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Chronic Endometritis: Old Problem, Novel Insights and Future Challenges

Elena Puente, M.D.¹, Luis Alonso, M.D.², Antonio Simone Laganà, M.D.^{3*}, Fabio Ghezzi, M.D.³, Jvan Casarin, M.D.³, Jose Carugno, M.D., F.A.C.O.G.⁴

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Abstract

Chronic endometritis (CE) is a poorly investigated pathology which has been related to adverse reproductive outcomes, such as implantation failure and recurrent miscarriage. In this paper, we aim to provide an overview of diagnosis, etiology, pathophysiology and treatment of CE, its impact on endometrial microenvironment and its association with infertility. We present a narrative review of the current literatures, synthesizing the findings retrieved from searches of computerized databases. CE is more prevalent in infertile patients. Effective antibiotic treatment of CE seems to improve the pregnancy and live birth rate in patients with unexplained recurrent pregnancy loss (RPL), and increase ongoing pregnancy rate in patients with recurrent implantation failure. In order to increase the diagnostic accuracy, immunohistochemistry is recommended besides the conventional histology. In addition, hysteroscopy could be considered as gold standard tool for diagnosis, considering its high correlation with histological findings. CE, as the chronic inflammation of endometrium, is usually asymptomatic and probably underestimated. Interaction of bacteria with endometrial microenvironment promotes changes in leukocyte population, cytokine production and growth factors which support its negative impact on endometrial receptivity. Nevertheless, standardization of the criteria for histopathological diagnosis and immunohistochemistry technique needs to be defined.

Keywords: Endometritis, Hysteroscopy, Immunochemistry, Reproductive Outcomes

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Introduction

Assisted reproductive techniques undergo great advances, including improved means of tissue culture, updated criteria of embryonic selection and extended culture to blastocyst, leading to reach pregnancy rates up to 66% in the selected patients (1-4). In this regards, many factors involved in the implantation are not yet fully understood and it seems that endometrium plays much more relevant role than the other recognized factors (5-7).

Endometrium is a unique tissue that undergoes monthly cyclical changes resulting in menstruation, proliferation, secretion and decidualization under the influence of ovarian steroids. Endometrium contains a large variety of immunocompetent cells, natural killers (NKs), macrophages, T cells and neutrophils, whose composition and density fluctuates periodically (8). The cycle-dependent changes in these subpopulations of leukocytes and their mediators probably play a crucial role in implantation. In contrast, antibody-bearing B-lymphocytes and plasma cells are rarely found in endometrial tissue (9).

Chronic endometritis (CE) is defined as localized inflammation of the endometrial mucosa characterized by the presence of edema, increased stromal cell density, dissociated maturation between epithelial cells and stroma fibroblasts, as well as the presence of plasma cell infiltrate in the stroma (10). These changes at the level of endometrial microenvironment could affect endometrial receptivity (11).

CE is often asymptomatic or present with non-specific clinical symptoms, such as pelvic pain, dysfunctional uterine bleeding, dyspareunia, vaginal discharge, vaginitis, recurrent cystitis and mild gastro-intestinal discomfort (12). Nonspecific quality of the symptoms and importance of performing endometrial biopsy to confirm diagnosis makes it difficult to estimate the prevalence of this condition.

Based on endometrial biopsy of patients who subsequently underwent hysterectomy with benign pathology, prevalence of CE is 10-11% of the general population (13, 14), 3-10% of patients with abnormal uterine bleed-

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ing (15) and up to 72% of women with suspected pelvic inflammatory disease (PID) due to the sexually transmitted diseases (STDs) (16). As far as infertile patients are concerned, the prevalence varies greatly depending on the utilized biopsy method and investigated population. In a prospective study published by Cicinelli et al. (17) in a total number of 2190 diagnostic hysteroscopy with different indications, they found a prevalence of 20% (438 patients) with CE, among whom 37% were also infertile. However, Kasius et al. (18) reported a prevalence of only 2.8%, in a total of 678 women.

CE can be due to the presence of foreign bodies or structural pathology of the endometrial cavity, such as the presence of intrauterine device (IUD), submucous myomas, polyps, retained products of conception, incomplete abortion or infectious agents. The most frequent infectious agents are common bacteria frequently found in the urogenital area such as *Streptococcus* (27%), *E. coli* (11%), *Enterococcus faecalis* (14%) and *Ureaplasma urealyticum* (11%) (19). The presence of *Chlamydia trachomatis* is only 2.7%, and *Neisseria gonorrhoeae* is practically undetectable as causative in CE (20). These findings are in line with the results of the PEACH study (21), showing that 60% of women with PID present non-gonococcal or *Chlamydia* infection.

In certain areas of the world, *Mycobacterium tuberculosis* is highly prevalent. It is considered as the main cause of infertility in 40-75% of cases, since it causes implantation failure due to alteration of the immune response at the endometrial level, hormonal alterations and release of antiphospholipid antibodies (22). Today, it is well accepted that the uterus is not a sterile cavity, and that presence of the microorganisms is not equal to inflammation (23).

Asymptomatic presence of bacteria in the endometrial cavity in either transcervical samples, or cultures obtained in post-hysterectomy specimens, has been reported by several investigators (24, 25). More recently metagenomics, investigating hypervariable regions of the ribosomal 16S rRNA genes allow definition of genus order and species of bacteria, leading to confirm presence of up to 12 different bacterial types in up to 95% of endometrial biopsies performed in patients undergoing hysterectomy for non-cancer indications (26). As described by Espinoza et al. (27), accumulating evidences suggest that endometrium is continuously exposed to bacteria from the genital tract. Therefore, presence of pathology is also determined by interaction of the infectious agent with the endometrial microenvironment (28).

Considering that the published data has not yet been able to draw a firm conclusion in this regard, in this paper we aim to review the current pieces of evidence regarding diagnosis, impact on reproductive outcomes and management of CE.

Pathophysiology of the endometrial microenvironment, microbial and immune cross-talk

In the normal endometrium, B lymphocytes are only lo-

cated at the basal layer, representing less than 1% of the leukocyte population. Conversely, in CE a large population of B cells lymphocytes are present at the both basal layer of the endometrium and glandular epithelium, as well as in the lumen of endometrial glands (29). Recent data suggest that a lipopolysaccharide derived from *E. coli* is capable of inducing the in vitro expression of E-selectin, as an adhesin that promotes passage of B cells to the endothelium of endometrial microvascularization (30). In addition, E-selectin promotes expression of chemoattractant CXCL13, activating adhesion molecules of B cells and expression of CXCL1 at the glandular endometrium level. In this microenvironment, gram-negative bacteria within the endometrium induce an abnormal immune response with migration of circulating B lymphocytes to the endometrial stromal compartment (8). At the endometrial level, plasma cells of the stroma express multiple immunoglobulins (IgM, IgA1, IgA1, IgG1 and IgG2), while excess of these antibodies could negatively affect implantation of the embryo (31).

In a study performed by Di Pietro et al. (32), expression of the 25 genes encoding the proteins involved in inflammation, proliferation and apoptosis at endometrial was compared by real-time polymerase chain reaction (RT-PCR) during the implantation time window in 16 women with hysteroscopic and histological diagnosis of CE and 10 healthy women without endometritis; the results of this study suggested that endometrial expression of some genes is significantly altered. In particular, they found up-regulated gene expression of insulin-like growth factor binding protein 1 (IGFBP1), B-cell CLL/Lymphoma 2 (BCL2) and BCL2-associated X protein (BAX), while down-regulated gene expression of IL11, Chemokine (C-C MOTIF) Ligand 4 (CCL4), insulin-like growth factor 1 (IGF1) and caspase 8 (CASP8). These altered gene expressions could affect, at least in part, the embryonic implantation and they also explained presence of endometrial hyperplastic lesions.

In CE, stromal cells secrete IGFBP1 protein during the decidualization process, exerting a negative effect on the implantation process and counteracting effect of IGF2. Thus, an increase of IGFBP1 expression and reduction of IGF1 expression in CE may lead to unfavorable conditions for implantation and embryonic development (33).

In this scenario, CE can alter the production of cytokines, impair endometrial function and induce an abnormal pattern of the leukocyte population at the endometrial level, leading to altered secretion of paracrine factors involved in endometrial receptivity. As reported elsewhere, decrease in IL11 production by epithelial and stromal cells may lead to dysregulation of trophoblastic invasion, associating with infertility. Similarly, lower CCL4 activity in CE may lead to a decreased recruitment of NKs and macrophages, accounting for the observed implantation failure (34). In addition, downregulation of BCL2 and CASP8 (35), associated with upregulation of BAX (36), causes endometrial cell resistance to apoptosis and dis-

turb the correct process of implantation (35).

Diagnosis of chronic endometritis: current management and potential pitfalls

Diagnosis of CE represents a challenge for the gynecologist. The clinical manifestations of CE such as pelvic pain, vaginal discharge, dyspareunia and abnormal vaginal bleeding are non-specific, while about 25% of patients with CE are asymptomatic (37). Moreover, the peripheral blood inflammation markers, such as C-reactive protein (CRP), leukocytosis, leptin and IL6 do not predict presence of CE (8).

Histopathology of chronic endometritis

The accepted gold standard for diagnosis of CE is presence of the plasma cells in endometrial tissue. However, their histological identification is sometimes hampered by the presence of mononuclear cell infiltration, mitosis and proliferation of stromal cells, plasmacytoid appearance of stromal cells (fibroblasts and mononuclear cells) or decidual transformation of the endometrium during late secretory phase. Plasma cells are characterized by the presence of chromatin in the form of a clock face inside an eccentric nucleus with perinuclear halo (Fig.1) (15).

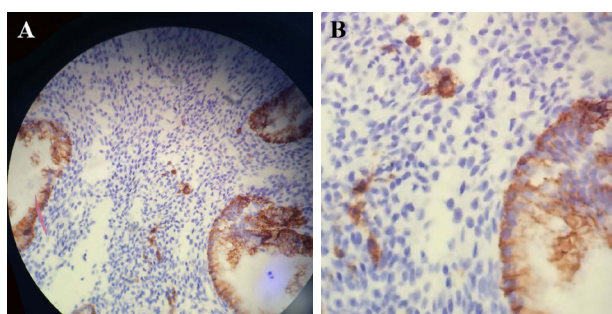


Fig.1: Immunohistochemistry of chronic endometritis. **A.** Fragment of endometrial biopsy specimen showing glandular cell surface syndecan 1 immunoreactivity. Plasma cells are highlighted by brown staining in the center of the picture (original magnification: $\times 400$) and **B.** More detailed picture of plasma cell syndecan 1 immunoreactivity.

Syndecan 1 is a proteoglycan of the transmembrane heparan sulfate type presenting on the surface of plasma cells and keratinocytes, while it is not expressed in mononuclear cells, lymphocytes or endometrial stromal cells. It is also known as CD138, facilitating detection of plasma cells and presence of CE, not affected by intra- and inter-observer variability (38).

It is recommended to include clinical immunohistochemistry and conventional pathology study to increase the accuracy of the CE diagnosis (15). Furthermore, it is important to obtain standardization of the current diagnostic techniques, considering that depending on the dilution of Syndecan 1, diagnosis of CE might differ. For a dilution of 1:1000, a prevalence of 2.8% was initially reported for CE in asymptomatic infertile women prior to *in vitro* fertilization (IVF) (39), which does not overlap with the prevalence of 30.3%, previously reported by Johnston-MacAnanny et al. (40), as well as the prevalence of 10% in the patients

with recurrent miscarriage (41). In addition, the menstrual cycle phase whereby the biopsy is performed and thickness of the biopsy have paramount importance: in particular, in 15% of the samples during secretory phase, plasma cells are present only in the basal layer of the stroma, which will be missed if not included in the biopsy. Finally, it is important to define number of the plasma cells required to establish diagnosis of CE: although most authors believe that there must be two or more plasma cells, the others recommend presence of five or more plasma cells in at least one of the three sections of biopsy (40).

Hysteroscopic findings of chronic endometritis

Hysteroscopy is a useful diagnostic modality in CE. Usual hysteroscopic findings for characteristic CE include presence of local or diffuse hyperemia, edema of the stroma and presence of micropolyps (less than 1 mm in size, Fig.2) (42).

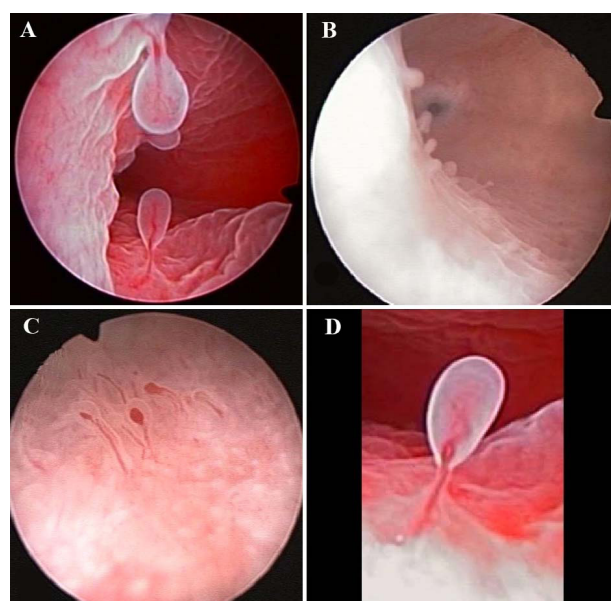


Fig.2: Different findings of chronic endometritis at the fluid hysteroscopy. **A.** Endometrial surface is completely covered by micropolyps, **B.** Isolated micropolyps on the lateral wall of the cavity, **C.** Endometrial mucosa appears thick, edematous, diffuse hyperemic, with presence of micropolyps, and **D.** Detailed image of an endometrial micropolymp appearance.

Cicinelli et al. (42, 43) reported that presence of endometrial micropolyps at hysteroscopy suggests the existence of CE. Interestingly, they obtained a positive diagnostic correlation of 93.4% with the pathology findings, following their criteria of hysteroscopic diagnosis. These findings have been replicated by others (44) with 86.5% correlation of hysteroscopic with histological diagnosis.

Chronic endometritis and reproductive outcomes

The implantation consists of a physiological process involving mediators of inflammation such as leukocytes, cytokines, chemokines and other endometrial factors. All these cells and their mediators play an essential role in the regulation of immunoresponse and growth of the trophoblast. The presence of CE can alter receptivity of the en-

ometrium creating an inadequate microenvironment that interferes with normal implantation. In particular, recent data (8) suggests that the endometrium of one third of infertile patients, presenting with CE, expresses high level of estrogen receptor, progesterone and Ki-67 nuclear marker of cell proliferation in both epithelial cells and stroma, in addition to the increased expression of anti-apoptosis genes such as BCL2 and BAX, all of which represent a proliferative phenotypic change of the endometrium even in the secretory phase. This increase in expression levels of estrogen and progesterone receptors was replicated by Wu et al. (33), suggesting that CE modifies stromal cells by altering the function of these hormonal receptors.

CE also modifies the pattern of uterine contractility in both of the periovulatory and mid-luteal phases of menstrual cycle (45). Physiologically, in the proliferative phase, there is anterograde contractility from the fundus to the cervix which facilitates removal of menstrual debris, followed by periovulatory and the luteal phase when there is predominance of retrograde contraction in the opposite direction, from the cervix to fundus, which favors migration of the spermatozoa to the fallopian tubes. Conversely, during CE, there is 3.3 times lower occurrence of retrograde contractility of the fallopian tubes (46). This "altered peristalsis" induced by the presence of CE could impair, at least in part, fertility and contribute to some of the symptoms such as pelvic pain and dysmenorrhea.

Implantation failure after *in vitro* fertilization and chronic endometritis

Impact of the CE presence in implantation is controversial, although many studies suggest a negative impact on the endometrial receptivity of plasma cells as well as IgM, IgG and IgA alterations in genes encoding proteins involved in the inflammatory response, proliferation and apoptosis.

Bouet et al. (47) reported a prospective observational study including 46 women with recurrent implantation failure (RIF), defined as failure to achieve pregnancy after transferring three good quality embryos in fresh or frozen cycle in women up to 35 years of age, or 4 embryos of good quality in women over 35 years. In this study, the authors excluded women with uterine cavity anomalies, presence of submucous myomas or endometrial polyp of more than 5 mm, as well as the patient who were treated with antibiotics within one month prior to biopsy or those who had unexplained vaginal bleeding. Hysteroscopy with endometrial biopsy was performed between days 6 and 12 of the cycle. Diagnosis of CE was confirmed by 1:100 immunohistochemical dilution, while they were considered positive with the presence of 5 or more plasma cells in 10 high power fields ($\times 400$). They found 14% prevalence of CE, with 80% correlation between hysteroscopic criteria and histological confirmation. Using a slightly different methodology, others (40) investigated retrospectively 33 women, defining implantation failure as the failure to achieve pregnancy after two cycles of IVF with transfer of at least one good-quality embryo. They

performed endometrial biopsy and immunohistochemical study dilution of 1:100, considering it negative with the presence of less than one plasma cell, reporting a prevalence of 30.3% CE. In a larger cohort analysis, Cicinelli et al. (48) included the patients who were younger than 40 years, normal responders at ovarian stimulation and normal karyotype, defining RIF after embryo transfer of at least six good quality embryos in three or more previous IVF/intracytoplasmic sperm injection (IVF/ICSI) cycles. Patients with follicle stimulating hormone (FSH) >10 on day 3, body mass index (BMI) >30, endometriosis, history of abortion, steroid use, autoimmune disease, antiphospholipid syndrome, thrombophilia or presence of anti-spermatozoid antibodies were excluded from the study. They performed hysteroscopy and biopsy in the follicular phase of the following cycle, obtaining an endometrial biopsy and cultured these cells. According to their data analysis, CE was diagnosed by hysteroscopy in 66% of the cases, by histology in 57.5%, and by positive culture in 45% of the cases. Higher rate of positive diagnosis could be explained probably by the experience of pathologist and hysteroscopist, regarding the diagnostic criteria of CE (40) and a selection bias since the authors' Institution is a referral center for women with suspected CE. The final concordance between hysteroscopic and histologic diagnosis of CE was 87%. Noteworthy to say that women who were included in both studies, performed by Johnston-MacAnanny et al. (40) and Cicinelli et al. (48), were treated with antibiotic. Patients included in the former study (40) were treated with 100 mg Doxycycline for two weeks, followed by Ciprofloxacin and Metronidazole 500 mg (twice daily) for two weeks in those with positive cultures. Those included in the latter study (48) were treated with Ciprofloxacin 500 mg (twice daily) for 10 days against gram negative bacteria and Amoxicillin-Clavulanic acid 1 g (twice daily) for 8 days against gram positive bacteria. If the cultures persisted positive, then the antibiotic protocol was repeated up to three times and if the cultures were negative, the patient would receive intramuscular single dose of Ceftriaxone 250 mg, followed by Doxycycline 100 mg (twice daily) and Metronidazole 500 mg (twice daily) for 14 days.

Regarding reproductive outcomes, Cicinelli et al. (48) found a live born rate of 61% in patients responding to antibiotics, whereas the live born rate was only 13% in patients who did not respond to antibiotic therapy. Conversely, in the study performed by Johnston-MacAnanny et al. (40), patients of the CE group improved pregnancy rate after good response to therapy, although the CE group had still lower pregnancy rate than non-CE group, despite a good response to treatment with antibiotics. These different results may probably be due to the other unrecognized endometrial abnormalities, which are not solved with antibiotic therapy.

Overall, both studies suggest that CE has a negative impact on endometrial receptivity, and adequate response to antibiotic therapy may significantly improve reproductive outcomes, as it was confirmed in a recent systematic

review and meta-analysis (49). Nevertheless, diagnostic hysteroscopy itself and endometrial biopsy may also play a positive role. In one hand, hysteroscopy could physically remove bacterial biofilms involved in the pathophysiology of CE; on the other hand, endometrial biopsy and the subsequent recovery process can promote secretion of cytokines and growth factors in the endometrium involved in embryo implantation.

Recurrent pregnancy loss and chronic endometritis

According to European Society of Human Reproduction and Embryology (ESHRE), recurrent pregnancy loss (RPL) is defined as the loss of two or more pregnancy, even not consecutive, occurring before 20 weeks of gestation, which is in agreement with the definition of the American Association of Reproductive Medicine (ASRM) guidelines (50). In patients with implantation failure, the aberrant endometrial microenvironment resulting from an anomalous pattern in the CE lymphocyte population has been linked to RPL. Kitaya et al. (41) reported a total of 58 women with RPL (three or more abortions), detecting presence of CE by immunohistochemistry in 9.3% of the patients. Using the same experiment, others (51) reported a prevalence of 42.9% CE on a total of 142 women with three or more abortions. McQueen et al. (52) studied 395 women with two or more abortions by week 10 or at least one pregnancy loss of more than 10 weeks, finding 9% CE prevalence diagnosed by endometrial biopsy. In the latter study, the patients were then treated with antibiotics: after the first course, there was adequate response in 94% of the cases, rising to 100% after administration of two courses of antibiotics. They reported an increase of live birth rate from 7% before treatment to 56% after receiving antibiotic treatment for two weeks.

Cicinelli et al. (53) performed a retrospective study of 360 women under the age of 40 with three or more abortions before 20 weeks gestation, excluding patients with severe male factor, endometriosis, uterine anomalies, metabolic or hormonal alterations, antiphospholipid syndrome and thrombophilia. Hysteroscopy was performed in the follicular phase. Patients with hysteroscopic diagnosis of CE had endometrial biopsy in the following cycle. They found 57.8% of patient with hysteroscopic sign of CE, out of which 91.3% were confirmed by histology and 68% had positive cell cultures. Confirming previous reports, after antibiotic treatment they found that live birth rate in women responding to antibiotic treatment was higher, compared to non-responder women, suggesting that presence of the infectious agents in the uterine cavity has a potential deleterious impact on the endometrial environment.

Similarly, a more recent case-control observational study (54) was performed in 107 women with two or more abortions before 20 weeks gestation, after ruling out other causes of pregnancy loss. In this study, investigators performed endometrial biopsy analyzed with hematoxylin eosin and CD138, defining CE as the presence of 1-5

plasma cells at immunohistochemistry test. Using these criteria, the prevalence of CE varied from 13% to 56% upon completion of an immunohistochemical study. They also found a trend towards a higher rate of pregnancy loss in women with untreated CE compared to patients without CE. Finally, Bouet et al. (47) published a prospective observational study, including 53 women with two or more unexplained pregnancy loss in pregnancies less than 14 weeks gestation. They performed hysteroscopy and endometrial biopsy, using syndecan 1, as a biomarker, considering that is positive with the presence of five or more plasma cells in 10 high power fields. They found a prevalence of 27% CE.

Conclusion

CE is associated with poor reproductive outcomes, including implantation failure and RPL. Accumulating evidences suggest that this condition modifies endometrial microenvironment at different levels: first of all, CE promotes changes on immunocompetent cell population in the endometrium. It also affects production of inflammatory cytokines, involved in NKs recruitment, which play a crucial role in local immune response during early pregnancy and favor implantation. In addition, CE has negative impact on normal endometrial decidualization, promoting proliferation, diminishing apoptosis and modifying the expression of sex steroid receptors, which affect endometrial receptivity.

Hysteroscopy, in expert hands, could be considered a good tool to combine with histology for diagnosis of CE. Nevertheless, a consensus about strict criteria is mandatory for diagnosis to combine immunohistochemistry with conventional histology. Finally, future investigation should be aimed to redefine the minimum volume of biopsy and the number of plasma cells needed for diagnosis.

There is still lack of the uniform definition of RPL. Obtaining that would allow more accurate analysis and comparison among different studies. Considering this scenario, part of conflicting data found by different authors can be due to this element.

Antibiotic treatment of CE improves implantation rates and decreases the rate of abortion, although there is a lack of well-designed prospective studies that corroborate this finding.

The metagenomics and a better understanding of the microbioma of the reproductive tract will allow researchers to develop therapies aimed to not only eliminate pathogenic flora but also establish a flora which favors reproductive success.

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Authors' Contributions

L.A., A.S.L.; Screened literature and selected relevant data included in the review. E.P.; Wrote the manuscript. F.G., J.Cas.; Edited the manuscript in terms of intellectual content. J.Car.; Supervised the review development and gave approval for the final version of the manuscript.

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Medical and Surgical Treatment of Reproductive Outcomes in Polycystic Ovary Syndrome: An Overview of Systematic Reviews

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Abstract

Polycystic ovary syndrome (PCOS) is a common, complex condition that affects up to 18% of reproductive-aged women, causing reproductive, metabolic and psychological dysfunctions. We performed an overview and appraisal of methodological quality of systematic reviews that assessed medical and surgical treatments for reproductive outcomes in women with PCOS. Databases (MEDLINE, EMBASE, CINAHL PLUS and PROSPERO) were searched on the 15th of September 2017. We included any systematic review that assessed the effect of medical or surgical management of PCOS on reproductive, pregnancy and neonatal outcomes. Eligibility assessment, data extraction and quality assessment by the Assessing the Methodological Quality of Systematic Reviews (AMSTAR) tool were performed in duplicate. We identified 53 reviews comprising 44 reviews included in this overview; the majority were moderate to high quality. In unselected women with PCOS, letrozole was associated with a higher live birth rate than clomiphene citrate (CC), while CC was better than metformin or placebo. In women with CC-resistant PCOS, gonadotrophins were associated with a higher live birth rate than CC plus metformin, which was better than laparoscopic ovarian drilling (LOD). LOD was associated with lower multiple pregnancy rates than other medical treatments. In women with PCOS undergoing *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI), the addition of metformin to gonadotrophins resulted in less ovarian hyperstimulation syndrome (OHSS), and higher pregnancy and live birth rates than gonadotrophins alone. Gonadotrophin releasing hormone (GnRH) antagonist was associated with less OHSS, gonadotrophin units and shorter stimulation length than GnRH agonist. Letrozole appears to be a good first line treatment and gonadotrophins, as a second line treatment, for anovulatory women with PCOS. LOD results in lower multiple pregnancy rates. However, due to the heterogeneous nature of the included populations of women with PCOS, further larger scale trials are needed with more precise assessment of treatments according to heterogeneous variants of PCOS.

Keywords: Infertility, Polycystic Ovary Syndrome, Review, Therapeutics, Treatment Outcome

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most important dilemmas in reproductive medicine. PCOS is a member of the World Health Organization group II ovulation disorders, and has a 9-18% prevalence among reproductive-aged women (1) and nearly 80% among

infertile anovulatory women (1, 2). There is an ongoing debate related to its definition, aetiology, diagnosis and treatment for its clinical phenotypes (3). Since first described by Stein and Leventhal (4), a number of reports and meetings have suggested diagnostic criteria for this condition (3, 5, 6). However, the criteria reported by ESHRE/ASRM in Rotterdam in 2003 are most com-

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monly used both in research and clinical care. These criteria propose that two out of three domains should be present to establish a diagnosis of PCOS. These domains are: an-/oligo-ovulation, hyperandrogenism (clinical $\pm$ biochemical) and polycystic ovary morphology on ultrasound examination, with exclusion of other causes of hyperandrogenism (6). In 2012, the National Institute of Health reinforced the need for identification of four phenotypes within the Rotterdam criteria in women with PCOS, which refer to the combination of diagnostic criteria (7). By using the possible combinations of these criteria, four different phenotypes of PCOS are now identified: i. Hyperandrogenism (clinical or biochemical) and chronic anovulation (H-CA), ii. Hyperandrogenism and polycystic ovaries on ultrasound (PCOm), but with ovulatory cycles (H-PCOm), iii. Chronic anovulation and polycystic ovaries without hyperandrogenism (CA-PCOm), and iv. Hyperandrogenism, chronic anovulation and polycystic ovaries (H-CA-PCOm). The identification of specific phenotypes in women with PCOS seems to be justified from the metabolic point (3).

This heterogeneous condition manifests with many clinical presentations, including infertility, pregnancy complications, and psychological and metabolic features. The reproductive problems associated with PCOS consist mainly of menstrual dysfunction, infertility and pregnancy complications. Many treatments are proposed by different guidelines for infertility with PCOS, and include clomiphene citrate (CC), letrozole and gonadotrophins. However, there is a lack of clarity around the relative efficacy of these different treatments. Despite the agreement between most guidelines of the importance and priority of lifestyle modification in PCOS and weight loss, where women are overweight or obese, there are still limited studies that compare lifestyle modification and pharmacological drugs for reproductive outcomes (8). With regards to pharmacological treatment in isolation, CC is recommended as first-line treatment for ovulation induction (OI) in infertile women with PCOS with the alternative treatment, letrozole, which has encouraging results in many recent trials (1, 2, 8-10). Although the insulin sensitizer metformin has been recently recommended as a first-line treatment (11), its role and specific indication are controversial (1-3). The second-line treatment is usually recommended as gonadotrophins or laparoscopic ovarian drilling (LOD) (2). Additional issues relating to treatment of reproductive outcomes which are still somewhat controversial include the best time to use *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) in women who failed to become pregnant after pharmacological treatment, and the potential benefit of modern techniques like *in vitro* maturation (IVM) (2, 3).

The aim of this review was to perform an overview to summarize and appraise the content, results and quality of systematic reviews that assess medical or surgical treat-

ments for reproductive outcomes in women with PCOS.

Materials and Methods

Inclusion criteria

The Participant, Intervention, Comparison, Outcomes and Studies (PICOS) framework was used for this review. This overview is part of a larger overview of systematic reviews. For this broader overview, we included any systematic review or meta-analysis where the assessment or management of PCOS was the primary focus of the review, either as interventions in PCOS or a comparison of women with and without PCOS for a specific outcome. Exclusion criteria were studies where PCOS was a secondary condition assessed as part of a broader topic. For this specific overview, we included any systematic review that assessed the effect of medical or surgical management of PCOS on reproductive outcomes. The specific inclusion criteria were: i. Published from 2009 onwards, as this was the date of publication of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement as a guideline for conducting systematic reviews (12), ii. Must have included a search strategy that contained at least key words or terms, iii. Must include the number of identified and included articles, and iv. The review needed to conduct some form of quality appraisal of the articles. The comparisons term was not applicable in this review context. The outcomes assessed were reproductive outcomes, specifically live birth, clinical pregnancy, miscarriage, ovulation, multiple pregnancy, menstrual cycle frequency, follicular size, pregnancy related outcomes (gestational diabetes, pregnancy-induced hypertension and pre-eclampsia), neonatal outcomes, costs and side effects. Only articles published in English were included. The protocol is registered in the International Prospective Register of Systematic Reviews PROSPERO (CRD42016052649).

Article selection

A comprehensive database search was conducted on the 17th of October 2016, which was last updated on 15th September 2017. The following electronic databases were used to identify relevant published literature: Medline in-process and other non-indexed citations [Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily and Ovid MEDLINE(R) 1946 to Present]; EMBASE (EBM Reviews - Cochrane Database of Systematic Reviews 2005 to September 15, 2017, EBM Reviews - ACP Journal Club 1991 to September 2017, EBM Reviews - Database of Abstracts of Reviews of Effects 1st Quarter 2016, EBM Reviews - Cochrane Central Register of Controlled Trials September 2017, EBM Reviews - Cochrane Methodology Register 3rd Quarter 2012, EBM Reviews - Health Technology Assessment 4th Quarter 2016, EBM Reviews - NHS Economic Evaluation Database 1st Quarter 2016); and CINAHL PLUS. The search strategy is documented in Supplementary Appendix 1 (See Supplementary On-

line Information at www.celljournal.org). This search was modified for EMBASE and CINAHL using their subject headings instead of the MeSH subject headings. The International Prospective Register of Systematic Reviews PROSPERO (<http://www.crd.york.ac.uk/PROSPERO/>) was additionally searched on the 15th September 2017 using the key words “PCOS” or “polycystic ovary syndrome”. In addition, experts in the field were asked to provide any potentially relevant studies for consideration. Two independent reviewers, who were not blinded to the names of investigators or sources of publication, identified and selected the articles that met the inclusion criteria (L.J.M, D.H or C.T.T). Disagreements between reviewers were discussed and resolved by consensus or arbitration with a third reviewer.

Data extraction

All eligible systematic reviews included were examined and extracted independently by two reviewers (L.J.M, M.G or C.T.T). Disagreements were discussed and resolved by consensus or arbitration with a third reviewer. The data extracted included information on author(s), year, country of author, inclusion criteria, study methodology, study outcomes, number of studies identified, number of participants in the review, whether a meta-analysis was conducted, and quality of identified articles in each review (as reported by the systematic review authors as overall quality of the entire study or evidence or reported as unclear if not summarized by the systematic review authors).

Data synthesis

A narrative description of the included reviews was performed. We presented results per reproductive outcome.

Quality assessment of systematic reviews

All included reviews were evaluated by two independent reviewers (L.J.M, M.G or C.T.T) using the Assessing the Methodological Quality of Systematic Reviews (AMSTAR) tool (13, 14). Disagreements were discussed and resolved by consensus or arbitration with a third reviewer. The AMSTAR tool contains 11 items to appraise the methodological aspects of the systematic reviews. Each item was scored 1 for “yes” and 0 for “no” or “not applicable” with a total score range from 0 to 11. The methodological quality for each review was classified as low [≤ 3], moderate [4-7] and high [8-11] (15).

Results

Characteristics of included reviews

The search yielded 978 citations, with 60 citations identified from PROSPERO and one citation identified from expert assessors, for a total of 1039 citations. There were 831 citations that remained after removal of duplicates. Based on a priori selection criteria, screening for title and abstract identified 276 articles for assessment of the full

text. Of these, 128 articles were excluded for the following: not conducting quality assessment, not in English, no search terms detailed or no identified search strategy (Supplementary Appendix 2) (See Supplementary Online Information at www.celljournal.org). We included 139 full-text articles for our final analysis, of which 53 articles were related to the theme of medical or surgical treatment on reproductive outcomes in PCOS, with the remaining eligible articles assessed in separate overviews of systematic reviews and excluded from this specific review. These 53 articles comprised 44 reviews (Fig.1).

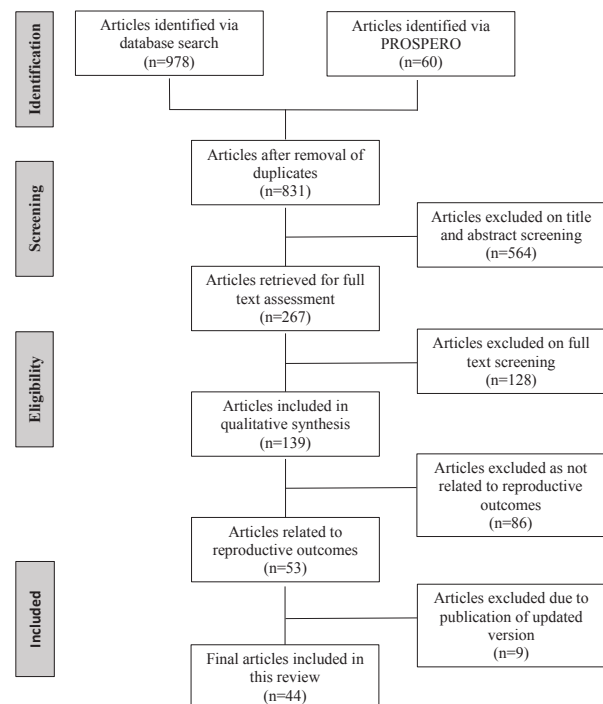


Fig.1: Study selection.

The characteristics of these reviews are summarized in Supplementary Appendix 3 (See Supplementary Online Information at www.celljournal.org). The number of included studies in each review ranged between none (16, 17) and 66 (18). The type of included studies in each review was only randomised controlled trials (RCTs) in 22 reviews (16, 18-38), RCTs and crossover trials until first inclusion in 11 reviews (17, 33, 39-47), RCTs and systematic reviews of RCTs in two reviews (48, 49), any study design in two reviews (50, 51), any study with control group in three reviews (52-54), RCTs and prospective studies in one review (55) and not stated in three reviews (56-58). Participants in the included reviews were treatment-naïve women in two reviews (27, 28), women resistant to CC in six reviews (19, 23, 29, 32, 33, 56), women whose treatment status was undefined in 32 reviews (16-18, 20-22, 24-26, 30, 31, 35-42, 44-51, 55, 57, 59), pregnant women with PCOS in four reviews (52-54, 58), adolescents with PCOS (11-19 years old) in one review (34), and women with PCOS not trying to conceive in one review (43). Twenty-two reviews were conducted according to prior guidelines for conducting systematic

reviews such as PRISMA, Meta-analyses Of Observational Studies in Epidemiology (MOOSE), Quality of Reporting of Meta-analyses (QUORUMS) or Cochrane (16, 17, 19, 23, 25, 27, 29, 32, 34, 36, 39-46, 50, 51, 58, 59). Meta-analyses were performed in 39 reviews (18-32, 34-43, 45-50, 52-59). The systematic reviews did not apply language restrictions in 28 reviews (16, 17, 19, 20, 24-26, 28, 29, 34, 36, 38-47, 50, 53-56, 58, 59), restricted the search to articles in English in 12 reviews (18, 21, 22, 27, 32, 33, 35, 37, 48, 49, 52, 57), restricted the search to articles in English and Chinese in two reviews (30, 31) and did not state if language restrictions were applied in two reviews (23, 51). The quality of included studies in each review was not reported by authors or was not able to be easily interpreted in 31 reviews (16, 17, 20-29, 32, 36, 38, 39, 42, 43, 45-47, 50-59), low or insufficient in eight reviews (18, 31, 34, 35, 37, 40, 41, 44), low to moderate in two reviews (19, 48) and low to high in three reviews (30, 33, 49).

Quality of included reviews

The quality of the included reviews are presented in Supplementary Appendix 4 (See Supplementary Online Information at www.celljournal.org). Seven reviews were of low quality (28, 30, 33, 36, 51, 52, 58), 22 reviews were of moderate quality (16, 20-27, 31, 32, 35, 37, 38, 40, 50, 53-57, 59) and 15 reviews were of high quality (17-19, 29, 34, 39, 41-49). Twenty reviews had pre-specified their clinical question and inclusion criteria (16-19, 29, 33, 34, 39-49, 55, 59). Nineteen reviews conducted study selection and data extraction in duplicate (17-19, 21, 23, 26, 27, 29, 32, 34, 37, 39, 42-45, 50, 55, 57). Twenty-eight reviews conducted a comprehensive literature search (16-19, 21, 24-26, 28-31, 34, 38-49, 53, 54, 59). Twenty reviews included grey literature searches (16, 17, 19, 25, 26, 29, 34, 38-47, 53, 54, 59). Twenty-four reviews listed included and excluded studies (16, 17, 19, 23-27, 29, 32, 34, 38, 39, 41-46, 48-50, 57, 59). Forty reviews described the

characteristics of the included studies (18-29, 32-59). Thirty-eight reviews assessed study quality (16-27, 29-35, 37-50, 54, 56, 57, 59, 60). Nineteen reviews used the scientific quality of their included studies in formulating results (18, 20-22, 24, 25, 29, 31, 32, 34, 35, 39, 40, 45-49, 57). Thirty-seven reviews combined the studies using appropriate methods (18-32, 35-43, 45, 46, 48-50, 52-59). Twenty-two reviews addressed the risk of reporting bias, and used a statistical test where appropriate (16-19, 32, 34, 35, 37-39, 41-44, 46, 47, 50, 52, 53, 55, 56, 58). Seven reviews addressed the potential for conflict of interest (16, 17, 29, 43, 47-49).

Types of interventions

Letrozole

Six reviews (three high quality (19, 39, 49) and three moderate quality (20, 27, 32) assessed interventions that contained letrozole, comprising a total of 89 trials and 14 008 participants. Of these, five assessed letrozole ± other OI drugs versus OI drugs, including letrozole alone (20, 27, 32, 39, 49) and one assessed letrozole versus LOD (19). The populations studied were women with PCOS who were treatment-naïve (27), CC resistant (32), or treatment-naïve ± CC resistant or unknown treatment status (20, 39, 49).

