there are no significant differences regarding these variables between two groups.

The results showed that life satisfaction significantly improved in the intervention group when comparing the results of the post-test with those of the pre-test (P<0.001), while this improvement was not significant in the control group (P=0.405). Moreover the quality of life showed no improvement in both groups when comparing the results of the post-test with those of the pre-test (Table 3).

Another finding of this study showed that life satisfaction displayed a significant increase in the intervention group in comparison to the control one (P=0.006), but there is no significant difference regarding this variable between two groups (Table 4).

Table 2: Comparison of demographic characteristics and the pre-test depression scores between the control and intervention groups

Variables	Control group Mean (SD) n (%)	Intervention group Mean (SD) n (%)	Total sample Mean (SD) n (%)	t or χ^2	P value
Age	29.25 (5.65)	32.33 (4.82)	30.49 (5.68)	t=1.62	0.11
Husband's age	34.63 (6.52)	37.60 (8.36)	35.11 (6.38)	t=1.10	0.27
Length of marriage (Y)	7.63 (5.89)	7.07 (5.65)	7.75 (5.36)	t=0.27	0.79
Infertility duration (Y)	3.93 (3.13)	5.27 (5.37)	4.45 (4.04)	t=0.83	0.41
BDI-II	21.87 (5.45)	20.87 (5.44)	19.43 (11.97)	t=0.51	0.61
Education					
Pre-high school	4 (25.0)	5 (33.3)	27 (23.5)		
High school	7 (43.8)	5 (33.3)	53 (46.1)	χ^2=0.41	0.81
Higher education	5 (31.2)	5 (33.3)	35 (30.4)		
Sum	16	15	115		

BDI-II; Beck Depression Inventory-II, t; Independent t test and χ^2; Chi-squared.

Table 3: Investigation of the differences of the variables between the pre-test and the post-test results of control and the intervention groups using paired-samples t test

Measurement	Group	Pre test		Post test		t	P value
		Mean	SD	Mean	SD		
SWLS	Intervention	22.66	4.48	26.13	4.10	5.56	P<0.001
	Control	21.06	4.73	21.68	5.79	0.086	0.405
SF-12	Intervention	31.00	5.02	33.53	4.83	1.96	0.069
	Control	30.87	4.42	31.06	5.02	0.15	0.876

SWLS; Satisfaction With Life Scale, SF-12; 12-item Short Form Health Survey and t; Independent t test.

Table 4: Comparison of the differences between the control and the intervention groups after controlling pre-test using ANCOVA

	Source	SS	df	MS	F	P value	Eta- squared	Observed power
SWLS	Pre-test	532.99	1	532.99	72.38	0.001>	0.72	
	Group	65.67	1	65.67	8.92	0.006	0.24	0.82
	Error	206.17	28	7.36				
	Total	18509.0	31					
SF-12	Pre-test	174.42	1	174.42	9.18	0.005	0.25	
	group	44.81	1	44.81	2.36	0.136	0.07	0.31
	Error	532.25	28	19.01				
	Total	753.93	31					

ANCOVA; Analysis of covariance, SWLS; Satisfaction With Life Scale, SF-12; 12-item Short Form Health Survey, SS; Sum of squares, df; Degree of freedom, MS; Mean squares and F; Function.

Discussion

Infertility is a physical illness caused by several physical and emotional factors. A number of different approaches for infertility treatment have led to many psychological treatment methods. The first finding of this study showed that there was a significant increase in life satisfaction in the intervention group as compared to the control group. This finding is in line with the results of the meta-analysis of Sin and Lyubomirsky (25). In another study (36), 55 students were placed into two groups, an intervention group of 28 and a waiting list of 27. The intervention group underwent a 10 week-long Wellness Promotion Intervention. The life satisfaction of these students showed significant improvement in the post-test, whereas the well-being of the students in the waiting list showed a decline, even though this decline was not significant (36). Life satisfaction could be considered as a cognitive and judgmental process which is based on comparing the individual's condition with what is considered as a proper standard (16). Lower life satisfaction in infertile women is verified in the previous study (17) that is more likely to be due to the fact that the infertile women find their conditions hopeless. Positive psychological interventions are certain treatment methods cultivating positive emotions, positive behaviors and positive cognitions (25). Considering the fact that these treatment methods emphasize on positive emotions and strengths, they both treat depression and enhance life satisfaction. For example, counting the blessings teaches the infertile women that even though they are deprived of having a blessing as important as having a child, they have thousands of other blessings in their lives which they should be thankful for.

Another finding of this study was that the quality of life showed no significant improvement both in the intervention and the control group. Since this study was the first of its kind, it had some limitations. The first limitation was the short duration of the treatment period. Although six weeks of the treatment saved time and expenses, it may not be enough time for some participants to see a positive result. It is recommended that the results of this short-term treatment to be compared with those of long-term treatments, so the best intervention method may be determined for the infertile women. The second limitation was related to the measurement tools. Due to limited number of questions regarding the quality of life factor, this was hard for the researcher to identify and separate the areas where the treatment was effective or ineffective. Moreover, life satisfaction in this study was measured using a five-question questionnaire, which may not be suitable to study the effectiveness of the treatment. It is recommended that in the future researches, a different questionnaire to be used. The third limitation in the study was that the sample participants were limited to the infertile women who were selected only from the residents of one city in Iran. Therefore, the results of this study may not apply to infertile women with different ethnicities. Finally this treatment method relies mostly on practice. However, those patients who were used to the structured sessions may not benefit from it.

Conclusion

This study showed that group positive psychotherapy could be beneficial in elevating life satisfaction in infertile women. Considering the low cost of positive psychotherapy due to a less number of the sessions and easy method, this treatment could be used comprehensively in all the infertility treatment centers. The results of the study revealed that, for elevation of the quality of life in infertile women, other interventions must be used. However, more research must be carried out and the limitations of this study must be considered.

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Original Article

Development and Validation of Attitude toward Gestational Surrogacy Scale in Iranian Infertile Couples

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Abstract

Background: Surrogacy is one of the most challenging infertility treatments engaging ethical, psychological and social issues. Attitudes survey plays an important role to disclosure variant aspects of surrogacy, to help meeting legislative gaps and ambiguities, and to convert controversial dimensions surrounding surrogacy to a normative concept that eliminates stigma. The aim of this study is to develop a comprehensive scale for gestational surrogacy attitudes.

Materials and Methods: Development process of gestational surrogacy attitudes scale (GSAS) performed based on a descriptive cross-sectional study and included a rich data pool gathered from literature reviews, a qualitative pilot study on 15 infertile couples (n=30), use of expert advisory panel (EAP) consisting of 20 members, as well as use of content validity through qualitative and quantitative study by the means of content validity ratio (CVR) and content validity index (CVI). Also internal consistence using Cronbach's alpha and test-retest reliability using intracalss correlation coefficient (ICC) were evaluated. Application of GSAS was tested in a cross-sectional study that was conducted on 200 infertile couples (n=400) at Royan Institute, Tehran, Iran, during 2014.

Results: Final version of GSAS had 30 items within five subscales including "acceptance of surrogacy", "Surrogacy and public attitudes", "Child born through surrogacy", "Surrogate mother", and "Intentional attitude and surrogacy future attempt". Content validity was represented with values of CVR=0.73 and CVI =0.98. Cronbach's alpha value was 0.91 for the overall scale, while ICC value due to test-retest responses was 0.89.

Conclusion: Acceptable level of competency and capability of GSAS is significantly indicated; therefore, it seems to be an appropriate tool for the evaluation of gestational surrogacy attitudes in Iranian infertile couples.

Keywords: Surrogate Mother, Gestational, Attitude, Scale, Validation

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Introduction

Having a child is a universal desire (1) and considered mostly as a basis of human motivation for the continuity and stability of marriage (2). Infertility is defined as absence of pregnancy after one year of regular unprotected intercourse (3). Almost 10 to 15% of couples experience infertility (4). Infertile couples face pervasive personal and social crisis such as depression, anxiety, dissatisfaction and low self-esteem. Furthermore destructive impacts of infertility have been seen on interpersonal relationships, quality of life and marital status that may eventually lead to divorce (5). Due to importance of having children in Iranian culture, the social consequences of infertility goes deeper in conflicts (6).

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In recent decades, there has been enormous improvement in infertility treatments. Surrogacy is one of the most challenging infertility treatment for which there has been considered lots of positive and negative outcomes (1), and includes two types of traditional and gestational surrogacy (7), although gestational type is the only type approved in Iran (8).

Choice of treatment for infertile couples is affected by their knowledge, understanding, expectations, as well as attitudes of the community they are living in; therefor, providing accurate information may have positive impact on a couple's decision-making (9, 10). Despite of consideration of surrogacy as legitimate treatment method, there is inadequate awareness in this respect in Iran (7, 11). People use attitudes to evaluate the objects by placing it within their existing knowledge structures, so attitudes influence the way they behave. It means that attitudes help an individual to decide whether behavior is appropriate or applicable. It is important to know about attitudes in order for predicting potential future behaviors (12).

Survey of knowledge, attitudes and decision-making patterns plays an important role in finding legal solutions in the process of converting surrogacy controversial approaches to a normative concept (11, 13). Nowadays, there is an increasing focus within the area of social psychology on developing methodologies that measure attitudes with self-report scales, while applications of these measures is required to continue development of methodologically strong and valid attitudes scales (12, 13). For ethical issues related to health-care, it seems to be essential to increase adequate awareness and to provide proper consultative services for infertile people (14). Furthermore disclosure of different aspects of surrogacy makes the relevant authorities to establish and to amend proportional regulations, leading to eliminate stigma in the community (9).

A few studies has been conducted in Iran in order to survey public opinion on gestational surrogacy (7). Scales used in the mentioned-studies are self-designed questionnaires with an indefiniteness in the expression of methods used in development for assessing validity and reliability of the scale. Therefore, we decided to conduct this study with the aim of achieving a valid gestational surrogacy attitudes scale which fulfilled all three cognitive, emotional and intentional aspects of attitude and conformed to cultural and logical aspects of surrogacy in Iranian infertile couples. This might be an opportunity to provide an organized and comprehensive scale that might best match their needs.

Materials and Methods

Development process of gestational surrogacy attitudes scale (GSAS) was performed based on a descriptive cross-sectional study at Royan Institute, Tehran, Iran, during 2014. GSAS in Persian language was passed through the following steps:

Development of a data pool

Initially literature review was conducted to the aim of development of concepts and questionnaire items, benefiting from related studies and an expert advisory panel (EAP) consisting of 20 members.

Pilot study

A pilot study was performed on 15 infertile couples (n=30) who attended the Infertility Clinic of Royan Institute, with a previous knowledge of surrogacy issue. They were asked two open-ended questions including "What is your opinion about gestational surrogacy?" and "What is your most concerning issues about surrogacy experience?". Data pool was enriched with their answers as well as the comments provided by the members of EAP.