The meta-analyses reported statistically significant results for higher live birth, pregnancy and ovulation after letrozole compared to CC followed by timed intercourse in overall women with PCOS, and higher live birth and pregnancy after letrozole in women with PCOS and body mass index (BMI) >25 kg/m² (20, 27, 39, 49). In women with CC resistance, letrozole with or without metformin resulted in higher live births compared to CC with metformin (32, 39), letrozole resulted in higher pregnancy and ovulation than anastrozole and higher ovulation than LOD (49). Long-term letrozole (10 days) resulted in higher pregnancy than short-term letrozole (5 days) (Tables 1, 2) (49).

Table 1: Results of main medical interventions

Review	Population	Outcomes assessed	Comparison	Outcomes with significant results	
Letrozole					
Abu Hashim et al. (32), 2015	CC resistant PCOS	Live birth Pregnancy Ovulation Miscarriage Multiple pregnancy OHSS	CC+metformin vs. Letrozole	Live birth/woman	OR: 0.21, 95% CI: 0.05 to 0.87
Franik et al. (39), 2014	PCOS, reproductive age	Live birth Pregnancy Miscarriage Multiple pregnancy OHSS	Letrozole vs. CC (BMI >25 kg/m ²)	Live birth/woman	OR: 1.67, 95% CI: 1.31 to 2.11
			Letrozole vs. CC (with or without adjuncts followed by timed intercourse)	Live birth/woman	OR: 1.64, 95% CI: 1.32 to 2.04
			Letrozole vs. CC (with or without adjuncts followed by IUI)	Pregnancy/woman	OR: 1.71, 95% CI: 1.30 to 2.25
			Letrozole vs. CC (overall with or without adjuncts followed by timed intercourse)	Pregnancy/woman	OR: 1.40, 95% CI: 1.18 to 1.65
			Letrozole vs. CC+rFSH and rFSH only	Pregnancy/woman	OR: 1.66, 95% CI: 1.23 to 2.22
			Letrozole+metformin vs. CC+metformin	Live birth/woman	OR: 4.5, 95% CI: 1.09 to 18.50

Table 1: Continued

He and Jiang (20), 2011	PCOS	Pregnancy Ovulation Miscarriage Multiple pregnancy OHSS Mature follicles	Letrozole vs. CC Letrozole vs. CC	Mature follicles/cycle Ovulation/cycle	SMD: 1.41, 95% CI: 1.54 to 1.28 RR: 1.29, 95% CI: 1.12 to 1.49
Misso et al. (49), 2012	PCOS	Live birth Pregnancy Ovulation Miscarriage Multiple pregnancies Adverse events Cost effectiveness	Letrozole long-term (10 days) vs. Letrozole short-term (5 days) Letrozole vs. Anastrozole Letrozole vs. Anastrozole Letrozole vs. CC Letrozole vs. LOD	Pregnancy/cycle Ovulation/cycle Pregnancy/woman Ovulation/woman Ovulation/cycle	Higher in long-term (10 days) Higher in letrozole Higher in letrozole OR: 2.90, 95% CI: 1.72 to 4.88 Higher in letrozole
Roque et al. (27), 2015	PCOS (therapy naïve)	Live birth Clinical pregnancy Ovulation Miscarriage Multiple pregnancy	Letrozole vs. CC Letrozole vs. CC	Live birth/woman Pregnancy/woman	RR: 1.55, 95% CI: 1.26 to 1.90 RR: 1.38, 95% CI: 1.05 to 1.83
CC					
Abu Hashim et al. (32), 2015	CC resistant PCOS	Live birth Pregnancy Ovulation Miscarriage Multiple pregnancy OHSS	CC+metformin vs. Gonadotrophins CC+metformin vs. Gonadotrophins CC+metformin vs. CC + NAC CC+metformin vs. Gonadotrophins CC+metformin vs. CC + NAC	Live birth/woman Ovulation/woman Ovulation/woman Pregnancy/woman Pregnancy/woman	OR: 0.33, 95% CI: 0.13 to 0.85 OR: 0.25, 95% CI: 0.15 to 0.41 OR: 8.93, 95% CI: 4.61 to 17.32 OR: 0.45, 95% CI: 0.27 to 0.75 OR: 5.28, 95% CI: 1.91 to 14.62
Brown and Farquhar (46), 2017	WHO group 2 anovulation	Live birth Pregnancy Ovulation Miscarriage Multiple pregnancy OHSS Adverse effects	CC vs. Placebo CC vs. Gonadotrophins CC vs. Gonadotrophins CC 5 day vs. CC 10 day CC 5 day vs. CC 10 day CC+DEX vs. CC Early CC vs. late CC CC+OCP vs. CC	Pregnancy/woman Live birth/woman Pregnancy/woman Live birth/woman Pregnancy/woman Pregnancy/woman Pregnancy/woman	OR: 5.91, 95% CI: 1.77 to 19.68 OR: 0.64, 95% CI: 0.41 to 0.98 OR: 0.61, 95% CI: 0.40 to 0.93 OR: 0.10, 95% CI: 0.02 to 0.45 OR: 0.18, 95% CI: 0.06 to 0.55 OR: 6.2, 95% CI: 2.20 to 17.48 OR: 2.81, 95% CI: 1.02 to 7.75 OR: 27.18, 95% CI: 3.14 to 235.02
Ding et al. (36), 2016	PCOS	Pregnancy Ovulation Miscarriage Number of follicles	Late CC vs. Early CC	Mature follicles/cycle	MD: 1.82, 95% CI: 0.86 to 2.78
Farquhar et al. (19), 2012	CC resistant PCOS	Live birth Pregnancy Ovulation Miscarriage Multiple pregnancy OHSS Costs	LOD vs. CC+metformin LOD vs. CC+metformin	Live birth/woman Costs	OR: 0.44, 95% CI: 0.24 to 0.82 MD: 3711.3, 95% CI: 3585.17 to 3837.43
Gill et al. (33), 2014	CC resistant PCOS, reproductive age	Pregnancy Ovulation	CC+metformin vs. CC CC+metformin vs. CC	Ovulation/woman Pregnancy/woman	Higher in CC+metformin Higher in CC+metformin
Palomba et al. (24), 2009	PCOS	Live birth Pregnancy Ovulation Miscarriage Adverse events	Metformin vs. CC+metformin Metformin vs. CC+metformin Metformin vs. CC+metformin	Live birth/woman Ovulation/woman Pregnancy/woman	OR: 0.23, 95% CI: 0.13 to 0.40 OR: 0.23, 95% CI: 0.15 to 0.34 OR: 0.23, 95% CI: 0.14 to 0.37
Siebert et al. (28), 2012	PCOS (therapy naïve)	Live birth Pregnancy Ovulation	Metformin vs. CC Metformin vs. CC CC+metformin vs. CC CC+metformin vs. CC	Live birth/woman Ovulation/woman Ovulation/woman Pregnancy/woman	OR: 0.48, 95% CI: 0.31 to 0.73 OR: 0.48, 95% CI: 0.41 to 0.57 OR: 1.6, 95% CI: 1.2 to 2.1 OR: 1.3, 95% CI: 1.0 to 1.6
Tang et al. (41), 2012	PCOS	Live birth Pregnancy Ovulation Miscarriage Multiple pregnancy Menstrual frequency	Metformin vs. CC (BMI ≥ 30) Metformin vs. CC (BMI ≥ 30) CC+metformin vs. CC (CC resistant PCOS) CC+metformin vs. CC (BMI < 30) CC+metformin vs. CC (BMI ≥ 30) Metformin vs. CC (BMI ≥ 30) Metformin vs. CC (BMI < 30) CC+metformin vs. CC CC+metformin vs. CC CC+metformin vs. CC CC+metformin vs. CC	Live birth/woman Ovulation/woman Ovulation/woman Ovulation/woman Ovulation/woman Pregnancy/woman Pregnancy/woman Pregnancy/woman Side effects Side effects (GIT)	OR: 0.3, 95% CI: 0.17 to 0.52 OR: 0.43, 95% CI: 0.36 to 0.51 OR: 4.86, 95% CI: 2.43 to 9.74 OR: 1.75, 95% CI: 1.27 to 2.39 OR: 1.78, 95% CI: 1.51 to 2.1 OR: 0.34, 95% CI: 0.21 to 0.55 OR: 1.94, 95% CI: 1.19 to 3.16 OR: 1.51, 95% CI: 1.17 to 1.96 OR: 1.76, 95% CI: 1.26 to 2.47 OR: 3.31, 95% CI: 2.11 to 5.20 OR: 3.4, 95% CI: 2.08 to 5.54
Thakker et al. (47), 2015	PCOS	Live birth Ovulation Miscarriage, Multiple pregnancy OHSS Menstrual regularity	NAC vs. Placebo (CC resistant PCOS) NAC vs. Placebo (CC resistant PCOS) NAC vs. Placebo (CC resistant PCOS)	Live birth/woman Ovulation/woman Pregnancy/woman	OR: 3.0, 95% CI: 1.05 to 8.6 OR: 8.4, 95% CI: 4.5 to 15.67 OR: 4.83, 95% CI: 2.30 to 10.13

Table 1: Continued

Xiao et al. (30), 2012	PCOS, <35 years	Pregnancy Ovulation Miscarriage	Metformin vs. CC Metformin+CC vs. CC	Ovulation/woman Pregnancy/woman	OR: 0.48, 95% CI: 0.26 to 0.87 OR: 1.56, 95% CI: 1.16 to 2.08
Insulin sensitizers					
Tang et al. (41), 2012	PCOS	Live birth Clinical pregnancy Ovulation Miscarriage Multiple pregnancy Menstrual frequency	Metformin vs. Placebo Metformin vs. Placebo (BMI < 30) Metformin vs. Placebo (BMI ≥30) Metformin vs. Placebo (BMI<30) Metformin vs. Placebo Metformin vs. Placebo Metformin vs. Placebo	Side effects (GIT) Menstrual frequency Menstrual frequency Pregnancy/woman Menstrual frequency Ovulation/woman Pregnancy/woman	OR: 4.27, 95% CI: 2.4 to 7.59 OR: 21.15, 95% CI: 1.01 to 445.0 OR: 1.57, 95% CI: 1.03 to 2.41 OR: 2.35, 95% CI: 1.44 to 3.82 OR: 1.72, 95% CI: 1.14 to 2.61 OR: 1.81, 95% CI: 1.13 to 2.93 OR: 2.31, 95% CI: 1.52 to 3.51
Feng et al. (52), 2015	PCOS, pregnant and took metformin to get conception	GDM, PE, Miscarriage, Premature delivery	Metformin during pregnancy vs. Placebo Metformin during pregnancy vs. Placebo	Miscarriage Preterm birth	RR: 0.32, 95% CI: 0.19 to 0.56 RR: 0.4, 95% CI: 0.18 to 0.91
Tan et al. (58), 2016	PCOS and pregnant	GDM, PIH/PE, Miscarriage Preterm delivery Fetal abnormality, Fetal birth weight	Metformin during pregnancy vs. Placebo Metformin during pregnancy vs. Placebo Metformin during pregnancy vs. Placebo Metformin during pregnancy vs. Placebo (Non RCTs) Metformin during pregnancy vs. Placebo (Non RCTs)	GDM Miscarriage Preterm birth GDM PIH/PE	OR: 0.28, 95% CI: 0.10 to 0.75 OR: 0.20, 95% CI: 0.12 to 0.31 OR: 0.33, 95% CI: 0.18 to 0.60 OR: 0.14, 95% CI: 0.09 to 0.24 OR: 0.28, 95% CI: 0.16 to 0.48
Zhuo et al. (54), 2014	PCOS and pregnant	GDM	Metformin during pregnancy vs. Placebo	GDM	OR: 0.19, 95% CI: 0.13 to 0.27
Zeng et al. (53), 2016	PCOS and pregnant	Live birth Miscarriage Preterm delivery GDM PIH/PE IUGR Fetal malformation Neonatal death Macrosomia	Metformin during pregnancy vs. Placebo Metformin during pregnancy vs. Placebo Metformin during pregnancy vs. Placebo Metformin during pregnancy vs. Placebo Metformin during pregnancy vs. Placebo Metformin during pregnancy vs. Placebo	GDM IUGR Live birth/woman Miscarriage PIH/PE Preterm birth	OR: 0.02, 95% CI: 0.14 to 0.87 OR: 0.17, 95% CI: 0.08 to 0.33 OR: 5.23, 95% CI: 3.12 to 8.75 OR: 0.19, 95% CI: 0.12 to 0.28 OR: 0.22, 95% CI: 0.13 to 0.38 OR: 0.37, 95% CI: 0.20 to 0.68
Li et al. (22), 2011	PCOS	Pregnancy Ovulation Menstrual regularity	Metformin vs. Thiazolidinediones (3 months duration) Metformin vs. Thiazolidinediones (6 months duration)	Side effects Side effects	OR: 8.88, 95% CI: 3.54 to 22.27 OR: 12.22, 95% CI: 3.53 to 42.31
Thakker et al.(47), 2015	PCOS	Live birth Ovulation Miscarriage, Multiple pregnancy OHSS Menstrual regularity	NAC vs. Metformin NAC vs. Metformin	Ovulation/woman Pregnancy/woman	OR: 0.13, 95% CI: 0.08 to 0.22 OR: 0.4, 95% CI: 0.23 to 0.71
Al Khalifah et al. (34), 2016	Adolescents with PCOS (11-19 year old)	Menstrual regulation	OCP vs. Metformin	Menstrual frequency	MD: 0.27, 95% CI: -0.33 to -0.21
Fang et al. (37), 2017	PCOS	Dominant follicles Menstrual regularity	Vitamin D + metformin vs. Metformin	Menstrual frequency	OR: 1.85, 95% CI: 1.01 to 3.39
Pundir et al. (38), 2017	PCOS	Live birth Clinical pregnancy Ovulation Miscarriage Menstrual regulation	Inositol vs. Placebo Inositol vs. Placebo Pioglitazone vs. Placebo Roziglitazone vs. Placebo	Ovulation/woman Menstrual frequency Menstrual frequency Menstrual frequency	RR: 2.3, 95% CI: 1.1 to 4.7 RR: 6.8, 95% CI: 2.8 to 16.6 OR: 8.88, 95% CI: 2.35 to 33.61 OR: 5.59, 95% CI: 2.20 to 14.19

BMI; Body mass index, CC; Clomiphene citrate, DEX; Dexamethasone, GDM; Gestational diabetes mellitus, GIT; Gastrointestinal tract, IUGR; Intra-uterine growth restriction, IUI; Intra uterine insemination, LOD; Laparoscopic ovarian drilling, MD; Mean difference, NAC; N-acetyl cysteine, OCP; Oral contraceptive pills, OHSS; Ovarian hyper-stimulation syndrome, OR: Odds ratio, PCOS; Polycystic ovary syndrome, PIH/PE; Pregnancy induced hypertension/Preeclampsia, RCT; Randomized controlled trial, rFSH: Recombinant follicle stimulating hormone, RR; Risk ratio, SMD; Standardized mean difference, and WHO; World Health Organization.

Table 2: Results of their interventions

Gonadotrophins					
Bordewijk et al. (45), 2017	PCOS and anovulatory women	Live birth Clinical pregnancy Ovulation	FSH+metformin vs. FSH in PCOS resistant	Live birth/woman	OR: 2.31, 95% CI: 1.23 to 4.34
		Multiple pregnancy Miscarriage OHSS Adverse effects	FSH+metformin vs. FSH in PCOS resistant	Pregnancy/woman	OR: 2.46, 95% CI: 1.36 to 4.46
Farquhar et al. (19), 2012	CC resistant PCOS	Live birth Pregnancy Ovulation	LOD vs. Gonadotrophins long-term	Costs	MD: -2235.0, 95% CI: -4433.16 to -36.84
		Miscarriage Multiple pregnancy OHSS Costs	LOD vs. Gonadotrophins short-term	Costs	MD: -1115.75, 95% CI: -1309.72 to -921.77
			LOD vs. Gonadotrophins	Multiple pregnancy	OR: 0.13, 95% CI: 0.03 to 0.52
Moazami Goudarzi et al. (23), 2014	CC-resistant PCOS	Live birth Pregnancy Miscarriage Multiple pregnancies	LOD vs. Gonadotropins	Live birth/woman	OR: 0.446, 95% CI: 0.269 to 0.74
			LOD vs. Gonadotropins	Multiple pregnancy	OR: 0.127, 95% CI: 0.028 to 0.579
Palomba et al. (40), 2014	PCOS	Live birth Pregnancy Miscarriage Multiple pregnancy OHSS	Gonadotrophins+metformin vs. Gonadotrophins in OI	Live birth/woman	OR: 1.94, 95% CI: 1.10 to 3.44
			Gonadotrophins+metformin vs. Gonadotrophins in OI	Pregnancy/woman	OR: 2.25, 95% CI: 1.50 to 3.38
			Gonadotrophins+metformin vs. Gonadotrophins in OI	Cancellation/cycle	OR: 0.41, 95% CI: 0.24 to 0.72
			Gonadotrophins+metformin vs. Gonadotrophins in OI	Gonadotrophins units	MD: 306.62, 95% CI: -500.02 to -113.22
			Gonadotrophins+metformin vs. Gonadotrophins in OI	Stimulation length	MD: -3.28, 95% CI: -6.23 to -0.32
Weiss et al. (29), 2015	CC-resistant ± failure PCOS Women treated with prior metformin use +/- CC Women with prior electro cautery of ovaries.	Live birth Clinical pregnancy Miscarriage Multiple pregnancy OHSS	rFSH vs. All urinary gonadotrophins rFSH vs. HMG rFSH vs. uFSH rFSH vs. All urinary gonadotrophins rFSH vs. HMG rFSH vs. uFSH	Gonadotrophins units Gonadotrophins units Gonadotrophins units Stimulation length Stimulation length Stimulation length	MD: -105.44, 95% CI: -154.21, -56.68 MD: -283.94, 95% CI: -449.10 to -118.78 MD: -88.4, 95% CI: -139.44 to -37.36 MD: -0.66, 95% CI: -1.04 to -0.28 MD: -2.28, 95% CI: -3.49 to -1.07 MD: -0.49, 95% CI: -0.88 to -0.09
Laparoscopic ovarian drilling (LOD)					
Farquhar et al. (19), 2012	CC resistant PCOS	Live birth Pregnancy Ovulation Miscarriage Multiple pregnancy OHSS Costs	LOD vs. Metformin LOD vs. other medical treatments	Pregnancy/woman Multiple pregnancy	OR: 2.47, 95% CI: 1.05 to 5.81 OR: 0.21, 95% CI: 0.08 to 0.58
Baghdadi et al. (56), 2012	CC resistant PCOS	Pregnancy Ovulation	Lean vs. obese PCOS Lean vs. Obese PCOS Lean vs. Obese PCOS Lean vs. Obese PCOS	Ovulation/cycle Ovulation/woman Pregnancy/cycle Pregnancy/woman	RR: 1.90, 95% CI: 1.46 to 2.48 RR: 1.43, 95% CI: 1.22 to 1.66 RR: 4.14, 95% CI: 2.08 to 8.23 RR: 1.73, 95% CI: 1.39 to 2.17
IUI/IVF/ICSI related interventions					
Luo et al. (57), 2014	PCOS undergoing COS/IUI	Live birth Clinical pregnancy Miscarriage	GnRH antagonist +IUI vs. Control IUI GnRH antagonist +IUI vs. Control IUI GnRH antagonist +IUI vs. Control IUI	LH Premature lutenization rate Progesterone	MD: 4.6, 95% CI: 0.9 to 8.31 OR: 4.36, 95% CI: 2.15 to 8.84 MD: 0.31, 95% CI: 0.24 to 0.37
Kollman et al. (18), 2016	PCOS	Live birth/ ongoing pregnancy Clinical pregnancy Miscarriage OHSS	Inositol vs. Placebo IVF Myo-inositol vs. D-chiro-inositol Antagonist vs. Agonist Mannitol vs. Placebo	Pregnancy/woman Pregnancy/woman OHSS OHSS	RR: 1.41, 95% CI: 1.05 to 1.89 RR: 2.86, 95% CI: 1.14 to 7.16 RR: 0.63, 95% CI: 0.49 to 0.80 RR: 0.54, 95% CI: 0.39 to 0.77

Table 2: Continued

Palomba et al. (59), 2013	PCOS undergoing IVF cycles	Live birth Pregnancy Miscarriage OHSS	Gonadotrophins+metformin vs. Gonadotrophins (Metformin stopping time until 12 weeks of gestation)	Live birth/woman	OR: 75.6, 95% CI: 8.03 to 711.5
			Gonadotrophins+metformin vs. Gonadotrophins	Miscarriage	OR: 0.50, 95% CI: 0.30 to 0.83
			Gonadotrophins+metformin vs. Gonadotrophins (Metformin stopping time until 12 weeks of gestation)	Miscarriage	OR: 0.08, 95% CI: 0.02 to 0.39
			Gonadotrophins+metformin vs. Gonadotrophins (Pretreatment length effect for long-term >3 weeks)	Miscarriage	OR: 0.41, 95% CI: 0.21 to 0.78
			Gonadotrophins+metformin vs. Gonadotrophins (Pretreatment length effect for short-term ≤ 3 weeks)	Miscarriage	OR: 0.20, 95% CI: 0.07 to 0.54
			Gonadotrophins+metformin vs. Gonadotrophins (Metformin stopping time until oocyte retrieval, ET and HCG injection)	OHSS	OR: 0.22, 95% CI: 0.11 to 0.42
			Gonadotrophins+metformin vs. Gonadotrophins (no pretreatment period)	OHSS	OR: 0.14, 95% CI: 0.05 to 0.38
			Gonadotrophins+metformin vs. Gonadotrophins (higher dose >1000 mg daily)	OHSS	OR: 0.40, 95% CI: 0.20 to 0.80
			Gonadotrophins+metformin vs. Gonadotrophins (lower dose ≤1000 mg/daily)	OHSS	OR: 0.15, 95% CI: 0.06 to 0.38
			Gonadotrophins+metformin vs. Gonadotrophins	OHSS	OR: 0.27, 95% CI: 0.16 to 0.46
			Gonadotrophins+metformin vs. Gonadotrophins (higher dose >1000 mg daily)	OHSS	WMD: -1.11, 95% CI: -1.86 to -0.36
			Gonadotrophins+metformin vs. Gonadotrophins	OHSS	WMD: -1.16, 95% CI: -1.96 to -0.37
			Gonadotrophins+metformin vs. Gonadotrophins	OHSS	WMD: -1.45, 95% CI: -2.37 to -0.53
			Gonadotrophins+metformin vs. Gonadotrophins (higher dose >1000 mg daily)	OHSS	WMD: -1.32, 95% CI: -2.40 to -0.23
			Gonadotrophins+metformin vs. Gonadotrophins (Pretreatment length effect for long-term >3 weeks)	Oocyte number retrieved	OR: 0.28, 95% CI: 0.12 to 0.62
			Gonadotrophins+metformin vs. Gonadotrophins (Metformin stopping time until pregnancy test)	Oocyte number retrieved	OR: 1.42, 95% CI: 1.24 to 2.75
			Gonadotrophins+metformin vs. Gonadotrophins (Pretreatment length effect for long-term >3 weeks)	Oocyte number retrieved	WMD: 0.85, 95% CI: 0.02 to 1.68
			Gonadotrophins+metformin vs. Gonadotrophins (higher dose > 1000 mg daily)	Oocyte number retrieved	WMD: -326.84, 95% CI: -505.99 to -147.69
			Gonadotrophins+metformin vs. Gonadotrophins (Metformin stopping time until pregnancy test)	Implantation/embryo	
			Gonadotrophins+metformin vs. Gonadotrophins (lower dose ≤1000 mg/daily)	Implantation/embryo	
				Stimulation length	
				Gonadotrophins units	
Huang et al. (21), 2015	PCOS undergoing IVF/ICSI in non-donor cycles	Live birth Clinical pregnancy Miscarriage Multiple pregnancy OHSS	Metformin vs. Placebo	OHSS	RR: 0.44; 95%CI 0.26 to 0.77
Tso et al. (42), 2014	PCOS and of reproductive age undergoing IVF or ICSI	Live birth Clinical pregnancy Miscarriage OHSS Side effects	Metformin vs. Placebo	Pregnancy/woman	OR: 1.52, 95% CI: 1.07 to 2.15
			Metformin vs. Placebo	Side effects	OR: 4.49, 95% CI: 1.88 to 10.72
			Metformin vs. Placebo (long protocol GnRH agonist)	OHSS	OR: 0.29, 95% CI: 0.18 to 0.49
				OHSS	OR: 0.29, 95% CI: 0.16 to 0.51
Pundir et al. (26), 2012	PCOS undergoing IVF with or without ICSI	Live birth Clinical pregnancy Ongoing pregnancy Miscarriage OHSS	GnRH antagonist vs. Agonist	Gonadotrophins units	WMD: -0.28, 95% CI: -0.43 to -0.13)
			GnRH antagonist vs. Agonist	Moderate and severe OHSS	RR: 0.59, 95% CI: 0.45 to 0.76
			GnRH antagonist vs. Agonist	OHSS (moderate & severe)	RR: 0.60, 95% CI: 0.48 to 0.76
			GnRH antagonist vs. Agonist	Stimulation length	WMD: -0.74, 95% CI: -1.12 to -0.36

Table 2: Continued

Siristatidis et al. (50), 2015	PCOS, PCO and control undergoing IVM	Live birth	IVM in (PCOS vs. Control)	Cancellation/cycle	OR: 0.15, 95% CI: 0.05 to 0.44
		Clinical pregnancy	IVM in (PCOS vs. Non PCOS)	Cancellation/cycle	OR: 0.18, 95% CI: 0.06 to 0.47
		Miscarriage	IVM in (PCOS vs. PCO)	Cancellation/cycle	OR: 0.25, 95% CI: 0.07 to 0.92
		Oocyte maturation	IVM in (PCOS vs. Non PCOS)	Implantation/embryo	OR: 1.73, 95% CI: 1.06 to 2.81
			IVM in (PCOS vs. Control)	Maturation/oocyte	OR: 0.74, 95% CI: 0.59 to 0.93
			IVM in (PCOS vs. Control)	Pregnancy/cycle	OR: 3.09, 95% CI: 1.46 to 6.53
			IVM in (PCOS vs. Non-PCOS)	Pregnancy/cycle	OR: 2.23, 95% CI: 1.45 to 3.43
			IVM in (PCOS vs. Control)	Pregnancy/woman	OR: 3.29, 95% CI: 1.42 to 7.62
			IVM in (PCOS vs. Non PCOS)	Pregnancy/woman	OR: 2.37, 95% CI: 1.53 to 3.68
Xiao et al. (31), 2013	PCOS	Clinical pregnancy	GnRH antagonist vs. GnRH agonist	Moderate-severe OHSS	OR: 0.36, 95% CI: 0.25 to 0.52
		Miscarriage OHSS	GnRH antagonist vs. GnRH agonist	Stimulation length	Shorter in GnRH antagonist group

CC; Clomiphene citrate, COS; Controlled ovarian stimulation, ET; Embryo transfer, FSH; Follicle stimulating hormone, GnRH; Gonadotrophins releasing hormone, HCG; Human chorionic gonadotrophin, HMG; Human menopausal gonadotrophin, ICSI; Intra cytoplasmic sperm injection, IUI; Intra uterine insemination, IVF; *In vitro* fertilization, IVM; *In vitro* maturation, LH; Luteinizing hormone, LOD; Laparoscopic ovarian drilling, MD; Mean difference, OHSS; Ovarian hyper-stimulation syndrome, OI; Ovulation induction, OR; Odds ratio, PCOS; Polycystic ovary syndrome, rFSH; Recombinant follicle stimulating hormone, RR; Risk ratio, uFSH; Urinary follicle stimulating hormone, and WMD; Weighted mean difference

Clomiphene citrate

Seventeen reviews, [seven high quality (19, 39, 41, 46–49), six moderate quality (16, 20, 24, 27, 32, 38) and four low quality (28, 30, 33, 36)] assessed interventions that contained CC, comprising a total of 203 trials with 26 731 participants. One review assessed CC versus LOD (19). One review assessed early follicular versus late luteal CC administration (36). The remaining 14 reviews assessed CC ± other OI drugs such as metformin, inositol, N-acetyl cysteine (NAC) and others versus other OI drugs, including CC. The populations studied were women with PCOS who were treatment-naïve (27), CC resistant (19, 32, 33) and women with PCOS who were treatment-naïve ± CC resistant PCOS or unknown treatment status.

The meta-analyses reported in overall women with PCOS that CC compared to placebo had statistically higher pregnancy and ovulation (46). Early follicular CC had higher pregnancy than late luteal CC (46) but with less mature follicles (36). Higher live birth, pregnancy, and ovulation resulted after CC compared to metformin mainly in women with BMI ≥30 kg/m² (28, 30, 41) while metformin resulted in higher pregnancy than CC in women with BMI <30 kg/m² (41). CC plus metformin was of more benefit than CC or metformin alone with regards to live birth (24), pregnancy and ovulation, but had higher gastrointestinal side effects (24, 28, 30, 33, 41). Higher live birth and pregnancy resulted after gonadotrophins compared to CC and 10 days of CC compared to 5 days of CC, respectively (46).

In women with CC resistant PCOS, gonadotrophins resulted in statistically higher live birth, pregnancy and ovulation than CC plus metformin (32, 46) which, in turn, resulted in higher live birth than LOD (19). In the same population of women, the addition of dexamethasone, NAC or contraceptive pills to CC resulted in higher live births, pregnancy and ovulation than CC alone (46, 47). Furthermore, the addition of metformin to CC resulted in more favourable outcomes compared with the addition of

NAC with regards to pregnancy and ovulation. However, the cost of treatment was greater for gonadotrophins followed by LOD then CC plus metformin (19).

Gonadotrophins

Ten reviews [six high quality (19, 29, 39, 45, 46, 49) and four moderate quality (23, 32, 40, 59)] assessed interventions containing gonadotrophins, which comprised 146 trials with 18 379 participants. Two reviews assessed gonadotrophins versus LOD (19, 23). Three reviews assessed the effectiveness of adding metformin to gonadotrophins during OI (40, 45) and IVF (59). Two reviews assessed gonadotrophins versus anti-oestrogens ± adjunctive drugs (32, 46). Two reviews assessed gonadotrophins versus aromatase inhibitors (39, 49). One review assessed the effectiveness of different types of gonadotrophins (29). The populations studied were women with CC resistant PCOS (19, 23, 29, 32) and women who were treatment-naïve ± CC resistant PCOS women or unknown treatment status.

The meta-analyses reported that in women with CC resistant PCOS, gonadotrophins resulted in statistically higher live births, multiple pregnancies, and costs of short- and long-term treatment in comparison to LOD (19, 23) and higher live births, pregnancy and ovulation in comparison to CC ± metformin (32, 46), but lower pregnancy in comparison to letrozole (39). Adding metformin to gonadotrophins, compared to gonadotrophins alone, resulted in higher live birth and pregnancy in OI (40, 45) and higher live birth, implantation rate, lower miscarriage, ovarian hyperstimulation syndrome (OHSS) and number of oocyte retrieved in IVF (59). Recombinant follicle stimulating hormone (FT.) resulted in lower dose and stimulation duration than other urinary gonadotrophins in OI (29).

Insulin sensitizers

Thirty reviews (12 reviews of high quality (18, 19, 34,

39, 41, 42, 44-49), 13 reviews of moderate quality (16, 21, 22, 24, 25, 32, 35, 37, 38, 40, 53, 54, 59) and five reviews of low quality (28, 30, 33, 52, 58) assessed interventions that contained insulin sensitizers comprising 398 trials with 45 031 participants. Four reviews assessed metformin versus placebo (18, 21, 41, 42). Four reviews assessed metformin during pregnancy (52-54, 58). One review assessed the effect of pre-gestational metformin on risk of miscarriage (25). One review assessed rosiglitazone, pioglitazone, and D-chiro-inositol versus placebo (41). One review assessed metformin versus thiazolidinediones (22). One review assessed LOD versus metformin (19). One review assessed NAC versus placebo or metformin (47). One review assessed oral contraceptive pills versus metformin (34). One review assessed the benefit of adding vitamin D to metformin (37). Three reviews had CC resistant PCOS women as participants (19, 32, 33) while the others did not clarify the treatment status.

The meta-analyses reported that, overall in women with PCOS, metformin resulted in higher live births, pregnancy, and gastrointestinal side effects with lower OHSS than placebo when used in addition to IVF (18, 21, 42) and higher pregnancy, ovulation, side effects and menstrual frequency in OI (41). Metformin had higher gastrointestinal side effects than thiazolidinediones (22). In women with CC resistant PCOS, NAC resulted in higher live births, pregnancy and ovulation than placebo, but lower pregnancy and ovulation than metformin (47). Oral contraceptive pills were better than metformin in improving menstrual frequency (34). Adding vitamin D to metformin improved menstrual frequency than metformin alone (37). Inositol resulted in higher pregnancy than placebo with more benefit of myo-inositol over D-chiro inositol in IVF (18), while inositol resulted in higher ovulation than placebo in OI. Rosiglitazone, pioglitazone and inositol improved menstrual frequency in OI (38). In women with PCOS who became pregnant, metformin intake during pregnancy resulted in higher live birth and lower miscarriage, preterm labour, gestational hypertension, preeclampsia, gestational diabetes and intrauterine growth retardation (52-54, 58).

Laparoscopic ovarian drilling

Six reviews [four high quality (19, 32, 39, 49) and two moderate quality (23, 56)] assessed ovarian ablation therapy and LOD as an intervention in PCOS comprising 97 trials with 13 617 participants. Three reviews had participants as CC resistant PCOS (19, 23, 56).

The meta-analyses reported that LOD resulted in lower live births than CC plus metformin and gonadotrophins, respectively (19, 23), higher pregnancy than metformin alone (19), lower ovulation than letrozole (49), higher costs than CC plus metformin but lower than gonadotrophins (19) and lower multiple pregnancy rate than other medical treatments (19). Pregnancy and ovulation were higher in lean women ($\text{BMI} < 25 \text{ kg/m}^2$) with CC resistant PCOS than in overweight and obese women ($\text{BMI} \geq 25 \text{ kg/m}^2$) undergoing LOD (56).

Intrauterine insemination, *in vitro* fertilization, intracytoplasmic sperm injection related interventions

Nine reviews [three high quality (17, 18, 42)] and six moderate quality (21, 26, 31, 50, 57, 59) assessed different interventions in women with PCOS undergoing assisted reproductive techniques [intrauterine insemination (IUI), IVF/ICSI] comprising 126 trials with 12 298 participants in eight reviews and 333 cycles in the ninth review which did not report on the number of participants (57). Three reviews assessed gonadotrophin releasing hormone (GnRH) antagonist as an adjuvant intervention in controlled ovarian stimulation plus IUI (57) and in comparison with GnRH agonist during IVF/ICSI (26, 31). Three reviews assessed the effect of metformin during IVF/ICSI (21, 42, 59). Two reviews assessed the use of IVM (17, 50).

The meta-analyses reported statistically significant results for lower progesterone, luteinizing hormone (LH) and premature luteinisation rate during IUI after GnRH antagonist (57) and lesser dose, duration of gonadotrophins and OHSS rate after GnRH antagonist during IVF/ICSI. Metformin compared to placebo in IVF resulted in higher live births (18, 59), pregnancy (18, 42), lower miscarriage (59), lower OHSS (18, 21, 42, 59), and lower oestradiol (E2), gonadotrophin dose and higher implantation rate (59); however, disadvantages included more, yet mild, gastrointestinal side effects (42). Compared to placebo, inositol resulted in higher pregnancy with better results after myo-inositol than D-Chiro inositol, while mannitol resulted in lower OHSS (18). IVM used in women with PCOS had higher pregnancy, lower cancelled cycles, higher implantation but lower mature oocytes than IVM in non-PCOS patients (50).

Other interventions

A low quality review reported that bariatric surgery improved menstrual frequency in women with PCOS in six trials and 264 participants (51). A high quality review reported that statins did not improve menstrual frequency or ovulation in women with PCOS not trying to conceive in four trials and 244 participants (43). A high quality review (44) assessed the use of antidepressants in women with PCOS, and identified no studies reporting on any of the primary reproductive outcomes with the exception of one RCT that reported on endocrine and metabolic outcomes between fluoxetine with sibutramine found no significant difference between both drugs (61). A moderate quality review assessed orlistat versus other anti-obesity drugs and found no difference in reproductive outcomes (55).

Discussion

We reported the first overview of systematic reviews on treatment for reproductive outcomes in women with PCOS. This review follows a process of systematic reviews proposed by the Cochrane collaboration that sum-

marizes evidence from more than one systematic review of different interventions for the same condition (62, 63). This type of review can be utilized as a rich source of data synthesis for developing and updating guidelines, and for health care policy makers. Our overview included 53 systematic reviews (9 older versions and 44 currently updated articles), 498 studies, and 56 057 participants. The quality of most included reviews was moderate to high, although the quality of included studies was variable.

Our results align with most current guidelines on PCOS. According to many guidelines, treatment of anovulation in PCOS should start with lifestyle modification before commencing pharmacological agents, especially in obese women with BMI >30 kg/m² (1, 3, 8, 10, 11). The first-line pharmacological agent is usually CC (2, 3, 11, 64, 65) and some guidelines propose letrozole as an alternative (1, 8, 10). Our results suggest that, overall, in women with PCOS (with or without CC resistance), letrozole resulted in higher live birth and clinical pregnancy rates than other OI drugs, especially CC. This is consistent with many reviews and RCTs (9, 20, 27, 32, 39, 49, 66-68), despite the fact that letrozole is an off-label drug in OI. Nevertheless, the issue of safety in pregnancy for both CC and letrozole has not been completely resolved. Most large retrospective studies found no evidence of any difference between these drugs (69). Metformin is recommended in many guidelines as an adjunctive treatment with CC in women with glucose intolerance and in obese women (1-3, 8, 10), while the National Institute for Health and Clinical Excellence Guidance (NICE) recommended metformin alone or with CC as a first-line treatment (11). Our results suggest that, overall, in women with PCOS, CC plus metformin also resulted in better reproductive outcomes than CC or metformin alone. The Australian National Health and Medical Research Council (NHMRC) evidence-based guidelines suggested that it is acceptable to use gonadotrophins as a first-line treatment (8). Our results suggest that the use of gonadotrophins resulted in higher live birth and clinical pregnancy rates than CC, overall, in women with PCOS.

CC is usually used for six months, which is recommended by many guidelines (1, 8, 11). After that, women are considered to be CC resistant, which necessitates a second-line treatment. Most fertility guidelines recommend low dose gonadotrophins or LOD as a second-line treatment (1-3, 8, 10, 11). CC plus metformin was also recommended by some guidelines, if not already used as a first-line treatment (8, 11). Gonadotrophins have the disadvantage of cost and increased rates of multiple pregnancies, while LOD has a risk with anaesthesia, decreased ovarian reserve, and the need to use adjuvant drugs for OI after surgery (3). Our results suggest that, in women with CC resistant PCOS, gonadotrophins resulted in better reproductive outcomes than many OI drugs with the disadvantages of increased multiple pregnancies and increased cost (19, 23, 32, 46). We found that women who used gonadotrophins had higher live birth than those who were prescribed CC plus metformin

or LOD respectively, and higher clinical pregnancy and ovulation rates than CC plus metformin. CC plus metformin resulted in higher live birth rate and lower cost than LOD. Gonadotrophins are more expensive than LOD. LOD has the advantage of lower rates of multiple pregnancies compared to other interventions, such as gonadotrophins, in CC resistant PCOS (19). LOD in lean women seem to have better reproductive outcomes than in overweight and obese women.