Expert advisory panel

A team of experts in maternity and infertility issues consisted of 20 members as follows: 10 Academic Board Members of Nursing and Midwifery School, Tehran University of Medical Sciences, Tehran, Iran, and 10 specialists who were closely dealing with infertile couples and working at the different parts of the Royan Institute such as Academic Departments, Consultants and Treatment Clinics. Subsequently a primary version of the scale with 30 items retrieved from literature reviews and pilot qualitative study was judged and commented by the members of EAP. The final version of scale was then obtained after applying modifications for passing reliability and validity tests.

Validity

Validation of the scale was performed in two ways of face validity and content validity. In face

validity, difficulty, irrelevancy and ambiguity of scale items were assessed by the experts, in which they scored and qualified the scale according to the mentioned criteria and their recommendations. Content validity was carried out by both qualitative and quantitative approaches. Experts discussed and qualified the scale based on qualitative criteria including grammar, wording, item-allocation and scaling. Quantitative content validity was determined by the content validity ratio (CVR) and content validity index (CVI). For calculating CVR, each item of GSAS was scored by the members of EAP using a 3-point Likert scale as follows: i. Essential, ii. Useful but not essential, and iii. Unessential. According to Lawshe's table, when there are 20 members in EAP, selected items are those with CVR= 0.42 or above (15).

According to Lawshe's recommendation for determination of the mean values by the experts, which is assigned to each item of the scale, answers to CVR assessment questionnaire must be scored as 2 for "essential", 1 for "useful but non-essential", and zero for "unessential". Items with mean values more than 1.5 are acceptable, even though with CVR lower than 0.42 (15).

To calculate CVI for each item by all 20 members of EAP, simplicity, specificity and clarity of criteria were evaluated using five-point Likert scale as follows: i. Totally relevant=4 points, ii. Relevant=3 points, iii. Semi-relevant=2 points , iv. Irrelevant=1 point and v. Totally irrelevant. CVI score was calculated by accumulating eligible answers with points of 3 and 4 that was followed by being divided by the total number of panels for each item. A CVI score equal to 0.79 or higher indicated the appropriateness of the content validity (16).

Reliability

Cronbach's alpha and test-retest were used for evaluating the reliability of the scale (12). In internal consistence measured by Cronbach's alpha, score equal to 0.7 or higher was considered as the acceptable reliability (17). Also stability of the questionnaire measured by test-retest method was determined. In order to define the coefficient for scale retesting, 15 infertile couples were randomly selected to answer the questionnaire twice within two weeks interval. Intracalss correlation coefficient (ICC) values equal to 0.4 or higher were considered acceptable (18).

Descriptive study

Eventually at the end of the development process, with the aim of assessing practicability of GSAS, a descriptive cross sectional study on 400 infertile men and women were performed by simple sampling method with ethical considerations. Infertile couples participated in both pilot and final descriptive study with signing informed consents. This study approved by Tehran University of Medical Sciences, Faculty of Nursing and Miwifery and Reproductive Epidemiology Research Center of Royan Institute Ethics Committee.

Results

Final version of GSAS was obtained with 30 items and each item was scored using five-point Likert scale; therefore, positive attitude expressions were scored as follows: I strongly agree=5, I agree=4, I am indecisive=3, I disagree=2, and I strongly disagree=1. Negative attitude expressions were scored in the reverse order of the above-mentioned scoring, and these 14 items with negative connotations included item numbers of 6 to 11, 14, 15, 17, 18, 22 to 24 and 26.

Surrogacy is a concept associated with the most challenging issues that each aspect could lead someone to various and countercurrent attitude trends which could not to be totalized, thus according to the literature reviews and opinions of EAP, GSAS splits into five subscales including: i. Item numbers 1 to 9 indicating acceptance of surrogacy, ii. Item numbers 10 to 13 indicating surrogacy and public attitudes, iii. Item numbers 14 to19 indicating child born through surrogacy, iv. Item numbers 20 to 26 indicating surrogate mother, and v. Item numbers 27 to 30 indicating intentional attitude and surrogacy future attempt. Therefore, these items may cover major issues related to gestational surrogacy and prevent interference of opposite tendencies in attitude assessment.

Maximum score of the scale is 150, indicating more positive attitudes, while minimum score is 30. Score range defers in the subscales due to number of questions as it consists of 9-45 for overall acceptance of surrogacy subscale, 4-20 for surrogacy and public attitudes and intentional attitude and surrogacy future attempt subscales, 6-30 for child born through surrogacy subscale, as well as 7-35 for surrogate mother subscale. Both the total and subscale scores were calculated using raw scores after the negative items are recoded.

Table 1: Mean values of CVR, EAP, as well as face validity consisting of difficulty, irrelevancy and ambiguity in case of validation of GSAS items

Item number	Items	CVR	EAP mean value of judjment	Difficulty	Irrelevancy	Ambiguity
1	Surrogacy is a good way to help infertile couples have a child with their own genetic characteristics	80	1.9	100	100	100
2	Surrogacy reduces psychological tensions in infertile couples	60	1.8	95	100	95
3	Surrogacy improves the life satisfaction of infertile couples	80	1.8	97.5	100	100
4	Surrogacy can prevent divorce and strengthen family structure	80	1.9	100	100	100
5	If there is no other infertility treatment option, surrogacy could be the last choice	80	1.9	95	100	95
6	I prefer to be voluntarily childless rather than to accept surrogacy	80	1.9	100	100	97.5
7	Adoption is better than surrogacy	80	1.9	100	100	97.5
8	Surrogacy could be followed by ethical and .social issues	60	1.7	92.5	100	92.5
9	Surrogacy is against religion	80	1.9	95	100	97.5
10	Mainly most traditional societies have negative attitudes toward surrogacy	80	1.8	95	95	95
11	Surrogacy must be hidden from others in order to prevent society to reject the child	100	1.9	90	92.5	92.5
12	I am not concerned about disclosure of surrogacy to friends and relatives	100	2	92.5	92.5	90
13	If mass media promotes public awareness about surrogacy, I will not be concerned about disclosure of the issue to the child and the others	80	1.9	95	95	92.5
14	Children born through surrogacy may have further risk of birth defects than others	60	1.9	97.5	100	97.5
15	Children born through surrogacy may have further risk of psychological problems than others	80	2	100	100	100
16	Disclosure of surrogacy is considered as an inalienable right of the child	80	2	100	100	100
17	Surrogate mother's identity must be hidden from the child	60	1.9	100	100	100
18	Close relationship of the child and surrogate mother will cause insecurity of parental relationship between commissioning couple and the child	60	1.7	95	92.5	95
19	Disclosure of surrogacy to the child is better to be after his/her adolescence stage	80	1.9	100	100	100
20	Only commissioning couple are truly parents of the child	80	2	100	100	100
21	Surrogate mother's role is as antenatal nanny	60	1.7	100	100	100
22	It seems that surrogate mother's intention is to get money rather than to be altruistic	80	1.8	100	100	100

Table 1: Continued

Item number	Items	CVR	EAP mean value of judjment	Difficulty	Irrelevancy	Ambiguity
23	Surrogate mother might be careless about the child during pregnancy	80	1.9	100	95	100
24	Emotional bonding may cause surrogate mother to avoid relinquishment of the child	60	1.9	100	95	100
25	I prefer involving an unfamiliar surrogate mother	100	2	100	100	100
26	There is no need to maintain contact with surrogate mother after delivery	100	2	100	100	100
27	If my physician recommends to get a surrogate, I will use this treatment	100	2	100	100	100
28	If I know that one of my relatives or friends decide to be a surrogate mother, I will support them	100	2	100	100	100
29	In case of use of surrogacy, I will disclose the truth to my child in future	60	1.7	100	100	100
30	After relinquishment of the baby, facing the surrogate mother never make me uncomfortable	80	1.7	100	10	100
	Total scale	73	1.8	98	98	98

CVR; Content validity ratio and EAP; Expert advisory panel.

Table 2: ICC and Cronbach's alpha values of subscales

Subscales	ICC	Cronbach's alpha
Overall acceptance of surrogacy	0.90	0.93
Surrogacy and public attitudes	0.86	0.90
Child born through surrogacy	0.73	0.91
Surrogate mother	0.87	0.91
Intentional attitudes and surrogacy future	0.82	0.93
Total scale	0.89	0.91

ICC; Intracalss correlation coefficient.

In this study, total values of CVR and CVI were 0.73 and 0.98, respectively, representing the substantial content validity of the scale. Mean value of the judgment status of expert panel was calculated above 1.5 for each item; therefore, they were all accepted in the final version of the scale. Reliability of GSAS has confirmed by Cronbach's alpha value that was 0.91 for the overall scale, and the value of ICC due to test-retest responses was found 0.89 (Tables 1, 2).

Application of GSAS was tested in a cross-sectional study on 200 infertile couples who were applied to receive infertility treatment service at Royan Institute (n=400) via simple sampling method. The mean age of men was 34 ± 5.52 and for women was 29 ± 5.12. They were all Muslim, among which 97.5% were Shiite. Among 200 infertile couples, 55 women (27.5%) and 49 men (24.5%) showed an education level of elementary school, 72 women (36%) and 65 men (32.5%) had a high-school diploma, as well as 73 women (36.5%) and 86 men (43%) had university degrees. Mean of infertility duration was 4.12 ± 2.74 years. Mean score of total attitude toward gestational surrogacy was 91/14 in women and 92/46 in men, indicating there was no significant difference between couples. Results of this study proved the practicability of the scale.

Discussion

Due to the importance of surrogacy issue, similar surrogacy attitudes surveys have been conducted around the world and the majority of them were used self-designed questionnaires in accordance with the objectives of their studies, but the validation methods are not clearly represented. One of the privileges of GSAS compared to the tools used in those studies is the application of an enriched scientific and statically validation process.

Here we pointed the most cited surrogacy attitudes-related articles to compare scale development approaches. Members of EAP also provided more erudite comments for developing a satisfactory scale in practice as compared to other sources used in this study. As results, Cronbach's alpha value indicates an excellent reliability of the scale in this study that is similar to the studies by Ahmari Tehran et al. (19), Rahmani et al. (20). However, in the studies by Saito and Matsuo (21) as well as Poote and van den Akker (22), Cronbach's alpha or other validity and reliability assessments was not applied. In order for item-designing and -scoring, Saito and Matsuo (21) and Minai et al. (23) used open ended questions, whereas present study and other studies used Likert scale (mostly 5-point). Furthermore, scale development in Poote and van den Akker's (22) study was based on theory of planned behavior (TPB). In the present study, 30 different pilot studies were employed that is similar to the study by Chliaoutakis et al. (24), whereas other studies, either included no pilot study, or had less pilot sample size. This study seems to be unique among previous surrogacy attitudes surveys. Firstly we performed qualitative pilot study in order to enrich data pool, and we also applied the qualitative and quantitative content validity approaches to obtain highly acceptable CVR and CVI values. Finally we attained the test-retest ICC values within satisfactory level.