Current recommendations state that IVF should be used in case of CC failure, which is defined by failure of conception after 6-9 months (1, 11). Our results support the current evidence for use of GnRH antagonists and addition of metformin to GnRH agonist to decrease OHSS (1). There is lack of data on use of IVM in PCOS (1), which is reported by one of included reviews (17). Another review by the same author reported higher pregnancy and implantation rates with lower cancellation rate in women with PCOS undergoing IVM compared to IVM in non-PCOS women (50).

Despite the large number of reviews and RCTs that have been conducted assessing different treatments for management of reproductive outcomes in women with PCOS, there are still a considerable number of research gaps. Recently, the international evidence-based guideline for the assessment and management of PCOS has issued new recommendations for the diagnosis and management of PCOS (70). These guidelines state that letrozole should be considered first-line pharmacological treatment for OI in women with PCOS with anovulatory infertility and no other infertility factors to improve ovulation, pregnancy and live birth rates. This is consistent with our results in this overview. They also stated that inositol (in any form) should currently be considered an experimental therapy in PCOS, with emerging evidence on efficacy highlighting the need for further research (70). Furthermore, research on the possible reasons for CC resistance and failure utilizing unified definitions is needed. This is particularly relevant given that some recent reviews revealed that the antiestrogenic effect of CC, specifically on endometrial tissue, is not enough rationale for resistance and failure (66). Furthermore, a recent crossover RCT found that there is no difference in clinical pregnancy and live birth rates between CC and letrozole when used as a second line treatment in women who failed to ovulate or conceive with CC or letrozole as first line of treatment (9). It is also important to note that a thorough study of the cost effectiveness of any of these treatments has not been performed, particularly in low income countries. Further investigation of metformin with regards to its cost effectiveness, safety, and effectiveness in non-obese women is also needed (1, 8). There is also a lack of data relating to the comparison between the use of LOD and medical treatment as a first line treatment, and the minimum efficient dose of LOD to induce ovulation without affecting ovarian reserve (1, 3, 11).

Limitations include our search strategy with reviews pub-

lished from 2009 onwards, coinciding with the PRISMA statement publication for conducting systematic reviews. While this would miss earlier reviews, later included reviews would be likely to be of higher quality and aligned with the PRISMA statement. We applied language restrictions including only articles in English, which might lead to bias in exclusion of other languages. We found insufficient data on the quality of included studies in each review. We did not perform a quality assessment of each of the individual trials within each systematic review and relied instead on the judgement of the authors which varied from cursory to comprehensive; although we note that performing a quality assessment of 498 total studies would have been an extensive task. We note that the actual effect of different treatments in each treatment status and PCOS phenotypes is still unclear. We also note wide variability in the definition of outcomes across reviews and included studies. For instance, although pregnancy was reported as clinical pregnancy in most included reviews, ongoing pregnancy was reported in some reviews (26, 45) and pregnancy was not predefined in others (22, 24, 36, 40, 51, 56). The definition of clinical pregnancy varied across the included studies within each review.

Conclusion

We report here a significant contribution to the literature in the overview and synthesis of systematic reviews that assessed medical and surgical treatments for reproductive outcomes in women with PCOS. In agreement with most recent international guidelines on management of PCOS, letrozole was superior to other OI agents as a first-line pharmacological treatment with gonadotrophins a second-line pharmacological treatment for anovulatory women with PCOS.

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Authors' Contributions

L.J.M., D.S.H., B.W.J.M., A.M., H.J.T., S.T., J.P., R.J.N., M.A.G., C.T.T.; Participated in conception and design. L.J.M., D.S.H., C.T.T.; Participated in identification and selection of included articles. L.J.M., M.A.G., C.T.T.; Participated in data extraction and quality assessment of included articles. M.A.G.; Drafted the manuscript, revising by all authors. All authors read and approved the final manuscript.

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Quality of Life and General Health in Pregnant Women Conceived with Assisted Reproductive Technology: A Case-Control Study

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Abstract

Background: Infertility affects different aspects of life including the quality of life (QOL) in infertile couples. Many infertile couples conceive via using assisted reproductive technology (ART). However, the effect of pregnancy and childbearing on QOL is not known in these couples. This study aimed to evaluate QOL and general health during pregnancy and after successful treatment of infertility, in women conceived with ART.

Materials and Methods: In this case-control study, QOL and general health were evaluated in 40 women conceived with ART and 40 women who conceived spontaneously and served as the control group. WHO quality of life-BREF (WHOQOL-BREF) inventory was used to evaluate QOL and General Health Questionnaire-28 (GHQ-28) was applied to evaluate general health. These two questionnaires were completed in the first and second trimester of pregnancy and results were compared between the two groups.

Results: Mean age of women was 29.4 ± 4.4 and 29.6 ± 5 years in ART and control group, respectively. QOL in women conceived with ART was similar to QOL in the control group in the first and second trimester of pregnancy while general health score (distress level) in women conceived with ART was significantly higher than that of the control group in both trimesters. Although distress level decreased in the second trimester in ART group, but yet, it was higher than that recorded for the control group.

Conclusion: After pregnancy, QOL in women conceived with ART is similar to women conceived spontaneously. However, these women experience higher distress level in the first and second trimester of pregnancy compared to women conceived spontaneously.

Keywords: Assisted Reproduction Technology, General Health, Infertility, Quality of Life

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Introduction

Infertility is defined by failure of getting pregnant after at least one year of regular and unprotected intercourse (1). Worldwide, about 1 out of 4 couples in developing countries, 1 out of 8 in developed countries and globally about 8-12% of couples are affected by primary or secondary infertility (2). In about 40% of infertile couples male factors, in 40% of them female factors and in 20% of cases combinations of both or unknown causes, are responsible for infertility (3).

Prevalence of primary infertility in a population-based study in an urban population in Iran was 17.3% which is higher than global infertility rate. In Iranian couples, female (56.1%) and male factors (29.1%) were the most common causes of infertility, followed by unexplained infertility (14.4%). Among female factors of infertility, ovulation disorders (39.7%) were the most common cause (4).

Infertility can profoundly affect different aspects of

life in infertile couples and due to its social, cultural and economic problems, it produces a severe crisis in infertile couples' life and causes severe distress and psychological (anxiety, depression, etc.) (5, 6) and financial problems (7). Infertility may also cause problems in couples relationship or even lead to divorce (7, 8). Infertility stigma for women in regions with traditional cultures like Middle East countries, is more prominent and stressful and causes various problems for them (9).

Above-mentioned factors affect deeply the quality of life (QOL) and general health in infertile couples (6, 10). Several studies investigated the relationship between depression, anxiety, QOL, general health and marital satisfaction in infertile couples and socio-demographic determinants of QOL (11-14).

Most of studies showed impaired QOL and general health in infertile couples where QOL was affected by factors such as the duration of infertility, age, education,

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income, residential place and cause of infertility (6, 10, 15-17). A previous study showed that more than half of infertile women have a degree of general health disorder (6). In a study, Maroufizadeh et al. (11) found that QOL in infertile couples was influenced by their own and their spouses' depression. In another study, Maroufizadeh et al. (13) showed that marital satisfaction in infertile patients was affected by their own and their spouses' perceived stress.

Following successful progresses in infertility treatment and achieving pregnancy, it is assumed that the above-mentioned problems may decrease and QOL may improve (18). Nevertheless, following successful conception, due to high risk pregnancy and concern of continuity of pregnancy distress may increase resulting in decrement of QOL. However, limited studies evaluated QOL and general health of infertile couples after conception and during pregnancy.

The main goal of this study was to evaluate QOL and general health in pregnant women conceived with assisted reproductive technology (ART).

Materials and Methods

This case-control study was conducted during 2013-2014 in a private clinic in Mashhad, Iran and 40 pregnant women conceived with ART and 40 pregnant women who conceived spontaneously were included.

Pregnant women who conceived with one of the ART methods including *in vitro* fertilization (IVF) or intra cytoplasmic sperm injection (ICSI), those who did not have a previous history of successful pregnancy or children and were in the first trimester (gestational age under 12 weeks) of pregnancy, were included in case group. Pregnant women, who did not have previous successful pregnancy or children (nulliparous) but conceived spontaneously and were in the first trimester of pregnancy, were enrolled as control group. The two groups were matched for age, education, income and gestational age.

Pregnant women with a history of chronic diseases, diabetes, cardiovascular diseases, seizure, or addiction and those with a history of psychiatric disorders as well as those who did not sign the informed consent, were excluded from the study.

To evaluate QOL, a Persian version of WHO quality of life- BREF questionnaire (WHOQOL-BREF) was used. Also, a Persian version of General Health Questionnaire-28 (GHQ-28) was used to evaluate general health in the participants. In the present study, participants completed these two questionnaires twice (once in the first and once in the second trimester of pregnancy).

WHOQOL-BREF inventory has 26 items and four domains including physical health, psychological health, social relationship and environment. Reliability and validity of the Persian version of WHOQOL-BREF was previously evaluated and approved (19). Two scoring

systems are used in WHOQOL-BREF. In the first method, the inventory is scored between 0 and 100 and in the second method, it is scored between 4 and 20. In the current study, both methods were used but in the Tables, only results from the first method are presented.

GHQ-28 is a self-administered inventory with 28 items that has been developed for screening of emotional distress and possible psychiatric morbidity. GHQ-28 evaluates psychological well-being in four subscales namely, somatic symptoms, anxiety/insomnia, social dysfunction and severe depression. Each subscale has seven questions and each item has four optional responses scored 0 to 3 as follows; score 0: "not at all" score 1: "no more than usual"; score 2: "rather more than usual" and score 3: "much more than usual." The total score of the GHQ-28 ranges from 0 to 84 and a higher score indicates a higher distress level. In each subscale, a score >6 was considered "abnormal condition".

Validity and reliability of the Persian version of GHQ-28 was previously assessed and confirmed (20). Two inventories were completed by participants in the first and second trimester of pregnancy. Demographic information including age, gestational age, education and economic status, etc. was recorded in a separate form.

Data analysis

Considering Nilforooshan et al. (21) study that mean of QOL score in case and control group was 170.52 ± 18.17 and 182.22 ± 18.08 , and by considering 95% CI and power of 80%, sample size was calculated by following formula:

$$n = \frac{(S_1^2 + S_2^2)(Z_{1-\frac{\alpha}{2}} + Z_{1-\beta})^2}{(\bar{X}_1 - \bar{X}_2)^2} = \frac{(18.17^2 + 18.08^2)(1.96 + 0.84)^2}{(182.22 - 170.52)^2} = 37/62$$

Sample size of 40 was considered in each group. Obtained data was analyzed using SPSS software version 22.00 for Windows (Armonk, NY: IBM Corp.) and STATISTICA Ver. 10.00. Numerical data are presented as mean $\pm$ standard deviation and categorical data as numbers/percentages. Normality of data was assessed by Kolmogorov-Smirnov test with the correction of Lilliefors.

For comparison of data with normal distribution between the two groups, Student's t test was used and for comparison of data obtained in the first and second trimester of pregnancy, paired t test was used. For comparison of data without normal distribution, non-parametric Mann-Whitney U test was applied. To compare qualitative and categorical data such as education and job between the two groups, Chi-square test was applied. A $P \leq 0.05$ was considered significant.

Ethical considerations

Institutional Review Board at Mashhad Branch of Islamic Azad University approved the study protocol and all participants signed written informed consent before enrollment (IR.IAU.NEYSHABUR.REC.1398.008).

Results

Eighty women in the first trimester of pregnancy participated in this study; of them, 40 conceived with ART and 40 conceived spontaneously (control group). Mean age of all women was 29.5 ± 4.7 years. Demographic characteristics of pregnant women in two study groups are depicted in Table 1. There were no significant differences in age, gestational age, education, job and income between the two groups.

There were no significant differences in QOL score between ART and control group neither in the first trimester nor in the second trimester of pregnancy. Also, there were no significant differences between the two groups in none of the four QOL sub-domains in the first and second trimesters of pregnancy. In the second trimester of pregnancy, QOL improved significantly in both groups compared to the first trimester ($P=0.006$ and $P=0.03$, respectively) while the differences in the four sub-domains of QOL were not significantly different between

the first and second trimesters, in each group (Table 2).

There was a significant difference in general health between the women conceived with ART and the control group in a way that general health score of women conceived with ART was significantly higher than those conceived spontaneously in the first as well as the second trimester of pregnancy ($P<0.001$). Also, a significant difference was observed between the two groups in all subscales of GHQ-28 in the first trimester of pregnancy while in the second trimester a significant difference was only found in somatic symptoms and anxiety ($P=0.001$ and 0.009 , respectively, Table 3).

In the second trimester of pregnancy, in the ART group, general health score and all its subscales except for somatic symptoms, were significantly lower than those of the first trimester of pregnancy while in the control group, total general health score and all its subscales were significantly higher than those of the first trimester of pregnancy (Table 3).

Table 1: Demographic characteristics of study participants in two study groups

Parameter	ART pregnancy group	Spontaneous pregnancy group	P value
Age (Y)	29.42 ± 4.39	29.57 ± 5.02	0.47
Education			
Primary	13 (32.5)	10 (25)	0.74
High school diploma	11 (27.5)	13 (32.5)	
University education	16 (40)	17 (42.5)	
Job			
Housewife	30 (75)	29 (72.5)	0.79
Employed	10 (25)	11 (27.5)	
Income*			
Below 5000	6 (15)	7 (17.5)	0.82
5000-10000	14 (35)	16 (40)	
10000-15000	12 (30)	12 (30)	
More than 15000	8 (20)	5 (12.5)	
Gestational age (weeks)	9.87 ± 1.91	9.55 ± 2.11	0.55

Data are presented as mean $\pm$ SD or n (%). *: Thousands rials and ART; Assisted reproductive technology.

Table 2: Quality of life score and scores of its four domains in two study groups in the first and second trimesters of pregnancy (0-100)

WHOQOL-BREF domain	ART pregnancy group	Spontaneous pregnancy group	P value	95% CI
First trimester of pregnancy				
Physical health	50.71 ± 12.05	51.61 ± 11.91	0.74	-4.44, 6.22
Psychological health	57.08 ± 19.29	60.83 ± 19.28	0.38	-4.83, 12.33
Social relationship	62.08 ± 15.20	65.83 ± 14.71	0.26	-2.91, 10.41
Environment	60.31 ± 12.40	63.35 ± 14.20	0.31	-2.88, 8.98
Overall feeling	66.25 ± 25.50	68.43 ± 23.34	0.69	-8.69, 13.07
Second trimester of pregnancy				
Physical health	51.42 ± 11.17	52.58 ± 12.68	0.66	-4.16, 6.48
Psychological health	59.37 ± 18.62	60.20 ± 20.71	0.85	-7.93, 9.60
Social relationship	61.66 ± 13.96	64.79 ± 13.80	0.31	-3.05, 9.30
Environment	60.78 ± 14.65	62.50 ± 14.41	0.59	-4.75, 8.19
Overall feeling	$73.43 \pm 22.32^*$	$74.06 \pm 19.69^*$	0.89	-8.74, 9.99

Data are presented as mean $\pm$ SD. *: Significant difference with first trimester of pregnancy in the same group, CI; Confidence interval, and ART; Assisted reproductive technology.

Table 3: GHQ-28 and its subscales scores in two study groups in the first and second trimesters of pregnancy

GHQ-28 domain	ART pregnancy group n=40	Spontaneous pregnancy group n=40	P value	95% CI
The first trimester of pregnancy				
Somatic symptoms	8.70 ± 3.00	5.60 ± 3.30	0.001*	-4.50, -1.69
Anxiety and insomnia	9.15 ± 3.36	5.80 ± 2.52	0.001*	-4.67, -2.02
Social dysfunction	8.55 ± 3.28	6.22 ± 2.61	0.001*	-3.64, -1.00
Severe depression	6.85 ± 3.14	5.47 ± 2.83	0.04*	-2.70, -0.04
Total general health	33.25 ± 7.41	23.10 ± 5.68	0.001*	-13.09, -7.20
The second trimester of pregnancy				
Somatic symptoms	9.65 ± 3.18†	6.80 ± 3.39†	0.001*	-4.31, -1.38
Anxiety and insomnia	7.80 ± 2.20†	6.35 ± 2.64†	0.009*	-2.53, -0.36
Social dysfunction	7.52 ± 2.63†	6.60 ± 2.76†	0.12	-2.12, 0.27
Severe depression	6.05 ± 2.36†	6.05 ± 3.39†	0.99	-1.30, 1.30
Total general health	31.02 ± 5.54†	25.80 ± 6.18†	0.001*	-7.83, -2.61

Data are presented as mean ± SD. ART; Assisted reproductive technology, CI; Confidence interval, *; Significant difference, GHQ-28; General Health Questionnaire-28, and †; Significant changes compared to the first trimester of pregnancy in the same group.

Table 4: Prevalence of psychiatric disorders based on GHQ-28 results in two study groups in the first and second trimesters of pregnancy

GHQ-28 domain	ART pregnancy group n=40	Spontaneous pregnancy group n=40	P value
The first trimester of pregnancy			
Somatic symptoms disorder	32 (80)	12 (30)	<0.001*
Anxiety and insomnia disorder	30 (75)	14 (35)	<0.001*
Social function disorder	29 (72.5)	20 (50)	0.03*
Severe depression disorder	22 (55)	9 (22.5)	<0.001*
Total general health disorder	38 (95)	21 (52.5)	<0.001*
The second trimester of pregnancy			
Somatic symptoms disorder	33 (82.5)	18 (45)	<0.001*
Anxiety and insomnia disorder	27 (67.5)	17 (42.5)	0.02*
Social function disorder	26 (65)	19 (47.5)	0.11
Severe depression disorder	18 (45)	12 (30)	0.16
Total general health disorder	39 (97.5)	26 (65)	<0.001*

Data are presented as n (%). *; Significant difference, ART; Assisted reproductive technology, and GHQ-28; General Health Questionnaire-28

Somatic symptoms score in the ART group was significantly higher in the second trimester of pregnancy ($P < 0.001$). However, the difference in GHQ-28 between the two study groups was significant and distress in the ART group was higher compared to the control group, in the second trimester of pregnancy (Table 4). Prevalence of general health disorders based on GHQ-28 in two study groups has been shown in Table 4.

Discussion

This study found that QOL in pregnant women conceived with ART was similar to women conceived spontaneously, in the first and second trimesters of pregnancy while general health in ART group was significantly superior to control group in the first and second trimesters. Women conceived with ART had significantly higher somatic symptoms, anxiety, social dysfunction and depression compared to the control group, in the first trimester of pregnancy. In the second trimester of pregnancy, all

GHQ-28 subscales were significantly reduced compared to the first trimester in the ART group while at the same time, distress increased in the control group. In the first trimester of pregnancy, in women conceived with ART, stress, anxiety and depression increase probably due to uncertainty about the continuity of pregnancy and in the second trimester, this uncertainty about stability of pregnancy decreases which may lead to reduced distress and anxiety.

Due to infertility and probably repeated treatment failures, couples face different problems such as financial problems and difficulties in social relations that affect different aspects of their life. Infertility has negative psychological effects such as anxiety, depression (5, 11) stress, hopelessness, etc. which reduce QOL of infertile couples.

Maroufizadeh et al. (14) showed that both men's and women's anxiety affect the marital satisfaction. Also, they found that in infertile couples, women's

anxiety has a significant effect on their partner marital satisfaction. They showed that in infertile couples, marital satisfaction of each member of each couple has an effect on his/her own depression. They also found that men's marital satisfaction has a significant effect on their partner depression symptoms while the wives' marital satisfaction has no effect on husbands' depressive symptoms (12).

Previous studies showed poor QOL in infertile couples in Iran and other countries in the world (15, 21-26). These studies showed that depression, anxiety, failure of previous treatments, female gender, lower educational level, younger age and unknown cause of infertility were associated with lower QOL (21, 27) while higher educational level, social support and coping strategy increased QOL in these couples (15). However, duration of infertility has no effect of QOL (27).

However, successful infertility treatment and conception may restore reduced QOL and increased distress level during pregnancy. In a study conducted in Canada, QOL was evaluated in 243 women conceived with ART and 3,309 women with spontaneous conception before the 25th week of pregnancy and during the 34th-36th weeks of gestational age as well as four months postpartum by using SF-12 questionnaire. This study reported lower physical and mental health for women conceived with ART during pregnancy (before the 25th week and during the 34th-36th weeks of pregnancy) compared to women conceived spontaneously while these indices were equal between the two groups four months postpartum (28). Findings of that study are different from ours as we did not observe any difference between the two groups in QOL in the first and second trimester. The reason of such discrepancy may be application of different questionnaires for evaluation of QOL (SF-12 vs. WHOQOL-BREF). Also, postpartum QOL was not evaluated in the present study.

Gameiro et al. (29) study done in Portugal, evaluated QOL in 66 women conceived with ART and compared it with QOL determined for 70 women conceived spontaneously, during the 24th week of pregnancy and four months postpartum by using WHOQOL-BREF inventory. In their study, physical health in women of both groups was similar during pregnancy and improved four months postpartum in both groups, although its improvement in the ART group was better. Psychological health score in women of the ART group during pregnancy was higher than the control group and four months postpartum reduced more than the control group as well. The same changes were observed for psychological health in men of the ART group. Although similar to our study, Gameiro et al. (29) used WHOQOL-BREF inventory but the findings were to some extent different that may be due to the larger sample size of their study.

Ahmadi et al. (30) evaluated QOL in 86 women conceived with ART and 162 women with natural conception by using SF-36 inventory in the last trimester of pregnancy and one month postpartum. In this study,

subdomains of physical functioning, role physical, general health and social functioning were significantly different between the two groups before childbirth and improved one month postpartum in both groups except for social functioning that did not improve in control group significantly. However, improvements in all QOL measures in the ARTs group, were greater, except for general health, than the control group. Ahmadi et al. (30) also applied SF-36 inventory which is different from what we used in the present work.

A study in Slovenia showed that women conceived with ART had positive emotion that improved by progression of pregnancy despite the existence of more medical problems during pregnancy. However, they tend to social isolation (31). They used QOL scale to evaluate QOL which has different items compared to WHOQOL-BREF inventory that was used in our study.

The main limitation of the current study was the small sample size and lack of assessment of QOL and general health in the last trimester of pregnancy and postpartum. Cross-sectional design of the study, use of self-report questionnaire and lack of evaluation of psychological factors such as depression, anxiety, stress and self-esteem, were other limitations of the current study.

Future studies with larger sample size which assess QOL and general health using other valid, approved inventories in all trimesters of pregnancy and postpartum are suggested to be conducted to identify possible changes in QOL in the third trimester of pregnancy and during postpartum.

Conclusion

It seems that in infertile women following treatment and after successful conception and during pregnancy, QOL is similar to women conceived spontaneously and is not different. Although during pregnancy these women have high distress levels but by progression of pregnancy and increasing certainty about pregnancy, distress level reduces.

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Authors' Contributions

M.S.Y.; Data analysis, data collection and approving the final manuscript. R.N.; Concept and design of study, interpretation of data, and approving the final manuscript. M.Gh.J.; Distributing questioners, data collection, data analysis, and approving the final manuscript. S.S.Y.; Drafting the manuscript, data collection and analysis and approving the final manuscript. All authors read and approved the final manuscript.

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A Comparison of Postpartum Depression in Mothers Conceived by Assisted Reproductive Technology and Those Naturally Conceived

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Abstract

Background: It is thought that mothers who conceive via assisted reproductive technology (ART) may be at greater risk of postpartum depression (PPD) because of the problems and psychological stresses associated with ART treatment. The aim of the present study is to determine the occurrence of PPD among mothers who conceive by ART in comparison with those who naturally conceive. The Edinburgh Postnatal Depression Scale (EPDS) was used to assess PPD.

Materials and Methods: This historical cohort study investigated 406 mothers with infants aged 3-9 months. Three hundred and eight women with natural pregnancies were selected as the control group from mothers who referred to Tehran healthcare centres for infant vaccinations. The ART group consisted of 98 women who conceived via ART at Royan Institute. Participants completed a general questionnaire that asked about education, occupation, number of children, delivery method, history of infant hospitalization, breastfeeding, mothers' and infants' ages, cause of infertility (ART group), and history of depression. A validated Persian version of the EPDS was used to measure depressive symptoms.

Results: The mean EPDS score in mothers who naturally conceived was 8.38 ± 0.35 in comparison with mothers who conceived via ART (7.59 ± 0.63). The proportions of women who reported PPD were 26.0% for the control group and 20.4% for the ART group. There was no statistically significant difference in PPD between the control and ART groups ($P=0.26$).

Conclusion: The occurrence of PPD in mothers who conceived via ART was similar to those who conceived naturally.

Keywords: Assisted Reproductive Technology, Edinburgh Postnatal Depression Scale, Natural Pregnancy, Postpartum Depression

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Introduction

Pregnancy is a welcomed event, usually associated with psychological and behavioral changes, especially for women who have become pregnant by an assisted reproductive technology (ART) (1). Postpartum depression (PPD) is important because it reduces the ability of a mother to care for her infant and decreases the quality of the relationship between the mother and her infant (2). Moreover, it increases the risk of future depression for the mother, and could negatively affect the mother and child relationship (3). Kettunen et al. (4) reported that

infants having symptoms and illnesses, especially from infantile colic, were more common among depressed than nondepressed mothers.

The prevalence of PPD has been reported as 10-15% in different countries; however, in a systematic review carried out by Halbreich and Karkun, the prevalence of PPD was 0-60% in 40 countries (5). In two Iranian cities (Tabriz and Bousher) this value was determined by the Edinburgh Postnatal Depression Scale (EPDS), with estimates of 34.7 and 15.5% as reported by Sehhatie Shafaei et al. (6) and Bagherzadeh et al. (7), respectively.

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In recent years, the impact of infertility on the mental health of couples has been more widely considered. Of note, infertility is a very unpleasant experience for many couples (8). Seibel and Taymor have reported that the frequency of psychological problems is 20-25% in infertile couples (9). The experience of using fertility methods is unpleasant and difficult for both couples, but it is harder for women because they must take most of the medications (10). Moreover, infertility treatments usually cause high levels of stress for couples (11). It has been shown that women who undergo ART experience anxiety and depression (12). Depression, anxiety, and health problems are common reactions at the time of medical treatments in infertile couples, with estimates of 19.1% in women and 14.6% in men. Multiple failures in these treatments can negatively affect self-esteem and increase depression symptoms in infertile women (11).

Studies have shown that mothers who conceive via ART are more emotionally vulnerable and have higher levels of distress compared to those who conceive naturally (13, 14). Fisher and colleagues have reported that those who conceive via ART show significantly more early parenting difficulties, which can negatively affect interactions between the mother and her infant. They conclude that these mothers need more support during pregnancy and after birth (15).

Based on our searches, there has been no study in Iran that compared PPD in mothers who conceived via ART with those who conceived naturally. Therefore, we designed this study to use the EPDS to compare the frequency of PPD and its risk factors among these groups.

Materials and Methods

This historical cohort study investigated 406 mothers of 3-9-month-old infants. We used convenience sampling methods for patient selection. The control group consisted of 308 mothers who had natural pregnancies and referred to the health centers affiliated with three main medical universities in Tehran, Iran for child vaccinations. The ART group consisted of mothers with 3-9-month-old infants conceived by ART and selected from the registry data bank at Royan Institute, Tehran, Iran. From these, we selected 98 mothers as the ART group.

The study was approved by the Ethics Committee of Royan Institute. All mothers signed a consent form before completing the questionnaire. The questionnaire was completed by each of the control group mothers. The ART mothers were contacted by telephone to complete the questionnaire. Questions that pertained to the mothers' and infants' ages, education, occupation, number of children, delivery method, history of infant hospitalization, breastfeeding, causes of infertility in women with infertility issues, and history of depression, along with the EPDS, were answered by each mother.

The EPDS is one of the most important screening tools used to identify PPD. It is a short, 10-item questionnaire that has a score from 0 to 30. Questions 1, 2, and 4 are scored from 0 to 3, whereas questions 3 and 5-10 are scored from 3 to 0 (16). Although it was developed for English-speaking populations, the EPDS has been validated in non-English populations. Montazeri et al. (16) validated the Persian version of the EPDS in an Iranian population and reported that the questionnaire was acceptable, reliable, and valid for this population with a Cronbach's alpha coefficient of 0.86 and test-retest reliability (interclass correlation coefficient) of 0.80. Based on the EPDS scores, we categorized the mothers into two groups according to their scores: non-depressed (score: 0-9) and depressed (score: ≥ 10). Mothers with total scores of ≥ 10 should be further examined for depression (17).

The sample size was calculated based on at least 4% of the clinical differences of 2.6% depressed for the ART group and 6.7% depressed for the control group in EPDS between the ART and control groups. In order to determine the sample size, the power to detect the difference and type one error were set to 0.8 and 0.05, respectively.

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) software, version 20. Results were presented as proportions (percentages) and mean $\pm$ standard error (SE) or standard deviation (SD). One-way ANOVA, followed by Tukey and Dennett's tests for multiple comparisons, were used to compare depression scores among education levels, causes of infertility, numbers of pregnancies and breastfeedings. The chi-square test was applied to compare the numbers of depressed individuals between the control and ART groups. The t test was used to compare continuous variables between normal and depressed samples. $P < 0.05$ were considered statistically significant.

Results

The mean age of the mothers was 28.87 ± 5.18 years (range: 17-51 years) and the infants had a mean age of 5.37 ± 1.30 months (range: 3-9 months). The percentage of mothers who reported PPD were 26.0% in the control group and 20.4% in the ART group, which was not statistically significant ($P = 0.26$).

The mean $\pm$ SE score for EPDS in mothers who conceived naturally was 8.38 ± 0.35 and it was 7.59 ± 0.63 for mothers who conceived via ART. The difference was not statistically significant ($P = 0.27$).

Our results showed that among mothers who conceived naturally, education level, occupation, and history of depression were significantly related to PPD. However, among mothers in the ART group, the type of delivery, history of infant's hospitalization, and history of depression had a statistical correlation with PPD (Table 1).

Table 1: Comparison between the studied variables and PPD scores in the control and ART groups

Variables	Naturally conceived	ART conceived
Education	P=0.01*	P=0.12
Less than diploma (n=58)	9.98 (0.88)	6.92 (1.09)
Diploma (n=131)	8.79 (0.53)	9.12 (1.04)
Higher than diploma (n=119) ^a	7.15 (0.52)	6.30 (1.06)
Occupation	P<0.001*	P=0.59
Employed (n=71)	5.69 (0.64)	6.95 (1.45)
Housewife (n=236)	9.14 (0.39)	7.76 (0.69)
Type of delivery	P=0.21	P=0.016*
Vaginal (n=102)	7.73 (0.59)	12.33 (3.84)
Cesarean (n=205)	8.67 (0.43)	7.44 (0.52)
History of infant hospitalization	P=0.21	P=0.016*
No (n=96)	9.04 (0.60)	10.03 (1.32)
Yes (n=210)	8.09 (0.43)	6.35 (0.61)
History of depression	P=0.015*	P=0.03*
No (n=15)	12.20 (1.63)	12.12 (1.23)
Yes (n=287)	8.21 (0.36)	7.18 (0.65)
Number of last pregnancies	P=0.99	P=0.56
One (n=277)	8.33 (0.36)	8.02 (0.77)
Two (n=18)	8.27 (1.50)	6.45 (1.08)
Three or more (n=2)	8.50 (6.50)	6.85 (2.72)
Number of children	P=0.36	P=0.13
One (n=172)	8.57 (0.47)	8.19 (0.79)
Two or more (n=131)	7.92 (0.53)	6.13 (1.0)
Baby feeding status	P=0.17	P=0.49
Breastfeeding (n=181)	8.95 (0.44)	6.93 (0.87)
Breastfeeding plus milk powder (n=91)	7.78 (0.70)	8.76 (1.37)
Milk powder (n=34)	7.29 (1.0)	7.53 (1.16)

*; Significant at P<0.05, *; Significant between groups with education levels of less than diploma and higher than diploma, SE; Standard error, ART; Assisted reproductive technology, and PPD; Postpartum depression. Data are presented as mean $\pm$ SE.

Our results also showed that causes of infertility were not associated with PPD in the ART group (Table 2).

Table 2: Comparison of PPD scores and causes of infertility

Causes of infertility	n (%)	Mean score ($\pm$ SE)	P value
Male factor	57 (58.2)	8.31 (0.86)	0.26
Female factor	19 (19.4)	4.73 (1.26)	
Both (male and female) factors	14 (14.3)	7.07 (1.24)	
Unexplained	8 (8.1)	10.12 (2.36)	

SE; Standard error and PPD ;Postpartum depression.

Discussion

We evaluated the occurrence of PPD among mothers who conceived via ART in comparison with those who conceived naturally due to the impact of mothers' PPD in caring for their infants. In our study, the occurrence of PPD was 20.4% in the ART group and 26% in the control group. These results were consistent with other studies in Iran (16-18). Montazeri et al. (16) reported 22% of women with PPD at 6-8 weeks and 18% at 12-14 weeks after childbirth. Another Iranian study reported the level of PPD in women at 30% (18).

About 13-19% of mothers who have recently given birth experience depression during the postpartum period (19). These differences in the numbers of women with PPD are probably due to differences in body mass index (20), age (21), race/ethnicity (22), cultural, social and economic status, mental health perceptions, and other environmental factors (poverty, social support or perception, nutrition, stress, and biological vulnerability) (5).

PPD is a multidimensional disorder. The determination of factors (biological, psychological, and social) that predispose a mother to PPD will help identify at-risk mothers (23). One of the factors that has been suggested to increase PPD is infertility. However, in the literature, we have found three meta-analyses on PPD and none reported pregnancy via ART as a potential risk factor for PPD (24-26). In a systematic review, Ross et al. (27) showed that the risk of a higher prevalence of PPD in mothers who become pregnant via ART was very low or unchanged in comparison to those with natural pregnancies. It seemed that women who have conceived through ART usually have a more intense emotional attachment to the fetus than women with spontaneous pregnancies (28). Although the mean EPDS score in mothers who conceived naturally was slightly higher than those who conceived via ART, we found no significant difference between PPD in both groups. However, Monti et al. (29) reported that the average PPD scores in mothers who conceived via ART were higher than those who conceived naturally, but the difference between the two groups was not statistically significant by using a cut-point of more than 12. This finding was similar to those reported by Chatziandreou et al. (30) and Listijono et al. (23). The results of meta-analyses by Gressier et al. (31) showed no increased risk of significant post-partum depressive symptoms after medically assisted conception.

Many studies have reported that the risk of PPD in mother with a history of depression is more than those without any history of depression (3, 5). Silverman et al. (32) reported that women with a history of depression were 20 times more likely to have PPD. The results of another study showed that the risk of PPD in women who had depression before delivery was 6 times higher than those without depression (33). The results of our study showed that the history of depression in mothers in both groups (control and ART) had a significant relationship with PPD, which was consistent with the results reported by Silverman et al. (32), Sadr et al. (34), and Davé et al. (35).

Our study showed a significant relationship between PPD and type of delivery in mothers who conceived by ART, which did not agree with the results reported by Sadr et al. (34). These findings supported the results of a study by Rahmani et al. (36). Kettunen et al. (4) reported a relationship between complicated delivery and PPD, especially with pain during delivery.

Vigod et al. (37) reported in their systematic review that mothers who gave birth to an infant of very pre-term or very low birth weight (LBW) had higher levels

of depression throughout the first postpartum year. The results of our study showed a significant relationship between infant hospitalization and PPD in mothers who conceived by ART. Mothers in the ART group appeared to worry more about their infants than the control group mothers because of the difficulties with conceiving (infertility, cost of infertility treatment).

The results of our study showed no significant relationship between occupational and educational status in both groups. Two previous studies have shown that working mothers probably have a protective factor for PPD (38, 39). Lewis et al. (40) observed that employed women reported less symptoms of depression than stay-at-home mothers, regardless of their weekly working hours. Sadr et al. (34) showed that there was no significant relationship between PPD and occupational and educational status.

Limitations of this study included the relatively low sample size in the ART group and limited access to this patient population since some of these women lived in other cities and only referred to Royan Institute for infertility treatment before pregnancy. In addition, we did not have consents from all of the women who became pregnant after ART at Royan Institute and could not enroll them in this study. Further studies with larger sample sizes are recommended.

Conclusion

This study reveals that the occurrence of PPD in mothers who conceived naturally is similar to those who conceived via ART. Our study has also provided evidence that levels of education, occupation, type of delivery, history of infant hospitalization, and history of depression are risk factors for PPD in mothers. These factors, rather than conception via ART, should be given further prominence in interventions to prevent PPD in women.

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Authors' Contributions

F.M., E.A.: Conception and design of study, data collection, and drafting the manuscript. Z.E., Data collection. Sh.S., M.Ch., F.M., E.A., Z.E.: Analysis and/or interpretation of data. All authors read and approved the final version of the manuscript.

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Comparison of Laparoscopic Ovarian Drilling Success between Two Standard and Dose-Adjusted Methods in Polycystic Ovary Syndrome: A Randomized Clinical Trial

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Abstract

Background: One of the treatment methods for increasing the ovarian response to ovulation induction in polycystic ovary syndrome (PCOS) is laparoscopic ovarian drilling (LOD). The optimal amount of the electrosurgical energy discharged in the ovaries to achieve maximum treatment response with minimal follicle injury is unknown. This study was performed to compare the success level of LOD by means of standard and dose-adjusted treatment methods among infertile clomiphene-resistant PCOS women.

Materials and Methods: This randomized clinical trial was conducted on infertile clomiphene citrate-resistant PCOS women in the Gynaecology Department of Imam Reza Hospital between 2016 and 2017. The patients were randomly divided into two groups based on the ovarian cautery method. The two groups were examined and compared regarding the antral follicles, the serum levels of anti-Müllerian hormone (AMH), androgens, and mid-luteal progesterone one month after surgery. The regularity of cycles, ovulation, and pregnancy were examined monthly up to six months after surgery.

Results: In total, 60 women received bilateral LOD (n=30 per group). The level of AMH (P=0.73), testosterone (P=0.91), and dehydroepiandrosterone sulphate (DHEAS, P=0.16) did not differ at study entrance and one month after ovarian cautery [P=0.94 (AMH), P=0.46 (testosterone), and P=0.12 (DHEAS)] and for postoperative mid-luteal progesterone (P=0.31). Intragroup comparisons showed a statistically significant difference in the decrease in the number of antral follicles and testosterone in the standard group (P=0.02) and AMH level in the cautionary dose-adjusted group (P=0.04). We observed no difference in cycle regularity (P=0.22), ovulation (P=0.11), and pregnancy (P=0.40) between the two groups after six months.

Conclusion: The results indicated that there was no difference between the two methods of ovarian cautery with regards to establishing cycle regularity and ovulation. The standard treatment was effective in decreasing the numbers of antral follicles and testosterone levels, whereas the dose-adjusted method significantly affected the decrease in AMH levels (Registration Number: IRCT20171210037820N1).

Keywords: Anti-Müllerian Hormone, Infertility, Polycystic Ovary Syndrome

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Introduction

Polycystic ovary syndrome (PCOS) was initially reported by Stein and Leventhal (1) in modern medical texts when they described seven women who suffered from amenorrhea, hirsutism, and enlarged ovaries that contained several cysts. This syndrome is now considered to be a common, heterogeneous, and hereditary disorder which can affect women of reproductive age. The prevalence of PCOS may vary based on the applied diagnostic criteria (2, 3). The highest rate of PCOS has been reported at 52% among West Asian women having migrated to England (4); in other references, this figure

has been reported as 2-26% (3). Infertility involves 40% of the cases affected by PCOS (5).