Content validity is an essential step in the development of new empirical measuring devices because it represents a beginning mechanism for linking abstract concepts with observable and measurable indicators (25); however, none of the mentioned studies reported CVR and CVI values. As a result, studies based on only relevance or representativeness, as judged by experts, cannot offer any support for validity. Therefore, it is important to abandon content validity, to clarify subscales, and to develop the adequacy of content sampling from the content domain for GSAS (26).

In this study, we tried to take into account these most important criteria and other prestigious methods for development and validation of the scale. Therefore, after obtaining satisfactory levels in validity and reliability process, our findings significantly indicated the adequate level of competency and capability of applied scale in disclosure of tendencies toward major challenging aspects of GSAS.

Conclusion

According to the results, GSAS provides enough admissibility and validity in evaluation of gestational surrogacy attitude in Iranian infertile couples, so seems to be useful in surveys with similar sociocultural backgrounds. Further studies within different populations are suggested to determine if the scale can accurately identify attitude toward gestational surrogacy in variable demographic characteristics and cultural backgrounds.

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Original Article

A Preliminary Study: N-acetyl-L-cysteine Improves Semen Quality following Varicocelectomy

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Abstract

Background: Surgery is considered the primary treatment for male infertility from clinical varicocele. One of the main events associated with varicocele is excessive production of reactive oxygen species (ROS). N-acetyl-L-cysteine (NAC), an antioxidant that scavenges free radicals, is considered a supplement to alleviate glutathione (GSH) depletion during oxidative stress. Despite beneficial effects of NAC in other pathological events, there is no report on the effect of NAC in individuals with varicocele. Therefore, the aim of this study is to evaluate the outcome of NAC on semen quality, protamine content, DNA damage, oxidative stress and fertility following varicocelectomy.

Materials and Methods: This prospective clinical trial included 35 infertile men with varicocele randomly divided into control (n=20) and NAC (n=15) groups. We assessed semen parameters, protamine content [chromomycin A3 (CMA3)], DNA integrity [terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL)] and oxidative stress [2', 7'-dichlorodihydrofluorescein-diacetate (DCFH-DA)] before and three months after varicocelectomy.

Results: Percentage of abnormal semen parameters, protamine deficiency, DNA fragmentation and oxidative stress were significantly decreased in both groups compared to before surgery. We calculated the percentage of improvement in these parameters compared to before surgery for each group, then compared the results between the groups. Only percentage of protamine deficiency and DNA fragmentation significantly differed between the NAC and control groups.

Conclusion: The results of this study, for the first time, revealed that NAC improved chromatin integrity and pregnancy rate when administered as adjunct therapy post-varicocelectomy (Registeration Number: IRCT201508177223N5).

Keywords: DNA Fragmentation, Protamines, Oxidative Stress, Varicocele, NAC

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Introduction

Production, maturation, and transport of sperm occur in the male reproductive tract (1). Molecular and structural anomalies in this system may result in male infertility (2). The most common structural anomaly associated with the reproductive tract is abnormal enlargement of the pampiniform plexus of veins within the scrotum, commonly referred to as varicocele (3). Although the association between male infertility and varicocele has been known since the past century, a limited number of studies exist that

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report the molecular and genetic bases of varicocele. Therefore, further research in this field may open new strategies for treatment of male infertility due to varicocele (4).

One of the key events in the pathology of varicocele is excessive production of reactive oxygen species (ROS) (5). Oxidative stress results from an imbalance between ROS production and antioxidant capacity (6). However, it is important to bear in mind that ROS acts as a double-edged sword. Although it serves as a key signal molecule in physiological processes, ROS also has a role in pathological processes (7). In pathological conditions, two roles have been envisaged for overproduction of ROS: i. ROS induced damage to sperm membrane reduces sperm motility and ability of the sperm to fuse with the oocyte, and ii. ROS directly damages sperm DNA and subsequently effects genomic integrity of the embryo (8, 9). Therefore, antioxidant therapy may overcome the deleterious effects of ROS in individuals with varicocele (10). Varicocelectomy, especially through microsurgery, has been shown to restore testicular volumes and semen parameters, as well as reduce the degree of DNA fragmentation (11, 12). Despite these beneficial effects of varicocelectomy, fewer studies have focused on the role of antioxidants as adjunct therapy along with varicocelectomy (13).

N-acetyl-L-cysteine (NAC) is a derivative of the naturally occurring amino acid L-cysteine that has free radical scavenging activity. Therefore, it is supplemented to alleviate glutathione (GSH) depletion during oxidative stress (14, 15).

Previous studies have shown both *in vivo* (6) and *in vitro* (16, 17) addition of NAC may improve semen parameters and thereby improve male fertility. Despite these beneficial effects of NAC, there has been no report on the effect of NAC in individuals with varicocele. Therefore, the aim of this study is to evaluate the effects of NAC on semen quality, protamine content, DNA damage, oxidative stress and fertility following microsurgical varicocelectomy.

Materials and Methods

This prospective clinical trial carried out between 2011 and 2013, was approved by the Ethics Committee for Research Involving Human Subjects at Royan Institute and the Isfahan Fertility and Infertility Center. All individuals gave informed consent prior to participation in the study.

Inclusion and exclusion criteria

Inclusion criteria included male gender, age younger than 45 years, primary infertility, and left-sided varicocele (grades II and III) diagnosed by palpation and Doppler duplex ultrasound. Exclusion criteria included grade I varicocele, azoospermia, recurrent varicocele, leukocytospermia, urogenital infections, testicular size discrepancy, abnormal hormonal profile, anatomical disorders, Klinefelter's syndrome, cancer, fever in the 90 days prior to surgery, seminal sperm antibodies, excessive alcohol and drug consumption, previous history of scrotal trauma or surgery, and occupational exposure.

We included female partners who were less than 35 years of age that had normal ovulatory cycles and patent tubes (hysterosalpingography or laparoscopy) in this study. Individuals with endometriosis, cycle irregularity, or gross anatomical abnormalities were excluded.

Patient selection

This study was designed similar to a blinded clinical trial. A total of 40 individuals with grades II and III varicocele enrolled in this study. Following microsurgery, the patients were randomly allocated to the control or treatment groups. In the control group, individuals received no drug after varicocelectomy (n=20). In the treatment or NAC group (n=15), the individuals received three tablets of NAC (200 mg daily) post-varicocelectomy for three months based on a previous study (6). In this study, five individuals were excluded from the treatment group due to lack of compliance with NAC use, according to the study protocol. All parameters assessed in this study were carried out by a single trained individual unaware of treatment assignment. Duration of infertility was 2.1 ± 0.2 years and duration of marriage was 3.8 ± 0.3 years in individuals with varicocele.

We initially aimed to include a group in which the individuals did not want to undergo surgery, as either a control (without surgery) or treatment

(without surgery+NAC) group. However, there were few individuals that refused to undergo surgery since the majority of these individuals had referred for infertility treatment.

Prior to surgery and at three months post-surgery, all participants provided semen samples by masturbation after 3-4 days of abstinence. Semen samples were analyzed according to World Health Organization (WHO) criteria (18). After immobilizing the sperm with a fixing solution, we evaluated the sperm concentration by a Makler counting chamber. Sperm were expressed as million/ml. Sperm motility and morphology were assessed by the Computer Aided Sperm Analysis (CASA) system (LABOMED, SDC313B). Sperm morphology was evaluated by Diff-Quik staining. DNA fragmentation and protamine deficiency were assessed with the TUNEL assay (19) and chromomycin A3 (CMA3) staining (20).

Assessment of sperm morphology (Diff-Quik staining)

A sperm suspension (20-30 µl) was smeared on the slide, allowed to air dry and stained with the prepared kit (18). Briefly, slides were immersed for 30 seconds into methanol (fixative), eosin (stain basic proteins) and a thiazin-like stain (stain DNA), respectively. Subsequently, slides were dipped into water to remove excess dye and allowed to air dry. We evaluated 200 sperm per slide.

Assessment of DNA fragmentation and protamine deficiency sperm by TUNEL and CMA3 staining

DNA fragmentation was evaluated with the aid of a terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) kit (Apoptosis Detection System Fluorescein, G3250, Promega, Mannheim, Germany) according to Kheirollahi-Kouhestani et al. (19). On each slide, 500 sperm were assessed under an epifluorescent microscope (BX51, Olympus, Japan) at ×100 magnification. Sperm with red heads were considered to have intact DNA. Those with green heads were considered to have fragmented DNA.

The percentage of sperm with protamine deficiency was assessed with CMA3 staining, according to Nasr-Esfahani et al. (20). On each slide, we assessed 500 sperm under an epifluorescent microscope (BX51, Olympus, Japan) at ×100 magnifications. Those sperm that stained light yellow were considered as CMA3 positive or protamine deficient; however, sperm that stained dark yellow were considered to have normal protamine content.

Assessment of reactive oxidative species by DCFH-DA

ROS status was assessed using a 2', 7'-dichlorodihydrofluorescein-diacetate (DCFH-DA, D6883, Sigma Co., USA) probe according to Kiani-Esfahani et al. (21). Briefly, a 2.5 mM stock solution of H2DCF-DA was prepared in dimethyl sulfoxide and stored at -70°C. A total of one million sperm were treated with 5 µM H2DCFDA for 30 minutes, while percentages of ROS positive sperm were defined by flow cytometery.

Statistical analysis

The Kolmogorov-Smirnov Z test was used to assess normal data distribution. The student's t test was carried out using the Statistical Package for the Social Studies (SPSS11.5, Chicago, IL, USA). For comparison between control and NAC groups, we used the independent t test. For comparison between the pre- and post-surgery in control and NAC groups, the paired t test was used. The differences with values of $P<0.05$ were considered statistically significant.

Results

The study population consisted of 35 individuals with grades II and III varicocele. The mean ages of male participants was 30.1 ± 4.4 (range: 22-45) years; for females, it was 26.6 ± 4.9 (range: 17-35) years. During this study, individuals with varicocele were randomly assigned to control (non-NAC) and NAC groups. In order to show that there were no significant differences between the two groups before treatments, the sperm parameters between control and NAC groups were compared. The results showed no significant difference between the two groups (Table 1). Age range of females (27.4 ± 5.7 vs. 26.05 ± 4.2 years) and males (30.7 ± 1.4 vs. 29.6 ± 0.7 years) were also similar between the two control and NAC groups.