Clomiphene citrate is the first treatment option for inducing ovulation in these women (6-8); however, drug resistance has been observed in around 20% of such cases (8). Clomiphene resistance is defined as three cycles of ovulation failure or six cycles without pregnancy (9). One of the alternatives used among clomiphene-resistant women is laparoscopic ovarian drilling (LOD); particularly, in cases which the patient has other surgical indications or when she is unable to

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attend the frequent visits required for treatment with gonadotropins (10).

Historically, the surgical treatment of infertile PCOS women reported by Stein and Leventhal (1) in 1935 was ovarian wedge resection via laparotomy, and it showed promising results. However, three decades later, this method was abandoned due to the risk of pelvic adhesions following surgery and has been replaced by ovulation-inducing medications such as clomiphene and gonadotropins (11). In 1984, the surgical treatment of infertile PCOS women improved remarkably with the introduction of LOD that had an ovulation success rate of 92% and pregnancy success rate of 80% (12). LOD, as a less harmful and less-invasive method compared to ovarian wedge resection, uses electrocautery (diathermy) or laser beam and has played a significant role in the treatment of infertile PCOS women (11).

The beneficial effects of this method seem to be related to the destruction of the androgen generating stroma, which results in reduced production of androgens in the ovary and its reduced concentration in the blood circulation. Clomiphene citrate-resistant women may respond better to medical therapy after this type of surgery. Sensitivity to exogenous gonadotropins also increase in such cases (13).

Several studies have evaluated ovarian cauterization. In the initial studies, it was hypothesized that a higher energy level would result in a more efficient procedure. Subsequently, lower temperatures with a fixed number of drilled points, regardless of the ovary's size or unilateral ovarian cautery have been reported with the intent to reduce a possible risk of ovarian atrophy and adnexal adhesions. With such fixed doses of temperature, the optimal amount of ovulation may not be achieved or the clinical manifestations of the disease may persist in individuals with enlarged ovaries (14). Armar et al. (15) reported the first descriptive research on ovarian drilling with 4 drills at a dose of 640 joules per ovary; this method was later widely accepted and used in various studies. Many authors subsequently examined and compared the effects and consequences of changes in the number of ovarian drills or the appropriate thermal dose based on the ovarian size during laparoscopic ovarian cauterization (14-22). However, in some studies, the relationship between the number of ovarian drills and adnexal adhesion was not confirmed (23). Nevertheless, the optimal amount of electrosurgical energy required during LOD to achieve the maximal fertility outcome without causing any risk to the follicles and ovaries has not been established (24).

We designed this study because of the inadequate number of studies in this area (particularly in Iran) and by taking into consideration the influence of genetic, regional and nutritional factors on PCOS. We sought to compare the effect of ovarian cauterization between the standard and dose-adjusted (based on the ovarian volume) methods in Iranian women with infertile clomiphene-resistant PCOS.

Materials and Methods

This randomized clinical trial was conducted in the Gynaecology Department of Imam Reza Hospital, Mashhad, Iran from 2016 to 2017. All infertile clomiphene-resistant PCOS women who visited the Gynaecology Department enrolled in this study. The sample size of this study was calculated at 30 women according to the following formula and by taking into consideration information from a previously published study (14), with an alpha error=0.05, beta error=0.8, P1=0.6, and P2=0.9.

$$n = \frac{Z_{1-\alpha/2} + Z_{1-\beta})^2 (P_1(1-P_1) + P_2(1-P_2))}{(p_1 - p_2)^2} =$$

The achieved power of this study was 37% based on the antral follicle count (AFC).

Ethical observations

At study initiation, the study protocol was fully described to each patient and they were free to withdraw from the study at any time. Their data was regarded as confidential. All patients signed a written informed consent to participate in the study. The Ethics Committee of Mashhad University of Medical Sciences approved this study (IR.MUMS.fm.REC.1395.335). The study was registered in the Iranian Registry for Clinical Trials (IRCT20171210037820N1).

The inclusion criteria were: all women aged 18 to 35 years, not pregnant despite two years without contraception, diagnosed with PCOS based on the Rotterdam criteria, having ruled out other reasons of infertility except for ovulation disorder (normal sperm analysis of the spouse, normal uterine tubes in hysterosalpingography or laparoscopy), clomiphene-resistant, and provided consent to participate in this study.

Exclusion criteria were: withdrawal during the study, patients lost to follow-up, presence of any other pathology during laparoscopy (e.g., endometriosis or adhesion) suggestive of other aetiologies for infertility.

Initially, we recorded the patients' demographic characteristics and paraclinical data by means of an interview and the patient's records. We divided the patients into two groups according to a table of random number generator with equal sizes of groups: standard method (group A) and ovarian cautery based on the ovarian volume or the dose-adjusted method (group B). One radiologist performed the transvaginal ultrasonography (TVS) for group B patients by using a Honda sonography device (Honda Electronics, Japan) to measure ovarian volume. This volume was measured on the basis of a cubic centimetre and at three perpendiculars.

A gynaecology laparoscopist performed each laparoscopy via an Olympus laparoscopic machine (Olympus Europa SE & Co., Germany) in the gynaecology theatre of Imam Reza Hospital with patients under general anaesthesia and in the lithotomy position. Abdominal entry was done by the closed technique and

via a Veress needle. Only patients who had any history of abdominal surgery had an open laparoscopic procedure. A triple puncture laparoscopy was performed with 3 trocars. The abdominal and pelvic environment, and the patency of the tubes were examined. Patients with adhesions, endometriosis, or any pathology in the pelvic area were excluded from the study.

Next, the ovarian cautery was performed. The utero-ovarian ligament was caught with an Atraumatic Grasper (Aesculape Inc., USA) and the ovary was separated from the intestines. Afterwards, the ovarian cautery was carried out with a 4-millimetre monopolar needle electrode (with a straight needle) and with a Vallylab generator that had a voltage of 30 (in both groups) as follows: using the CUT energy, a puncture with the depth of 4 millimetres was initially created on the ovarian capsule and then the coagulation button was activated. After the cautery of each ovary and before releasing the utero-ovarian ligament, the ovary was rinsed with cold normal saline serum to prevent any adhesion or injury to the adjacent viscera. The ligament was then released and examined with regards to the possibility of mechanical injury.

In group B (on the basis of ovarian volume), the measurement of energy was based on the following model (15, 16, 18, 25) that used 640, 450, 600, and 800 joules for each ovary (mean: 625 joules) and ovarian volume means of 8 and 10 cm³. The dose of 60 joules was chosen for each cubic centimetre of the ovarian mass. The ovarian mass was multiplied by 60 joules. In order to achieve the correct time, we multiplied the ovarian volume by 2 and measured the number and time of each puncture as follows: Energy=Power (voltage)×Time (number of punctures×time of each puncture) and by taking into account that the generator's energy for all individuals was 30 joules.

In group A, based on the size of the ovary, we created either 4 drills of 5 s or 5 drills of 4 s with a voltage of 30 in order to achieve an energy of 600 joules per ovary (4×5×30=600).

The patients were followed for six months from the first menstrual cycle after the operation. Hormonal levels of anti-Müllerian hormone (AMH), testosterone, dehydroepiandrosterone sulphate (DHEAS), and progesterone were obtained on the third day of the first menstrual cycle after the operation and the progesterone level was measured at the mid-luteal phase of the same cycle. All tests were performed in the same laboratory and without charge.

TVS was also performed from the sixth day of the first menstrual cycle after the operation, every three days up to the 16th day or until the observation of a dominant follicle. Ovulation was confirmed by the observation of an 18 mm dominant follicle or pregnancy. TVS was performed by the same radiologist as before the LOD. In the event of anovulation, subsequent sonographies were not performed. Cycle regularity and the occurrence of pregnancy were examined after six months.

Patients who did not menstruate until a month after ovarian cautery (menstrual cycle over 35 days) were administered 100 mg of intramuscular micronized progesterone to re-establish the menstrual cycle, and the investigations were performed though most of these cases had no ovulation.

Statistical analysis

We performed SPSS software (version 16) analyses of the raw data. If the quantitative variables were normally distributed, we used the t test and paired t test; for non-normally distributed data, the corresponding non-parametrical tests were used. The chi-square test was used to examine qualitative variables. Repeated measure ANOVA was performed to assess the interaction and overall effect of before/after assessments in the two groups. The significance level was set at P<0.05.

Results

In this study, 60 infertile clomiphene-resistant PCOS women received LOD by two methods: standard and dose-adjusted on the basis of the ovarian volume. The demographic, clinical and sonographic characteristics did not differ between the two study groups (Table 1, Fig.1).

Table 1: Comparison of demographic, clinical, and sonographic data of infertile clomiphene-resistant PCOS women between the two treatment groups

Variable	Standard n=30	Dose-adjusted n=30	P value
Age (Y)	26.36 ± 4.69	28.53 ± 5.84	0.11
History of infertility (Y)	4.42 ± 2.77	4.84 ± 2.73	0.62
Type of infertility			0.79
Primary	17 (56.6)	18 (60)	
Secondary	13 (43.4)	12 (40)	
Clinical manifestations			0.58
Oligomenorrhea	20 (66.7)	19 (63.3)	
Oligomenorrhea+Hirsutism	1 (3.3)	3 (10)	
Hirsutism	9 (30)	8 (26.7)	
Regularity of cycles			0.61
Regular	9 (30)	8 (26.7)	
Irregular	21 (70)	22 (73.3)	
Sonography findings			--
Volume of right ovary (cm ³)	--	15.02 ± 7.46	
Volume of left ovary (cm ³)	--	13.34 ± 5.87	
Endometrial line (mm)	--	6.56 ± 1.93	
AFC	16.33 ± 2.53	16.80 ± 1.99	0.43
Hormonal profile			
AMH (ng/ml)	7.87 ± 4.86	7.46 ± 4.45	0.73
Testosterone (ng/dl)	80.52 ± 40.80	81.46 ± 29.14	0.91
DHEAS (µg/dL)	173.86 ± 73.32	201.34 ± 77.76	0.16

Data represented as mean ± SD or frequency (%) as appropriate. PCOS; Polycystic ovary syndrome, AMH; Anti-Müllerian hormone, AFC; Antral follicle count, and DHEAS; Dehydroepiandrosterone sulphate.

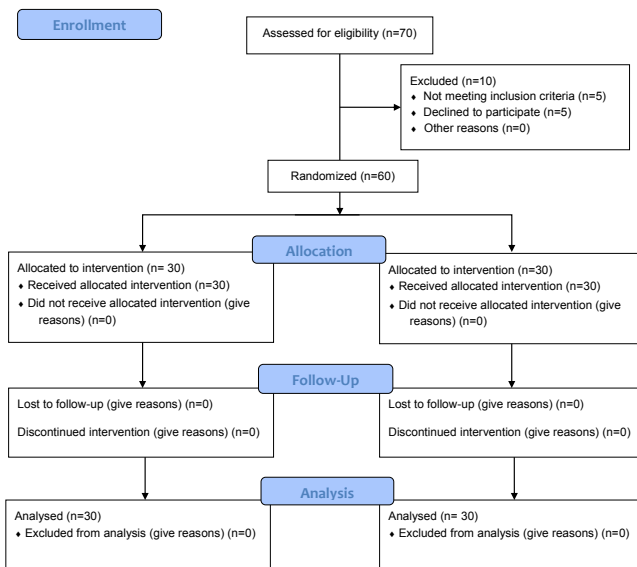


Fig.1: Flowchart.

Table 2 shows the mean number of points and the time of cautery in the dose-dependent group for each ovary.

Table 2: Mean numbers of points and cautery time per ovary in the dose-dependent group

Variable	Right ovary	Left ovary
No. of points	5.93 ± 1.66	5.59 ± 1.68
Time (seconds)	4.07 ± 0.45	4.24 ± 0.57

Data are presented as mean ± SD.

Table 3 shows a comparison of the mean AFC and serum levels of AMH, testosterone, and DHEAS between the two groups, before and after the operation.

Table 4 displays the mean changes in the AFC and serum levels of AMH, testosterone, and DHEAS before and after the operation in each of the studied groups. Repeated measure ANOVA revealed that there was no interaction, nor any difference between the two study groups in terms of AFC ($P=0.14$), AMH ($P=0.71$), testosterone ($P=0.67$), and DHEAS ($P=0.12$).

The number of antral follicles before the operation was not significantly different between the two groups (independent t test, $P=0.43$). The same result was obtained after the operation ($P=0.10$). Intra-group comparisons showed that the decrease in the number of antral follicles was significant in the standard treatment group (paired t test, $P=0.02$); however, we did not observe any difference in the dose-adjusted group ($P=0.24$).

Before the intervention, the two groups were matched in terms of AMH level (independent t test, $P=0.73$). We observed the same result after the intervention (Mann-Whitney test, $P=0.94$). In the intra-group comparison, there was a significant decrease in the AMH level in the dose-adjusted group (paired t test, $P=0.04$); however, this difference was not observed in the standard treatment group (paired t test, $P=0.17$).

Before the intervention, the testosterone level was similar in the two groups (independent t test, $P=0.91$). The same result was obtained after the intervention ($P=0.46$). However, the decrease in testosterone level in the standard treatment group was meaningful (paired t test, $P=0.02$), but this difference was not observed in the dose-adjusted group (paired t test, $P=0.14$).

Table 3: Comparison of the hormone profile and AFC between the study groups

Variable	Before surgery			After surgery		
	Standard n=30	Dose-adjusted n=30	P value	Standard n=30	Dose-adjusted n=30	P value
AFC	16.33 ± 2.53	16.80 ± 1.99	0.43	15.10 ± 2.97	16.27 ± 2.55	0.10
AMH (ng/ml)	7.87 ± 4.86	7.46 ± 4.45	0.73	7.08 ± 4.28	6.71 ± 3.32	0.94
Testosterone (ng/dl)	80.52 ± 40.80	81.46 ± 29.14	0.91	71.28 ± 36.17	77.37 ± 26.68	0.46
DHEAS (µg/dL)	173.86 ± 73.32	201.34 ± 77.76	0.16	160.51 ± 60.36	189.13 ± 80.33	0.12

AFC; Antral follicle count, AMH; Anti-Müllerian hormone, and DHEAS; Dehydroepiandrosterone sulphate. Data represented as mean ± SD.

Table 4: A comparison of the changes in the hormone profile and AFC before and after the operation in the two groups

Variable	Standard			Dose-adjusted		
	Before surgery	After surgery	P value	Before surgery	After surgery	P value
AFC	16.33 ± 2.53	15.10 ± 2.97	0.02	16.80 ± 1.99	16.27 ± 2.55	0.24
AMH (ng/ml)	7.87 ± 4.86	7.08 ± 4.28	0.17	7.46 ± 4.45	6.71 ± 3.32	0.04
Testosterone (ng/dl)	80.52 ± 40.80	71.28 ± 36.17	0.02	81.46 ± 29.14	77.37 ± 26.68	0.14
DHEAS (µg/dL)	173.86 ± 73.32	160.51 ± 60.36	0.16	201.34 ± 77.76	189.13 ± 80.33	0.08

AFC; Antral follicle count, AMH; Anti-Müllerian hormone, and DHEAS; Dehydroepiandrosterone sulphate. Data represented as mean ± SD.

In addition, both at study initiation and study termination, the level of DHEAS did not significantly differ between the two studied groups (independent t test, $P=0.16$ at study initiation, $P=0.12$ at study termination). In the intra-group comparisons, the level of DHEAS decrease was not significant in either group (paired t test, $P=0.16$ in the standard group and $P=0.08$ in the dose-adjusted group).

The status of cycle regularity and the occurrence of ovulation and pregnancy among patients were examined from the first post-surgical cycle up to six months. The obtained results are presented in Table 5.

There were regular menstrual cycles reported in 25 (83.3%) patients in the standard treatment group and 21 (70%) patients in the dose-adjusted group. Accordingly, there was no statistically significant difference observed between the two groups (chi-square test, $P=0.22$).

Ovulation occurred in 26 (86.7%) patients in the standard group and in 21 (70%) patients from the dose-adjusted group, which was not statistically significant (chi-square test, $P=0.11$).

Finally, 11 (36.7%) patients in the standard group and 8 (26.7%) patients in the dose-adjusted group became pregnant during 6 months, which indicated no meaningful difference between the two groups (chi-square test, $P=0.40$).

Intragroup comparisons on cycle regularity indicated a significant increase after the operation compared to before the operation in the standard treatment (30 vs. 83.3%) and dose-adjusted (26.7 vs. 70%, $P<0.001$) groups.

No case of premature ovarian failure was observed in our study population. Cycle regularity was experienced in 9 (30%) patients in the standard group before the operation and in 25 (83.3%) patients after the operation. In the dose-dependent group 8 (26.7%) patients had cycle regularity before the operation and 21 (70%) had cycle regularity after the operation. This was a significant change in both groups ($P<0.001$).

Table 5: A comparison of cycle regularity, ovulation, and pregnancy between the study groups

Variable	Standard n=30	Dose-dependent n=30	P value
Regularity of cycles			0.22
Regular	25 (83.3)	21 (70)	
Irregular	5 (16.7)	9 (30)	
Ovulation			0.11
Yes	26 (86.7)	21 (70)	
No	4 (13.3)	9 (30)	
Pregnancy			0.40
Yes	11 (36.7)	8 (26.7)	
No	19 (63.3)	22 (73.3)	

Data represented as frequency (%).

We measured progesterone levels in both groups in the first postoperative menstrual cycle. There was no significant difference between the two groups ($P=0.11$). However, the mean progesterone level in patients with (12.44 ± 2.20) and without ovulation (2.93 ± 0.20) was significantly different ($P<0.001$). No case of early ovarian failure was observed in the studied subjects.

Discussion

The results of our study indicated no difference in the number of antral follicles after the operation in both groups. However, the decrease in the number of antral follicles was significant in the standard treatment group. Such results corresponded to those reported by Nasr et al. (26) who observed a meaningful decrease in the number of antral follicles in the ovarian cautery group that had a fixed dose. However, no decrease in the numbers of follicles and the ovarian volume was observed in the ovarian cautery performed with a Harmonic scalpel group. The authors believe that the decrease in the number of antral follicles or the ovarian volume is caused by the adjacent thermal destruction created by the use of electrocautery. The creation of one puncture destroys the ovarian tissue as deep as 4 mL; thus if 4 punctures are made in each ovary, 3.2 mL of the ovarian tissue will be destroyed. The Harmonic scalpel minimizes the amount of ovarian tissue destruction (0.50 mL), which is about 1/8 of the destruction done by the electrocautery. Salem et al. (27) have considered the decrease in the number of follicles as the undesired consequence of LOD. They indicated that the amount of AMH and numbers of antral follicles were reliable indicators of the ovarian reserve. Their measurement in clomiphene-resistant PCOS women without ovulation could be a useful indicator to assess the treatment outcome of LOD. In our study, the dependency of the energy used by the cautery to the ovarian volume might have led to the selection of a more appropriate amount of energy for the ovarian cautery, and therefore caused less damage to the ovarian tissue.

AMH is one of the new predictive indicators of ovarian reserve (28). This hormone can be used as a substitute for determining the age of ovaries because it is related to the number of initial antral follicles, which can reflect the number of residually stored follicles (29). The current study findings indicated a decrease in the AMH, testosterone, and DHEAS levels in both the standard and dose-adjusted groups. The decrease in the amount of AMH in the dose-adjusted group and the decrease in the amount of testosterone in the standard treatment group were statistically significant. These findings did not fully correspond to the results of similar studies. This could be due to the difference in study design, sample size, or genetic and regional differences among the studied patients. Sunj et al. (30) had a vast inclusion criteria that included variables such as weight, acceptable hormonal range, infertility period, etc. in selection of their study population. This could result in decreased generalizability of the achieved results. In their study, only women with an infertility period of fewer than 3 years participated in the research, while the mean infertility period in our study was 4.7 years. Therefore, one of the reasons for the heterogeneity of the results might be the difference in patient selection due to differences in the inclusion criteria. The results of another study on the changes in AMH, testosterone, and free androgen index by unilateral (dose-adjusted) and bilateral (fixed dose) ovarian diathermy revealed a significant decrease in AMH, testosterone, and LH levels in both treatment groups. Amer et al. (31)

and Elmashad (32) also reported significant decreases in AMH levels following LOD. However, Farzadi et al. (33) reported no such relationship. The meaningful decreases in serum levels of FSH, LH, AMH, testosterone, and free androgen index following LOD were also reported in the study by Salem et al. (27).

Given that the increase in androgens in PCOS is the result of the insulin's ability to increase the secretion of androgens in ovarian theca cells, the remarkable decrease in the level of androgens after drilling among patients who receive cauterization with a volume dependent dose might be justified by the hypothesized destruction of androgen generating stromal cells. It is believed that the effects of LOD on androgen levels are influenced by the amount of energy entrapped by the ovaries and, for this reason, low doses may be less successful (13).

Both groups had a nonsignificant decrease in DHEAS levels. A review of previous literature has revealed that the existing data on DHEAS are ambiguous. LOD seems to have a minimal effect on adrenal function, even among women affected by hyperinsulinemia, and the improvement of hyperandrogenism is probably secondary to the decrease in LH concentration and reduced androgen production by the ovarian stroma (34).

In our study, the regularity of menstrual cycles increased from 30 to 83.3% in the standard treatment group. The regularity of menstrual cycles in the dose-adjusted group increased from 26.7 to 70%. This was a significant increase in both groups. The intra-group changes were different compared to the Zakherah et al. (14) study. In their study, the cycle's regularity was higher in the dose-adjusted cautery group (87.9%) compared to the fixed-dose cautery group (75.4%); however, similar to our study, its effect on the regulation of the cycles was significant.

In a study by Nasr et al. (26), the occurrence of regular cycles after LOD was similar in both groups (92.8%) and higher than our study results. Takeuchi et al. (35) reported that a regular menstrual pattern was established in 94% of the patients and the rate of oligomenorrhea decreased to 6%. Felemban et al. (16) observed that the occurrence of regular cycles was 80.4% and oligomenorrhea was 19.6% in patients after ovarian cautery. However, Salem et al. (27) reported that among 37 clomiphene-resistant PCOS patients, the cycles regularization was 16.22% three months after the ovarian cautery and 54.06% after six months. Some authors believe that such differences could be due to the different definitions used for the diagnosis of PCOS or the differences in the study populations (26).

In our study, ovulation occurred in 86.7% of patients in the standard treatment group and 70% of those in the dose-adjusted group; 36.7% of patients in the standard treatment group and 26.7% in the dose-adjusted group became pregnant. The findings of our study contradicted those reported by Zakherah et al. (14). In the latter study, the rate of ovulation (81.8 vs. 62.2%) and pregnancy (51.7 vs. 36.8%) in the volume-dependent ovarian cautery group was significantly higher than the fixed thermal

dose group. The authors concluded that the adjusted thermal dose on the basis of ovarian volume (60 joules/cm³) in LOD resulted in improved fertility consequences in comparison to the fixed thermal dose (600 joules per ovary) among clomiphene-resistant PCOS patients. The difference between the results of this study and our research might be due to the differences in sample size or racial and regional characteristics. As with our study, the measurement of the ovarian volume was not done in the standard treatment group. Possibly, the ovarian volume of these patients was more or identical to the patients of the dose-adjusted group; therefore, the same intervention might have been done for the patients in both groups.

In a study by Salem et al. (27), 4 (10.81%) pregnancies occurred after three months and 18 (48.65%) after six months, which were less than our study. They mentioned various reasons for the low rate of pregnancy occurrence among their study patients, which included the existence of subtle aetiologies such as hyperprolactinemia, minor anatomical problems, and male reasons such as varicocele. He also mentioned inadequate drilling to induce optimal changes in fertility parameters.

Ramezani et al. (36) examined the cumulative effect of pregnancy after cauterization of polycystic ovaries in clomiphene-resistant patients at Imam Khomeini Hospital in Karaj, Iran, with the following pregnancy rates after surgery: 14.7% (6 months), 36.8% (12 months), 58.8% (18 months), and 76.6% (24 months). However, in this study, the fixed dose method was used for ovarian cauterization in all patients. Although the rate of pregnancy after 6 months (14.7%) was less than the pregnancy rate achieved in our study (26.7% for the dose-adjusted group and 36.7% for the standard group), the rate of pregnancy after 12 months was very close to that of our standard treatment group after 6 months.

Our study had certain limitations; the small sample size which led to a low power, decreased cooperation of patients for the ultrasound study and the postoperative lab tests, as well as the impossibility of performing TVS in all subjects due to limited facilities in this center.

Conclusion

The results of this study indicated a significant decrease in antral follicles and testosterone in the standard treatment group in comparison to the dose-adjusted group along with a significant decrease in AMH level in the dose-adjusted group. The changes in DHEAS were insignificant in both groups.

Cycle regulation, and the occurrence of ovulation and pregnancy showed that both methods were efficient; however, there were no statistically significant differences. In terms of the effects of ovarian cautery on these variables, neither of the two methods was superior. It is possible that the small number of samples examined and the differences in the sample selection method or the racial and regional differences might have led to the difference in the results of our study with previous researches. Therefore, conducting similar regional studies with a larger sample sizes are highly recommended.

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Authors' Contributions

L.H., M.A., Gh.Gh.; Study design. Y.D., M.J., Gh.Gh.; Data gathering and writing manuscript draft. L.H., M.A., M.J., Gh.Gh.; Analysis and interpretation. All authors read and approved the final manuscript.

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The Effect of Vaginal Sildenafil on The Outcome of Assisted Reproductive Technology Cycles in Patients with Repeated Implantation Failures: A Randomized Placebo-Controlled Trial

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Abstract

Background: The aim of this study was to investigate the effects of vaginal sildenafil on the outcome of patients with at least two unsuccessful *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) attempts.

Materials and Methods: In this randomized placebo-controlled trial study, a total of 66 infertile women aged ≤ 38 years, with a history of normal ovarian reserve, two prior consecutive failed IVF/ICSI attempts, human chorionic gonadotropin (hCG) day endometrial thickness < 7 mm in all prior IVF/ICSI cycles, normal endometrial appearance by either hysteroscopy, hysterosonography, or hysterosalpingography enrolled in this study. The conventional gonadotropin-releasing hormone (GnRH) protocol was used for ovarian stimulation. The patients were randomly divided into three groups: vaginal sildenafil (suppository-100 mg/daily), vaginal placebo/sildenafil (suppository-100 mg/daily), and vaginal placebo (suppository). Each patient underwent colour Doppler ultrasound on day 14 of their previous cycle to investigate any abnormalities in the uterus and adnexa. Endometrial thickness, echo pattern, uterine artery resistance, and pulsatility indices were recorded pre- and post-treatment. The primary outcome measures were implantation, chemical and clinical pregnancy rates. For data analysis, SPSS version 20 software was used. In all tests, the significance level was considered less than 0.05.

Results: There was no significant difference between three groups in endometrial thickness on the hCG injection day. The chemical pregnancy in women who received sildenafil (alone or in combination with placebo) showed a two-fold increase in comparison to the placebo group. This increase was clinically meaningful, but according to sample size, it was statistically non-significant. The results of our study showed that the implantation was higher in women who received placebo/sildenafil compared to the other groups. The abortion rate was not statistically significant among the groups.

Conclusion: Vaginal sildenafil may conceivably improve chemical pregnancy rates in repeated IVF failure patients. Further randomized clinical trials using oral or vaginal sildenafil with higher sample size are recommended (Registration number: NCT03192709).

Keywords: Outcome, Repeated Implantation Failure, Sildenafil Citrate, Thin Endometrium

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Introduction

Successful implantation of an embryo requires a receptive endometrium, a good quality embryo, and embryo-endometrial synchronization in all species (1).

Endometrial receptivity during the implantation win-

dow is often assessed by ultrasonography markers such as endometrial thickness, echogenic pattern, blood flow, and biochemical markers (2). An appropriately thickened endometrium is a crucial factor for embryo implantation and can predict pregnancy outcome with high sensitivity and specificity (3). Moreover, several studies have reported a

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significant positive correlation between a triple-layered thickened endometrium of 7 mm (preferably >9 mm) and pregnancy rate (3-5).

Despite significant advances in ovarian stimulation protocols, treatment of repeated, unresponsive thin endometrium is still a challenge in assisted reproductive cycles, which usually results in cycle cancellation or repeated implantation failures (6).

There is a growing body of evidence that the endometrial growth has a relationship with the state of uterine blood flow (2). Treatment options, such as low-dose aspirin, oestradiol administration and gonadotropin therapy, low dose human chorionic gonadotropin (hCG), vitamin E, pentoxifylline, L-arginine, luteal phase support with gonadotropin-releasing hormone (GnRH) agonist, intrauterine granulocyte colony-stimulating factor (G-CSF), vaginal sildenafil, and recent application of stem cell therapy are suggested for management of a thin lining endometrium (7).

Sildenafil citrate (Viagra®, Pfizer, NY, USA) is a 5-phosphodiesterase inhibitor that increases smooth muscle relaxation and vasodilation by preventing cGMP breakdown (8). Sildenafil citrate potentiates uterine blood flow and, in conjunction with oestrogen, it leads to the oestrogen-induced proliferation of the endometrium (9).

It has been reported that vaginal sildenafil significantly reduced peripheral natural killer cell (NK-cell) activity and improved successful pregnancy rates in women with histories of recurrent miscarriages. Although the mechanism of influence by sildenafil on natural killer cell activity is unclear, it seems that enhancement of uterine artery flow has an effective influence on the local endometrial NK-cell population (10). However, adverse effects that include myocardial infarctions and strokes have been associated with this drug in the normal healthy population (11, 12).

This comparative pilot study evaluated the effect of vaginal sildenafil suppositories on endometrial proliferation and IVF outcome in infertile patients with a history of repeated IVF failure.

Materials and Methods

Patients

This phase II randomized, double-blind, placebo-controlled trial was performed at Royan Institute, Reproductive Biomedicine Research Center, Tehran, Iran, between February 14, 2014 and November 14, 2016 (NCT03192709). The assessors and patients were not aware of the treatment allocated. The Institutional Review Board and Ethics Committee of Royan Institute, Tehran, Iran reviewed and approved this study in compliance with the Declaration of Helsinki (EC/88/1045). Informed consent was obtained from all patients prior to their participation in the study.

The study population consisted of 66 infertile women aged ≤ 38 years. The inclusion criteria was met when the women had normal ovarian reserve [blood anti-müllerian hormone (AMH) levels >1.5 ng/mL] with at least two

prior cycles with follicle stimulating hormone (FSH) <10 mIU/mL; a history of two prior consecutive failed IVF/ICSI attempts with at least a transfer of two good quality fresh or frozen-thawed embryos; hCG day endometrial thickness <7 mm in all prior IVF/ICSI attempts; and normal endometrial appearance according to either hysterosonography, hysterosalpingography, or hysteroscopy. Women were excluded if they had a history of myomectomy or Asherman's syndrome.

Treatment cycle

A preliminary colour Doppler transvaginal sonography with a 4-8 MHz probe (ProSound Alpha 10; Aloka, Japan) was performed by an expert radiologist on day 14 of the patient's prior menstrual cycle to investigate the uterine and adenexes for any abnormal findings. The endometrial parameters of endometrial thickness, endometrial pattern, pulsatility index (PI), and resistance index (RI) were measured. The uterine artery PI and RI were obtained through flow velocity waveforms from the ascending branch of the uterine artery at the point near to the internal cervical orifice and calculated as previously described (13). In the subsequent cycle, ovarian stimulation was performed with the long protocol using a GnRH agonist (14).

In the IVF treatment cycle, the patients were randomly assigned to three groups according to a random allocation sequence generated by a randomized block design. The size of each block was 3. In group A, the sildenafil (vaginal suppositories, 100 mg/day, Parnian Daroo, Co., Tehran, Iran) were administered from the first day of the FSH injection until the day of oocyte retrieval. In group B, placebo (vaginal suppositories, Parnian Daroo, Co., Tehran, Iran) was initiated from the first day of the HMG injection until 2 days before the hCG injection, after which sildenafil vaginal suppositories were initiated and continued until the day of oocyte retrieval. In group C, the placebo was given from the first day of HMG injection until the day of oocyte retrieval. Participants received 10 000 IU of hCG (Choriomon, IBSA, Switzerland) when at least two dominant follicles were 18 mm in diameter. Endometrial thickness, pattern, PI and RI were measured on the day of hCG administration and compared with the data obtained in the previous cycle without sildenafil citrate treatment. Oocytes were retrieved 36 hours later via transvaginal ultrasound-guided needle aspiration.

Embryo transfer was performed after 48 hours of oocyte retrieval. Progesterone in oil (100 mg, IM daily) or intravaginal progesterone (400 mg, twice daily) was used for luteal support and maintained until the pregnancy test was conducted. Serum hCG levels were measured on the 14th day following oocyte retrieval. Vaginal ultrasound confirmation of pregnancy was performed at 4-6 weeks after embryo transfer.

A chemical pregnancy was determined by a positive β -hCG test result. A clinical pregnancy was confirmed by ultrasonography visualization of one or more gestational sacs or definitive clinical signs of pregnancy. The spontaneous abortion was defined as a pregnancy

loss of an intrauterine pregnancy before 22 weeks' gestation (15).

Suppositories containing 100 mg of sildenafil were prepared from the oral tablets by a local pharmacy (Par-nian Daroo, Iran). We defined the endometrial thickness threshold cut-off of <7 mm as a thin endometrium based on other studies (8).

Statistical analysis

Data are expressed as mean $\pm$ standard error (SE) and proportion. Continuous and categorical outcome variables were compared between three intervention groups by one-way analysis of variance (ANOVA) and the chi-square test. All statistical analyses were performed using SPSS version 22 for Windows 7 (IBM Analytics, Armonk, NY). The significance level was set at 0.05.

Results

During the recruitment process, we enrolled 66 patients and allocated 22 patients to each study group. A total of 10 patients were lost to follow up for measuring clinical pregnancy for the following reasons: all of the embryos of their treatment cycle were cryopreserved ($n=3$), they had no oocytes at retrieval ($n=2$), or no embryos to transfer ($n=5$). The flow diagram explicitly shows the number of participants at the beginning, intervention allocation, follow-up, and analysis (Fig.1).

Table 1 shows the baseline characteristics of the three study groups. There were no significant differences among the study groups. The majority of patients had

normal endometrial patterns, which were similar between the three study groups (Table 1). Uterine artery PI and RI in the left and right were not statistically significant among the study groups ($P>0.05$). Table 1 shows the results of the ovulation stimulation cycle and include initial endometrial thickness, number and type of ampoules used, time interval for ovulation stimulation, and numbers of total and metaphase II (MII) oocytes. These parameters did not differ significantly among the three intervention groups. Table 2 shows numbers of embryos transferred in total and by grade (A, B and C). These values were not different among the groups. The generated embryos were graded as good (A and B) or poor (C) according to their morphological features, cleavage stage, multi-nucleation, equal size blastomeres, and fragmentation rate (16).

Of note, the embryo transfer days were similar between the three groups. All of the embryos were transferred either two or three days after ovum pickup.

Comparisons after the interventions

The endometrial thickness and patterns after the interventions were not statistically different between the study groups. Additionally, the three intervention groups were not different in left and right uterine artery PI and RI. Implantation rate was not statistically significant over the three groups ($P=0.290$). Clinical pregnancy rates were 33.3 (sildenafil), 33.3 (sildenafil+placebo), and 17.6 (placebo) as seen in Table 2. Although the clinical pregnancy rates varied among the intervention groups, they were not statistically different (Table 3). Vaginal administration of sildenafil had no adverse effects on the patients during the study.

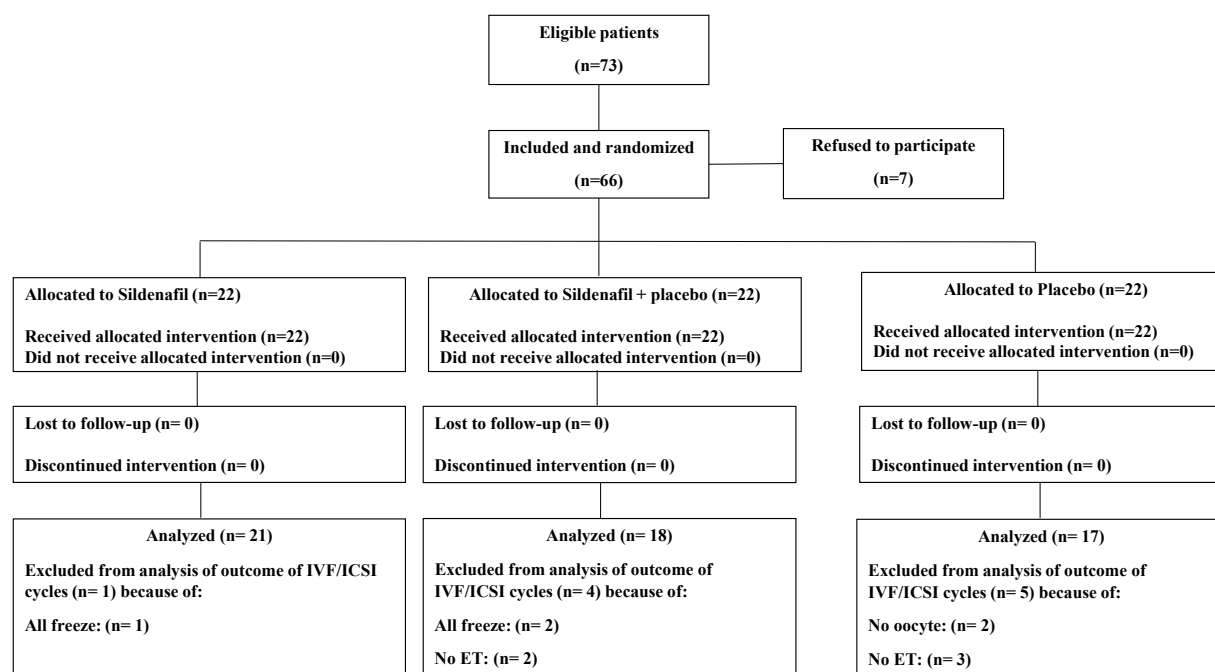


Fig.1: Flowchart of participants in this randomized controlled trials. IVF/ICSI; *In vitro* fertilization/intracytoplasmic sperm injection and ET; Embryo transfer.

Table 1: Comparison of baseline characteristics and cycle related factors between the sildenafil, sildenafil + placebo, and placebo groups prior to intervention

Variable	Sildenafil n=22	Sildenafil+placebo n=22	Placebo n=22	P value
Age (Y)	33.2 ± 4.6	31.7 ± 4.8	32.8 ± 4.6	0.568
BMI (kg/m ²)	24.7 ± 3.7	26.2 ± 3.6	25.2 ± 2.9	0.379
Infertility duration	8.8 ± 4.9	10.5 ± 5.1	8 ± 4.1	0.220
Infertility type				
Primary	19 (86.4)	20 (90.9)	17 (77.3)	0.438
Secondary	3 (13.6)	2 (9.1)	5 (22.7)	
Infertility reason				
Tubal factor	1 (4.5)	1 (4.5)	1 (4.5)	0.910
Male factor	14 (63.6)	11 (50)	11 (50)	
Endometriosis	0 (0)	0 (0)	1 (4.5)	
Unexplained	1 (4.5)	2 (9.1)	1 (4.5)	
Two or more	6 (27.3)	8 (36.4)	8 (36.4)	
Endometrial pattern				
Normal	18 (81.8)	18 (81.8)	19 (86.4)	0.683
Heterogenic	2 (9.1)	3 (13.6)	3 (13.6)	
Ecogene	2 (9.1)	1 (4.5)	0	
Uterine artery PI				
Right	2.4 ± 0.7	2.5 ± 0.7	2.9 ± 0.9	0.134
Left	2.5 ± 0.8	2.7 ± 0.9	2.9 ± 1	0.440
Uterine artery RI				
Right	80.1 ± 19.3	69.7 ± 32.7	74.2 ± 31.2	0.474
Left	76.5 ± 25.2	66.4 ± 35.7	74 ± 31	0.527
Endometrial thickness	8.0 ± 2.4	8.9 ± 2.0	7.6 ± 2.1	0.146
Type of gonadotropins				
FSH (75 IU/mL)	5 (22.7)	8 (36.4)	3 (13.6)	0.209
FSH+LH (75 IU/mL)	17 (77.3)	14 (63.6)	19 (86.4)	
Ovulation duration	9.9 ± 2.1	10.3 ± 2.2	9.1 ± 1.3	0.100
Ampoules (n)	9.1 ± 12.2	9.6 ± 14.6	7.8 ± 8.5	0.871
Oocytes (n)	11.5 ± 5.6	11.6 ± 6.7	8.1 ± 5.5	0.098
MII (n)	9.3 ± 5.1	9.4 ± 5.8	6.3 ± 4.1	0.079

BMI; Body mass index, PI; Pulsatility index, FSH; Follicle stimulating hormone, LH; Luteinizing hormone, RI; Resistance index, and MII; Mature metaphase II.

Table 2: Comparison of treatment cycle outcomes between the sildenafil, sildenafil+placebo, and placebo groups after intervention

Variable	Sildenafil n=22	Sildenafil+placebo n=22	Placebo n=22	P value
Endometrial pattern				
Normal	17 (77.3)	21 (95.5)	20 (90.9)	0.263
Heterogenic	3 (13.6)	0	2 (9.1)	
Ecogene	2 (9.1)	1 (4.5)	0	
Uterine artery PI				
Right	2.1 ± 0.6	2.2 ± 0.7	2.0 ± 0.5	0.515
Left	2.2 ± 0.4	2.3 ± 0.7	2.1 ± 0.3	0.357
Uterine artery RI				
Right	79.8 ± 7.0	82.3 ± 5.9	80.0 ± 5.0	0.318
Left	78.2 ± 18.1	79.0 ± 18.2	81.5 ± 4.0	0.747
Endometrial thickness	10.1 ± 2.4	10.30 ± 2.5	9.60 ± 2.5	0.656
Embryo (n)	4.8 ± 3.3	4.80 ± 2.7	3.60 ± 3.2	0.322
ET (n)	2.5 ± 1.2	2.20 ± 1.4	2.10 ± 1.6	0.603
ET grade				
A	2.1 ± 2.0	2.13 ± 2.3	2.14 ± 2.2	>0.999
B	1.0 ± 1.0	1.0 ± 1.1	1.0 ± 1.1	0.957
C	0.4 ± 0.8	0.10 ± 0.3	0.40 ± 1.0	0.555

PI; Pulsatility index, RI; Resistance index, and ET; Embryo transfer.

Table 3: Comparison of reproductive outcomes between the sildenafil, sildenafil+placebo, and placebo groups after intervention

Variable	Sildenafil n=21	Sildenafil+placebo n=18	Placebo n=17	P value
Chemical pregnancy	7/21 (33.3)	6/18 (33.3)	3/17 (17.6)	0.490
Clinical pregnancy	4/21 (19.0)	6/18 (33.3)	3/17 (17.6)	0.464
Implantation rate	5/56 (8.9)	8/51 (15.7)	3/47 (6.4)	0.290
Miscarriage rate	0	1 (16.7)	0	0.562

Data are presented as n (%).

Discussion

The importance of the endometrial pattern as a predictor of treatment cycle outcome in IVF-treated patients is well-documented (17). In addition, endometrial quality is also an important factor for the successful implantation of the fetus (18). Endometrial thickness increases with high pregnancy rates; however, pregnancy rates are not predictable only based on endometrial thickness (19). Uterine arterial blood flow seems to affect endometrial growth and the outcome of pregnancy. Studies have shown that sildenafil widens the vasculature by its effects on the smooth muscles of the arteries (10, 20). Sildenafil citrate is an inhibitor-5-phosphodiesterase type that, by preventing the effect of cGMP, exacerbates the effect of NO on the smooth muscle of the arteries (20, 21). According to the results of some studies, the use of sildenafil during the proliferative phase of the cycle improves uterine blood flow and endometrial growth, and results in a higher level of implantation and pregnancy in patients with repeated IVF failure and Ashermen syndrome (8, 22). Also the administration of sildenafil (50 mg intravenous) in a sterilized animal model (sheep) has been shown to exacerbate uterine flow (23). Several studies have shown a correlation between “thin endometrium” and low implantation rates (9, 24). The results of current study showed increased endometrial thickness in the three groups on the day of hCG injection, but the increase was not statistically significant. The results of previous studies have shown that sildenafil citrate (vaginal or oral alone or with oestradiol) is significantly effective in improving endometrial thickness (25-27). Sher and Fisch (22) reported that the use of sildenafil vaginal suppository could reduce the adverse effects of headache and low blood pressure compared with oral sildenafil. In another study, they reported that vaginal sildenafil (25 mg, 4 times per day) improved endometrial thickness (≥ 9 mm) in 70% of the patients (8). In a prospective study, Takasaki et al. (25) compared the effects of vaginal vitamin E, L-arginine, and vaginal sildenafil citrate on endometrial thickness in patients with endometrial thickness less than 8 mm and right arterial resistance in their radial vessels (RA-RI ≥ 0.81). The results showed that vitamin E, L-arginine, and sildenafil citrate significantly improved RA-RI and endometrial thickness in these patients; however, the improvement in endometrial thickness in patients treated with sildenafil citrate was more than the other two groups. Several studies (26-28) showed that vaginal sildenafil significantly improved endometrial

thickness in patients with history of poor endometrial thickness in the previous cycles. Jerzak et al. (10) reported that endometrial thickness significantly increased after administration of oral sildenafil (25 mg, 4 times per day) in women with a history of abortion. Dehghani Firouzabadi et al. (29) recommended oral sildenafil administration as an appropriate solution for improving endometrial admission in patients with unsuccessful cycles from low endometrial thickness. Their results showed that the triple line endometrial pattern in the sildenafil citrate + oestradiol group was significantly higher than in the oestradiol-only group, while the intermediate pattern of the endometrium was not significantly different between the two groups. Fetih et al. (30) reported that sildenafil vaginal gel significantly increased endometrial thickness and uterine blood flow, and might improve pregnancy rate in patients with clomiphene citrate (CC) failure due to thin endometrium. The results reported by Chanona et al. (31) showed that the use of vaginal sildenafil in patients whose endometrial thickness was equal to or less than 7 mm in the failed assisted reproductive technology (ART) cycles led to an increase in implantation and pregnancy. Zinger et al. (32) reported that two patients with a history of curettage and secondary infertility were treated with sildenafil after removing adhesions by surgery (due to thin endometrial thickness in previous IVF cycles), and both patients became pregnant during the first cycle of sildenafil administration. Increases in their endometrial thicknesses was also shown in transvaginal ultrasonography.

Several treatment modalities have been offered to patients with “thin” endometrium, including hormonal manipulation by oestrogen and gonadotropin therapy, low-dose hCG, tamoxifen, L-arginine or sildenafil, vitamin E, pentoxifylline, low-dose aspirin, hysteroscopic adhesiolysis, intrauterine infusion of growth factor such as G-CSF, and the recent application of regenerative medicine. Despite the large variety of treatment, most options lead to only minor modifications in the endometrium thickness and subsequent pregnancy, and when this modality fails, patients are eventually candidates for surrogacy. Treatment of thin endometrium remains a challenge and future investigations are required to further clarify and ideally manage patients with thin endometrium (7).

In the present study, the clinical and chemical pregnancy rates in the two groups of women who were taking sildenafil alone and the women who took placebo

and sildenafil showed a twofold increase compared to the placebo-only-treated women, which according to the sample size, this increase is not significant. The results reported by Dehghani Firouzabadi et al. (29) in a study of 80 patients showed that the chemical pregnancy rate was higher but not statistically significant in the patients who used sildenafil; this finding was consistent with the results of our study. However, AbdelKader Fahmy et al. (33), with a sample size of 70 patients, reported a significantly greater chemical pregnancy rate in the sildenafil group.

In this study, the clinical pregnancy rate was higher in the sildenafil and sildenafil+placebo groups than in the placebo group. Although this increase was not statistically significant, it was clinically shown to be twofold. These results were consistent with the findings of AbdelKader Fahmy et al. (33) and Kim et al. (28). The study by AbdelKader Fahmy et al. (33) reported a 2.5-fold increase in pregnancy in the sildenafil group, but this difference was not statistically significant. Kim et al. (28) reported that the use of vaginal sildenafil plus oral oestrogen pills in the luteal phase of patients treated with an IVF-ET cycle increased the pregnancy rate by two-fold, but this increase was not statistically significant. Mangal and Mehriishi (34) showed that the rate of pregnancy in the patients who used vaginal sildenafil in three successive intra uterine insemination (IUI) cycles was significantly higher in the third cycle than in the group who used oestradiol valerate, while this difference was not significant in the first and second cycles. The findings from a retrospective study by Margreiter et al. (35) showed a significant improvement in the rate of implantation, endometrial thickness and pregnancy, and decreased abortion in the group of patients treated with vaginal sildenafil.

The endometrial receptivity is an important stage in the ART cycles, the results of our study showed a higher rate of implantation in the women who took sildenafil+placebo than in the other two groups, which was consistent with the results of Dehghani Firouzabadi et al. (29).

In the present study, 2 out of 7 cases of pregnancy ended in abortion in the sildenafil group, 1 out of 6 cases of pregnancy in the sildenafil+placebo group, and 0 of 3 cases of pregnancy in the placebo group. The rate of abortion was negligible in the sildenafil group. Follow-up evaluations showed a molar pregnancy in the sildenafil group. Dzieciol et al. (36) reported that sildenafil citrate increased uterine tissue perforation during the uterus preparation for fertility.

In our study, higher numbers of MII oocytes were retrieved in the sildenafil groups compared to the placebo group, which was clinically important; however, one-way ANOVA results showed that this finding was not statistically significant. To our knowledge, there is only one study (abstract available) by Vidal et al. (37) that provided evidence of the benefit of sildenafil supplementation in the first days of hyperstimulation induction in terms of the numbers of mature and fertilized oocytes. Therefore, more research on this topic needs to be undertaken.

Conclusion

Vaginal sildenafil might conceivably improve chemical and clinical pregnancy rates in repeated IVF failure patients. Since this was a pilot study, we recommend that clinical trials should be conducted with vaginal or oral sildenafil on larger numbers of these patients.

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Authors' Contributions

A.M.; Contributed to conception, study design and evaluation. F.Z.; Participated in data collection, extensively in interpretation of the data and the conclusion, and drafting of manuscript. N.J., Sh.J.S.; Participated in data interpretation, conclusion and drafting of manuscript. M.S.; Participated in acquisition of data and data interpretation. M.Ch.; Contributed to study design and data analysis. F.A.; Participated in acquisition of data, interpretation and conclusion. All authors reviewed and approved the final manuscript.

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Dietary Phytoestrogen Intake and The Risk of Endometriosis in Iranian Women: A Case-Control Study

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Abstract

Background: Endometriosis is an important gynecologic disease affecting reproductive-age women. Based on the effect of phytoestrogens on inflammatory, immunological and hormonal factors, limited studies have suggested that phytoestrogen consumption could probably modulate endometriosis risk. The aim of this study was to evaluate the relationship between phytoestrogen intake and endometriosis risk.

Materials and Methods: In the present case-control study, 78 women with a laparoscopically confirmed endometriosis and 78 normal pelvis women (as the control group), were recruited. Common dietary intake was recorded by a validated 147-item semi-quantitative food frequency questionnaire (FFQ). Type of phytoestrogen in each dietary item was analyzed by the database from the United States Department of Agriculture (USDA). A logistic regression model was used to determine the association between phytoestrogen intake and endometriosis risk.

Results: Higher intake of total phytoestrogen (P-trend=0.01), total isoflavones (P-trend=0.002) specially formononetin (P-trend=0.04) and glycitein (P-trend=0.04), total lignan (P-trend=0.01) specially secoisolariciresinol (P-trend=0.01) and lariciresinol (P-trend=0.02) and matairesinol (P-trend=0.003), and total coumestrol [third quartile odds ratios (OR): 0.38; 95% confidence intervals (CI): 0.15-0.96; P-trend=0.1] was related to reduced endometriosis risk. Among food groups, only isoflavin (OR: 0.48; 95% CI: 0.44-0.63), lignan (OR: 0.66; 95% CI: 0.62-0.94), coumestrol (OR: 0.64; 95% CI: 0.51-0.99), phytoestrogen (OR: 0.46; 95% CI: 0.38-0.83) in dairy products and coumestrol in fruits (OR: 0.69; 95% CI: 0.03-0.77) were negatively associated with endometriosis risk.

Conclusion: Phytoestrogens have a major impact on the level of hormones, and immune and inflammatory markers; thus, it can play an important role in the control and prevention of many diseases. Due to the inflammatory nature of endometriosis and the effect of hormones on the progression of the disease, the role of phytoestrogens consumption in the progression and regression of the disease should be assessed in future works.

Keywords: Case-Control Study, Endometriosis, Phytoestrogen

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Introduction

Endometriosis is an important gynecologic disease affecting reproductive-age women (1). The prevalence of endometriosis is approximately 10.8 per 1000 women of reproductive age (2). Endometriosis development was shown to be highly related to prolonged exposure to estrogens in the absence of progesterone. The diet has a strong effect on hormonal activity, inflammatory markers and the immune system, therefore, plays an important role in the pathogenesis of endometriosis (3-5). The results of some studies showed that nutrition and diet have a major impact on endometriosis risk (6-9). Phytoestrogens are estrogenic components that exist in multiple foods of plant origin. Dietary sources of

phytoestrogens have been identified in various food stuffs including fruits, vegetables, spinach, sprouts, beans, cabbage, soybean, grains, and oilseeds (such as flaxseed). Main classes of phytoestrogens consist of isoflavones, coumestans, lignans, and flavonoids. Isoflavones have several subgroups including genistein, daidzein, glycitein, formononetin, biochanin A, and their glycosides. Mammalian lignans include secoisolariciresinol, matairesinol, pinorensinol, lariciresinol, syringaresinol, arctigenin, and 7-hydroxy matairesinol (10). Lignan and isoflavonoid glycosides are converted by gut microflora to hormone-like substances with poor estrogenic activity (0.1% that of estradiol). Therefore, phytoestrogens may demonstrate poor estrogenic activity in low-estrogen

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environments such as that observed in menopause and have antiestrogenic activity in high-estrogen environments such as that observed in endometriosis or endometrial cancer (11, 12). These substances bind competitively to estrogen receptors, thus blocking endogenous estrogens binding (11). High intake of phytoestrogen is associated with lower C-reactive protein (CRP) concentration (13) and suppressed the immune response (14).

Phytoestrogens stimulate sex hormone-binding globulin (SHBG) production in the liver (15). High levels of SHBG bind to the free estrogen and diminish the concentration of estrogens available for binding to estrogen receptors (16).

With regard to the role of inflammatory, immunologic and hormonal factors in the pathogenesis of this disease, we hypothesized that phytoestrogen intake can reduce the risk of endometriosis. Our study evaluated the association between dietary phytoestrogen intake and endometriosis risk using a food frequency questionnaire (FFQ).

Materials and Methods

Between May 2016 and February 2017, the present case-control study was conducted on 156 infertile women in clinic Arash Hospital, Tehran, Iran. The sample size was determined using the information obtained from a pilot study with 20 patients and the following formula: all infertile women who underwent diagnostic laparoscopy during the period of the study were allocated. The case group consisted of 78 endometriosis women for whom the disease was confirmed by laparoscopy and histology examinations. Control group included 78 infertile women with a normal pelvis. Women in the two groups were comparable in demographic and personal characteristics.

Inclusion criteria were as follows: i. Age between 15-45 years, ii. The absence of a history of chronic disease (such as cancer, diabetes, stroke, heart disease, etc.), iii. Being from Iranian race, iv. Not being pregnant, v. Not using medications affecting food absorption, appetite and basal metabolism of the body, and vi. No smoking and vii. Lack of mental retardation.

The medical Ethics Committee of Tarbiat Modares University approved the study (IR.TMU.REC.1395.358) also, before enrolment of the participants, a written informed agreement was obtained from each one. In the beginning, a socio-demographic questionnaire including questions about socioeconomic status, age, smoking, education, habitat, and ethnicity was completed by women, then participants' dietary information was obtained using FFQ.

Dietary assessment

Dietary data were collected using FFQ as a validated semi-quantitative questionnaire with 147 food items. Trained dietitians questioned participants regarding their intake frequency for each food item consumed during the past year on a daily, weekly, or monthly basis; all these were converted to daily intakes. Then, by applying the manual for household measures, portion sizes of the consumed food were transformed to grams (17). The validity and reliability of the FFQ for food groups intakes were assessed and were found to be acceptable

(18). Type of phytoestrogen per 100 gram of each dietary item was analyzed by the database from the United States Department of Agriculture (USDA) (19) and Tables and databases available from other studies (19-25). Total consumption of phytoestrogen was calculated as the sum of isoflavin, lignan, and coumestrol. We excluded individuals with dissimilar nutrient intake and those with daily energy intake of >4300 or <670 kcal.

Statistical analysis

Statistical analysis of data was performed by using Statistical Package for Social Science (SPSS, version 21, SPSS Inc., Chicago, IL, USA). Odds ratio [adjusted for age, total energy intake, body mass index (BMI), educational level, and income], with 95% confidence intervals (95% CIs) were calculated using logistic regression models to assess the strength of the associations between the phytoestrogen intake and the risk of endometriosis. Dietary phytoestrogen, isoflavin, lignan, and coumestrol intake was categorized into quartile categories, based on the distribution of control subjects. To calculate the linear trend in the odds of dietary variable quartile, median factor score of each quartile was entered into the logistic regression analysis, and the lowest quartile of intake was used as the reference category for all regression analyses. T test, Mann-Whitney, and chi-square were used to compare other variables. A P value below 0.05 was considered statistically significant.

Result

Table 1 compares the demographic characteristics of healthy women and subjects with endometriosis. There were no statistically significant differences in the women's age, BMI, parity, educational, marital status, occupation, income, and age at menarche between the two groups.

Table 1: Demographic and anthropometric characteristics of women with and without endometriosis

Characteristic	Case group	Control group	P value
Age (Y)*	31.01 ± 6.56	29.35 ± 7.00	0.13
BMI**			0.19
<25	50 (64.1)	50 (64.9)	
25-29.9 (overweight)	24 (30.8)	23 (29.9)	
≥30 (obese)	4 (5.1)	4 (5.2)	
Education**			0.58
Lower than university	42 (53.8)	38 (49.4)	
University	36 (46.2)	39 (50.6)	
Age at menarche***	13.49 ± 2.38	13.35 ± 1.64	0.70
Marital status**			0.90
Unmarried	22 (28.2)	21 (27.3)	
Married or cohabiting	56 (71.8)	56 (72.7)	
Parity**			0.06
Parous	32 (41)	42 (54.6)	
Nulliparous	46 (59)	35 (45.5)	
Occupation**			0.09
Housewife	61 (78.2)	68 (88.3)	
Employed	17 (21.8)	9 (11.7)	

*; Values are given as mean ± SD and compared using Student's t test, **; Values are given as a number (%) and compared using Chi-squared test, ***; Values are given as mean ± SD and compared using Mann-Whitney test, and BMI; Body mass index.

Table 2 summarizes the ORs for endometriosis by daily phytoestrogen intake according to quartile of intake. We observed inverse associations between consumption of phytoestrogen (OR: 0.68; 95% CI: 0.51-0.91, P-trend=0.01) and total isoflavones (OR: 0.38; 95% CI: 0.33-0.83; P-trend=0.002) and endometriosis risk, but this difference was more related to formononetin (OR: 0.57; 95% CI: 0.27-0.97; P-trend=0.04) and glycitein (OR: 0.68; 95% CI: 0.67-0.98; P-trend=0.04).

High consumption of lignan was associated with a lower risk of endometriosis (OR: 0.49; 95% CI: 0.46-0.52; P-trend=0.01). Among the sub type of lignan, only secoisolariciresinol (OR: 0.54; 95% CI: 0.36-0.77; P-trend=0.01), lariciresinol (OR: 0.64; 95% CI: 0.32-

0.74, P-trend=0.02) and matairesinol (OR: 0.30; 95% CI: 0.22-0.52; P-trend=0.003) were related to reduced risk of endometriosis. The intake of coumestrol in the third quartile was associated with reduced risk of endometriosis (OR: 0.38; 95% CI: 0.15-0.96; P-trend=0.15).

Table 3 demonstrates an association between the sub-type of phytoestrogen in each food group and risk of endometriosis. Among food groups, only isoflavin (OR: 0.48; 95% CI: 0.44-0.63), lignan (OR: 0.66; 95% CI: 0.62-0.94), coumestrol (OR: 0.64; 95% CI: 0.51-0.99) and phytoestrogen (OR: 0.46; 95% CI: 0.38-0.83) in dairy products and coumestrol in fruits (OR: 0.69; 95% CI: 0.03-0.77) were associated with endometriosis.

Table 2: Adjusted odds ratios (OR) of endometriosis and corresponding 95% confidence intervals (CI) according to the subtype of phytoestrogen intake

Phytoestrogen	Quartile, OR (95% CI)*				OR	P trend
	1	2	3	4		
Total isoflavones	1.00	0.69(0.32-1.91)	0.77(0.27-1.64)	1.00(1.00-1.03)	0.38(0.33-0.83)	0.002
Genistein	1.00	0.54(0.30-1.80)	0.92(0.27-1.63)	0.92(0.26-1.54)	0.45(0.43-1.01)	0.55
Daidzein	1.00	1.00(0.99-1.06)	0.46(0.20-1.19)	0.53(0.31-1.89)	0.44(0.29-1.01)	0.18
Formononetin	1.00	0.67(0.19-1.23)	0.38(0.08-0.56)	0.35(0.18-1.17)	0.57(0.27-0.97)	0.04
Glycitein	1.00	0.71(0.11-0.81)	1.00(1.00-1.46)	0.35(0.07-0.81)	0.68(0.67-0.70)	0.04
Total lignans	1.00	0.69(0.44-1.07)	0.79(0.66-1.90)	1.00(1.00-1.01)	0.49(0.46-0.52)	0.01
Secoisolariciresinol	1.00	0.75(0.30-1.88)	0.45(0.18-1.12)	0.38(0.15-0.96)	0.54(0.36-0.77)	0.01
Pinoresinol	1.00	0.84(0.34-2.09)	0.56(0.23-1.38)	0.42(0.17-1.07)	0.49(0.40-1.02)	0.08
Lariciresinol	1.00	0.54(0.21-1.36)	0.36(0.14-0.91)	0.27(0.10-0.70)	0.64(0.32-0.74)	0.02
Matairesinol	1.00	0.38(0.16-1.33)	0.78(0.57-3.76)	0.34(0.13-0.86)	0.30(0.22-0.52)	0.003
Coumestrol	1.00	1.04(0.42-2.59)	0.77(0.31-1.88)	0.38(0.15-0.96)	0.48(0.36-1.03)	0.15
Total phytoestrogens	1.00	0.56(0.22-1.37)	0.55(0.22-1.37)	0.27(0.10-0.70)	0.68(0.51-0.91)	0.01

BMI; Body mass index and *: Odds ratio adjusted for age, energy intake, BMI, educational level, and income. Quartile 1 used as the reference category.

Table 3: Adjusted odds ratios (OR)* of endometriosis and corresponding 95% confidence intervals (CI) according to phytoestrogen from the dietary item

Food group	Isoflavones	Coumestrol	Lignans	Total phytoestrogens
Soy products	0.76(0.71-1.03)	0.76(0.71-1.24)	0.76(0.68-1.07)	0.74(0.71-1.03)
Legume	0.63(0.58-1.13)	0.45(0.24-0.86)	0.45(0.42-1.14)	0.62(0.59-1.08)
Nut	0.99(0.97-1.14)	0.99(0.99-1.03)	0.99(0.99-1.03)	0.99(0.98-1.01)
Dairy product	0.48(0.44-0.63)	0.64(0.51-0.99)	0.66(0.62-0.94)	0.46(0.38-0.83)
Vegetable	0.99(0.92-1.05)	0.63(0.32-1.15)	0.97(0.99-1.02)	0.97(0.96-1.02)
Fruit	0.99(0.93-1.20)	0.69(0.03-0.77)	1.01(1.00-1.01)	1.00(1.00-1.01)
Cereals and breads	0.99(0.86-1.04)	0.99(0.01-3.24)	0.94(0.87-1.01)	0.96(0.92-1.01)
Meat	0.99(0.99-1.03)	0.63(0.12-1.68)	0.99(0.72-1.15)	0.99(0.99-1.03)
Beverages, nonalcoholic	0.99(0.90-1.19)	0.98(0.66-1.53)	0.99(0.99-1.01)	0.99(0.99-1.01)

BMI; Body mass index and *: Odds ratio adjusted for age, energy intake, BMI, educational level, and income.

Discussion

Our findings suggest that higher intake of phytoestrogen such as isoflavin, lignan, and coumestrol is associated with a reduced risk of endometriosis. All subtypes of phytoestrogen in dairy products and coumestrol in fruits were related to reduced endometriosis risk.

Recently, some studies discussed associations between phytoestrogen and endometriosis. One of such studies (26), consumption of phytoestrogen (25 to 250 mg/

day), used for the treatment of endometriosis. Genestein inhibits the activity of tyrosine kinase, and in this way, plays an important role in growth factors signaling. While in another study (27), soy isoflavones supplementation maintained endometriosis and induced conversion of the disease to the malignant form (i.e. mixed Müllerian tumor). Moreover, using an animal model, it was shown that pharmacologic genistein, but not dietary form, helps to the maintenance of the implants (28). In a Japanese study, higher levels of urinary genistein and daidzein

were related to reduced advanced endometriosis risk (29). However, to the best of our knowledge, no study has assessed possible associations between dietary phytoestrogen intake and women endometriosis risk.

Results of some studies demonstrated that dietary consumption of phytoestrogens was associated with reduced risk of endometrial, breast, colorectal and prostate cancer (30-33).

It was indicated that increased urinary excretion of phytoestrogen was associated with decreased CRP levels (13). Soy consumption is associated with a decrease in serum nitric oxide, E-selectin, interleukin-18 and CRP concentrations (34).

Animal studies showed that ginseng has an anti-proliferative effect on mammary tissue in rats exposed to prepubertal estrogens (35). Diadzein induces mitochondrial-dependent apoptosis by increasing caspase-9 activity and decreasing cyclin D expression that can affect cell cycle regulation (36). Phytoestrogens have antioxidant effects, that lead to reduced production of reactive oxygen species (ROS) (37). Genistein can suppress lymphocyte proliferation and antigen-specific immune response but enhance the cytotoxic responses mediated by NK and cytotoxic T cells and production of cytokines from T cells (38).

Regarding the effect of phytoestrogens on inflammatory, immunological and hormonal factors, phytoestrogen consumption can reduce the risk of endometriosis. Bioavailability, absorption, and estrogenic characteristics of phytoestrogens are dependent on the compound's bioactivity, which is metabolized in to compounds by intestinal microflora (39). Also, the phytoestrogen content in diet is dependent on environmental and genetic factors for example variety, harvest, food processing, cooking and growth locations (40). Up to now, Iranian dietary phytoestrogen has not been measured. Our calculations were done based on the phytoestrogens found in the USDA food composition Table.

A limitation of this study was the problem of convincing the participants to answer many questions. Also, as it was a case-control study, the probability of selection and recall bias including under- and over-reporting of the specific food items might have affected our results.

Conclusion

Phytoestrogens have a major impact on the level of hormones, and immune and inflammatory markers; thus, it can play an important role in the control and prevention of many diseases. Due to the inflammatory nature of endometriosis and the effect of hormones on the progression of the disease, the role of phytoestrogens consumption in the progression and regression of the disease should be assessed in future works.

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Authors' Contributions

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

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Evaluation of Enkephalin-Degrading Enzymes in Sperm from Heroin-Addicted Men

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Abstract

Background: The aim of this study was to investigate two enkephalin-degrading enzymes, aminopeptidase N (APN/CD13) and endopeptidase (NEP/CD10), gene and protein expression levels in sperm samples of fertile and heroin-addicted men, and the correlation between their expressions and semen quality.

Materials and Methods: In this case-controlled study, semen was collected from 24 normozoospermic healthy (as a control group) and 24 heroin-addicted men donors (as case or addiction group). Sperm cells isolated by Cook Medical gradient (40-80%) and followed up by swim-up techniques were used for real-time quantitative polymerase chain reaction (qPCR) and flow cytometry techniques to assess *APN/CD13* and *NEP/CD10* genes and proteins subsequently. Semen parameters were analyzed by computer-assisted sperm analysis.

Results: The findings revealed that there were significant differences in sperm total motility (41.07 ± 3.63 vs. 63.03 ± 3.31 %, $P=0.0001$), progressive motility (35.21 ± 2.64 vs. 20.93 ± 3.22 %, $P=0.001$) and viability (69.9 ± 4.69 vs. 86.81 ± 1.26 %, $P=0.002$) in the addicted group vs. control ones. *APN* and *NEP* gene expression levels in the addicted group decreased compared with the control ones (1.00 ± 0.67 vs. 0.36 ± 0.13 , $P=0.008$ and 1.07 ± 0.11 vs. 0.52 ± 0.12 0.002, respectively). Flow cytometry analysis showed that the average percent of APN/CD13 in heroin consumers significantly decreased compared with the healthy ones, while NEP/CD10 rate between two groups was similar. We also observed that duration of drug dependence is correlated with sperm viability ($r=-0.627$, $P=0.016$) and motility ($r=-0.410$, $P=0.05$), *NEP* ($r=-0.434$, $P=0.049$), and *APN* ($r=-0.641$, $P=0.002$) gene expression levels.

Conclusion: We conclude that semen quality and enkephalin-degrading enzymes were altered in heroin-addicted men. other confirming the internal validity of our estimates.

Keywords: Addiction, Aminopeptidase N, Endopeptidase, Heroin, Sperm Quality

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Introduction

Infertility is one of the most serious social problems that has an effect on a huge percentage of couples. In general, the basic cause of infertility refers to the male partner in approximately 40-45% of cases (1). In some national data and available sources, the number of drug abusers in Iran is estimated to be between 1,200,000 and 2,000,000 people (2-4) with a mean age of 33 years (5). This means that they are generally young in reproductive age. Heroin, from the opioid group, is morphine O-acetylated at

position 3 and 6 (diacetylmorphine) (6). Heroin addiction has been linked to some degenerative diseases including aging, abscesses, arthritis, other rheumatologic disorders, and immunological disorders (7).

The activity of the opioid system is mediated by endogenous opioid peptides (EOPs). EOPs or opiate alkaloids carry out their function through three types of opioid receptors [the delta-opioid receptor (DOR), the mu opioid receptor (MOR) and the kappa opioid receptor (KOR)] on membranes (8, 9). EOPs control

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male reproductive function at different levels (1): i. At the hypothalamus, they inhibit the secretion of gonadotropin-releasing hormone (GnRH) and suppress the rate of luteinizing hormone (LH) from the pituitary gland (decrease the levels of testosterone), ii. At the testes, leydig cells produce EOPs through LH affect, and these peptides perform an inhibitory effect on sertoli cells [inhibiting the production of Androgen-binding protein (ABP)] and iii. In germ cells, somatic cells of the testes and iv. In sperm cells (regulates sperm motility). As a result, EOPs may be involved in human reproductive function by a direct effect on sperm. However, EOP levels are controlled by enzymatic degradation by aminopeptidase N (APN) and endopeptidase neutral N (NEP). These enzymes are present in both sperm and seminal fractions (10), and their activity in semen is particularly high compared with other body tissues (11). Interestingly, APN activity levels were found to be altered in semen from sub-fertile patients, suggesting that this enzyme may play an important role in male fertility (12).

The effect of opiates on spermatozoa in some aspects is still unknown or controversial. Some studies have been performed on the effect of certain drug abuse on the human and mouse sperm parameters (13-15). In our previous study, we showed the deleterious effects of kerack, (unlike to crack cocaine), consumption in Iran on testis structure, sperm parameters, and particularly sperm morphology in the adult mouse. Also, It down-regulated the expression of CatSper genes, resulting in depression of sperm motility (16). Nazmara et al. (17) reported that heroin is strongly associated with abnormalities in histone-to-protamine transition and with human semen quality, particularly sperm morphology and motility.

Sperm motility is considered a key functional parameter that controls reproduction (18) and is widely used as an indicator of semen quality (19) since sperm must move to reach the oocyte and then penetrate it using sperm movement. More reports on heroin (13) and nicotine (20) consumption in animals and opioids in human (14, 17) showed decreasing in sperm parameters.

Since decreased motility (asthenozoospermia) is a common abnormality among opiate drug addicts (17), it is likely that the opioid system involves the sperm movement. Previous studies have suggested expression of enkephalin-degrading enzymes in human sperm and semen (1, 10, 12, 21). Subiran et al. (10) reported Enkephalin-degrading enzymes were expressed in human sperm including messenger RNA of both enzymes and APN and NEP, (in a small number of sperms), were detected in spermatozoa at the protein level. In addition, the activity of the enkephalin-metabolizing enzyme aminopeptidase N was measured in various fractions of human semen from normal and subfertile patients by Irazusta et al. (12). They reported that the activity of aminopeptidase N was lower in males with asthenozoospermia as compared with normal semen. It has been described that the inhibition of some of those enzymes can significantly improve sperm motility (21). In the present study, we investigated

the expression of two enkephalin-degrading enzymes APN and NEP in heroin-users' sperm cells to assess any correlation between expression of abovementioned and sperm motility.

Materials and Methods

Participants

In this case-control study, participants were interviewed after written informed consent. The data on personal information (e.g. name, age, marital and parental status), history of addiction [duration of heroin consumption, heroin use (mg/day), cigarette smoking, and alcohol drinking], and medical status (e.g. medications, special illness and surgery) were obtained via a structured questionnaire.

Based on the medical files and questionnaires, twenty-four 20-50-year-old men with normal body mass index (BMI) who just used heroin for at least 12 months -without using other drugs during that interval- were selected as a case or heroin-addicted group. They were introduced from addiction treatment centers before entering treatment programs and should have met the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) criteria for addiction.

Also, 24 age-BMI-matched men with a normal semen analysis according to World Health Organization (WHO) 2010 criteria volunteered to participate in this study as a control group. They were male partners of married couples without any illicit drug use who had attended the Shahid Akbar-Abadi Obstetrics and Gynecology Hospital of the Iran University of Medical Science for female infertility consultation.

Subjects with medical problems known to be associated with subfertility in both groups, illicit-drug users in control group, and individuals that started treatment with other drugs in addicted ones were excluded from the study.

Sperm preparation

Semen samples were obtained from donors by masturbation after 2 to 3 days of abstinence into sterile containers and allowed to liquefy at 37°C for 30 minutes before processing. Semen volume, as well as sperm concentration, viability (by eosin B staining), morphology (by Papanicolaou staining method), and motility were measured in each sample by CASA (22).

For eosin B staining (0.5% in saline), 20 microliters the sperm suspension was mixed with 7 µl eosin and observed under a light microscope (×400 magnification). Then, 200 sperm were counted and the percentage of live spermatozoa was recorded. With the staining, red sperm heads were considered as dead (23, 24).

Sperm morphology was assayed by the Papanicolaou method. First, smears were prepared and stained with the Papanicolaou method based on the protocol. Then, the morphology of 200 spermatozoa was surveyed

under $\times 1000$ oil immersion lens. With the staining, the nuclei turn blue, and the acrosome and tail become pink. Abnormal morphology was reported in five fields of vision randomly, and the percentage of abnormal morphology was recorded (16).

Before RNA extraction or flow cytometry, spermatozoa were isolated from semen on cook medical gradient (40-80%), followed by a swim-up in washing medium supplemented by Albumin 10% to recover motile cells without any contaminant leukocytes or other debris, and then they were visually examined under a light microscope.

Real-time quantitative polymerase chain reaction technique

All primers (Table 1) were designed by primer 3 (Online: [Http://primer3.sourceforge.net](http://primer3.sourceforge.net)), and then, primers were blasted in NCBI.

Table 1: Sequences of the designed primers used for real-time quantitative polymerase chain reaction

Gene	Primer sequence (5'-3')
APN	F: CCACCTTTCTGACATTGCC R: CAGGGGCTGTACGTTTTTA
NEP	F: GCCTCAGCCGAACCTACAAG R: AGTTTGCACAACGTCTCCAAG
β -actin	F: GCAAGCAGGAGTATGACGA R: CAAATAAAGCCATGCCAATC

The RNA of spermatozoa was isolated with the RNeasy Mini kit (Qiagen, Germany). First strand cDNA synthesis was carried out using QuantiNova Reverse Transcription Kit (Qiagen, Germany). Then real-time quantitative polymerase chain reaction (qPCR) was performed using QuantiNova SYBR Green PCR Kit (Qiagen, Germany).

The thermal cycling program included an initial incubation at 95°C for 2 minutes, followed by 60 cycles of 95°C for 5 seconds and 60°C for 30 seconds. Three replicates of each reaction were performed, and the cycle threshold (Ct) values were averaged. Expression values were normalized to the average expression of the house-keeping gene (β -actin) and compared with a calibrator (control group) by the comparative Ct method ($2^{-\Delta\Delta\text{Ct}}$) (10).

Flow cytometry analysis

Flow cytometry was performed on a BD Biosciences FACSCalibur in order to rate of presences of surface expression of enkephalinase and aminopeptidase on sperm cells. Sperm was stained directly for APN and NEP. Briefly, 1×10^6 sperm in 2 ml phosphate buffered saline (PBS, Sigma, USA) was centrifuged in 4°C for 10 minutes ($\times 300$ g). The pellet was suspended in 100 μl washing medium and then was added 20 μl antibody [PE Mouse Anti-Human CD13 and PE Mouse Anti-Human CD10 (BD Biosciences USA)]. Samples were incubated at 4°C for 1 hour. Next, sperm was centrifuged three times

at 4°C for 10 minutes ($\times 300$ g). Finally, the pellet was suspended in 300 μl PBS and analyzed using the flow cytometer.

Statistics analysis

Statistical analysis was done with statistical software (Ver. 16.0, Chicago, SPSS Inc.). The normal distribution was evaluated with the Kolmogorov-Smirnov test. The results were analyzed by performing independent-samples t test and Mann-Whitney U test. $P \leq 0.05$ was considered as statistically significant and mean $\pm$ SE was also calculated for each variable. The partial correlation and multiple regression analyses were done between APN and NEP levels and other parameters.

Ethical considerations

This study has been approved by the medical Ethics Committee of Iran University of Medical Science (code: IR. IUMS.rec.1394.9211313202). All human trials were carried out in accordance with the Declaration of Helsinki guidelines.

Results

Demographic information

Twenty-four healthy and twenty-four men addicted to heroin participated in the study. The mean ages in addicted and control groups were 35.44 ± 1.3 years and 33.91 ± 1.79 respectively, which there was no significant difference in average age. Although the range of BMI was normal in both groups, this parameter in the addicted men ($22.34 \pm 0.38 \text{ kg/m}^2$) was significantly lower than the healthy ones ($28.69 \pm 0.91 \text{ kg/m}^2$) ($P \leq 0.01$). All subjects in the addicted group were smoker, so there was a statistical difference between groups in this case ($P \leq 0.01$).

Semen quality analysis

According to our data, although semen volume, sperm concentration, and normal morphology were the same between groups, there was a significant decrease in sperm viability and motility rates in the addicted group (Table 2).