Table 1: Comparison of pre-surgery semen parameters and ages in men with varicocele in the control and N-acetyl-L-cysteine (NAC) groups

Parameters	NAC group (n=15)	Control group (n=20)
Concentration (10^6/ml)	23.94 ± 5.9	29.7 ± 6.9
Sperm motility (%)	36.94 ± 5.5	34.92 ± 4.68
Abnormal morphology (%)	99.28 ± 0.2	99.3 ± 0.16
Volume (ml)	3.9 ± 0.5	3.47 ± 0.36
Age (Y)	30.73 ± 1.4	29.64 ± 0.74

Comparison of sperm parameters before and after surgery in control and NAC groups

In the NAC group, sperm concentration prior to surgery (23.9 ± 5.9) compared to after surgery (45.4 ± 7.1, P<0.01), percentage of sperm motility prior to surgery (36.9 ± 5.5) versus after surgery (58.2 ± 5.4, P<0.01) and normal morphology prior to surgery (0.71 ± 0.1) versus after surgery (2.71 ± 0.3, P<0.01), showed significant improvement (Fig.1A). Similarly, in the control group, sperm concentration (29.7 ± 6.9 vs. 42.4 ± 7.02, P<0.05) and normal morphology (0.7 ± 0.1 vs. 1.9 ± 0.2, P<0.01) also significantly improved following surgery. Unlike the NAC group, however, the percentage of sperm motility (34.9 ± 4.6 vs. 43.6 ± 4.9, P=0.1) did not increase following surgery in the control group (Fig.1B).

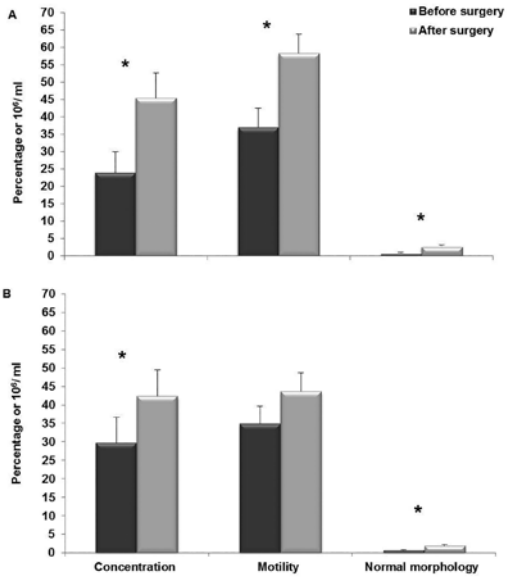

Fig.1: Comparison of sperm parameters before and after surgery in **A.** N-acetyl-L-Cysteine (NAC) and **B.** Control group.
*; Indicate significant difference before and after surgery.

Comparison of protamine content, DNA integrity, and reactive oxygen species status before and after surgery between control and NAC groups

In the NAC group, percentages of normal protamine content (48.7 ± 4.1 vs. 63.5 ± 1.6, P<0.01), percentages of DNA integrity (80.6 ± 1.8 vs. 89.8 ± 1.4, P<0.01), percentage of ROS-negative sperm (77.2 ± 7.5 vs. 92.3 ± 2.6, P<0.05), and intensity of sperm ROS (40.02 ± 7.1 vs.78.1 ± 14.6, P<0.01) significantly increased following surgery (Fig.2A). Similarly, in the control group, percentages of normal protamine content (48.7 ± 2.9 vs. 53.2 ± 3.1, P<0.01), percentages of DNA integrity (82.2 ± 1.7 vs. 85.9 ± 1.7, P<0.01) and intensity of sperm ROS (37.2 ± 3.6 vs. 61.3 ± 5.3, P<0.01) also increased significantly following surgery. Unlike the NAC group, the percentage of ROS-negative sperm (57.6 ± 6.6 vs. 60.9 ± 6.4, P=0.3) did not increase following surgery in the control group (Fig.2B).

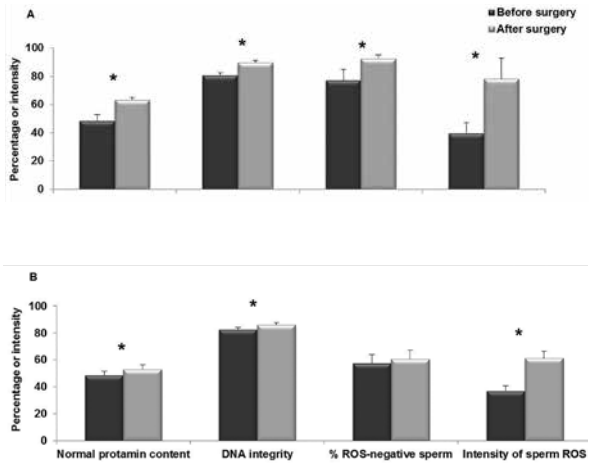

Fig.2: Comparison of different parameters before and after surgery in **A.** N-acetyl-L-cysteine (NAC) and **B.** Control groups.
*; Indicate significant difference before and after surgery and ROS; Reactive oxygen species.

Comparison of percentage of improvement of sperm parameters, status of chromatin and reactive oxygen species between NAC and control groups

In order to evaluate improvement in these parameters between the NAC and control groups, we calculated the difference between the mean values of these parameters before and after surgery, divided by the mean values of these parameters before surgery, times 100. There was no significant difference in improvement of sperm

concentration (220.5 ± 75.9 vs. 149.7 ± 81.3, P=0.5), percentage of sperm motility (100.4 ± 29.5 vs. 44.6 ± 21.8, P=0.1), abnormal morphology (100 ± 16.3 vs. 81.8 ± 27.1, P=0.5), percentage of ROS-negative sperm (36.6 ± 23.1 vs. 16.5 ± 9.5, P=0.3) and intensity of sperm ROS (76.6 ± 29.5 vs. 64.9 ± 19.5, P=0.7) observed between the NAC and control groups, respectively (Figs.3, 4). However, we found significant differences in percentages of improvement of normal protamine content (45.6 ± 13.5 vs. 11.9 ± 4.7, P<0.05) and DNA integrity (11.8 ± 2.01 vs. 4.7 ± 1.3, P<0.01) between the NAC and control groups, respectively (Fig.4).

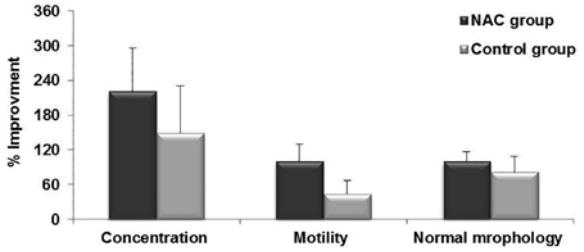

Fig.3: Comparison of percentage of improvement in semen parameters in N-acetyl-L-cysteine (NAC) and control groups.

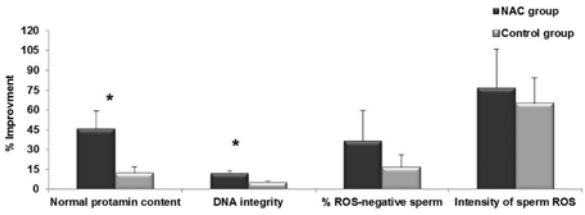

Fig.4: Comparison of percentage improvement in different parameters in N-acetyl-L-cysteine (NAC) and control groups.
*; Indicate significant difference between NAC and control groups and ROS; Reactive oxygen species.

Clinical pregnancy

The percentage of clinical pregnancy in the NAC group was 33.4% (5/15), in the control group, this result was 10% (2/20).

Discussion

Oxidative stress induced by heat stress is considered the central element that contributes to the etiology of infertility in individuals with varicocele (22). Therefore, surgical varicocele repair is expected to be beneficial to these individuals by alleviating heat, and thereby oxidative stress (10). A second approach to alleviate the varicocele associated ROS is antioxidant therapy (10, 13). In order to improve the efficiency of surgical treatment, concomitant therapy with antioxidants has been suggested (13).

We aimed to evaluate the effect of NAC on semen quality, protamine content, DNA damage, oxidative stress and fertility following varicocelectomy, as well as to compare these parameters in individuals with varicocele who did not use NAC after surgery. NAC is one of the oldest and most powerful antioxidants that treat various diseases, including respiratory disorders, heart disease, heavy metal poisoning, overdose with acetaminophen and epilepsy (6, 23).

The results of this study showed that sperm parameters (concentration, motility and normal morphology) significantly improved after surgery compared to before surgery in both the NAC and control groups, with the exception of the percentage sperm motility which insignificantly improved in the control group. Despite controversies on the degree of improvement of each semen parameters post-varicocelectomy (24), this insignificant improvement of motility in the control group was consistent with previous reports (25). However, the results of this study suggested that NAC might have an additional value by improving sperm motility post-varicocelectomy. In contrast to these results, Comhaire et al. (26) reported that although NAC improved sperm concentration and acrosome reaction, it had no effect on motility and morphology.

Despite the importance of semen parameters in fertility, many researchers have suggested that other sperm function characteristics should be considered along with these parameters when assessing fertility (27, 28). Therefore, in this study, we have assessed genomic integrity and ROS production.

Sperm DNA becomes susceptible to damage by three postulated routes: i. Improper packaging of DNA during spermiogenesis, ii. Oxidative stress and iii. Apoptosis (29). We have assessed DNA damage, ROS production and sperm nuclear maturity before and after surgery in the NAC and control groups. Of note, all parameters improved after surgery in both groups, except for the percentage of ROS negative sperm in the control group. The

percentage of sperm motility did not significantly improve before and after surgery in the control group. Therefore, this lack of significant improvement in motility post-surgery in the control group might be related to the lack of significant improvement in percentage of ROS negative sperm. In addition, the improved sperm motility in the NAC group was associated with a reduced percentage of sperm producing ROS or increased percentage of ROS negative sperm, which might be related to NAC treatment. The existence of this correlation might be explained by the cascade of events that begin with ROS associated with lipid peroxidation (30), which in turn, reduces membrane fluidity. This prevents axonemal protein phosphorylation and leads to sperm immobilization (30, 31). NAC may break these chains of events.

In this study, there was higher mean ROS intensity in semen samples after surgery compared to before surgery. This contrasted expectations since ROS production should decrease post-surgery. This was likely due to leakage of ROS or reduced production of ROS after loss of enzymes in the sperm of these individuals, which were in their final stage of apoptosis. This supposition has been previously presented by Aitken et al. (32) who reported that initially ROS positive sperm progressively become TUNEL positive. This indicated that in individuals with varicocele, despite higher production of ROS, the ROS might leak from these cells or sperm in the final stage of apoptosis, hence the enzymes that produced ROS were not as efficient. This might account for the reduced intensity of ROS.