Table 2: Sperm parameters in the study population

Parameters	Healthy men	Heroin addicted men	P value
Semen volume (ml)	3.63 ± 0.42	3.22 ± 0.35	0.457
Sperm concentration ($\times 10^6/\text{ml}$)	115.79 ± 16.5	113.51 ± 18.9	0.928
Sperm total motility (%)	63.03 ± 3.31	41.07 ± 3.63	0.0001
Sperm progressive motility (%)	35.21 ± 2.64	20.93 ± 3.22	0.001
Sperm normal morphology (%)	9.48 ± 1.54	12.11 ± 1.52	0.233
Sperm viability (%)	86.81 ± 1.26	69.9 ± 4.69	0.002

Data are presented as mean $\pm$ SE.

Changes in enkephalinase and aminopeptidase genes expression

As shown in the Table 3, *APN* and *NEP* gene expression levels in the addicted group (0.36 ± 0.13 , 0.52 ± 0.12) decreased compared with the control ones (1.00 ± 0.67 , 1.07 ± 0.11) ($P \leq 0.01$).

Table 3: Enkephalinase and aminopeptidase gene expression levels in healthy and heroin addicted men

Gene expression level	Healthy men	Heroin addicted men	P value
<i>APN</i> ($2^{-\Delta\Delta Ct}$)	1.00 ± 0.67	0.36 ± 0.13	0.008
<i>NEP</i> ($2^{-\Delta\Delta Ct}$)	1.07 ± 0.11	0.52 ± 0.12	0.002

Data are presented as mean $\pm$ SE.

Flow cytometry analysis

Figure 1 shows flow cytometry analysis of cell surface protein enkephalinase and aminopeptidase in sperm of healthy and addicted men. The results demonstrated that the mean *APN*/CD13 rate in the control group (63 ± 12) was significantly higher than addicted ones (24 ± 12) ($P \leq 0.05$), while *NEP*/CD10 ratio in the control (4.3 ± 1) and addicted (3.7 ± 2) group did not show a significant difference.

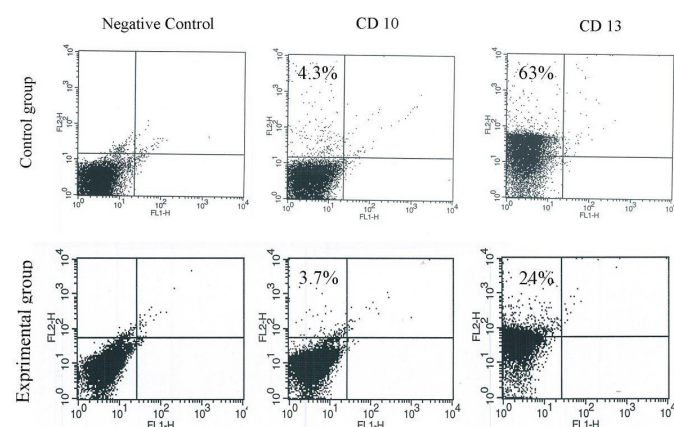


Fig.1: Flow cytometry analysis. It showed that the average percent aminopeptidase N (*APN*/CD13) in control group ($63 \pm 12\%$) significantly decreased compared to heroin addicted ones ($24 \pm 12\%$), while the average percent endopeptidase (*NEP*/CD10) in control ($4.3 \pm 1\%$) and addicted group ($3.7 \pm 2\%$) showed no significantly decrease.

Correlation between enkephalinase and aminopeptidase genes expression and sperm motility

As shown in Table 4 multivariate regression analysis proved that total motility is significantly correlated to duration of heroin dependence, whereas did not relate to other demographic data like age, BMI, cigarette smoking, and the amount of heroin daily use. Based on partial correlation test, sperm viability ($r = -0.627$, $P = 0.016$) and total motility ($r = -0.410$, $P = 0.50$), *NEP* ($r = -0.434$, $P = 0.049$) and *APN* ($r = -0.641$, $P = 0.002$) gene expression levels were significantly negative correlation with duration of heroin dependence (Table 5).

Table 4: Correlation between motility and demographic data

Parameters	Unstandardized coefficients		Standardized coefficients	P value
	Beta	SE	Beta	
Age (Y)	1.221	0.731	0.437	0.108
BMI (kg/m ²)	-0.262	1.082	-0.056	0.811
Duration of heroin consumption (Y)	-1.650	0.750	-0.799	0.040
Heroin use (mg/day)	1.821	2.468	0.134	0.468
Cigarette smoking	-0.427	10.109	-0.448	0.658

BMI; Body mass index and SE; Standard error.

Table 5: Partial correlation between duration of dependence and other studied parameters

Parameters	r-value	P value
Sperm viability (%)	-0.627	0.016
Sperm total motility (%)	-0.410	0.050
<i>APN</i> ($2^{-\Delta\Delta Ct}$)	-0.641	0.002
<i>NEP</i> ($2^{-\Delta\Delta Ct}$)	-0.434	0.049

Adjusted with cigarette smoking. *APN*; Aminopeptidase and *NEP*; Neutral endopeptidase.

Discussion

The present study showed that enkephalin-degrading enzymes (*NEP* and *APN*) and sperm viability and motility were reduced in men addicted to heroin. We also showed that there was a significant negative correlation between *NEP* and *APN* gene expression levels with the duration of heroin dependence.

Infertility is one of the most important problems of human societies throughout the world. Considering the role of recreational heroin consumption as an idiopathic etiology of male infertility and increasing consumption of illicit drugs, especially among young people of reproductive age, socio-medical studies on this issue has been done less yet. The living conditions, lack of cooperation, simultaneous use of various drugs and legal and ethical problems in sampling in addicted people make the research difficult and complicated in this area. Therefore, research in this area can be very valuable. Our findings suggest a remarkable association among heroin addiction, asthenozoospermia and decreased *APN* and *NEP* mRNA levels. In addition, the duration of drug dependence is one of the main factors contributing to the detrimental effects of heroin on impaired male fertility.

Decreased in heroin users BMI may be caused by caloric malnutrition (25), inhibition of androgen production (26), or disorders of the gastrointestinal tract (27). Although our finding appears to be consistent with a number of studies (16, 26, 27), Diamond et al. (25) showed drug and alcohol abuse did not change the BMI in adolescent males. The average BMI may affect spermatogenesis. However, multivariate regression analysis showed that duration of heroin consumption can be more effective than BMI in semen parameters.

In this study, reduced sperm motility was observed in addicted men. Our finding is in line with other studies who mentioned that opioids such as heroin, kerack, and morphine can impair sperm parameters in mice and human. They also showed those alterations were dose-dependent (8, 13, 14, 16, 17). The opioid system likely influences reproductive function by the central nervous system (28), the pituitary gland, and the testis (29), exerting a direct action on the spermatozoa (30).

Reduced total and progressive sperm motility may be caused directly by heroin because of alteration of the enkephalin-degrading enzymes. Researchers scrutinized the presence of APN/CD13 in the sperm head, neck and along the tail (19, 31). APN/CD13 was acknowledged to play a critical role in the sperm binding to oocyte due to being in the sperm head and control its motility by existence along the tail and in the neck. In the fact that opioid levels in semen are in charge of degrading enzymes like APN/CD13, alteration of these enzymes could regulate sperm motility. Indeed, an adequate level of enkephalin as a delta opioid agonist is essential to sperm motility, but this effect depends on opiate concentrations (10). We proposed that heroin directly affects sperm motility by two mechanisms; first, a higher concentration of mu opioid agonists not only bind mu opioid receptors but also have an affinity to delta-opioid receptors. Heroin bind to the receptors occupies the enkephalin-binding site on the sperm and could deactivate the receptors and cause to accumulate opioids in semen. Second, Reducing the *APN* gene and subsequently, its protein in heroin consumers may result in opioid accumulation in the semen microenvironment. Indeed, the occupation of receptors and degrading enzymes deficiency or inactivity may affect the sperm quality and male fertility. Agirregoitia et al. (8) reported the inhibitory effect of the delta-antagonist naltrindole on sperm motility.

Notwithstanding the level of *NEP* gene expression was statistically different between groups, its protein amount was similar in addicted and healthy men. In addition, a significant negative correlation between the *NEP* gene level and BMI was observed. Our data was proved by previous studies (10, 32, 33). Given that just a few cells after capacitation express NEP protein on sperm membranes (32), could justify the same protein amount between groups. This similarity may be due to the fact that NEP act by a mechanism that does not involve the opioid system which was suggested by Subiran et al. (10) who revealed naloxone does not affect sperm motility when incubated with thiorphan an enkephalin degradation inhibitor for NEP. Our data showed that the amount of *NEP* gene and protein do not relate to each other. We hypothesize that this may be due to: i. Epigenetic regulators or the presence of NEP mRNAs in the profile of RNAs' mature spermatozoa that interfere with embryogenesis; or ii. Since spermatozoa have a complex RNA package (34, 35), sperm cells could retain NEP messenger RNA for carrying epigenetic information in the zygote.

Based on the literature, NEP is the main peptidase that can hydrolyze tachykinins, which are present in spermatozoa and interfere in the regulation of sperm motility (12, 36, 37). Thus, alterations of NEP can cause slowly developing changes in sperm motility. Opioids and tachykinins, as bioactive peptides, could operate as signal molecules between spermatozoa and their environment, acting in an autocrine and/or paracrine fashion (38).

In addition, heroin can impair semen quality and alter sperm microenvironment by semen acidification and leukocytospermia (17), which probably affects the structure and function of these surface-expressed enzymes and influences semen parameters.

The main limitation in the present study were: i. Sampling of heroin-addicted men was complicated because (a) heroin consumers usually take various addiction and narcotic drugs like opiate, kerack or methamphetamines, (b) the majority of addicted men had no trends to provide semen. Hence, we selected persons who used only heroin for at least 12 months -without using other drugs, ii. In addition; cigarette smoking was a major interference variable, which was controlled by partial correlation, iii. Socio-economic and family backgrounds were different among the participants. For example, diet may affect sperm parameters, and iv. We could not follow up the fertility status after treatment.

Conclusion

We conclude that semen quality and enkephalin-degrading enzymes were decreased in heroin-addicted men and there is a significant negative correlation between *NEP* and *APN* gene expression levels with the duration of heroin dependence. This study may increase our understanding of the effects of drugs and toxins on male infertility.

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Authors' Contributions

S.R.-M., Z.N., Z.Z., M.M.; Gathered the semen and analysis, performed the research. M.R., P.S.; Cases section base on criteria medically and contributed to performing experimental procedures. M.N., H.R.A., M.A.; Performed the molecular design and analysis. M.K., H.R.A., M.A., M.M.; Designed the research study, wrote the manuscript. M.K.; Was responsible for overall supervision. All authors performed editing and approving the final version of this manuscript.

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Ameliorative Effect of Crocin on Sperm Parameters and *In Vitro* Fertilization in Mice under Oxidative Stress Induced by Paraquat

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Abstract

Background: Paraquat (PQ) is an herbicide that is genotoxic and cytotoxic for male germ cells. In this study, we investigated the protective role of crocin (Cr) against the destructive effects of PQ on sperm quality and *in vitro* fertilization (IVF) outcomes.

Materials and Methods: In this experimental study, a total of 28 male mice (20-25 g) were divided into four groups: control, which received intraperitoneal (IP) injections of 0.1 ml normal saline per day; PQ group received IP injections of PQ (5 mg/kg/day); experimental (PQ+Cr group) received PQ along with IP injections of Cr (200 mg/kg/day); and positive control (Cr) received IP injections of Cr (200 mg/kg/day). In the last two weeks of the treatment period (35 days of treatment), 16 non-pregnant mice were stimulated to receive adult oocytes. At the end of the treatment period, after euthanizing the mice, the sperms were extracted from the epididymis of each mouse and prepared for evaluation of sperm parameters and IVF.

Results: In the PQ+Cr group, Cr caused a significant increase in the average number of sperms and the mean percentage of motile and viable sperms. There was a significant decrease in the mean number of immature and DNA-damaged sperms compared to the PQ group ($P < 0.001$). IVF evaluation in the PQ+Cr group showed that the mean percentage of fertilization, two- and four-cell embryos, blastocysts, and hatched embryos significantly increased. Cr caused a significant decrease in the mean percentage of the arrested embryos compared to the PQ group ($P < 0.001$). However, the Cr group did not have any toxic effects on sperm quality or IVF results.

Conclusion: The findings of this study showed that Cr, due to its effective and potent antioxidant properties, could reduce or suppress the destructive effects on sperm parameters and IVF caused by PQ.

Keywords: Crocin, *In vitro* Fertilization, Mice, Paraquat, Sperm

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Introduction

Paraquat (PQ) (N, N'-dimethyl-4,4'-bipyridinium dichloride) is a pyridine compound that contains an ammonia sodium moiety, which activates oxidation. Oxidation begins with the methylation of chloromethane. PQ is a nonselective contact herbicide that is used to control annual weeds (1).

In general, the fertility rate of men exposed to toxins in the workplace is significantly lower (2). PQ is a highly effective, fast-acting, and nonselective herbicide widely used throughout the world. Human exposure by either respiratory or systemic routes leads to the accumulation of PQ in the lungs, resulting in pulmonary oedema, bronchial and alveolar destruction, and ultimately fibrosis with a high mortality, which is in part caused by the lack of a specific antidote to PQ (3). Chronic exposure to PQ is associated with liver damage, kidney failure, and Parkinsonian lesions, in addition to fibrosis (4). Upon entering the cells, PQ undergoes cyclic single-electron reduction/

oxidation through its quaternary ammonium nitrogen atoms and bipyridyl ring, and produces reactive oxygen species (ROS) and PQ radicals. Redox cycling is believed to play an important role in initiating lung damage and fibrosis by paraquat. The mechanism by which oxidative signals from PQ interact with the pathways that underlie the lung fibrogenic response is poorly understood (5). PQ reduces the cellular oxidation cycle with its tangible presence in this process, which results in the production of ROS (6). In a rat model, free radicals have been shown to accumulate in the testicular torsion, causing inflammation and damage to the tissues by membrane lipid peroxidation (7). Because of the scant amount of cytoplasmic antioxidant enzymes, the sperm cannot repair the damage caused by oxidative stress. Studies have shown that antioxidants such as vitamin C have extensive ameliorative effects on sex hormonal status and can protect sperm from ROS-induced abnormalities. These compounds also inhibit ROS produced by leukocytes, improve the quality of semen, and prevent DNA fragmentation and premature

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sperm production (8). Accordingly, antioxidants such as *Fumaria parviflora* on testicular injury induced by torsion/detorsion in adult rats have confirmed reduced DNA damage and apoptosis in sperms, as well as increased implantation and pregnancy (9).

Carotenoids, by acting as biological antioxidants, protect cells and tissues from the damaging effects of free radicals and singlet oxygen, and play a significant role in human health. *Crocus sativus* L., commonly known as saffron, is a stemless herb of the Iridaceae family. The major bioactive compounds in saffron are crocin, safranal, and picrocrocin. Crocin, glycosyl esters of crocetin, are unusual water-soluble carotenoids, and are responsible for the characteristic colour of saffron (10). Numerous studies have shown that crocin (Cr) can produce a variety of pharmacological effects, such as protection against cardiovascular diseases (11), inhibition of tumour cell proliferation (12), and neuroprotection (13). Saffron has a role in sexual enhancement (14). It also has been reported that Cr inhibits lipid peroxidation in the kidneys (15). The antioxidant and radical scavenging activities of Cr have also been reported in several *in vitro* models (16). In the current study, we aimed to evaluate the protective capacity of Cr on quality and fertilization potential of mice sperm against the toxicity caused by paraquat.

Materials and Methods

We purchased PQ with formulation of SL20%, (Exir Co., Iran). Cr was purchased from Sigma-Aldrich (USA) in the form of a powder.

In this experimental study, 28 adult mice (20 to 25 g) were randomly divided into four equal groups and allowed to adjust to their surroundings for one week before the start date of the experiments. All the ethical issues were carried out based on guidelines of the Ethics Committee of Urmia University, Faculty of Veterinary Medicine (ethics number: ECVU-173-2018). The mice were kept on a 12-hour light/12-hour dark schedule with free access to adequate water and food. The animals were fed with pellets and wheat, and tap water was used as drinking water. The treatment period lasted 35 days.

The mice were assigned to the following experimental groups: control group, which received intraperitoneal (IP) injections of normal saline (0.1 ml/day); PQ group received PQ (5 mg/ kg/day, IP) (17); experimental (PQ+Cr) group received Cr (200 mg/kg/day, IP) two hours before PQ (5 mg/ kg/day, IP) (18); and positive control (Cr) group received Cr (200 mg/kg/day, IP).

At the end of the treatment all mice were weighed one hour before the beginning of the sampling. They were anesthetized by ketamine (40 mg/kg) and xylazine (5 mg/kg), and were euthanized by dislocation of their neck vertebrae.

Sperm preparation

To obtain sperm from the testicles, the abdominal

skin was first sterilized with 70% ethanol. After cutting off the surrounding connective tissues, the tail of each epididymis was removed from the testes, and we placed them in sterile test tubes that contained 1 cc of human tubal fluid (HTF) medium (Sigma-Aldrich, USA) with bovine serum albumin (BSA, 4 mg/ml), which had been previously placed in an incubator to equilibrate. The sperm were incubated in a CO₂ incubator at 37°C. After 30 minutes, the sperm were released and spread in the medium (19).

Evaluation of sperm parameters

Evaluating sperm motility

In order to evaluate sperm motility, a 10 µl sperm suspension (HTF) and 190 µl distilled water (1:20 dilution) were placed on a pre-heated Neobar slide and covered with a cover slip. Motility was observed under a light microscope (Nikon, Japan) and we counted 10 microscopic fields for each specimen at ×400 magnification (20).

Sperm counts

We placed 10 µL of the diluted (1:20) sperm on a neobar slide, after waiting for 5 minutes and counted the number of sperm viewed by an optical microscope at ×400 magnification. We calculated the numbers of sperm according to the following formula: $n \times 50000 \times d$ where: n is the number of sperm counted in 5 squares of the Neobar slide and d is the inverse of the dilution of suspension that contained the sperm (in this study d=20).

Evaluation of sperm viability and morphology

Sperm viability was evaluated as follows. We used eosin-nigrosin staining to detect the nonviable sperm. These sperm are permeable to dye (eosin) because of plasma membrane damage. We dissolved 20 µl of the sperm in 20 µl of the eosin solution on the slide; after 20 to 30 seconds, we added 20 µl of the nigrosin solution. After the appropriate incubation period, we observed sperm viability with a light microscope at ×400 magnification. The nigrosin sperm (n=400) were counted in each sample and the viability percentage was computed (21). For the sperm morphology assessment, we used both the aniline blue and eosin-nigrosin stains. Sperm that appeared abnormal by aniline blue staining were counted and the results were expressed as percentages. With the eosin-nigrosin staining, spermatozoa that contained cytoplasmic debris were counted as immature sperm (22).

Evaluation of sperm nucleus maturity

Aniline blue staining was used to evaluate the maturity of the sperm nucleus. In spermatogenesis, a basic protein (protamine) is instead of histones of chromatin in the sperm nucleus. Immature sperm have remnants of histone that take up aniline blue stain, which is an important indicator of sperm maturity. Air-dried smears of the sperm

samples were fixed with 3% glutaraldehyde in phosphate-buffered saline (PBS) for about 30 minutes. Then, the slides were stained with aniline blue for 5 minutes. The slides were washed with distilled water and examined with a light microscope at $\times 400$ magnification. The percentages of mature sperm (colourless) and immature sperms (blue) were determined (23).

Assessment of sperm DNA damage

Acridine orange (AO) staining was used to evaluate any break in the double-stranded DNA of the mice sperm. The prepared semen samples were dried, then fixed for 2 hours using a Carnoy's solution, and subsequently stained with AO for 10 minutes. After the slides were washed with water, we examined them with a fluorescence microscope that had a 460 nm filter. The healthy double-stranded DNA showed a green fluorescent colour, whereas the DNA from single-stranded denatured DNA had a yellow to red colour. The results of the DNA damage were presented in percentages (23).

Sample preparation steps for *in vitro* fertilization

One hour after sperm capacitation, 6×10^6 sperm/ml of medium were added to the fertilization drops. After 4 to 6 hours, we observed male and female pronuclei formation (the percentage of zygotes), and, after 24 hours the percentage of two-cell embryos, and within 4-5 days the percentage of blastocysts and hatched embryos were investigated (24).

Stimulation of ovulation

After 35 days, the mice were prepared for IVF. After ensuring the setting of the light cycle (12 hours light/12 hours dark) of the female mice that is essential for the regulation of the sexual cycle and lasts for at least 2 weeks. Female mice ovaries were stimulated to obtain mature oocytes. The female mice received injections of 0.2 ml of 10 IU of pregnant mare serum gonadotropin (PMSG, Folligon, The Netherlands) and after 46-48 hours, they received IP injections of 0.2 ml of 10 IU of human chorionic gonadotropin (hCG, Folligon, The Netherlands). Ovulation occurred 10-12 hours after the hCG injection. The oocytes were removed from the ampullae of the oviducts by dissection, and subsequently transferred to fertilization droplets (HTF medium) (19).

Ovulation and *in vitro* fertilization

Between 10-12 hours after the hCG injection (the next morning), 7 female mice were anesthetized by injections of ketamine (40 mg/kg) and xylazine (5 mg/kg), and then they were euthanized by displacement of the neck vertebrae. After sterilization of the abdominal area, the uterine tubes were detached (Fig.1A, B) and placed in a 37°C equilibrated medium. Then, the oocytes were dissected from the fallopian tubes, washed, and transferred to fertilization droplets under mineral oil that included HTF

medium with 4 mg/ml of BSA (Fig.2). The number of potentially active sperm increased to about 1 million/ml in the medium. Fertilization occurs approximately 4-6 hours after addition of the sperm, and with the observation of two pronuclei. The resultant fertilized oocytes (zygotes) were washed, transferred into fresh medium, and equilibrated (19).

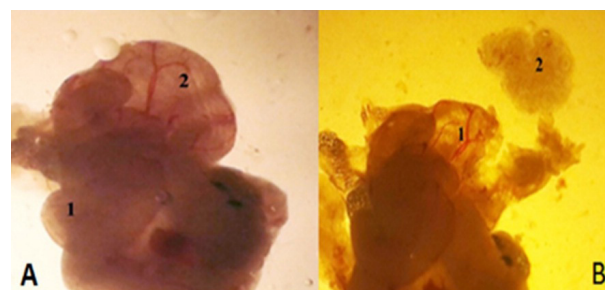


Fig.1: Dissected fallopian tube for obtaining oocyte mass. **A.** Fallopian tubes (1), Ampulla (2) and **B.** Ampulla (1), Detached oocyte masses (2) (magnification: $\times 40$).

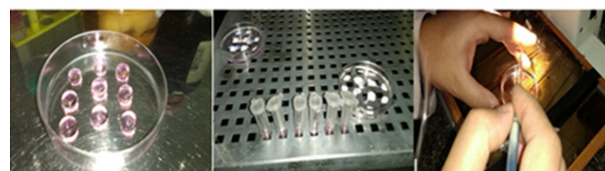


Fig.2: The dissecting steps.

We evaluated the amounts of fragmentation 24 hours after culture, and embryonic development was studied on the fifth day of fertilization. The embryos were examined for the degree of fragmentation, the rate of foetal growth duration or the number, and the type of arrested embryos. We defined the arrested embryos as: type I (embryos with perfect fragmentation and complete necrosis); type II (embryos with fragmentation in several blastomeres); and type III (embryos with a scanty amount of lysis, fragmented blastomeres, and cytoplasmic vesicles).

Statistical analysis

Data obtained from the sperm evaluation and IVF were analysed by Minitab[®] software (version 16). All data were compared by nonparametric statistical analysis with the Kruskal-Wallis H test. A $P < 0.001$ was considered significant.

Results

Quantitative evaluation of the sperm

The average number of sperm in the PQ group ($24.33 \pm 1.45\%$) was significantly different than the control ($40.33 \pm 2.90\%$) and PQ+Cr ($31.00 \pm 1.73\%$) groups ($P < 0.001$). There was a significant difference between the treatment and the Cr ($39.33 \pm 1.20\%$) groups ($P < 0.001$, Table 1). No significant difference existed between the control and Cr groups.

Evaluation of sperm motility

The average percentage of sperm motility showed a significant difference in the PQ ($72.33 \pm 2.72\%$) compared with the control ($88.00 \pm 2.64\%$) group ($P < 0.001$). The mean percentage of sperm motility was $81.00 \pm 1.51\%$ in the treatment group, which was a significant difference compared with the control group ($P < 0.001$). The Cr group ($83.66 \pm 2.60\%$) had no significant difference with the control group; however, the treatment group showed a significant difference with the Cr group ($P < 0.001$, Table 1).

Evaluation of immature sperm

There was a significant difference in average percentage

of immature sperm in the PQ group ($15.66 \pm 0.66\%$) compared with the control ($5.33 \pm 0.88\%$) and PQ+Cr ($9.33 \pm 0.88\%$) groups ($P < 0.001$). The Cr group ($4.66 \pm 0.88\%$) was not significantly different from the control group (Fig.3A, Table 1).

Evaluation of sperm viability

The results showed a significant difference in the number of viable sperm between the PQ group ($72.00 \pm 5.29\%$) and the control ($89.33 \pm 2.90\%$) groups ($P < 0.001$). The mean number of viable sperm showed no significant difference in the treatment group ($80.00 \pm 2.62\%$) compared to the control group and no significant difference with the Cr group ($88.33 \pm 3.52\%$) as seen in Figure 3B and Table 1.

Table 1: Average percentage of data from sperm quality parameters in the different groups

Group	Count $\times 10^6$	Motility	Viability	Immaturity	DNA damage
Con	40.33 ± 2.90^{ab}	88.00 ± 2.64^{ab}	89.33 ± 2.90^a	5.33 ± 0.88^{ab}	2.56 ± 0.46^{abc}
25%	35.00	84.00	84.00	4.00	1.8.00
Media	41.00	87.00	90.00	5.00	2.5.00
75%	45.00	93.00	94.00	7.00	3.4.00
PQ	24.33 ± 1.45^{bc}	72.33 ± 2.72^{bc}	72.00 ± 5.29^c	15.66 ± 0.66^{bc}	19.00 ± 2.51^c
25%	22.00	67.00	62.00	15.00	16.00
Media	24.00	74.00	74.00	15.00	17.00
75%	27.00	76.00	80.00	17.00	24.00
PQ+Cr	31.00 ± 1.73^c	81.00 ± 1.15	80.00 ± 2.64	9.33 ± 0.88^c	13.33 ± 3.38
25%	28	79.00	75.00	8.00	9.00
Media	31	81.00	81.00	9.00	11.00
75%	34	83.00	84.00	11.00	20.00
Cr	39.33 ± 1.20	83.66 ± 2.60	88.33 ± 3.52	4.66 ± 0.88	6.66 ± 1.20
25%	37.00	79.00	83.00	3.00	5.00
Media	40.00	84.00	87.00	5.00	6.00
75%	41.00	88.00	95.00	6.00	9.00

Data are presented as mean $\pm$ SEM. Con; Control group, PQ; Paraquat group, and Cr; Crocin group. The superscript letters "a, b, and c" indicate a significant difference with the PQ, PQ+Cr, and Cr groups respectively ($P < 0.001$).



Fig.3: Evaluation of sperm viability, maturity and DNA damage. **A.** Mature sperm are pale (1) immature sperm are light to dark and blue colour (2) (aniline blue, magnification: $\times 400$), **B.** Nonviable sperm are pink to red colour (1) and the viable sperm are achromatic (2) (eosin-nigrosin, magnification: $\times 400$), and **C.** Sperm without (1) and with (2) DNA damage [Acridine orange (AO), $\times 400$ magnification considering the camera magnification of $\times 300$].

Evaluation of the number of sperm with damaged DNA

A significant difference in the average number of DNA-damaged sperm was observed in the control group ($2.56 \pm 1.20\%$) compared with the PQ ($19.0 \pm 2.51\%$), PQ+Cr ($13.33 \pm 3.38\%$), and Cr ($6.66 \pm 1.20\%$) groups ($P < 0.001$). The PQ group also showed a significant difference compared with the Cr group ($P < 0.001$). The average number of the damaged sperm was not significantly different between the treatment and the Cr groups (Fig.3C, Table 1).

In vitro fertilization

Percentage of fertilization

The results of the IVF test showed a significant difference between the PQ group (52.66%) in comparison with the control group (89.87%, $P < 0.001$). The PQ+Cr group showed a significant difference with the PQ group (69.83%, $P < 0.001$). Crocin, alone, did not have a significant effect on fertilization percentage compared to the control group, but it showed a significant difference with the PQ and PQ+Cr (89.48%) groups ($P < 0.001$, Table 2).

Percentage of two-cell embryos

A comparison of the percentage of two-cell embryos that indicated the onset of cleavage showed that the percentage of these embryos were 91.42% in the control group and 77.71% in the PQ group, which was significantly different ($P < 0.001$). The percentage of two-cell embryos in the PQ+Cr group (78.63%) did not show any significant difference with PQ group. There was no significant difference between the Cr (88.77%) and the control group (Fig.4, Table 2).

Percentage of four-cell embryos

A comparison of the percentage of four-cell embryos, which indicated the onset of fragmentation revealed that PQ caused a significant difference in the percentage of these embryos, from 82.76% in the control group to 62.60% in the PQ group ($P < 0.001$). Co-administration of Cr and PQ improved the percentage of four-cell embryos to about 63.04% compared to the PQ group, but that difference was not significant. The Cr group (82.99%) showed no significant difference with the control group (Fig.4, Table 2).

Blastocyst percentage

The percentage of embryos that reached the blastocyst stage after 120 hours showed a significant difference between the PQ (35.80%) and the control (66.23%) groups ($P < 0.001$). The PQ+Cr group (47.91%) was significantly different compared with the control and Cr groups ($P < 0.001$). The Cr group (71.45%) was significantly different from the PQ and PQ+Cr groups ($P < 0.001$), but the Cr group had no significant difference with the control group (Fig.4A-C, Table 2).

Percentage of hatched embryos

PQ caused a significant difference in the percentage of hatched embryos, from 59.78% in the control group to 25.45% in the PQ group ($P < 0.001$). The PQ+Cr group had 39.45% hatched embryos, which differed from the percentages of hatched embryos in comparison with the other groups ($P < 0.001$). The Cr group did not show any significant difference with the control group (59.63%, Fig.4A, B, Table 2).

Table 2: Average percentage of obtained data from *in vitro* fertilization (IVF) parameters in the different groups

Groups	Zygote	2-cell	4-cell	Blastocyst	Hatching	Total arrest	Arrest type I	Arrest type II	Arrest type III
Con	89.87 ± 1.60^{ab}	91.42 ± 1.98^{ab}	82.76 ± 5.11^{ab}	66.23 ± 1.39^{ab}	59.78 ± 2.05^{ab}	35.63 ± 3.45^{ab}	15.53 ± 0.62^{ab}	20.86 ± 2.56	63.09 ± 2.47^{ab}
25%	86.84	87.87	72.72	63.63	57.57	31.15	14.280	16.16	58.33
Median	90.47	91.66	86.11	66.66	57.89	33.33	16.160	21.42	64.28
75%	92.30	94.73	89.47	68.42	63.88	42.42	16.160	25.00	66.66
PQ	52.66 ± 4.95^{bc}	77.71 ± 1.61^c	62.60 ± 2.83^c	35.80 ± 1.37^{bc}	25.45 ± 1.55^{bc}	64.18 ± 1.37^{bc}	69.80 ± 0.82^{bc}	18.51 ± 1.74	11.67 ± 2.41^c
25%	46.66	76.00	57.14	33.33	23.80	61.90	68.750	15.38	7.14
Median	48.83	76.19	64.00	36.00	24.00	64.00	69.230	18.75	12.50
75%	62.50	80.95	66.66	38.09	28.57	66.66	71.420	21.42	15.38
PQ+Cr	69.82 ± 0.90^c	78.63 ± 3.08^c	63.04 ± 4.55^c	47.91 ± 2.79^c	39.45 ± 3.60^c	52.07 ± 2.79^c	62.69 ± 2.86^c	16.18 ± 1.90	18.72 ± 2.69^c
25%	68.290	73.33	57.14	44.00	35.71	46.66	57.14	14.28	13.33
Median	69.760	78.57	60.00	46.42	36.00	53.57	62.28	14.28	21.42
75%	71.420	84.00	72.00	53.33	46.66	56.00	66.66	20.00	21.42
Cr	89.48 ± 3.27	88.77 ± 2.27	82.99 ± 1.44	71.45 ± 1.98	59.63 ± 3.76	28.53 ± 1.99	13.38 ± 3.06	20.71 ± 4.01	65.89 ± 5.82
25%	83.87	85.00	80.76	67.50	52.38	26.19	7.69	15.38	57.14
Median	89.36	88.46	82.50	73.07	61.53	26.92	14.28	18.18	63.63
75%	95.23	92.85	85.71	73.80	65.00	32.50	18.18	28.57	76.92

Data are presented as mean $\pm$ SEM. 2-cell; Two-cell embryo, 4-cell; Four-cell embryo, Con; Control group, PQ; Paraquat group, PQ+Cr; Paraquat and crocin group, and Cr; Crocin group. The letters "a, b, and c" in a column indicate a significant difference with the PQ, PQ+Cr, and Cr groups, respectively ($P < 0.001$).

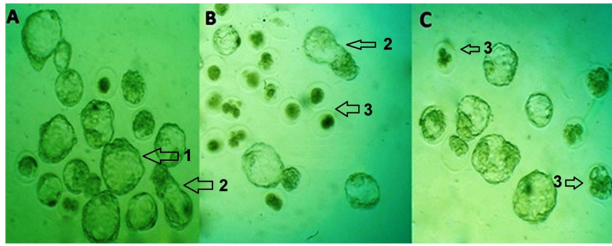


Fig.4: Zygotes and early embryos in different stages of development or arresting. **A.** In the control group, several embryos are seen in the blastocyst and hatching stages, **B.** In the PQ group, several hatched embryos are present at the blastocyst stage, and numerous infertile oocytes and arrested embryos are present and **C.** In the paraquat plus crocin (PQ+Cr) group, several hatched embryos are present in the blastocyst stage. There are several arrested embryos present, (magnification: $\times 300$). 1; Blastocyst, 2; Hatched embryo, and 3; Arrested embryo.

Percentage of total arrested embryos

The total number of arrested embryos showed a significant difference between the control (35.63%) and the PQ (64.18%) groups ($P < 0.001$). The PQ+Cr group (52.07%) had a significantly different percentage of arrested embryos compared to the PQ group ($P < 0.001$). However, in the Cr group there was no significant difference with the control group (Table 2).

Percentage of type I arrested embryos

A comparison of the type I arrested embryos showed that this parameter was significantly different in the PQ group (69.80%) compared with the other groups ($P < 0.001$). There was no significant difference between the control group (15.53%) and the Cr groups (13.38%), but there was a significant difference between the PQ+Cr (62.69%) and the control and Cr groups ($P < 0.001$, Table 2).

Percentage of types II and III arrested embryos

The percentage of type III arrested embryos in the PQ group had a significant difference with the control and Cr groups ($P < 0.001$). There was no significant difference between the groups for type II arrested embryos. However, there was no significant difference in the percentage of type III arrested embryos between the PQ and the PQ+Cr groups (Table 2).

Discussion

In the present study, we evaluated the experimental groups in two sections: spermatogenesis and early embryonic growth. The results of the first part of the experiment showed that PQ could significantly decrease sperm quality; thereby, in the second part, the PQ group had decreased percentages of fertilization, two-cell embryos, four-cell embryos, blastocysts, and hatched embryos. This group also had increased percentages of whole arrested embryos, and types I, II, and III arrested embryos. In this study, PQ could significantly reduce the number of sperm, the average percentages of sperm motility, and sperm viability, and could significantly increase the percentages of immature sperm and those with damaged DNA, which

significantly differed from the control and Cr groups. The results of the two sections of the experiments showed that Cr in the experimental group significantly improved the damage induced by paraquat. In most of the studied parameters, we observed a significant difference between the PQ+Cr and PQ groups.

In previous studies, it has been reported that oxidative stress in animals can cause infertility by affecting the genital organs (25). In a study conducted on men, it has been shown that psychological stress can reduce both motility and sperm quality, and lead to infertility (26). Genital damage is one of the known side effects of PQ poisoning, which has a toxic effect on reproductive systems of both sexes, and it can disrupt the process of spermatogenesis and oogenesis (27). The effects of low doses of PQ on the spermatozooids of Sprague-Dawley rats were studied. The researchers noted that the mean number of sperm decreased on the seventh and fourteenth days after the injection, and abnormal sperm significantly increased. Sperm mortality also increased with higher doses. In this study, it was found that PQ has genotoxic and cytotoxic effects on male germinal cells (28).

The first indication of an increase in ROS is the loss of sperm motility (29). The production of free radicals in mitochondria damage the DNA of mitochondria, and it can also damage the mitochondrial region of the middle part of the spermatozoid (30). Mitochondrial damage of the middle part of the sperm leads to a progressive decrease in sperm motility in terms of decreasing the numbers of motile sperm and decreasing the vehemence of motility (31).

Antioxidants in semen are categorized within the endogenous antioxidant group. Several studies have shown that antioxidants do not reduce sperm motility; however, they increase Sperm capability (32). Laboratory studies have also confirmed the role of antioxidants in reducing ROS production and improving the evolutionary ability of the foetus (33). Results of a study revealed that administration of citrus flavonoid extract significantly increased the total antioxidant capacity (TAC) and superoxide dismutase (SOD) levels, and sperm percentage, viability, and motility, and decreased MDA levels. This suggested that citrus, as an antioxidant, may be promising for enhancement of healthy sperm parameters (34).

In the present study, the number of fertilized oocytes in the PQ group significantly differed from the control and Cr groups, which indicated that PQ had a negative effect on fertility. This might be due to an increase of free radicals in the testicular tissue and semen, and ultimately damage the membrane of the gametes; it can reduce the percentage of IVF. However, Cr with PQ compensates for this failure.

Cr appears to have an antioxidant activity when tested *in vitro*. This activity is linked to half of its sugar content (35). In one study, the antioxidant activity of Cr was evaluated and showed that administration of Cr reduced the level of MDA and increased ferric reduction antioxi-

dant power (FRAP) following stress from renal ischemic reperfusion (36). In one study, the results of IVF showed an increase in fertilization, two-cell embryos, and blastocysts in the group that received Cr and cyclophosphamide (CP) in comparison with the CP-only group. It was also shown that in mice that received Cr along with CP the overall numbers of the arresting embryos was decreased in comparison with the CP-alone group. In this study, administration of Cr along with CP reduced MDA malondialdehyde and sperm with damaged DNA in comparison with the CP group (19).

In the present study, the average number of blastocytes and embryos at the hatching stage in the PQ group significantly differed from the control group. In both cases, the combined use of PQ with Cr ameliorated this effect. This finding showed the antioxidant effect of crocin, while the use of Cr alone did not show any negative effect on any of the above parameters. The PQ group had a significantly different number of arrested embryos compared to the control group, and this effect might be due to the destructive effect of PQ on the membrane and genome of the embryos, whereas Cr has a recovery effect. PQ also caused a negative effect on the percentage of the type I arrested embryos, and Cr ameliorated the percentage of the type III arrested embryos, which was due to its antioxidant properties.

The onset of fragmentation was also caused by the effect of PQ on the fertilized oocytes, which might be due to the effects of PQ on genetic and intracellular factors. This decrease was also observed in the four-cell zygotes, while Cr showed a better protective effect on two-cell zygotes. The effect of PQ on the cellular stage was such that Cr could not compensate for it at this stage. The two factors that protect the sperm DNA against oxidation are the density of the DNA nucleus and presence of antioxidant agents in semen plasma (37). In a study on diabetic men, it was found that the mechanisms that led to the damage of sperm DNA with increasing ROS, which could be a factor in the glycosylation of the final products of advanced glycation end products (38). In addition, the increase of oxidative stress and high fragmentation of DNA continuously occurs with apoptosis (39).