In order to further differentiate between the role of surgery and antioxidant therapy, we calculated the percentage of improvement relative to before surgery for sperm parameters, sperm protamine content, DNA integrity, and ROS in each group and compared them between the NAC and control groups. The percentage of improvement was calculated by the difference between the mean values of a parameter before and after surgery divided by its initial value before surgery. The results revealed no significant difference for percentage of improvement for the semen parameters between the NAC and control groups. However, among the sperm functional parameters assessed, the percentage of improvement for the normal protamine content and DNA fragmentation significantly differed between the NAC and control groups, despite no initial difference between the two groups before surgery. These results suggested that despite the similar process of surgery in the two groups, the difference between percentages of improvement in the two groups was due to antioxidant activity of NAC. Possibly other etiological factors might account for this difference, which were improved by NAC, or NAC might overcome the secondary side effects of surgery. However, the role of NAC on its own in treatment of varicocele has yet to be elucidated.

Despite the higher rate of pregnancy in the NAC group compared to the control group, we did not compare clinical pregnancy rates between the groups. Due to the limited number of cases, further study would be warranted.

Conclusion

NAC can scavenge free radicals, increase GSH production and reduce disulfide bonds, as well as viscosity and elasticity of semen, which are important for fertility. This, in conjunction with the results of the current study (improved sperm parameters, DNA integrity and chromatin packaging), may account for the higher pregnancy rate in NAC group. In order to reach this conclusion, additional studies are recommended.

Acknowledgements

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Original Article

Correlation of *Adiponectin* mRNA Abundance and Its Receptors with Quantitative Parameters of Sperm Motility in Rams

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Abstract

Background: Adiponectin and its receptors (AdipoR1 and AdipoR2), known as adiponectin system, have some proven roles in the fat and glucose metabolisms. Several studies have shown that adiponectin can be considered as a candidate in linking metabolism to testicular function. In this regard, we evaluated the correlation between sperm mRNA abundance of adiponectin and its receptors, with sperm motility indices in the present study.

Materials and Methods: In this completely randomized design study, semen samples from 6 adult rams were fractionated on a two layer discontinuous percoll gradient into high and low motile sperm cells, then quantitative parameters of sperm motility were determined by computer-assisted sperm analyzer (CASA). The mRNA abundance levels of *Adiponectin*, *AdipoR1* and *AdipoR2* were measured quantitatively using real-time reverse transcriptase polymerase chain reaction (qRT-PCR) in the high and low motile groups.

Results: Firstly, we showed that adiponectin and its receptors (AdipoR1 and AdipoR2) were transcriptionally expressed in the ram sperm cells. Using Pfaff based method qRT-PCR, these levels of transcription were significantly higher in the high motile rather than low motile samples. This increase was 3.5, 3.6 and 2.5 fold change rate for *Adiponectin*, *AdipoR1* and *AdipoR2*, respectively. Some of sperm motility indices [curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), wobble (WOB) and straightness (STR)] were also significantly correlated with *Adiponectin* and *AdipoR1* relative expression. The correlation of *AdipoR2* was also significant with the mentioned parameters, although this correlation was not comparable with adiponectin and AdipoR1.

Conclusion: This study revealed the novel association of adiponectin system with sperm motility. The results of our study suggested that adiponectin is one of the possible factors which can be evaluated and studied in male infertility disorders.

Keywords: *Adiponectin*, *AdipoR1*, *AdipoR2*, Sperm Motility

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Introduction

Adiponectin is a member of the adipose-secreted proteins, called adipocytokines. Adiponectin was initially described as a 30 kDa adipocyte complement-related protein (1). It is a 244-amino acids protein and the most abundant adipose-derived hormone secreted by adipocytes in white adipose tissue with relevant roles in lipid metabolism and glucose homeostasis (2). Adiponectin also plays role on stimulation of fatty acid oxidation in the liver and skeletal muscle, suppression of hepatic gluconeogenesis, stimulation of glucose uptake in skeletal muscle and increasing insulin secretion (3). Following production, the actions of adiponectin are supported by two distinct but structurally related adiponectin receptors (AdipoR), AdipoR1 and AdipoR2 (4). The metabolic importance of these receptors is now firmly established. So that, AdipoR1$^{(-/-)}$ and AdipoR2$^{(-/-)}$ mouse models exhibited various disorders due to aberration in the fat and glucose metabolisms (5).

In addition to the well-known metabolic effects, it has been shown that adiponectin could affect the reproductive system, in part, through central actions on the hypothalamus-pituitary axis (6). Hypothalamic neurons secrete gonadotropin-releasing hormone (GnRH) with a pulsatile pattern, stimulating the release of pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These gonadotropins regulate testicular steroidogenesis and spermatogenesis (7). AdipoR1 and AdipoR2 are generally expressed in human hypothalamus and pituitary (8), so adiponectin can presumably be involved in the modulation of the endocrine reproductive axis in humans. Adiponectin and its receptors are also expressed by different cell types of the male gonad, suggesting a possible regulation of testicular function by adiponectin, through endocrine and/or paracrine actions. In chicken, presence of the adiponectin system (adiponectin, AdipoR1 and AdipoR2) was demonstrated in peritubular and seminiferous tubule cells (9). In line with this, testicular expression of adiponectin receptors was found to be higher at mRNA or protein levels in adult compared to prepubertal chickens and rats (9, 10), suggesting that sexual maturation is likely associated with an increased sensitivity to the changes in plasma adiponectin levels. A significant, positive relationship was also reported between plasma adiponectin and high-density lipoprotein cholesterol in men (11) which may contribute to testosterone production. In this regard, Laughlin et al. (12) showed that, in the men and women, serum adiponectin is positively related to testosterone and high-density lipoprotein.

In general, the previous studies demonstrated a close relationship between adiponectin system and reproductive function. So, we hypothesized that the sperm *Adiponectin*, *AdipoR1* and *AdipoR2* mRNA abundances might correlate with sperm motility indices.

Materials and Methods

Semen samples and spermatozoa preparations

In this completely randomized design, testicles of 6 adult rams were collected from an official abattoir and transferred to the laboratory at room temperature (20-25°C). All procedures to sacrifice the animals were carried out at abattoir in accordance with Iranian government rules. Semen collection was carried out within the first 2 hours after the slaughter of the ram. Epididymis-testicle complexes were dissected into two parts: testicle, epididymis. Sperm was obtained by slicing the tissue of the cauda epididymis with a scalpel; the fluid was collected by sampler and the volume was estimated. To prohibit contamination, epididymis samples were carefully dissected free of blood clots and extraneous tissues. Care was taken to no cut blood vessels.

Semen samples were washed with Hepes-buffered tissue culture medium (Hepes TCM, Gibco, Life technologies, USA)+10% bovine serum albumin (BSA, Gibco, Life technologies, USA) and sperm suspensions were centrifuged at 500 g for 2 minutes. The supernatant was then discarded. This procedure repeated two times. The sperm of 6 rams was subsequently separated into low and high motility categories, as described below.

Sperm separation procedures

Sperm suspension was layered on a two-layer discontinuous Percoll gradient, consisting of 1 ml of 45% (v/v) and 2 ml of 90% (v/v) Percoll (Uppsala, Sweden) in a 15 ml conical plastic tube (Falcon No. 2095, Fisher Scientific, Pittsburg, USA). The tube was centrifuged at 700 g for 20 minutes. After centrifugation, the separated fractions in the tube were carefully transferred into a new set of tubes, and the volume of each fraction was determined.

Spermatozoa evaluation

The assessment of motility parameters was carried out, using computer-assisted sperm analysis (CASA,

HooshmandFanavar, Iran). Samples were diluted (10-20×10^6 cells/ml) in the same Hepes TCM medium with 320 mOsm/kg, and kept warm in the 37°C incubator during examination. Subsequently, 5 µl sample drop was placed into a Makler counting cell chamber (20 µm depth) and evaluated. Evaluation was carried out on both groups of the separated sperm by Percoll gradient.

The CASA settings were selected as follow: 6 vision-fields per sample, 20 frames per second with the time analysis of less than 15 seconds per frame, 0-180 µm/second analysis power for sperm velocity, and magnifying power of ×4 for microscope objective lens.

In CASA analysis results, sperm motility was divided into classes A, B, C and D as rapid motility, slow motility, non-progressive motility and immotility, respectively. Besides, the followed sperm motion parameter indices were studied: curvilinear velocity (VCL), the time-average of velocity along the actual trajectory for a spermatozoon in micrometers per second, straight line velocity (VSL) representing the average velocity of sperm from the first to last position of a sperm head in a track by micrometers per second. A straight-line path from the first to last position of a sperm head was plotted, and velocity along this trajectory was termed VSL (micrometers per second). The average path of sperm cell motion was also computed, and average-time of velocity along the average path was calculated and named as average path velocity (VAP, micrometers per second). For each centroid location of sperm, there was a deviation from the average path, called as the amplitude of lateral head displacement (ALH, micrometers). Beat cross frequency (BCF) was the frequency of sperm cell's head cross, through the sperm cell's average pathway in Hertz. Similarly, there were points where the curvilinear path intersects the average path, and the number of such intersections was termed as BCF (number per second). The linearity (LIN) represents the linearity of a curvilinear path in percentage. The wobble (WOB) was the measure of the actual path oscillation with regard to the average path, and the mean angular displacement (MAD) was the average time for the instantaneous turning angle absolute values of the sperm head, along with the curvilinear trajectory in degree (13).

RNA extraction and cDNA synthesis of sperm cells

Total RNA isolation was carried out on sperm cells, according to the acid guanidiniumthiocyanate-phenol-chloroform single-step extraction protocol, as described earlier (14). Treatment of total RNA with RNase-free DNase (Sinaclon Bioscience, Iran) was performed to avoid amplification of contaminating genomic DNA. The quality and integrity of the purified RNA was controlled by measurement of the A260/A280 nm ratio as well as agarose gel electrophoresis. Only RNA samples showing integrity by electrophoresis and exhibiting an A260/A280 ratio of >1.9 were used for synthesis of cDNA.

Total RNA was reverse transcribed into cDNA using moloney murine leukemia virus reverse transcriptase (M-MLV RT, Sinaclon Bioscience, Iran). The reverse transcribed mixture was incubated at 75ºC for 15 minutes to denature the RNA, and then stored at -20ºC.

Real-time quantitative reverse transcriptase-polymerase chain reaction analysis

The levels of all three transcripts (*Adiponectin*, *AdipoR1* and *AdipoR2*) were determined by real-time reverse transcriptase polymerase chain reaction. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was selected as a housekeeping gene to normalize the difference of input load of cDNA between the samples. Specific primers for cDNA of *Adiponectin*, *AdipoR1*, *AdipoR2* and *GAPDH* were designed using primer BLAST (http://www.ncbi.nlm.nih.gov/primerblast). Nucleotide sequences of the selected primer pairs and the length of amplified product are presented in the Table 1.

Table 1: Characteristics of the used primers

Gene	NIH GenBank accession no.	Product length (bp)	Primer sequence 5´- 3´
GAPDH	NM_001190390.1	116	F: GTTCCACGGCACAGTCAAGG R: ACTCAGCACCAGCATCACCC
Adiponectin	KJ159213.1	132	F: CGGCACCACTGGCAAATTCC R: TGGTCGTGGGTGAAGAGCAG
AdipoR1	KJ159212.1	131	F: CAGGGGTGCAGGAGGAACTT R: GTGGGCTGAAGCTTGGTTGG
AdipoR2	KF921623.1	155	F: GCATCGCAGCCATCATCGTC R: GATGGTGGCAGCCTTCAGGA

Real-time quantitative reverse transcriptase-PCR (qRT-PCR) analysis was performed on Rotor-Gene Q 6000 System (Qiagen, USA) using SYBR premix EX Tag II (Takara, China). One microliter of cDNA was added to the master-mix (0.5 µM of each specific primer, and 10 µl of SYBR premix EX Tag IIReady Mix) in a total volume of 20 µl. Aliquot of each reaction mixture was analyzed by electrophoresis in 1.5% agarose gel and stained with 0.5 µg/ml ethidium bromide. The relative quantification of three gene transcripts was determined in low and high motile sperm groups. Reaction condition was performed as 95°C for 5 minutes, 45 cycles of 95°C for 40 seconds, 63°C for 30 seconds and 72°C for 30 seconds. The PCR amplification was performed in triplicate for each sample.

Gene expression data were normalized to *GAPDH* (as internal reference gene). Data were analyzed using LinRegPCR software version 2012.0 (Amsterdam, Netherland), to give the threshold cycle (Ct) number . Mean efficiency values (E) for each gene were also determined from the amplification profiles of individual samples with this software (15). The mRNA level of each target gene relative to *GAPDH* was estimated for each sample in two experimental groups by following formula: $E_{GAPDH}^{(\text{Ct high motile})}/E_{Adiponectin}^{(\text{Ct high motile})}$. Then, the comparison was statistically done between groups. To determine fold change for each gene, the relative gene expression of high motile group relative to low motile group were calculated as following (16, 17).

$$\textbf{Ratio} = \frac{E_{GAPDH}^{(\text{Ct high motile})}}{E_{Adiponectin}^{(\text{Ct high motile})}} \div \frac{E_{GAPDH}^{(\text{Ct low motile})}}{E_{Adiponectin}^{(\text{Ct low motile})}}$$

To ensure product homogeneity, the melting curve analysis was performed after the real-time PCR procedure. The fluorescence signals were recorded continuously during temperature ramp (65-95°C).

Statistical analysis

Differences between experimental group means were analyzed through paired t test with SPSS, version 16.0 (SPSS Inc., USA). The Pearson correlation procedure was used to evaluate correlation between the level of mRNA abundance and all quantitative sperm motion parameters for the indicated genes. All results are shown as mean ± SEM and differences were considered significant at $P< 0.05$.

Results

The results of CASA evaluation for sperm motility and sperm motility pattern are given in the Tables 2 and 3. After separation on Percoll gradient, the remaining sperm phase, in 45% Percoll, had significantly lower motile sperm cells (Table 2). The high motile sperm groups were also significantly better in other sperm motility parameters, such as VCL, VSL, VAP, LIN, WOB and STR (Table 3). This result showed that separation procedure was performed well. After separation, we analyzed the mRNA abundance of three genes between high and low motile sperm groups. As presented in the Figure 1, the mean level of *Adiponectin*, *AdipoR1* and *AdipoR2* gene abundances was significantly higher in high motile group than low motile. In the next step and for more evaluation, the correlation analysis was performed between the level of mRNA abundance and all sperm motion parameters for all three genes in motile and immotile sperm groups. In this analysis, all samples from high and low motile groups were considered together and the general correlation between motility indices and the level of mRNA abundance was calculated. The results of this analysis showed that mRNA abbundance for *Adiponectin* gene had a significant positive correlation with the class A of sperm motility, percent of progressive motile sperms, percent of motile sperms, VCL, VSL, VAP, LIN and WOB (Table 4). The amount of mRNA for *AdipoR1* gene also showed a significant positive correlation with class A of sperm motility, percent of progressive motile sperms, LIN, WOB and STR (Table 4). For *AdipoR2* gene, significant correlation was only observed with WOB.

Table 2: Concentration, motility and progression of Percoll separated sperm samples (evaluated by CASA). Results are given as mean ± SE

Groups	Sperm density (Mill/ml)	Motile sperm (%)	Progression (%)			
			Fast progressive (class A)	Slow progressive (class B)	Non progressive (class C)	Non motile (class D)
High motile (n=6)	12.07 ± 2.56^{ns}	76.40 ± 2.27**	58.53 ± 3.52****	12.01 ± 4.66^{ns}	5.85 ± 0.51^{ns}	23.60 ± 2.2**
Low motile (n=6)	13.46 ± 1.73	58.49 ± 4.47	29.36 ± 2.41	16.34 ± 6.67	9.01 ± 3.85	44.00 ± 3.84

CASA; Computer-assisted sperm analyzer, ns; Not significant, **; P<0.01 and ****; P<0.0001.

Table 3: Sperm motility pattern parameters of percoll separated sperm samples (evaluated by CASA). Results are given as mean ± SE

Groups	Sperm motility pattern								
	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	MAD (°)	ALH (µm)	BCF (Hz)	LIN (%)	WOB (%)	STR (%)
High motile (n=6)	80.93 ± 9.66*	57.13 ± 8.47**	65.58 ± 8.66*	20.20 ± 3.28^{ns}	3.03 ± 0.18^{ns}	2.49 ± 0.77^{ns}	56.53 ± 2.56**	71.24 ± 1.59***	71.52 ± 1.95**
Low motile (n=6)	49.75 ± 7.78	23.65 ± 3.06	32.39 ± 5.66	13.86 ± 3.18	2.67 ± 0.26	1.73 ± 0.75	36.31 ± 3.48	54.64 ± 2.66	57.19 ± 2.97

CASA; Computer-assisted sperm analyzer, VCL; Curvilinear velocity, VSL; Straight-line velocity, VAP; Average path velocity, MAD; Mean angular displacement, ALH; Amplitude of lateral head displacement, BCF; Beat cross frequency, LIN; Linearity, WOB; Wobble, STR; Straightness, ns; Not significant, *; P<0.05, **; P<0.01 and ***; P<0.001.

Table 4: Correlations between the amount of relative gene abundance for *Adiponectin*, *AdipoR1* and *AdipoR2* with quantitative sperm motion parameters

Groups	Sperm motility pattern											
	MS (%)	Class A (%)	SPM (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	MAD (°)	ALH (µm)	BCF (Hz)	LIN (%)	WOB (%)	STR (%)
Adiponectin relative abundance	0.51*	0.66*	0.56*	0.54*	0.60**	0.61**	-0.10^{ns}	0.03^{ns}	0.18^{ns}	0.53*	0.64**	0.06^{ns}
AdipoR1 relative abundance	0.19^{ns}	0.44*	0.44*	0.47^{ns}	0.19^{ns}	0.23^{ns}	0.67^{ns}	-0.014^{ns}	0.56^{ns}	0.46*	0.55**	0.47*
AdipoR2 relative abundance	0.21^{ns}	0.36^{ns}	0.26^{ns}	0.10^{ns}	0.15^{ns}	0.16^{ns}	-0.03^{ns}	0.11^{ns}	0.28^{ns}	0.32^{ns}	0.45*	0.26^{ns}

MS; Motile sperm; Class A; Sperm with fast progressive motility, SPM; Sperm with progressive motility (class A+class B), VCL; Curvilinear velocity, VSL; Straight-line velocity, VAP; Average path velocity, MAD; Mean angular displacement, ALH; Amplitude of lateral head displacement, BCF; Beat cross frequency, LIN; Linearity, WOB; Wobble, STR; Straightness, ns; Not significant, *; P<0.05 and **; P<0.01.

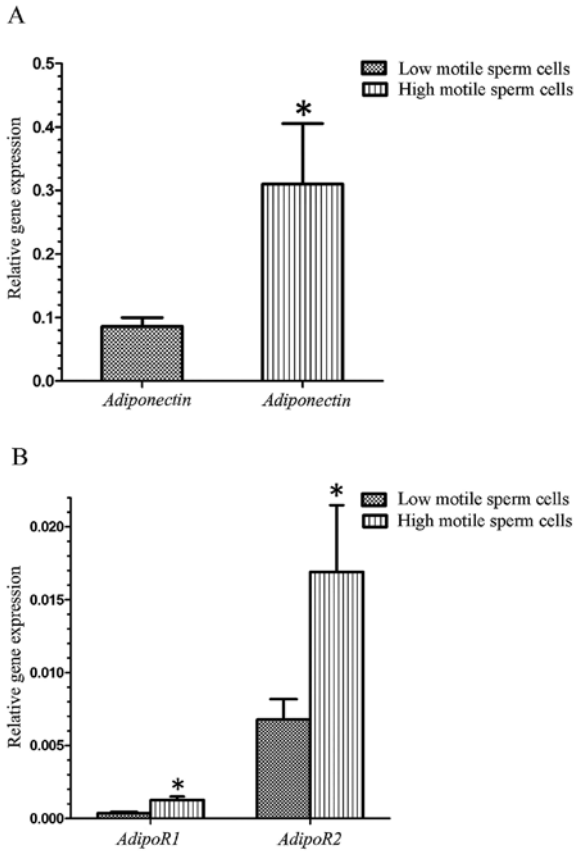

Fig.1: Relative abundance of **A.** *Adiponectin*, **B.** *AdipoR1* and *AdipoR2* mRNA in low and high motile sperm groups.
*; Significant difference between two groups.

Discussion

In the present study, associations between sperm mRNA abundance of *Adiponectin* and its receptors, *AdipoR1* and *AdipoR2*, with quantitative parameters of sperm motility were evaluated in rams. Adiponectin is a plasma protein with about 0.01% of total serum proteins concentration (18). The primary amino acid sequences of Adiponectin are highly conserved across the species (19). For example, bovine adiponectin shows 92% homology with human Adiponectin and 82% homology with murine Adiponectin (20). Adiponectin is synthesized as a single monomer which undergoes multimerization to provide three multimer forms with different molecular weights (MWs): i. Low molecular weight (LMW) Adiponectin composed of three monomers that are combined to form a trimmer, ii. Middle molecular weight (MMW) Adiponectin, as a hexamer formed by two trimmers, and iii. High molecular weight (HMW) multimer of Adiponectin, comprised of 12-18 monomers.

Ejaculated sperm retains a complex and specific population of RNAs. It was recently proposed that these RNA molecules may have important roles in the sperm development, chromatin repackaging and even zygote development (21). Studies on sperm RNA are available for humans (22), stallion (23), cattle (24) and boars (25). Analysis of the mRNA profiles in the normal and abnormal sperms is a growing field, which can become a diagnostic and prognostic tool to evaluate male fertility and can eventually lead to identify specific genetic pathways which are necessary for production of the fertile sperm. For example, studies have been conducted to compare the genetic profiles of sperm samples from normal fertile men and teratozoospermic patients (26, 27).