Conclusion

According to the findings of this study, we concluded that crocin, as an antioxidant, protected the male genital organs against the impacts of oxidative stress induced by PQ and significantly ameliorated both sperm quality and IVF outcomes in paraquat-treated mice. However, this study should be performed at the serological and molecular levels because there is not an adequate knowledge about the effects of PQ poisoning on *in vivo* embryo development.

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Authors' Contributions

F.S.K.: Data collection and evaluation, performed the study and drafted the manuscript. R.Sh.: Contributed to the conception and study design, technical support, supervision of the manuscript, provided final approval of the manuscript, and statistical analysis. Gh.R.N.: Conducted the IVF procedure and statistical analysis. M.R.: Contributed to the scientific review and assisted with discussion and writing some sections of the manuscript. All authors edited and approved the final version of this manuscript.

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Effect of Zinc Supplementation on Urate Pathway Enzymes in Spermatozoa and Seminal Plasma of Iraqi Asthenozoospermic Patients: A Randomized Clinical Trial

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Abstract

Background: Uric acid (UA) is crucial for sperm metabolism as it protects seminal plasma against oxidative damage. Zinc also plays a central role in sperm metabolism. The current study was designed to investigate the role of zinc supplementation on qualitative and quantitative properties of seminal fluid, in parallel with the UA level and urate pathway enzymes in the semen of patients with asthenozoospermia.

Materials and Methods: The study was designed as a randomized clinical trial of 60 asthenozoospermic subfertile men. The current study, which was conducted during one year, involved 60 fertile and 60 asthenozoospermic subfertile men belonging to Hilla City, Iraq. Semen samples were obtained from the participants before and after treatment with zinc supplements. The levels of UA, xanthine oxidase (XO), adenosine deaminase (ADA) and 5'-nucleotidase (5'-NU) activities were determined in spermatozoa and seminal plasma of both groups.

Results: UA levels ($P=0.034$) and 5'-NU activity ($P=0.046$) were significantly lower but ADA ($P=0.05$) and XO ($P=0.015$) activities were significantly higher in infertile men than in healthy men. Treatment with zinc sulfate induced an increase in UA ($P=0.001$) level and 5'-NU activity ($P=0.001$), but a decrease in ADA ($P=0.016$) and XO ($P=0.05$) activities.

Conclusion: Zinc supplementation restores UA levels and the activities of enzymes involved in the urate pathway (XO and ADA) in the seminal plasma and spermatozoa of patients with asthenozoospermia, to reference values. Supplementation of Zn compounds enhances the qualitative and quantitative properties of semen (Registration number: NCT03361618).

Keywords: Adenosine Deaminase, 5'-Nucleotidase, Uric Acid, Xanthine Oxidase, Zinc Supplementation

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Introduction

Male infertility as an underlying cause of subfertility, is observed in approximately 20% infertile couples. Although the percentage reaches up to 40% couples, both female and male factors are accounted. Thus, half of all infertility cases are caused by male-related factors (1). Asthenozoospermia, or low sperm motility, may be caused by sperm structural or functional deficiencies, a harmful effect of seminal plasma, or a combination of these factors. There are numerous factors, such as oxidative stress and nutritional insufficiency, contributing to male infertility (2). Although zinc is found in most food types, the World Health Organization (WHO) estimates that 33% of the world population suffer from zinc deficiency (3). Zinc is a fundamental micronutrient essential for different biochemical functions in mammals. Zinc has two forms: the first is found in the muscles, most of which is inadequately exchangeable and closely bound

to high molecular weight ligands, such as nucleic acids, nucleoproteins, and metalloproteins; and the second form is freely exchangeable and is tightly bound to citrate and amino acid (4). Zinc is involved in cell differentiation and proliferation by regulating protein synthesis, nucleic acid metabolism, and secretion of growth hormone, testosterone, prolactin, and other steroid hormones. Zinc acts as a structural component of several enzymes that participate in DNA synthesis and transcription. It is also attached to zinc-binding proteins of more than thousands of transcription factors where these factors supply a platform for interaction with proteins or nucleic acids (5).

The level of production of reactive oxygen species (ROS) in male reproductive tract, is of crucial importance because of the possible noxious properties of high concentrations of ROS; these noxious effects affect the physical properties of semen quality (6). Normal levels of ROS are essential for the regulation of normal sperm

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functions (7), motility, hyperactivation, and capacitation and acrosome reaction and sperm-oocyte fusion (8). Conversely, elevated concentrations of ROS can negatively affect semen quality. Pathological effects of ROS include increased lipid peroxidation (LPO) levels, decreased sperm motility, DNA damage, and apoptosis (9). Oxidative stress-induced sperm damage has been explained to be a significant contributing factor in 30-80% of all cases of male subfertility (10). Men with subfertility who produce high concentrations of ROS, are seven times less likely to create a pregnancy compared with those producing low concentrations of ROS. ROS production can be aggravated by environmental, infectious, and lifestyle etiologies (11).

Uric acid (UA) is the final compound of nucleotide catabolism. It reacts with oxidants as an essential water-soluble antioxidant. Consequently, UA has a possible function in resisting spermatozoal oxidative damage. The regulatory effector (adenosine) and cell energy compound, adenosine triphosphate (ATP), were maintained by adenine nucleotide catabolism that embodies central pathways of the intermediary metabolism. In most tissues, an ideal adenine nucleotide pool is provided via a specialized mechanism that correlates with adenosine 5' monophosphate (AMP) metabolism (12). Two fundamental enzyme sequences normally participate in the catalysis of the original AMP metabolism pathway. The first is AMP deaminase, which catalyzes the deamination of AMP to produce inosine monophosphate (IMP). The second is 5'-nucleotidase (5'-NU), which catalyzes the dephosphorylation of AMP to produce adenosine. The catabolism process further includes the conversion of adenosine to inosine via adenosine deaminase (ADA) catalytic activity. Xanthine oxidase (XO) catalyzes the terminal degradation of purine bases that generate UA, which is the final product of purines catabolism. ROS are produced during the enzymatic reaction of XO (13).

The proposed defensive role of UA against ROS in human seminal plasma has not been adequately tested. Only few research studies investigating the levels of UA in seminal fluid and the antioxidative resistance function of UA (14), have been published in scientific journals.

Furthermore, considerable controversies and inconsistencies exist in the literature. Although UA is an essential part of the total antioxidant status of human seminal fluid, which is reduced in subfertile subjects (12), another study (14) documented that UA levels were reduced in patients with normozoospermia. Consequently, the accurate UA level in seminal fluid is still undetermined.

Although few reports have investigated the association between subfertility and UA levels in semen, to the best of our knowledge, no study has reported the effects of asthenozoospermia treatments, such as oral zinc supplementation, on the activity of urate-related enzymes, which are important in fertility of the humans. The present study was designed to investigate the effect

of zinc treatment on the qualitative and quantitative properties of semen, as well as UA concentrations and urate-related enzymes in the seminal fluid of men with asthenozoospermia.

Materials and Methods

Objectives

The primary objective was to determine the effect of zinc treatment on the qualitative and quantitative properties of semen. The secondary objective was to investigate the effect of zinc treatment on the UA concentrations and urate-related enzymes in the seminal fluid of men with asthenozoospermia.

Study design

The trial was designed as a randomized clinical trial. The randomized trial was designed as a parallel group, superiority trial with 1:1 allocation ratio. No placebo was used in the current study. Double blind trial was applied for both the investigator and subject. The time of random allocation was completed immediately after the assessment for eligibility. Sixty male partners (age 32.8 ± 3.57 years) with subfertility were included in the present study. All couples were consulted at the Infertility Clinic of the Babylon Teaching Hospital of Maternity in Babylon governorate, Hilla City, Iraq from July 2011 to July 2012. Sample size was estimated according to Kadam and Bhalerao (15) method.

$$n = [2(Z_{\alpha} + Z_{1-\beta})^2 \sigma^2] / \Delta^2$$

n=number of samples

Z_{α} , is 1.96 (constant that obtained from ref (15)).

$Z_{1-\beta}$, is 0.8416 (constant that obtained from ref (15), when a study has 80% power.

σ = population standard deviation, it is equal to 1.23 according to published article.

Δ = the difference in effect of two interventions. i.e., it is equal to $3.66 - 3.04 = 0.62$.

The method that was used to generate the random allocation sequence, includes using a random-numbers table. Type of randomization is block randomization works by randomizing participants within blocks and allocates an equal number to each group. Professor Abdul Razzaq Alsalman generated the random allocation sequence, enrolled participants, and assigned participants to interventions. Figure 1 shows the flow of participants recruitment in this trial. A physical examination was completed, and complete medical history was recorded for each participant. Subjects who were administered with antioxidant supplementation or any other medication during the study period, were excluded from the study. The study was approved by The Institutional Research Ethics Committee [Ethics Committee (University of Babylon/College of Science), Reference number of approval: 545], and informed consent was obtained from all individual participants

included in the study. The criteria for inclusion in the study were presence of asthenozoospermia, absence of varicocele, female factor infertility, and endocrinopathy. Smokers were excluded from the study because of their distinguished low antioxidant concentrations and elevated seminal ROS concentrations. The selection criteria for inclusion into the fertile group, were as follows: those with children born within the previous year, absence of asthenozoospermia, endocrinopathy, and varicocele. All seminal analyses were performed based on the 2010 WHO recommendation. These analyses included checking for semen pH, sperm motility, semen volume, semen concentration, normal sperm, and round cell morphology (16). No changes were made to the methods during the study period.

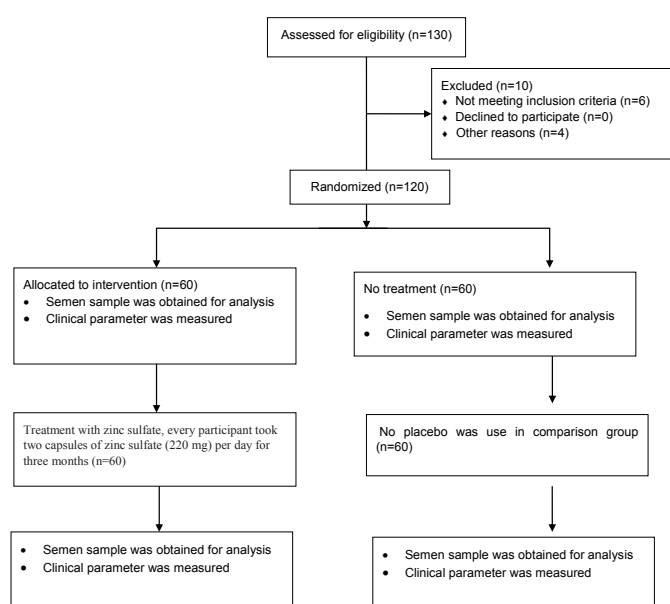


Fig.1: Flow of participants recruitment in the present trial.

Preparation of spermatozoa and seminal plasma for biochemical analysis

Spermatozoa were separated from the seminal plasma 1 hour after semen collection. Subsequently, 2 ml of seminal fluid was centrifuged at 1600 g for 15 minutes at 2°C and stored at -30°C until biochemical assessment. The pellet was washed with ten volumes of NTPC medium (a medium composed of NaCl, NaH₂PO₄, Na₂HPO₄, Tris, EDTA, CaCl₂, and D-glucose) and centrifuged at 1600 g for 10 minutes at 2°C. This washing process was repeated thrice. The resultant pellet was vigorously mixed with 0.1% Triton X-100 and was then recentrifuged at 8000 g for 30 minutes in a refrigerated centrifuge. The supernatant was used for biochemical assessments.

Semen samples were obtained from participants before and after treatment with two capsules of zinc sulfate (220 mg) per day for three months. The collected samples were categorized into three groups: group 1 (G1): healthy fertile subjects; group 2 (G2): patients with subfertility before

treatment; and group 3 (G3): patients after treatment.

Preparation of NTPC medium

The medium contained Tris buffer (20 mM, 0.242 g/100 ml), D-glucose (1.5 mM, 0.0027 g/100 ml), Ethylenediaminetetraacetic acid (EDTA) (0.4 mM, 0.0148 g/100 ml), NaCl (113 mM, 0.66 g/100 ml), Na₂HPO₄ (2.5 mM, 0.0355 g/100 ml), NaH₂PO₄ (2.5 mM, 0.3 g/100 ml), and CaCl₂ (1.7 mM, 0.0188 g/100 ml). Finally, pH was adjusted to 7.4 using 0.1 M HCl. D-glucose was procured from Sigma, USA. Other chemicals were obtained from BDH Chemicals Ltd, Poole, Dorset, UK.

Reagents and solutions

All reagents and solutions were obtained from standard commercial suppliers, were of analytical grade and were used without further purification.

Biochemical methods

Determination of the adenosine deaminase activity

ADA was measured using the protocol described by Martinek (17), in which the ammonia produced by deamination reaction reacts with hypochlorite to form an intermediate, monochloramine, which in turn reacts with added phenol to form blue-color indophenols that have maximum absorption at 640 nm. The reaction mixture was composed of 0.5 ml of buffered substrate (adenosine; pH=7.05) and 0.05 ml of specimen incubated for 3 minutes at 37°C in a water bath. Then, 2.5 ml of phenol reagent and subsequently, 2.5 ml of hypochlorite reagent were added. Absorption was read at 640 nm, against the reagent blank. Test units activity of ADA activity is obtained from the calibration curve.

Determination of xanthine oxidase activity

XO activity was determined using Hadwan et al. (18) method. This method is based on the reaction between H₂O₂ and thiamine to produce fluorescent thiochrome with excitation and emission wavelengths of 370 and 425 nm, respectively. Reaction mixture consisted of 30 µl of specimen, 0.3 mM xanthine and 50 mM 3-aminotriazole dissolved in 1000 µl of 50 mM sodium phosphate buffer (pH=7.4). XO activity was obtained from the standard curve plotted for concentration of hydrogen peroxide against fluorescence intensity.

Determination of 5'-nucleotidase activity

U activity was done using Hadwan et al. (19) method, in which phosphate is liberated by the reaction of molybdate in the acidic medium leading to formation of a complex of phosphomolybdate, which is in turn reduced to unstable molybdenum blue. A volume of 0.2 mL of a specimen was taken, and then 0.1 mL of 0.02 M MnSO₄ and 1.5 mL of 40 mM (pH=7.5) barbitone buffer, were added. One unit of activity of 5'-NU is defined as the release

of 1 μmol inorganic phosphate per minute. The level of UA in serum was enzymatically measured using the Biomeghrib® kit (Morocco).

Statistical analysis

SPSS 21 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Results are expressed as mean, standard deviation (SD), standard error (SE), and range. Data were analyzed using one-way analysis of variance (ANOVA). The Kolmogorov Smirnov test was used to verify if data followed normal distribution. A significance level of $P \leq 0.05$ was considered to estimate differences in mean values of the following three groups: G1 (healthy donors), G2 (patients before treatment), and G3 (patients after treatment).

Results

Table 1 presents the baseline values of the semen parameters. The ejaculated seminal fluids after treatment were reported in Table 1 in order to easily compare it with the baseline data. The patients in the present study were classified into three groups: G1 (healthy donors), G2 (patients before treatment), and G3 (patients after

treatment). Seminal parameters were significantly decreased in the subfertile group (G2) compared to healthy donor group (G1). Seminal parameters were included normal sperm count ($P=0.023$), progressive sperm motility ($P=0.001$), and semen volume ($P=0.042$). The results presented significant improvements in ejaculate properties in the group treated with zinc compared to the same group before treatment with zinc. Seminal parameters were included the normal sperm count ($P=0.03$), progressive sperm motility ($P=0.005$), and semen volume ($P=0.037$).

The UA levels and 5'-NU, ADA, and XO activities of seminal plasma and spermatozoa in the patients and healthy groups are presented in Tables 2, 3, 4, and 5, respectively. As compared with healthy subjects (G1), the spermatozoa and seminal plasma of patients with asthenozoospermia (G2) indicated decreases in UA levels and 5'-NU activity but increases in ADA and XO activities. However, zinc supplementation restored the seminal plasma concentration of UA ($P=0.034$) and activities of 5'-NU ($P=0.046$), ADA ($P=0.05$) and XO ($P=0.015$) to normal levels in treated patients (G3).

Table 1: Parameters of ejaculated seminal fluids in asthenozoospermic patients and healthy subjects

Name of group	Volume (mL)	Sperm count ($\times 10^6$)	Progressive sperm motility (%)	Normal sperm form (%)
Healthy donors (G1)	2.8 ± 0.53	77 ± 9	69 ± 8	38 ± 9
Patients before treatment (G2)	$1.83 \pm 0.66^*$ ($P=0.042$)	$47 \pm 21^*$ ($P=0.023$)	$21 \pm 9^*$ ($P=0.00$)	21 ± 11 ($P=0.005$)
Patients after treatment (G3)	$2.39 \pm 0.9^{**}$ ($P=0.037$)	70 ± 15 ($P=0.03$)	$39 \pm 14^{**}$ ($P=0.05$)	$33 \pm 7^{**}$ ($P=0.041$)

Data are presented as mean $\pm$ SD. *: Significance versus group I (healthy donors) and **: Significance versus group II (patients before treatment).

Table 2: Uric acid levels in seminal plasma ($\mu\text{M/L}$) and spermatozoa ($\mu\text{M}/10^8$ spermatozoa) of asthenospermic patients and healthy subjects

Name of group	Source	Mean $\pm$ SD	$\pm$ SE	95% confidence interval for mean		Compared groups		P value
				Lower bound	Upper bound			
G1	Seminal plasma	143.90 ± 44.44	5.73	124.68	163.11	1	2	0.034*
							3	0.185
G2	Seminal plasma	109.85 ± 53.48	6.9	86.72	132.98	2	1	0.034*
							3	0.001*
G3	Seminal plasma	164.98 ± 60.84	7.85	138.66	191.29	3	1	0.185
							2	0.001*
G1	Spermatozoa	77.61 ± 22.47	2.9	59.33	95.89	1	2	0.05*
							3	0.952
G2	Spermatozoa	53.65 ± 14.27	1.84	43.58	63.71	2	1	0.05*
							3	0.05*
G3	Spermatozoa	76.87 ± 24.17	3.12	53.45	100.3	3	1	0.952
							2	0.05*

*, Significance versus group I (healthy donors), SD; Standard deviation, and SE; Standard error.

Table 3: 5'-nucleotidase activity in seminal plasma (U/l) and spermatozoa (U/10⁸ spermatozoa) of asthenospermic patients and healthy subjects

Name of group	Source	Mean $\pm$ SD	$\pm$ SE	95% confidence interval for mean		Compared groups		P value
				Lower bound	Upper bound			
G1	Seminal plasma	9.57 $\pm$ 2.68	0.34	6.71	12.43	1	2	0.046*
							3	0.062
G2	Seminal plasma	5.85 $\pm$ 1.86	0.24	3.18	8.52	2	1	0.046*
							3	0.000*
G3	Seminal plasma	13.04 $\pm$ 3.60	0.46	10.49	15.59	3	1	0.062
							2	0.000*
G1	Spermatozoa	17.09 $\pm$ 6.88	0.89	15.38	21.80	1	2	0.045*
							3	0.943
G2	Spermatozoa	11.30 $\pm$ 5.12	0.66	9.81	15.78	2	1	0.045*
							3	0.050*
G3	Spermatozoa	16.89 $\pm$ 5.96	0.76	13.89	19.98	3	1	0.943
							2	0.050*

*, Significance versus group I (healthy donors), SD; Standard deviation, and SE; Standard error

Table 4: Adenosine deaminase activity in seminal plasma (U/l) of asthenospermic patients and healthy subjects

Name of group	Mean $\pm$ SD	$\pm$ SE	95% confidence interval for mean		Compared groups		P value
			Lower bound	Upper bound			
G1	22.39 $\pm$ 4.87	0.62	15.68	29.10	1	2	0.050*
						3	0.476
G2	41.18 $\pm$ 12.42	1.06	29.29	53.08	2	1	0.050*
						3	0.016*
G3	15.92 $\pm$ 5.01	0.64	19.93	29.90	3	1	0.476
						2	0.016*

*, Significance versus group I (healthy donors), SD; Standard deviation, and SE; Standard error

Table 5: Xanthine oxidase activity in seminal plasma (mU/l) and spermatozoa (mU/10⁸ spermatozoa) of asthenospermic patients and healthy subjects

Name of group	Source	Mean $\pm$ SD	$\pm$ SE	95% confidence interval for mean		Compared groups		P value
				Lower bound	Upper bound			
G1	Seminal plasma	128 $\pm$ 34.10	4.04	98.06	188.28	1	2	0.015*
							3	0.555
G2	Seminal plasma	218 $\pm$ 53.11	6.85	173.88	263.64	2	1	0.015*
							3	0.050*
G3	Seminal plasma	151 $\pm$ 46.78	6.03	97.09	201.02	3	1	0.555
							2	0.050*
G1	Spermatozoa	110.65 $\pm$ 38.27	4.94	77.46	143.83	1	2	0.035*
							3	0.436
G2	Spermatozoa	199.88 $\pm$ 57.97	7.48	151.40	248.34	2	1	0.035*
							3	0.050*
G3	Spermatozoa	143.94 $\pm$ 63.40	8.18	97.18	205.07	3	1	0.436
							2	0.050*

*, Significance versus group I (healthy donors), SD; Standard deviation, and SE; Standard error

Discussion

The results of the current study showed that supplementation of zinc enhanced the semen quality in infertile men. Our findings verified the data reported by a previous study (6) which linked the enhancement of semen quality to the biological properties of zinc, such as spermatogenesis induction, stimulation of sex organs growth, activation of 5 α -reductase enzyme that is necessary for the conversion of testosterone into the chemically active form, 5 α -dihydrotestosterone and increment of the activity of Zn-containing enzymes that play significant roles in sperm motility such as lactate dehydrogenase and sorbitol dehydrogenase. Fallah et al. (3), highlighted the importance of Zn content of seminal plasma for men's health, normal sperm function, fertilization and germination. On the other hand, highly toxic levels of zinc may have harmful effects on sperm quality.

Compared with the control group, UA concentrations were significantly decreased in the spermatozoa and seminal plasma of the patients with asthenozoospermia. Former studies did not report the main reason underlying decrement of UA levels in patients with asthenozoospermia (20). However, decreased UA could be related to elevated peroxynitrite levels in the seminal plasma of patients with asthenozoospermia. UA acts as a scavenger of peroxynitrate, to produce nitrated UA (21).

Oxidative stress might be the main reason for the depletion of UA in the spermatozoa and seminal plasma of patients with asthenozoospermia; UA was speculated to have ROS scavenging activity, and regular treatment with UA was documented to enhance antioxidant capacity (22). Mikami et al. (23) demonstrated a significant reverse correlation between oxidative stress and UA levels. UA reacts with ROS and convert them to an oxidized form, in mammalian tissues. Its action as an antioxidant is a so-called comprehensive mechanism in mammalian tissues, where it may offer an oxidizable co-substrate role to any attacking ROS, therefore, protecting the macromolecules from oxidative stress injury (20). In addition, UA preserves the integrity of cell membranes by preventing membrane LPO. It also participates in the stabilization of vitamin C antioxidant activity in the seminal plasma (24).

Administration of zinc salt supplements increases the UA concentration in asthenozoospermic seminal plasma to the reference range; this may be attributed to two mechanisms. First, it improves the total antioxidant status (6). Second, they induce the production of metallothioneins, which are low molecular mass zinc-binding proteins (5) that remove peroxynitrite from seminal plasma.

Hydrolysis of ATP produces adenosine that adapts to various reproductive functions, such as those involving contraction, steroidogenesis, and

maintenance of fluid composition. Interestingly, adenosine might act as a key capacitative modulator for mammalian spermatozoa to achieve fertilization (25). Extracellular nucleotide levels are influenced by cell surface ectonucleotidases. 5'-NT (EC 3.1.3.5) is a glycoprotein tightly bound to the membrane of mammalian spermatozoa and is an ectoenzyme with its active site facing the external medium (26). The 5'-NT of seminal plasma is a metalloprotein containing two zinc atoms per subunit of dimeric protein. Removal of the two zinc atoms from the enzyme molecule, results in a completely inactive apoenzyme (27). This enzyme is generally used in diagnosing plasma membrane abnormalities. The development of sperm fertilization and migration primarily depends upon the plasma membrane. A decrease in 5'-NT activity is usually regarded as a damage to the membrane architecture caused by elevated ROS concentrations in biological samples (28). Extracellular AMP is hydrolyzed by 5'-NT to free phosphate and adenosine. The present study demonstrates significant depletion of 5'-NT activity in the semen of patients with asthenozoospermia. This decrease may cause spermatozoal damage owing to exposure to ROS, which may consequently disturb membrane integrity and function. The most commonly proposed reason for this depletion has been the oxidative modification of 5'-NT sulfhydryl (-SH) groups and the reaction with LPO end-product. This impression comes from a previous study which reported powerful inhibition of 5'-NT activity by damaged sulfhydryl groups' compared with several other enzymes (29). Also, decreased 5'-NT activity may be caused by elevated NO levels in the asthenozoospermic semen. Siegfried et al. (30) reported that NO interacts with ecto-5'-NT and S-nitrosylation of 5'-NT probably results in inhibition of the enzyme activity. Overproduction of NO may cause an impairment of 5'-NT activity *in vivo*.

The enzyme that is subsequently produced in nucleotide catabolism is ADA, (EC. 3.5.4.4), also known as adenosine aminohydrolase. This enzyme irreversibly deaminates 2'-deoxyadenosine and adenosine to deoxyinosine and inosine. ADA is widely distributed among prokaryotic and eukaryotic cells. It is essential for the proliferation, maturation, and differentiation of lymphocytes (31). The active site of this metalloenzyme consists of Zn²⁺ that is present in the deep site of cavity and coordinates with enzyme substrate and four amino acid residues (His 15, 17, and 214 and Asp 295). Zn²⁺ is considered a sole cofactor of ADA activity (32).

The ADA values in the present study were found to be significantly elevated in asthenozoospermic patients compared with controls. The proposed mechanisms for increased ADA activity could be related to the increase of leukocyte levels and inflammatory conditions in seminal plasma in patients with asthenozoospermia (2). A previous study also reported that ADA activity increased in inflammatory diseases, indicating activation

and proliferation of T-cells. Thus, ADA is regarded as a T-cell activation marker (33). Erkiş et al. (34) documented that ADA increases the overproduction of ROS, such as H_2O_2 , O_2^- , NO, and 1O_2 . The overproduction of ROS generates oxidative stress, which amplifies inflammatory responses by propagating LPO adjacent to the membrane; this may initiate the development of spermatozoal dysfunction.

Decreased 5'-NT activity and increased ADA activity inevitably lead to decreased adenosine levels. These conditions lead to increased oxidative stress and generate unwanted complications because the amount of remaining adenosine is insufficient to perform its physiological functions. To exhaust extracellular adenosine, adenosine receptors play a role in the lowering vascular tone (35). In addition, adenosine is an essential anti-inflammatory agent, which suppresses tumor necrosis factor- α (TNF- α) production in monocytes and macrophages, thereby inhibiting the liberation of arachidonic acid and leukotriene production in neutrophils (36). Adenosine acts as an endogenous activator in antioxidant enzyme pathways (37). The 5'NU/ADA dynamic ratio was found to be increased in the group treated with zinc, indicating that adenine nucleotide metabolism may tend to stimulate adenosine production and therefore increase the pool of adenosine. The zinc supplementation decreased ADA activity owing to its anti-apoptotic and antioxidant properties.

The doses used in the present study were the same as those used in previous studies. Zinc sulfate ($ZnSO_4$) was used as an antioxidant in previous clinical studies (38). The dosage of $ZnSO_4$ used in previous clinical trials ranged from 66 to 500 mg, and the treatment duration ranged from 13 to 26 weeks. The results of these clinical trials have indicated positive benefits of $ZnSO_4$. No negative results for use of $ZnSO_4$ were reported in the previous studies; hence, investigators inferred that the dosage used was safe.

XO is a metalloenzyme containing iron, sulfur, and molybdenum in its active site; it has various functions and is widely distributed in the endothelial cells of sinusoids and capillaries (12). XO has two forms and functions: xanthine dehydrogenase (more predominant) and XO. The predominant form of XO oxidizes hypoxanthine to xanthine and UA via its dehydrogenase activity and generates NADH, whereas the minor XO with oxidase activity, produces O_2^- . The predominant XO could be modified to oxidase XO either by reversible oxidation of thiol groups in its active site or by an irreversible proteolytic attack. The formation of O_2^- by XO was intensively studied in experimental oxidative stress in seminal fluids (39).

The results of the present study showed a significant elevation in XO activity in asthenozoospermic samples compared with the control group. Increased XO activity could be related to the conversion of the

dehydrogenase form of XO into the oxidase form, by reversible oxidation of thiol groups or by irreversible proteolytic attack caused by elevated peroxynitrite levels. Also, increased activity of ADA that elevates the xanthine pool could contribute to increased XO activity. Thus, the high activity of XO can be explained by the high xanthine levels present in the semen of patients with asthenozoospermia, because xanthine is one of the substrates of XO. Therefore, the elevated levels of xanthine require elevated XO activity, which might tend to generate high oxidative stress.

An increase in the activity of the ADA and XO enzymes with contradictory low levels of UA in patients group, is attributed to the paradoxical effect of elevated oxidative stress on ADA enzyme, XO enzyme and UA.

Supplementing Zn as an inhibitor of XO activity has been paid great attention. Zinc supplementation restores XO in the seminal plasma and spermatozoa of patients with asthenozoospermia to reference values. You et al. (40) documented that Zn compounds act as XO inhibitors. XO is a rate-limiting enzyme in the degradation pathway of purine nucleotide. Because XO is considered one of the major generators of ROS, decreases in its activity may contribute to the reduction of LPO levels by dietary zinc supplementation. A limitation of the present trial was that the measurements of enzymes were completed without blinding the biochemist to the investigational groups, which has the potential for bias. Also, serum zinc status of subjects was not determined, and the study lacked a placebo-treated group. However, potential bias was reduced by random assignment of participants and through following the standardized protocol by the investigator. Although, zinc sulfate was previously used several times to treat asthenozoospermia, the present study should be repeated in different target populations to establish the external validity.

Conclusion

Zinc supplementation restores UA levels and the activities of enzymes involved in the urate pathway (XO and ADA), in the seminal plasma and spermatozoa of patients with asthenozoospermia, to reference values. Supplementation of Zn compounds enhances the qualitative and quantitative properties of semen.

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Authors' Contributions

All the authors made important contributions to the design of the study. M.H.H.; Wrote the manuscript,

and contributed to the investigation and elucidation of the data. L.A.A., A.R.S.A.; Participated in its design and coordination and assisted with preparing the draft of the manuscript. A.R.S.A.; Contributed to the implementation of the protocol. A.H.A.; Designed experiments, analyzed data and co-wrote the manuscript. All the authors have been involved in drafting and revising the manuscript, have read, and approved the final manuscript.

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Relationship between Sperm Parameters with Sperm Function Tests in Infertile Men with at Least One Failed Cycle after Intracytoplasmic Sperm Injection Cycle

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Abstract

Background: Imbalance between production of reactive oxygen species (ROS) and total antioxidant capacity in testis, epididymis, and seminal fluid can eventually lead to infertility. Abnormal sperm chromatin packaging, and DNA fragmentation is considered as the main underlying causes of infertility. Therefore, we aimed to assess relationship between sperm parameters with DNA damage, protamine deficiency, persistent histones, and lipid peroxidation in infertile men with at least one failed cycle after intracytoplasmic sperm injection (ICSI).

Materials and Methods: In this experimental study, semen samples were collected from infertile men with at least one failed cycle after ICSI (n=20). Sperm parameters, DNA damage, protamine deficiency, persistent histones, and lipid peroxidation were assessed using computer-assisted sperm analysis (CASA) system, sperm chromatin structure assay (SCSA) and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays, chromomycin A3, aniline blue, and BODIPY C11 staining, respectively.

Results: A negative significant correlation was observed between sperm concentration with percentage of sperm persistent histone ($r=-0.56$, $P=0.02$), while positive significant correlations were found between percentage of sperm persistent histones with abnormal sperm morphology ($r=0.54$, $P=0.02$), CMA3-positive spermatozoa ($r=0.6$, $P=0.008$) and intensity of lipid peroxidation ($r=0.6$, $P=0.01$). In addition, a significant correlation was observed between sperm DNA damage with intensity and percentage of lipid peroxidation ($r=0.62$, $P=0.009$, $r=0.77$, $P=0.007$). Correlation between CMA3-positive spermatozoa and intensity of lipid peroxidation ($r=0.5$, $P=0.03$) were also significant.

Conclusion: Observed significant correlations between sperm functional tests in infertile men with at least one failed cycle after ICSI, indicated that the reduction of oxidative stress by antioxidant supplementation may be considered as one therapy approach for improvement of sperm function and increase the chance of successful clinical outcomes in next assisted reproductive cycle.

Keywords: DNA Damage, Intracytoplasmic Sperm Injections Spermatozoa, Lipid Peroxidation, Protamines

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Introduction

One of the main byproducts of sperm metabolism is reactive oxygen species (ROS). Distinct roles have been envisaged for ROS at physiological and pathological levels. According to literature, a basal level of ROS are needed for processes such as sperm capacitation, acrosome reaction and sperm-oocyte fusion. But, uncontrolled or excess production of ROS can have devastating effects on sperm functions. Several studies have demonstrated that induction of lipid peroxidation cascades and fragmentation of DNA are two main pathological consequences of ROS production in sperm (1, 2).

Lipid peroxidation could lead to formation of electrophilic lipid aldehydes such as malondialdehyde, acrolein and 4-hydroxynonenal (4HNE). These aldehydes further increase ROS level through binding to nucleophilic centers of proteins, such as succinic acid dehydrogenase in the mitochondrial electron transport chain (1, 2) and thereby, induce a vicious cycle in production of ROS. The consequence of excessive ROS production is oxidation-induced apoptosis which is dose- and time-dependent (3, 4). In this regard, Aitken (5) has recently proposed a two-steps hypothesis which accounts for how DNA fragmentation occurs in sperm. The first step is a

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defect in the chromatin remodeling taking place during differentiation of spermatid to spermatozoa. This defect lead to relaxation of chromatin compared to when the chromatin is tightly packed. The second step is free radicals attack to the relaxed chromatin configuration. In addition, he stated “free radical attack might occur at any time during the life of a spermatozoon from its differentiation during spermiogenesis to its maturation and storage in the epididymis”. Collectively, these three fundamental aspects may account for etiology of DNA fragmentation in sperm (5-7). In this regard, several studies showed that there are significant correlations between sperm DNA fragmentation (SDF) with low quality of embryo, failed pregnancy and reduced implantation rate in infertile men candidate for assisted reproduction techniques (7). Considering importance of three aforementioned intrinsic factors in relation to DNA damage in sperm, we assessed sperm lipid peroxidation as an important class of generated biomolecules by oxidative stress, DNA fragmentation as a indicator of apoptotic sperm, and protamine deficiency as a chromatin maturity marker in infertile men with at least one failed cycle after ICSI.

Materials and Methods

Patients

This experimental study was performed from April 2016 to April 2018, and approved by the Ethics Committee of Royan Institute (IR.ACECR.ROYAN.REC.1396.270). Couples were informed of the study design and all the participants signed a written consent. Semen samples were obtained from 20 individuals referred to Isfahan Fertility and Infertility Center (IFIC) with male factor infertility.

Inclusion and exclusion criteria

Inclusion criteria: Couples with male factor infertility and at least one previous failed cycle after ICSI, without sign of varicocele and/reported genetic defect.

Exclusion criteria: Infertile couples with female factor infertility, individuals with leukospermia or varicocele, urinary infection, klinefelter syndrome, cancer and excessive alcohol or drug abuse.

Semen collection and analysis

Semen samples was collected from 20 infertile men with previous failed cycles after ICSI by masturbation after 3-7 days of abstinence. Part of the semen sample was used for assessment of sperm parameters (concentration, motility, morphology) with computer-assisted sperm analysis (CASA) system (Video Test, Version Sperm 2.1©, Russia) according to World Health Organization (WHO) criteria (8). The remaining portion was used for assessment of lipid peroxidation (BODIPY C11 staining), persistent histones (Aniline blue staining), protamine deficiency (CMA3 staining) and DNA fragmentation (SCSA and TUNEL assays).

Assessment of sperm lipid peroxidation

The level of lipid peroxidation in sperm was evaluated by BODIPY C11 loading BODIPY 581/591 C11 (D3861, Molecular Probes) according to Aitken et al. (9). Briefly, the sperm concentration was adjusted to 2×10^6 /ml. Equal volume of diluted sperm was mixed with equal volume of BODIPY C11 to have a final concentration of 5 mM. The mixture was maintained at 37°C for 30 minutes. Then, each sperm was washed twice at 650 g for 5 minutes. For positive control, oxidative stress was induced by hydrogen peroxide (H_2O_2 , 100 μ M) after the addition of H_2O_2 to sperm suspensions for each sample. Percentage of lipid peroxidation in sperm and intensity of lipid peroxidation in BODIPY C11 positive spermatozoa population were assessed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Intensity expresses the average of color emission intensity in cell population in the fluorescence channel. If the lipid peroxidation in the cell is further expressed, the intensity of the color (BODIPY-C11 dye) will increase.

Assessment of sperm persistent histones

Percentage of persistent histones in sperm samples was assessed by aniline blue staining according to Nasr-Esfahani et al. (10) protocol. Semen samples were washed twice with phosphate-buffered saline (PBS, Merck, Germany) at 300 g for 5 minutes, and two smears for each sample were prepared and air-dried at room temperature. Afterwards, slides were fixed in a solution of 3% glutaraldehyde in 0.2 M phosphate buffer (14 ml of 0.2 M NaH_2PO_4 plus 36 ml of 0.2 M Na_2HPO_4 , pH=7.2) for 30 minutes. Then, slides were stained with solution of 5% aniline blue in 4% acetic acid (pH=3.5) for 5 minutes. Lastly, stained smears were placed in alcohol 50, 70 and 100% for 30 seconds, respectively. For each sample, a minimum of 200 sperm cells were counted. Spermatozoa with unstained nucleus were considered as normal persistence of histones while spermatozoa with dark blue nuclei were considered as abnormal with retention of persistence of histones.

Assessment of sperm protamine deficiency

Percentage of protamine deficiency was assessed by chromomycin A3 (CMA3) staining according to Razavi et al. (11). Briefly, washed samples with PBS was fixated by Carnoy's solution (methanol:1:3glacial acetic acid) and incubated at 4°C for 5 minutes. Afterward, the sperm suspension was smeared on the slides. Then, prepared smears were treated for 20 minutes with CMA3 solution (McIlvaine buffer), and washed using PBS. Microscopic analysis of the slides was performed by an Olympus fluorescent microscope (Japan) with appropriate filters (460-470 nm). About 200 sperm cells were assessed and sperm with bright yellow color was considered as CMA3-positive spermatozoa with deficient protamine.

Assessment of sperm DNA fragmentation

Sperm DNA fragmentation was assessed by two procedures; sperm chromatin structure assay (SCSA)

and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) according to Evenson (12), and Kheirollahi-Kouhestani et al. (13) with minor alternation.

For TUNEL assay: washed semen samples were fixed by 4% paraformaldehyde for 25 minutes and treated with 0.2% Triton X-100 for 5 minutes. Then, samples were washed with PBS and stained with a detection kit of DNA fragmentation (Apoptosis Detection System Fluorescein; Promega, Mannheim, Germany) according to the manufacturer's instructions. For each sample, one positive control with an additional step [treatment of sperm with DNase I (1,000 U) after permeabilization with 0.2% Triton X-100] was considered for each sample. Finally, a minimum of 10,000 sperm were analyzed using BD Cell Quest Pro software, and the result was reported as TUNEL-positive spermatozoa for each sample.