In the present study, the mRNA abundances of all three components of adiponectin system were significantly higher in the high motile sperm groups. The mRNA abundances also positively correlated with some of the most important parameters of sperm motility pattern, especially for *Adiponectin* and *AdipoR1*. Adiponectin and the relevant receptors play major roles in sperm morphology and function, contributing to increase fertility. A recent study by Kasimanickam et al. (28) showed that Adiponectin, AdipoR1, and AdipoR2 were immunolocalized in the acrosomal, postacrosomal, equatorial, and tail regions of bull sperm. In this study, serum Adiponectin concentration and sperm mRNA expressions for *Adiponectin* and its receptors showed a significant positive correlation with sire conception rate. In ram, transcripts for adiponectin system components, have been detected in the testis, all parts of epididymis, vesicular and bulbourethral glands (29). Expression of Adiponectin receptors was also reported in porcine epididymis (30).

Our results showed a novel evidence for the presence of Adiponectin and its two receptors in at least sperm cells from cauda-epididymides. In this context, Rahmanifar and Tabandeh (29) also determined that *Adiponectin*, *AdipoR1* and *AdipoR2* were expressed in all parts of epididy-

mides (caput, corpus, and cauda), while *AdipoR2* mRNA expression was higher than *AdipoR1*. These results, in addition to the finding reported by Kasimanickam et al. (28) indicating that gene expression of *Adiponectin* and its receptors during pre- and post-capacitation in spermatozoa, provide evidences of possible production of fertile sperm by local actions of Adiponectin at the testis level.

In this regard, it should be noted that a sperm-specific ATP-binding cassette (ABC) transporter regulates intracellular lipid metabolism in rodents (31). Kitajima et al. (32) showed that Adiponectin and its receptors (AdipoR1 and AdipoR2) increased cholesterol efflux and reconstituted high-density lipoprotein-induced efflux, at least partially through an ABCA1 pathway. In that study, AdipoR1- and AdipoR2-transfected cells showed greater cholesterol efflux when treated with Adiponectin. In contrast, down-regulation of adiponectin receptors decreased reconstituted high-density lipoprotein-induced cholesterol efflux. Adiponectin related signaling pathways in the sperm cell are not well studied until now. But Adiponectin and its receptors might participate in cholesterol efflux via a sperm-specific ABC transporter and thereby affect sperm hyperactivation and capacitation (33).

The positive correlation of obesity with male infertility could be the evidence of clinical importance of Adiponectin in the fertility. In rodents, the obesity leads to sub-fertility caused by reduced sperm motility (34). Obese men have also reduced sperm concentration and total sperm count (35). In an interesting study performed by Thomas et al. (36), normal-weight men showed higher concentrations of Adiponectin in the seminal plasma and blood serum. In addition, Adiponectin concentration in seminal plasma positively correlated with sperm concentration and normal morphology of spermatozoa. Hammoud et al. (37) also found that asthenozoospermia and oligozoospermia were increased due to high body-mass index and worsened from overweight to obese men. One possible reason can be the correlation between plasma Adiponectin and testosterone concentrations. In this regard, Ribot et al. (38) confirmed that the diet-induced obesity in rats leads to decrease in the effective production of Adiponectin. Studies showed that Adiponectin played roles in gonadal steroidogenesis. As a paralog of Adiponectin, CTRP3 (a member of the C1q/TNF-related protein superfamily) was expressed at high level in the adipose tissue. In adult mouse testis, CTRP3 was expressed in Leydig cells and contributes to increase testosterone production by up-regulating Cyp11A1 and Star protein expressions (39). Interestingly, Adiponectin has been shown to regulate the expression of steroidogenic genes (*Star*, *Cyp11Aa1* and *Cyp19A1*) in human, rat, chicken and swine ovary (40, 41), suggesting that Adiponectin might affect steroidogenesis in Leydig cells through regulation of steroidogenic gene expressions as well. This finding was also confirmed by Erdemir et al. (42). In this study, the male fat rats, had significantly lower levels of testosterone compared to the controls. In terms of pathologic evaluation, Johnsen Score (a 1-10 degree score for microscopic evaluation of spermatogenesis quality in testicular tissue) was significantly lower in the fat male rats.

Conclusion

The findings indicated that the products of *Adiponectin* gene may be involved in the physiology of sperm cell movement. Although the exact role of Adiponectin in the male reproductive system remains hypothetical, demonstrated expression of this gene in epididymal spermatozoa in this study and all parts of epididymidis suggests a possible role of Adiponectin in maturational spermatozoa changes, as they transit the duct. Moreover, considering the demonstrated correlation between obesity and fertility impairment, the results of such studies will help to find the molecular mechanisms involved in the pathogenesis of this disorder.

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Case Report

Herlyn Werner Wunderlich Syndrome with Hematocolpos: An Unusual Case Report of Full Diagnostic Approach and Treatment

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Abstract

Herlyn-Werner-Wunderlich (HWW) syndrome is an uncommon combined müllerian duct anomalies (MDAs) and mesonephric duct malformation of female urogenital tract characterized by uterus didelphys and obstructed hemi-vagina and ipsilateral renal agenesis (OHVIRA) syndrome.

We present a rare and unusual case of this syndrome in a 19 year-old female who suffered from hypomenorrhoea and abdominal pain. She had an obstructed hemi-vagina on right side which led to marked distention of ipsilateral cervix, while proximal hemi-vagina compressed the contralateral side causing its partial obstruction resulting in hypomenorrhoea. Understanding the imaging findings of this rare condition is important for early diagnosis in order to prevent complications which may lead to infertility.

Keywords: Amenorrhea, Dysmenorrhea, Hematocolpos, Vagina, Infertility

Citation: **Bhoil R, Ahluwalia A, Chauhan N. Herlyn werner wunderlich syndrome with hematocolpos: an unusual case report of full diagnostic approach and treatment. Int J Fertil Steril. 2016; 10(1): 136-140.**

Introduction

Herlyn Werner Wunderlich (HWW) syndrome, also known as obstructed hemi-vagina and ipsilateral renal agenesis (OHVIRA) syndrome, is an uncommon combined müllerian duct anomalies (MDAs) (Table 1) and mesonephric duct malformation of female urogenital tract.

Table 1: MDAs classification

Class	Description
I	Segmental müllerian agenesis or hypoplasia
II	Unicornuate uterus
III	Uterus didelphys
IV	Bicornuate uterus
V	Septate uterus
VI	Arcuate uterus
VII	Uterus with internal luminal changes (T shaped uterus - diethylstilboestrol exposure related)

MDAs; Müllerian duct anomalies.

The exact incidence of this syndrome is unknown (1); however, the incidence of uterus didelphys (Fig.1) as a part of this syndrome is about 1/2000 to 1/28000 that is accompanied by unilateral renal agenesis with the incidence of approximately 1/1100, while 25 to 50% of affected women have showed to have genital abnormalities (2-5).

Although HWW syndrome includes variability of the anatomic structures like uterine, cervical, vaginal and/or renal anomalies, it is characterized by the presence of uterus duplicity and OHVIRA syndrome.

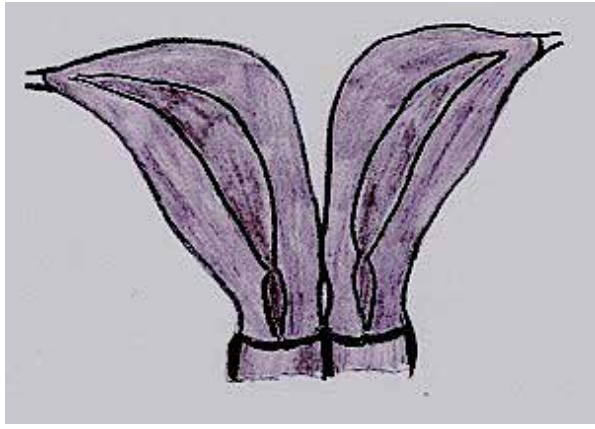

Fig.1: Class III MDAs-uterine didelphys. MDAs; Müllerian duct anomalies.

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Case report

A 19 year-old unmarried female presented to Dr. Rajendra Prasad, Government Medical College, Kangra, HP, India, in June 2013. She complained of abdominal pain gradually increasing in intensity and scanty periods since the last 6 months. Patient reached menarche at 16 years with normal menstrual cycles until 6 months ago. She also complained of periodic pain in lower abdomen accompanying her menstrual cycles beginning from around the time of her menarche. Initially for the first three-four months, she was being symptomatically managed for dysmenorrhea, but ultrasound scans done in a referral center revealed multiple cystic lesions in bilateral adnexa with low level internal echoes suggestive of endometriosis. Thereafter she was being managed medically for endometriosis (in the scans, her uterus was reported as normal). Her urine pregnancy test was negative.

An ultrasound scan done for pelvic organs at our institute revealed uterus didelphys (Fig.2) and a cystic fluid collection with low level internal echoes arising from the pelvis consistent with associated haematocolpos (Fig.3). Cystic lesion was noted in the right adnexa consistent with endometrioma. Right kidney was not visualized (Fig.4).

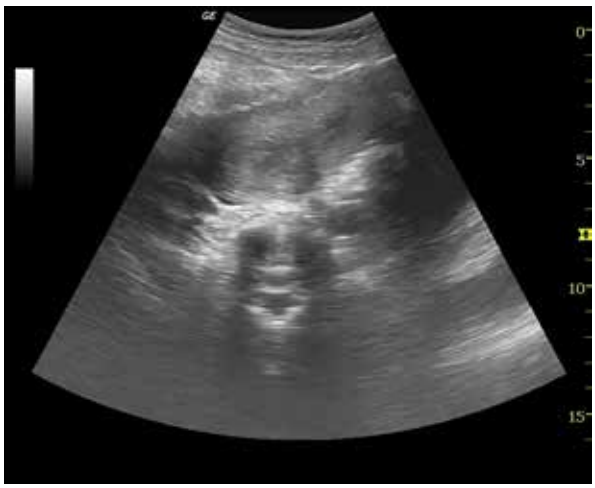


Fig.2: Transverse ultrasound image showing two uterine cavities with echogenic endometrium.

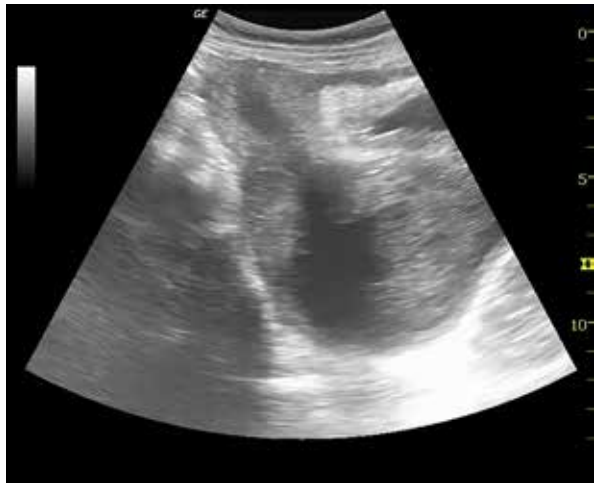


Fig.3: Longitudinal ultrasound image depicting a cystic lesion posterior to urinary bladder with low level echoes and communication with endometrial cavity through the cervix.

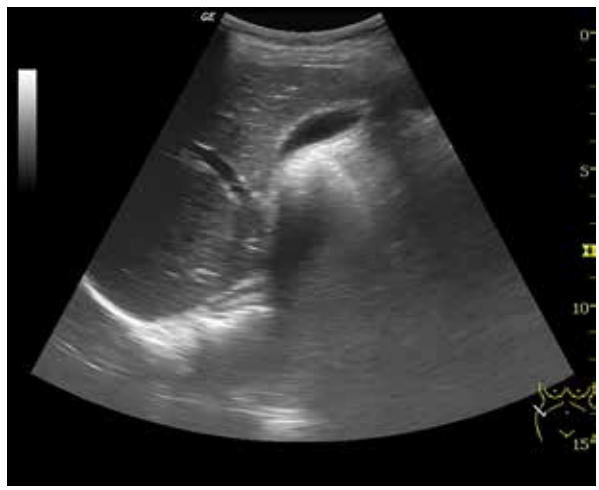


Fig.4: Transverse ultrasound of right hepatorenal space showing absent kidney in the right renal fossa.

Subsequently magnetic resonance imaging (MRI) was performed to better characterize the pelvic anatomy and better identify the anatomic location of this pelvic fluid collection. MRI revealed uterine didelphys with two separate cervices (Fig.5). The right cervix and proximal hemi-vagina were distended that led to the comparison of the left cervix and hemi-vagina (Fig.6A). The left endometrial cavity appeared normal; however, the left cervix/hemi-vagina was constricted in its lower part due to pressure from the distended cervix and hemi-vagina on the right side resulting in partial obstruction of menstrual blood outflow, as seen in the patient (Fig.6B). The high T2 MRI signal characteristics in

conjunction with low level internal echoes seen on the ultrasound were consistent with hematocolpos.

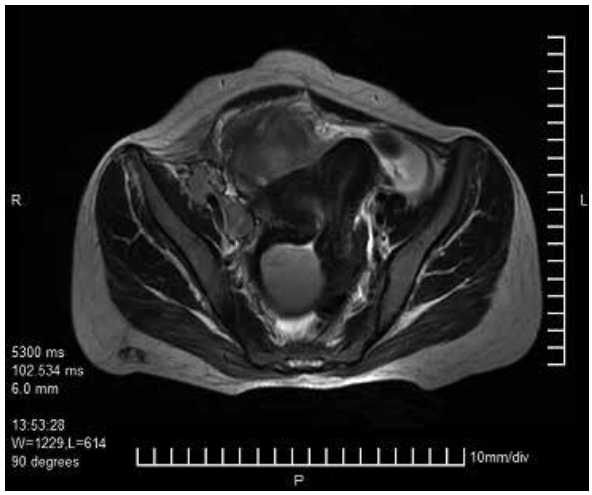
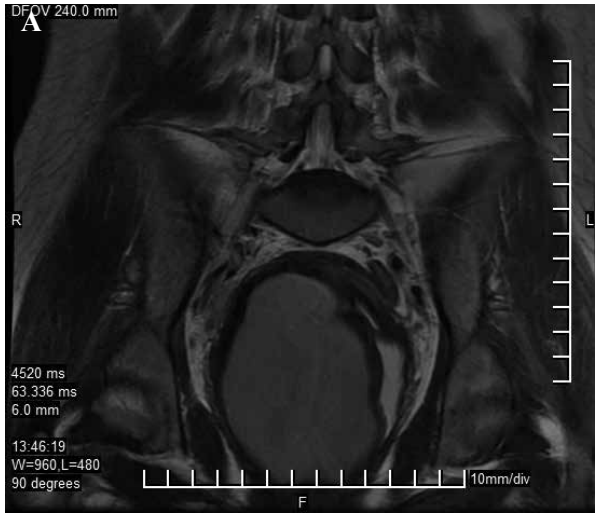


Fig.5: Axial T2W MRI image showing two uterine cavities with distended right cervix and hemi-vagina. MRI; Magnetic resonance imaging.

A right adnexal cystic lesion with blood products was seen suggestive of endometriotic cyst (Fig.7).

Subsequent gynecological examination revealed an obstructed right hemi-vagina and a fluid wave palpable through inferior septum. This hematocolpos was surgically drained and about 400 ml of old blood was evacuated. The patient recovered uneventfully and no further surgery was done.

A written consent was taken from the patient for publication of this report.



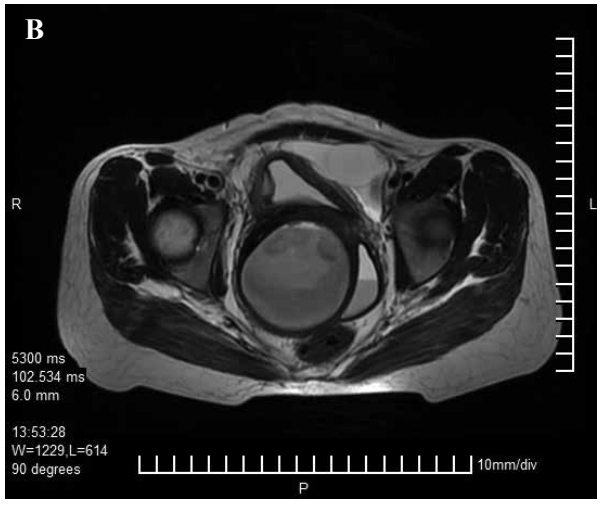


Fig.6: A. Coronal and **B.** Axia T2W MRI images showing distended right cervix and hemi-vagina compressing the normal left hemi-vagina which shows differential signal intensity resulting in layering.

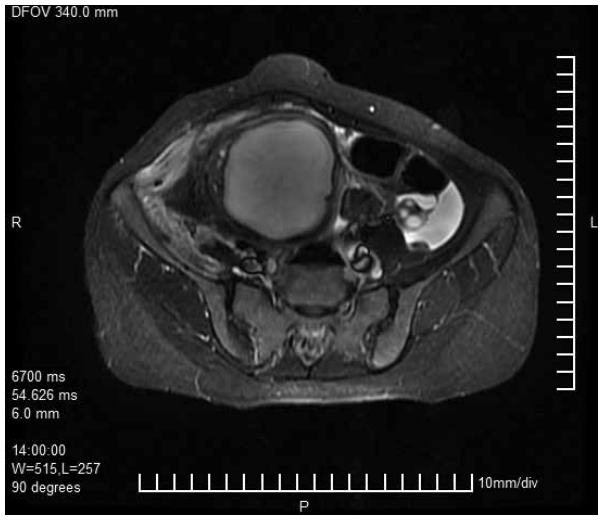


Fig.7: Axial T2W MRI image showing large hyperintense right adnexal cyst (endometriotic cyst). MRI; Magnetic resonance imaging.

Discussion

Most common type of MDAs is the lateral fusion defects which range from symmetric/asymmetric to obstructed/unobstructed fusion anomalies. A useful classification based on the degree of failure of normal development was proposed by Buttram and Gibbons (6).

Development of urinary system and müllerian duct system are closely related with which accounts for the frequent association of anomalies involving both the systems (2, 3).

Uterine didelphys results from complete failure of fusion of the müllerian ducts and their normal differentiation to form a cervix and uterus during the 8th week of gestation (7). Uterine didelphys (Class III MDA) occurs in case of complete failure of fusion as also seen in our case.

The Wolffian duct gives rise to the ipsilateral ureteric bud and thus is responsible for the formation of the kidney. Accordingly, in the absence of the Wolffian duct on one side, the kidney and ureter (of the same side) will fail to fuse (3, 4). On the side on which the Wolffian duct is missing, the müllerian duct is displaced laterally and fails to adequately fuse with the urogenital sinus, leading to the formation of a blind sac, imperforate or obstructed hemivagina (3), right side in the present case. The distal part of vagina which arises from the urogenital sinus is not affected and develops normally.

Patients with OHVIRA syndrome are usually asymptomatic until puberty, when they present with acute lower abdominal pain. Diagnosis is usually made soon after menarche (most patients are diagnosed from 2 months to 2 year after menarche) and the presenting symptoms are pelvic pain, dysmenorrhea, foul-smelling discharge and pelvic mass (7, 8). If not treated, complications leading to infertility, endometriosis, pelvic adhesions, and pyosalpinx or pyocolpos may present in the late phase with a high miscarriage rate (7).

The choice of investigation for the diagnosis and operative planning of OHVIRA syndrome are ultrasound and MRI, both of which have an added advantage of being non-invasive (1-5).

The role of computed tomography (CT) is limited due to radiation exposure and limited soft-tissue resolution. Ultrasound may reveal uterine didelphus and pelvic fluid collection with low level internal echoes, contiguous with the endocervix (haemato/pyocolpos). Due to retrograde menstruation, features of endometriosis in form of well defined, unilocular or multilocular, predominantly cystic masses containing diffuse, homogeneous, low level internal echoes (endometrioma/chocolate cyst) may also be seen (9).

MRI plays an important role in characterizing the didelphic uterus, obstructed hemivagina, and ipsilateral renal agenesis (1, 10). MRI findings of OHVIRA syndrome are characterized by iso/high T1W signal and high T2W signal that indicate pelvic fluid collection is contiguous with the endocervix along with didelphic uterus and an absent kidney on the affected side (1, 2).

MRI is far better than ultrasound for characterizing anatomical relationships due to its multiplanar capabilities and larger field of view (2). However, the gold standard for diagnosis is laproscopy through which has the added benefit of performing therapeutic drainage of hematometra/hematocolpos, vaginal septotomy and marsupialisation (10). Treatment usually involves surgery in the form of excision of the vaginal septum which helps in relieving obstruction (11). Surgical intervention also decreases the chances of pelvic endometriosis due to retrograde menstrual seeding. About 87% of patients go on to have a successful pregnancy; however, 23% of patients carry the risk of subsequent abortion (12).

The rarity of OHVIRA syndrome complicates its diagnosis, and hence clinicians and radiologists should consider MDAs among the differential diagnosis in young female patients presenting with abdominal symptoms, especially when associated with renal anomaly/agenesis. Understanding the imaging findings is critical for early diagnosis in an attempt to prevent complications such as endometriosis or adhesions from chronic infections with subsequent infertility.

Acknowledgements

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