For SCSA assay: sperm concentration was adjusted to 2×10^6 in 1ml of TNE [Tris HCl (Merck, Germany)/NaCl (Merck, Germany)/EDTA (Merck, Germany)] buffer. For test group, 400 μ l acid-detergent solution was added to 200 μ l of diluted sample in TNE buffere and after 30 seconds, 1200 μ l of acridine orange staining solution was mixed with this suspension, while for control group, only 1200 μ l of acridine orange (Sigma, St. Louis, USA) staining solution was added to 200 μ l of diluted sample. Finally, a minimum of 10,000 sperm for each sample were counted using a FACSCalibur flowcytometer, and the percentage of DNA damage was reported as SCF (14).

Statistical method

For statistical analysis, correlation coefficients were carried out with the Statistical Package for the Social Sciences software (SPSS 16, SPSS, Chicago, IL, USA). The mean, standard error, and range of variables were presented according to descriptive analysis. $P < 0.05$ was considered significant.

Results

Description of sperm parameters, and couples age were presented in Table 1. Mean of female and male age were 32.5 ± 6.4 and 37 ± 6.2 , respectively. Mean of sperm concentration, percentage of sperm total motility, and

abnormal morphology were 46.1 ± 5.4 , 34.5 ± 5.09 , and 98.1 ± 0.4 respectively.

Table 1: Description of sperm parameters, semen volume, and couples age (n=20)

Parameters	Minimum	Maximum	Mean $\pm$ SE
Female age (Y)	20	49	32.5 ± 6.1
Male age (Y)	29	51	37 ± 6.1
Sperm concentration (10^6 /ml)	8	80	46.1 ± 5.4
Sperm total motility (%)	5.2	72	34.5 ± 5.09
Sperm abnormal morphology (%)	95	100	98.1 ± 0.4
Semen volume (ml)	2	5.5	4.1 ± 0.2

The correlations analysis between sperm parameters with sperm functional tests such as DNA fragmentation, protamine deficiency, persistent histones, and lipid peroxidation (Table 2) show that there is a negative significant correlation between sperm concentration with percentage of sperm persistent histones ($r = -0.56$, $P = 0.02$), while positive significant correlations were observed between percentage of sperm abnormal morphology with percentage of sperm persistent histones ($r = 0.54$, $P = 0.02$) and intensity of lipid peroxidation ($r = 0.62$, $P = 0.01$). Other correlations were not significant at $P < 0.05$ level.

In addition, correlations between sperm functional tests were analyzed together and results are presented in Table 3. There were significant correlations between percentage of persistent histones with percentage of CMA3- positive spermatozoa ($r = 0.6$, $P = 0.008$) and intensity of sperm lipid peroxidation ($r = 0.6$, $P = 0.01$). We also observed significant positive correlations between percentage of DNA fragmentation assessed by SCSA (DFI) with DNA fragmentation assessed by TUNEL ($r = 0.83$, $P < 0.001$), percentage ($r = 0.77$, $P < 0.001$) and intensity of lipid peroxidation ($r = 0.62$, $P = 0.009$). In regard to TUNEL test, we observed a positive significant correlation between DNA fragmentation assessed by TUNEL with percentage of lipid peroxidation ($r = 0.84$, $P < 0.001$). In addition, there was a positive significant correlation between percentage of CMA3- positive spermatozoa with intensity of lipid peroxidation ($r = 0.5$, $P = 0.03$).

Table 2: Relationship between sperm parameters with sperm functional tests such as DFI and TUNEL⁺ and deficient protamine spermatozoa, persistent histones, lipid peroxidation (n=20)

Parameters	Concentration (10^6 /ml)	Total motility (%)	Abnormal morphology (%)
Persistent histones (%)	-0.56*	-0.50	0.54*
DFI (%)	-0.11	-0.45	0.37
TUNEL ⁺ (%)	-0.22	-0.37	0.23
CMA3 ⁺ (%)	-0.43	-0.28	0.47
Lipid peroxidation (%)	-0.19	-0.43	0.32
Lipid peroxidation (intensity)	-0.43	-0.45	0.62*

The asterisks at the end of the correlation indicate that the correlation is significant at $P < 0.05$. DFI; DNA fragmentation index, TUNEL; Terminal deoxynucleotidyl transferase dUTP nick end labeling, and CMA3; Chromomycin A3.

Table 3: Correlation between sperm lipid peroxidation, DNA fragmentation and chromatin status

Parameters	Blue-stained (%)	DFI (%)	TUNEL ⁺ (%)	CMA3 ⁺ (%)	Lipid peroxidation (%)
Persistent histones (%)	1	0.45	0.44	0.60**	0.30
DFI (%)	0.45	1	0.83**	0.30	0.77**
TUNEL ⁺ (%)	0.44	0.83**	1	0.33	0.84**
CMA3 ⁺ (%)	0.60**	0.30	0.33	1	0.06
Lipid peroxidation (%)	0.30	0.77**	0.84**	0.6	1
Lipid peroxidation (Intensity)	0.60**	0.62**	0.34	0.50*	0.23

The asterisks at the end of the correlation indicate that the correlation is significant at *P<0.05 and **P<0.01. DFI; DNA fragmentation index, TUNEL; Terminal deoxynucleotidyl transferase dUTP nick end labeling, CMA3; Chromomycin A3, and LO; Lipid peroxidation.

Discussion

Oxidative stress has been reported in 30-80% of infertile men and has toxic effects on sperm functions. Oxidative stress is mainly mediated through endogenous generation of hydrogen peroxide. Medium or moderate concentrations of hydrogen peroxide could result in sperm immobilization due to depletion of ATP and reduction of the phosphorylation in axonemal proteins while high concentrations of hydrogen peroxide can induce apoptosis in sperm (15).

In the normal condition, numerous antioxidants present in seminal plasma and sperm, support male gametes against oxidative stress. However, reduced antioxidant capacity and excessive generation of ROS, prone sperm to damage in infertility condition. One of the main reasons of sperm susceptibility to damage is the low volume of cytoplasm and high content of unsaturated fatty acids (16, 17). Excessive production of ROS could also be related to mitochondrial dysfunction leading to lipidperoxidation, the consequence of which is decreased sperm motility, increased DNA fragmentation and finally apoptosis (18, 19). High level of SDF is considered as one of the main factors contributing to male infertility and can result in failed fertilization, retarded embryonic development and consequently reduced implantation and pregnancy rates (7). Considering, traditional semen analysis is not sufficient for evaluation of sperm function and male fertility potential, we assessed sperm lipid peroxidation, protamine deficiency, and DNA fragmentation as sperm functional tests, in addition to sperm parameters, in infertile men with at least one failed cycle after ICSI.

Our results show a negative significant correlation between percentage of sperm persistent histones with sperm concentration while similar correlation was not observed between percentage of CMA3 positive spermatozoa with sperm concentration. According to literature background, aniline blue dye discriminates lysine-rich histones from arginine-and cysteine-rich protamine, while CMA3 dye compete with the protamines for binding to the minor groove of DNA in sperm (20, 21). Despite a positive significant correlation between these two markers, these results may suggest that aniline blue staining may be a better marker of sperm immaturity

compared to CMA3, but one should not ignore specific group of couples with at least one or more failed cycle after ICSI and low number of cases as one limitation of this study. Our data further indicate that the chance of selection and insemination of immature sperm increases with severity of oligozoospermia. In this regard, Simon et al. (22) demonstrated that percentage of sperm persistent histones can have adverse effect on embryo development and clinical pregnancy outcomes. Unlike result of the current study, they did not observe significant correlation between sperm concentration with percentage of sperm persistent histones but they observed positive significant correlations between this parameter with DNA fragmentation assessed by three different methods (Comet, TUNEL and FCCE assays). They concluded that assessment of chromatin condensation by aniline blue staining is a good predictor of assisted reproduction technique outcomes.

In addition, we also observed significant positive correlations between percentage of sperm abnormal morphology with percentage of sperm persistent histones and intensity of lipid peroxidation. These results suggest that abnormal sperm contain high level of excessive histones with more relaxed chromatin configuration compared to sperm chromatin that was packed with protamines, producing higher amount of hydrogen peroxide which prone sperm to lipid peroxidation. Based on previous study by professor Aitken group, lipid peroxidation by product not only exposed DNA to damage but also induces mitochondrial to produce higher amount of H₂O₂, the consequence of which DNA fragmentation and apoptosis. Therefore, antioxidant therapy to minimize the level of oxidative stress has been suggested for these type of patients. In this regard, we recently demonstrated that supplementation of One-Carbone Cycle, which improves chromatin remodeling and allowse proper exchange of histone to protamine to take place resulting in the reduction of sperm lipid peroxidation and DNA damage in varicocelized rat model (23). Similar to this study, other studies showed that antioxidant therapy can improve sperm parameters, and chromatin status, and level of oxidative stress (24-28). In this study, we did not assay effect of antioxidant therapy on infertile men with previous failed cycle after ICSI. Further studies are needed to confirm this result in this group of infertile men

with high population.

According to the literature background, the final consequence of the increased level of oxidative stress and protamine deficiency is fragmentation of DNA in sperm. Therefore, we assessed SDF by two methods; TUNEL, and SCSA and observed there was a strong significant correlation between these methods. In addition, there were significant correlations between percentage of DNA fragmentation assessed by two methods with percentage and intensity of sperm lipid peroxidation. This result shows that the intensity of lipid peroxidation in sperm is in line with the fragmentation of DNA. Based on previous proposed theory by professor Aitken group, the lipid peroxidation is induced mainly by H_2O_2 derived from mitochondrial and leucocytes (29). Therefore, supplementation with antioxidant may break lipid peroxidation chain and subsequently may improve semen quality. Indeed, vitamin E, lycopene and astaxanthin have been recommended in the hope of protecting sperm from lipid peroxidation damage and improving fertility outcomes in these individuals (30, 31). In this regard, it has been shown that sperm with fragmented DNA could successfully complete the fertilization process, but development to reach blastocyst or post implantation is severally retarded (32, 33). A second strategy, after antioxidant supplementation, to improve ICSI outcome in these type of couples is to take the advantage of novel sperm processing methods which minimizes the load of DNA damage is selected sperm population for insemination (34). However, if these two approaches fail to result in healthy delivery, use of testicular sperm instead of ejaculated sperm (35) has been recommended. It is also important to note that changes in lifestyle which reduced the production of excessive ROS and any other action like varicocelelectomy, is necessary and should be taken as the first measure in these couples (24, 36, 37).

Conclusion

The result of these studies clearly showed that there are strong significant correlations between oxidative stress, chromatin packaging and DNA fragmentation in sperm sample of infertile men with at least one failed cycle after ICSI. It seems a reduction of oxidative stress through clinical approach like varicocelelectomy and therapeutic approaches like antioxidant therapy and subsequently improvement of sperm function can be expected to provide satisfactory results in the next assisted reproduction cycle.

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Authors' Contributions

M.H.N.-E., M.T.; Conception, design, data analysis,

interpretation, manuscript writing and final approval of manuscript. F.B.; Semen analysis, prepared samples, carried out experimental, collected data, and manuscript writing. M.F.; Data analysis, interpretation, and manuscript writing. All authors read and approved the final manuscript.

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Effects of Alginate Concentration and Ovarian Cells on *In Vitro* Development of Mouse Preantral Follicles: A Factorial Study

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Abstract

Background: In the present study, the effects of alginate (ALG) concentration and ovarian cells (OCs) on the development and function of follicles were simultaneously evaluated.

Materials and Methods: In the first step of this experimental study, preantral follicles were isolated from the ovaries of 2-week-old mice, encapsulated in the absence or presence of OCs in 0.5, 0.75 and 1% ALG hydrogels, and cultured for 14 days. The morphology, diameter, survival and antrum formation rates of the follicles and the maturation of the oocytes were evaluated during culture. In the second step, preantral follicles were cultured in the best chosen ALG concentration, in both the absence and presence of OCs. Following these steps, the amount of DNA fragmentation, the expression levels of connexin 37 and connexin 43 proteins, the secretion levels of estradiol, progesterone and androstenedione by the follicles and the quality of mature (MII) oocytes were assessed.

Results: Our data revealed that in the absence of OCs, follicles of 0.5% group showed a higher survival rate than the 0.75 and 1% groups (71.87 vs. 52.52 and 40%, respectively, $P < 0.05$). Nonetheless, the antrum formation rate of the 1% group was higher and its oocyte degeneration rate was lower than that in the other groups. Furthermore, it was observed that co-culture of follicles with OCs relatively increased the follicle diameter, survival, antrum formation, and germinal vesicle (GV) to GV break down (GVBD)/MII transition rates. At last, the comparison of 0.5%-OCs and 0.5%+OCs groups indicated that the co-culture condition resulted in more progesterone production (1.8 ± 0.2 vs. 3.2 ± 0.4 ng/ml, respectively, $P < 0.05$) and also decreased oocytes' cortical granule abnormalities (100 vs. 40% for 0.5%-OCs and 0.5%+OCs groups, respectively).

Conclusion: The present study revealed that 0.5% ALG hydrogel is relatively suitable for preantral follicle culture, and in the presence of OCs, it mimics the natural ovarian condition better than the higher concentrations of ALG hydrogel.

Keywords: Alginate, Hydrogel, Ovarian Cells, Preantral Follicle, Tissue Engineering

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Introduction

Isolation and *in vitro* culture of immature ovarian follicles is widely used as a research tool to study the folliculogenesis process (1). It is also a potential alternative to preserve fertility in patients with cancer, who do not have enough time to undergo gonadotropin stimulation before chemotherapy treatments (2).

In general, follicles are cultured in attachment and non-attachment systems. In an attachment culture system, the follicle architecture and the communications of follicular cells are disrupted because of the attachment

of granulosa cells to the culture dish, as the mentioned phenomenon might negatively affect the growth and development of the follicles, especially in large mammals (3-5). Therefore, the non-attachment culture system is developed as an alternative way for follicle culture. In the non-attachment culture system, due to the use of a 3D natural or synthetic matrix, the natural structure of the follicles and gap junctions between the follicular cells are preserved well (3, 4). So, this system may be more successful than an attachment system, especially when applied to larger species such as domestic animals and primates (6, 7).

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In recent years, alginate (ALG) hydrogel has been largely used in biomedical and tissue engineering applications (8). ALG is a naturally-derived polysaccharide, which is produced by seaweed. It is ionically cross-linked with divalent cations such as calcium (Ca^{2+}) to form a gel with a mesh-like structure (9). ALG has been also used for the culture of isolated follicles and has yielded desirable results with follicles from different species (4, 5). However, previous studies have suggested that ALG physical characteristics, which are adjusted by its composition and concentration, could influence the follicles' survival rate, antrum formation, diameter, maturation, genes expression and hormonal secretions in a species- and stage-specific manner (5, 10-12).

Moreover, it is shown that the molecular support provided by different cell types could affect follicle growth and development as well as the physical mechanics of the matrix (13, 14). In this regard, recent studies have shown that ovarian cells (OCs) have a stimulatory effect on growth and development of follicles, *in vitro* (15, 16). The OCs could potentially be applied in follicle culture systems in forms of a feeder layer below the encapsulated follicles or they may be co-encapsulated with follicles inside the matrix (15-17). However, it seems that co-encapsulation of OCs with follicles is more practical for follicle culture, as it mimics *in vivo* follicular microenvironment and allows for paracrine signaling as well as the attachments and interactions of granulosa-OCs (17).

Although in many studies the impacts of different ALG concentrations and the rule of OCs on follicle development have been investigated, there is no study that evaluates these two parameters simultaneously to find the best ALG concentration for the culture of both preantral follicles and OCs. Hence, in the first step of the present factorial study, the morphology, diameter, survival, antrum formation and maturation of preantral follicles encapsulated and cultured in different concentrations of ALG hydrogel, in the absence or presence of co-encapsulated OCs (-OCs and +OCs, respectively), are evaluated. Then, in the second step, to understand the effects of OCs on the quality of cultured follicles and their oocytes, the preantral follicles were cultured in the best concentration of ALG in the absence or presence of OCs, and were investigated in terms of the amounts of DNA fragmentation in the follicular cells. Since gap junction proteins play a significant role in the folliculogenesis process via transferring nutrients, ions and some nucleotides among follicular cells and oocyte, the changes in their expression might somehow affect follicle development (18, 19). Therefore, the expression of connexin 37 (Cx37) and connexin 43 (Cx43), which are two main gap junction proteins in the follicle structure (20, 21), were also assessed in this study. Finally, the function of the follicles and the quality of the obtained metaphase II (MII) oocytes were examined by evaluating hormonal secretions, cortical granules and spindle/chromosome abnormality rates.

Materials and Methods

Study design

In the first step of this experimental study, preantral follicles were isolated from mice ovaries in five independent replicates, randomly allocated to encapsulate in 0.5, 0.75 and 1% ALG hydrogels in the absence or presence of OCs, and cultured for 13 days. The diameter and morphological appearance of developing follicles were analyzed on days 1, 6 and 13 of culture. Additionally, on day 13, the survival rate of the follicles was calculated and healthy follicles were evaluated with regard to their antrum formation rate. Then, antral follicles were induced by 2.25 IU/ml human chorionic gonadotropin (hCG, Choriomon, Switzerland) and 20-22 hours later, on day 14 of culture, the developmental stages of the obtained oocytes were assessed. After determining the best concentration of ALG based on the larger diameter, higher survival, antrum formation, and maturation rates, in the second step of the study this concentration was used for culturing preantral follicles in the absence or presence of OCs. On day 13 of culture, antral follicles were fixed for investigation of DNA fragmentation and assessment of Cx37 and Cx43 protein levels. Conditioned media from the follicle cultures were collected in three replicates for the measurement of estradiol, progesterone, and androstenedione secretions. Finally, after hCG induction, MII oocytes were collected and evaluated in terms of their cortical granule distribution, meiotic spindle organization, and chromosomal alignment.

Animals

Female NMRI mice (Pasteur Institute, Iran) were housed in the animal facility of Royan Institute under standard housing conditions, with controlled temperature (20-25°C) and lighting (12 hours light: 12 hours dark). They were handled pursuant to the ethical guidelines set by Royan Institute (ethical permission number: IR.ACECR.ROYAN.REC.1395.93).

Isolation and culture of ovarian cells

Twenty three-four-week-old immature mice were sacrificed by cervical displacement, and their ovaries were isolated in an aseptic condition and placed in ice-cold base medium containing Dulbecco's Modified Eagle's medium (DMEM, Gibco, UK), penicillin (Gibco, UK), streptomycin sulfate (Gibco, UK), sodium bicarbonate (NaHCO_3 , Sigma, USA) and 10% fetal bovine serum (FBS, Gibco, UK). Next, the ovaries were cleaned of the bursa and adipose tissue under a stereomicroscope (SZ61, Olympus, Japan). Oocytes and granulosa cells were removed from the ovaries by puncturing follicles with two 29G insulin syringes and then discarded. The remnants were chopped and incubated for 45 minutes at 37°C in 200 μl per ovary of collagenase solution containing 4 mg/ml collagenase IV (Gibco, UK) in serum-free base medium. During this time, the ovarian tissue pieces were pipetted up and down at least 20 times every 10-15 minutes to mechanically disrupt them. To stop the enzymatic activity an equivalent volume of base medium was added to the samples. The

isolated cell solution was then filtered through a sterilized 40 µm filter mesh (Falcon, Mexico) and centrifuged at 1800 rpm for 5 minutes. The obtained cells were washed and the final pellet was re-suspended in a known volume of base medium. The cells were transferred to T25 culture flasks containing 4 ml of base medium supplemented with 1% insulin-transferrin-selenium (ITS, Gibco, UK), 1% L-glutamine (Sigma, USA), 1% non-essential amino acids (Gibco, UK) and 0.1% β-mercaptoethanol (Sigma, USA), and then incubated at 37°C in a water-saturated atmosphere of 95% air and 5% CO₂ until they reach full confluency. Next, OCs were trypsinized and washed and the viable cells were counted with a trypan blue staining and a Neubauer chamber. Afterward, 1ml aliquots of the cells (5×10⁵ cells/ml) were stored in 10% DMSO (Sigma, USA)/FBS at -80°C for later use.

Isolation of follicles

A total of thirty 12-14-day-old mice were sacrificed by cervical displacement. Mouse ovaries were mechanically dissected under a stereomicroscope at 37°C, using two 29G needles attached to 1ml insulin syringes, and placed in alpha minimum essential medium (α-MEM, Gibco, UK) supplemented with penicillin, streptomycin, NaHCO₃, and 10% FBS. Only intact preantral follicles with 2-3 layers of granulosa cells and 100-130 µm in diameter were chosen and divided randomly into experimental groups.

Preparation of hydrogels

To make a 1.0% (w/v) ALG solution, 10 mg/ml alginic acid sodium salt (Sigma, USA), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma, USA) and 150 mM sodium chloride (NaCl, Sigma, USA) were dissolved in deionized water, filtered through a sterilized 0.22 µm filter (Millipore, USA) (22), then diluted with sterile 1X phosphate buffered saline (PBS, Takara, Japan) without calcium and magnesium to reach the final concentrations of 0.75 and 0.5% (w/v). In order to prepare hydrogels, cross-linking solution (50 mM calcium chloride (CaCl₂, Sigma, USA)/140 mM NaCl in deionized water) was mixed with hydrogel solutions.

Encapsulation and culture

In the first step of the study, groups of 109.83 ± 7.59 preantral follicles were individually encapsulated in 0.5, 0.75 and 1% ALG solutions and in the absence or presence of OCs, in five independent replicates. For cell encapsulation, about 5×10³ OCs per follicle were mixed with hydrogel solutions and pipetted in 5-µl droplets on sterile ultra-low attachment culture dishes (Dow Corning, USA). The concentration of OCs in each droplet was determined based on the best results obtained in our pilot study. Afterwards, follicles were individually placed in the 5-µl droplets, cross-linking solution was gently pipetted on top of each droplet, and then incubated at 37°C for 2 minutes. After incubation, the beads were washed

with α-MEM medium and then placed into 96-well plates (TPP, Switzerland). Each well contained one bead in 100 µl culture medium [α-MEM supplemented with 5% FBS, 1% ITS, 10 mIU/ml follicle stimulating hormone (FSH, Merck, Germany)]. Lastly, plates were incubated in a 5% CO₂ incubator at 37°C for 13 days and 50 µl of the medium was replenished every 3-4 days.

Assessment of follicle diameters, survival and antrum formation rates

Morphological features and the diameters of developing follicles were assessed on days 1, 6, 13 of culture. The diameters were determined as the mean of two perpendicular measurements of each follicle using ImageJ software (U.S. National Institutes of Health) (23). Moreover, on day 13 of culture, the survival rate of the cultured follicles and antrum formation rate of the survived follicles were evaluated observationally based on the morphological appearances of the follicles and their oocytes. In this regards, extrusion of the oocytes, their dark appearance and surrounding granulosa cells were considered the indications of degeneration. Also, antrum formation was determined as an observable transparent cavity within the granulosa cell masses.

Determination of oocytes meiotic maturation

In vitro maturation and ovulation of antral follicles were induced by 2.25 IU/ml hCG, on day 13 of culture. For evaluation of meiotic maturation of the oocytes, at 20-22 hours after induction the extruded cumulus-oocyte complexes (COCs) were removed from the culture wells and denuded by gentle pipetting, then the number of germinal vesicle (GV), GV breakdown/metaphase II (GVBD/MII), and degenerated oocytes were determined.

Histological processing

In the second step of the study, some preantral follicles were encapsulated in the ultimate concentration of ALG hydrogel from the first step, either in the absence or presence of OCs, and were cultured similar to the first step. On day 13 of culture, survived antral follicles were fixed in 4% paraformaldehyde overnight at 4°C; then the follicles were rinsed twice in PBS, dehydrated in increasing concentrations of ethanol and embedded in paraffin. Next, 5 µm thick slices were prepared and mounted on adhesion slides for DNA and protein analyses. Three sections per group, taken from the middle of three random follicles, were selected for either DNA fragmentation or Cx37 and Cx43 protein expression detection. To prepare the sections, they were first deparaffinized at 60°C for 30-40 minutes, washed in xylene solution for 20 minutes, and rehydrated by rinsing in serially diluted ethanol and water bath.

Detection of DNA fragmentation

Strand breaks of DNA in apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay utilizing the In Situ Cell

Death Detection Kit, TMR Red (Roche Diagnostics GmbH, Mannheim, Germany). The procedure was done according to the manufacturer's protocol and previous descriptions (24). In brief, after deparaffinization and rehydration, sections were pretreated with freshly prepared permeabilization solution [0.1% Triton X-100, 0.1% sodium citrate (Sigma, USA)] for 8 minutes at room temperature. After rinsing with PBS/0.05% Tween 20 (PBS-T, Sigma, USA), sections were incubated in TUNEL reaction mixture containing 50 µl enzyme solution (terminal deoxynucleotidyl transferase) and 450 µl label solution (nucleotide mixture in reaction buffer) at 37°C in a humid incubator for 1 hour. Finally, the sections were washed in PBS-T, counterstained in 40,6-diamino-2-phenylindole (DAPI, Sigma, USA), and examined under an inverted fluorescence microscope (Eclipse 50i; Nikon, Japan). All images were processed for publication by the Adobe Photoshop software (CS5.1, Adobe Systems Inc., San Jose, USA).

Red fluorescence was visualized in TUNEL-positive cells. Sections from mouse ovarian tissue were incubated with DNase I recombinant [5 U/ml in 50 mM Tris- HCl, 1mg/ml bovine serum albumin (BSA), pH=7.5] and were used as a positive control. Negative control sections were incubated with the label solution rather than TUNEL reaction mixture.

To quantify the DNA fragmented follicular cells, all cells found in the sections, either for TUNEL staining or DAPI counterstaining, were counted using ImageJ cell counter plugin; then, the percentage of TUNEL-positive cells was computed.

Assessment of Cx37 and Cx43 protein expression

After deparaffinization and rehydration, antigen retrieval was performed by incubating the sections of the antral follicles in 0.01 M sodium citrate buffer (pH=6.0, Sigma, USA) for 1 hour in a 96°C oven. The sections were washed two times in PBS-T, and incubated in 10% goat and donkey serums (Sigma, USA) diluted in PBS for 1 hour at 37°C to block non-specific protein bindings in Cx37 and Cx43 immunostainings, respectively. After two PBS washes, the sections were incubated overnight at 4°C with primary antibodies against Cx37 [Primary rabbit polyclonal antibody (ab181701, Abcam, UK)] and Cx43 [primary mouse monoclonal antibody (C8092, Sigma, USA)]. Both primary antibodies were diluted 1:400 in related blocking solutions. Then, the sections were rinsed with PBS carefully and incubated with secondary antibodies [(goat anti-rabbit IgG (H+L) cross-adsorbed (Alexa Fluor 488, A11034, Thermo Fisher Scientific, USA) and donkey anti-mouse IgG H&L (Alexa Fluor 488, ab150105, Abcam, UK)] for 1 hour at 37°C. Both secondary antibodies were diluted 1:1000 in related blocking solutions. Lastly, the sections were washed, counterstained with DAPI for 1 minute, and inspected under an inverted fluorescence microscope (Eclipse 50i, Nikon, Japan). Sections from rat heart tissue were used as a positive control. For the negative control, ovarian sections were processed without the primary antibodies.

Measurement of hormonal secretions

On day 13 of culture, the level of estradiol (E2), progesterone (P4) and androstenedione (A4) hormones were measured in conditioned media collected from 30 cultured antral follicles per group, in three replicates. The hormonal measurement was conducted using mouse ELISA kits (Bioassay Technology Laboratory, China) and according to the kits' instruction. Data were adjusted for every follicle by dividing each of the measured hormonal secretions by the number of the follicles. According to the kits' datasheets, the sensitivity of the assay for E2, P4 and A4 were 1.51 ng/L, 0.28 ng/ml and 0.022 ng/ml, respectively.

Evaluation of cortical granule distribution, meiotic spindle organization, and chromosomal alignment

Following the assessment of DNA fragmentation and protein expressions, cultured antral follicles were induced by hCG as was explained in detail in the determination of oocytes meiotic maturation section, and then a total number of 20 MII oocytes (10 oocytes per group) were collected in three replicates. Also, 10 *in vivo* matured MII oocytes were gathered as the control group. To get *in vivo*-developed oocytes, three 6-8-week-old NMRI mice were injected intraperitoneally by 7.5 IU of pregnant mare's serum gonadotropin (PMSG, Sigma, USA) followed 48 hours later by 7.5 IU hCG. After 18 hours, the mice were sacrificed, the COCs were isolated from their oviduct ampulla and then denuded. Afterwards, by using pronase (0.5 mg/ml in PBS, Sigma, USA) the zona pellucida of *in vivo*- and *in vitro*-developed oocytes were removed at 37°C and then oocytes were fixed in 4% paraformaldehyde for at least 1 hour. After washing in PBS with 0.01% Tween 20, oocytes were permeabilized in PBS containing 0.3% BSA (Gibco, UK) and 0.1% Triton X-100 (Sigma, USA) for 15 minutes at room temperature and blocked in PBS containing 0.3% BSA for 1 hour at 37°C. To stain the meiosis spindle and cortical granules, oocytes were incubated in the blocking solution containing anti-alpha tubulin antibody-microtubule marker (FITC) (1:100; Abcam, UK) and rhodamine-labeled Lens Culinaris Agglutinin (LCA) (1:500, Vector Laboratories, Burlingame, CA, USA) for 1 hour at 37°C. Finally, the stained oocytes were washed in PBS-T, counterstained with Hoechst 33342 (1 mg/ml in 1X PBS, Sigma, USA) for 5 minutes at 37°C and mounted on adhesion slides. Fluorescence labeling was detected using an inverted fluorescence microscope (Eclipse 50i, Nikon, Japan) and images were processed by Adobe Photoshop software. The lack of a cortical distribution was considered as the sign of cortical granule abnormality, and disorganized spindle or misaligned chromosomes were considered as an indicator of spindle abnormality.

Statistical analysis

Statistical differences in follicle survival rates, antrum formation rates, oocyte maturation rates, oocyte abnormality rates and DNA fragmentation were analyzed using

GENMOD procedure including function link logit in the model. GENMOD procedure produced odds ratio (OR) as the strength of difference between the groups. Data associated with follicle diameters were analyzed by MIXED procedure including RANDOM and REPEATED statements in the model to identify between and within covariances, respectively. Data pertaining to hormonal secretions were analyzed using GLM procedure. In addition, LSMEANS statement was included in the model to perform multiple comparisons. All analyses were conducted in SAS version 9.4 (SAS Institute Inc., NC, USA). Differences were considered significant at $P < 0.05$.

Results

The first step: determining the best concentration of alginate hydrogel

Assessment of the morphologies and diameters of the follicles on days 1, 6 and 13 of culture indicated no significant difference in follicles encapsulated in different concentrations of ALG, either in the absence or presence of OCs. On the other hand, follicles which were co-cultured with OCs had a more spherical shape and relatively larger diameters than the non-co-cultured ones. On day 13 of culture, for instance, the difference in the diameters of 0.5%-OCs and 0.5%+OCs groups was significant (347.18 ± 10.63 vs. 418.14 ± 21.89 μm , respectively, $P < 0.05$, Fig. 1A, B).

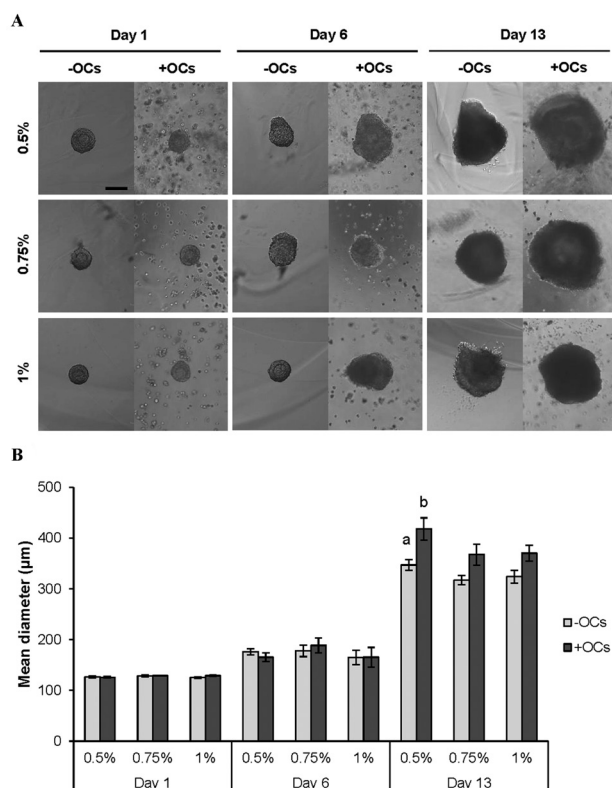


Fig. 1: Growth of preantral follicles encapsulated and cultured in 0.5, 0.75 and 1% alginate hydrogels in the absence or presence of ovarian cells (-OCs and +OCs, respectively). **A.** Morphological changes and **B.** Diameter of the survived follicles on days 1, 6 and 13 of culture. Data are presented as the mean diameter $\pm$ standard error. Data points a and b differ significantly ($P < 0.05$, scale bar: 100 μm).

Assessment of follicle survival rates on day 13 of culture indicated that there was a linear trend towards a better survival rate with lowering ALG concentration, in both the absence and presence of the OCs. However, the difference between 0.5%-OCs group and both 0.75%-OCs and 1%-OCs groups reached statistical significance (71.87 vs. 52.52 and 40%, respectively, $P < 0.05$, Table 1). Also, the comparison of -OCs and +OCs groups revealed that adding the OCs to all hydrogel beads had an affirmative effect on the follicle survival rate, and the difference between 1%-OCs and 1%+OCs groups was significant (40 vs. 63.91%, respectively, $P < 0.05$, Table 1).

Surprisingly, in the absence of OCs, the proportion of the follicles developed to antral stage was higher in 1% group as compared to the 0.75 % group (75 vs. 59.61%, respectively, $P < 0.05$); while in the presence of OCs there was no significant difference between the groups. On the other hand, all -OCs groups had a relatively lower antrum formation rate than the +OCs ones. Nevertheless, the difference reached statistical difference in 0.5 and 0.75% groups, only (69.59 vs. 88.57% and 59.61 vs. 77.65% for -OCs and +OCs groups, respectively, $P < 0.05$, Table 1).

The evaluation of oocytes obtained from 0.5, 0.75 and 1% ALG-cultured antral follicles showed that there was no significant difference between groups regarding the rates of GV and GVBD/MII oocytes, either in the absence or presence of OCs. However, 0.5% $\pm$ OCs groups had a higher rate of degenerated oocytes than the 1% $\pm$ OCs ones ($P < 0.05$). Also, it was clear that the oocytes of 0.5%+OCs and 0.75%+OCs groups were more likely to break down their GVs and develop to GVBD or MII stages as compared to the -OCs groups ($P < 0.05$, Table 1).

The second step: evaluation of the quality and function of cultured follicles

Based on the first step results, 0.5% ALG hydrogel is potentially best suited for preantral follicle culture, either in the absence or presence of the OCs. In the second step, the evaluation of DNA fragmentation in 0.5% ALG $\pm$ OC-cultured antral follicles revealed that although a negligible percentage of the follicular cells was TUNEL-positive in both groups, follicles that were co-cultured with OCs demonstrated a relatively lower percentage of DNA fragmentation compared to the non-co-cultured ones (2.2 ± 0.7 vs. $3.9 \pm 0.7\%$, respectively, Fig. 2A).

Immunofluorescence staining for Cx37 and Cx43 are displayed in Figure 2B and C as shown, the strong immuno-labeling of Cx37 and Cx43 were observed on granulosa cells of both -OCs and +OCs groups, while qualitatively, no remarkable difference was observed between the two groups.

Hormonal secretion data indicated that there was no significant difference between the groups in the levels of E2 and A4 hormones; however, the level of P4 in the +OCs group was significantly higher than that in the -OCs group (3.2 ± 0.4 vs. 1.8 ± 0.2 ng/ml, respectively, $P < 0.05$, Fig. 3A-C).

Table 1: Development of preantral follicles cultured in 0.5, 0.75 and 1% alginate hydrogels in the absence and presence of OCs for 14 days

Groups	Survival rate		Antrum formation rate		Oocyte maturation					
					GV		GVBD/MII		Degenerated	
	-OCs	+OCs	-OCs	+OCs	-OCs	+OCs	-OCs	+OCs	-OCs	+OCs
0.5%	69/96 (71.87) ^a	105/129 (81.39)	48/69 (69.59) ^a	93/105 (88.57) ^b	14/48 (29.16) ^a	8/93 (8.60) ^b	28/48 (58.33) ^a	69/93 (74.19) ^b	6/48 (12.5) ^a	16/93 (17.20) ^a
0.75%	52/99 (52.52) ^b	94/138 (68.11)	31/52 (59.61) ^{ab}	73/94 (77.65) ^b	12/31 (38.70) ^a	5/73 (6.84) ^b	17/31 (54.83) ^a	63/73 (86.30) ^b	2/31 (6.45)	5/73 (6.84)
1%	40/100 (40) ^{ba}	62/97 (63.91) ^b	30/40 (75) ^b	55/62 (88.70)	6/31 (19.35)	9/55 (16.36)	24/31 (77.41)	44/55 (80)	1/31 (3.22) ^b	2/55 (3.63) ^b

OCs; Culture in the absence of ovarian cells, +OCs; Culture in the presence of ovarian cells, GV; Germinal vesicle, GVBD; Germinal vesicle breakdown, and MII; Metaphase II. Data are presented as n (%). A vs. B in each column and a vs. b in each row differ significantly ($P < 0.05$).

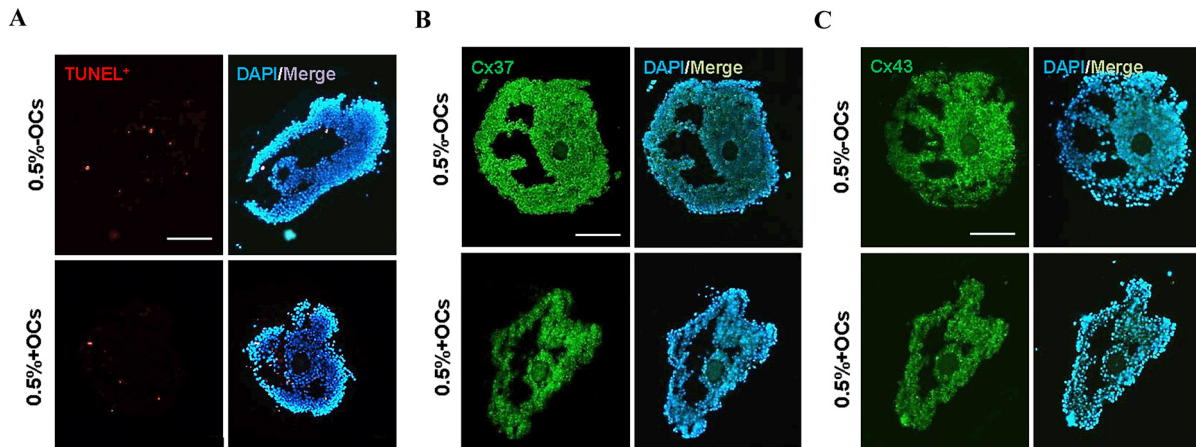


Fig.2: Quality assessment of antral follicles encapsulated and cultured in 0.5% alginate hydrogel in the absence or presence of ovarian cells (-OCs and +OCs, respectively), on day 13 of culture. **A.** TUNEL staining to detect DNA fragmentation in follicular cells. TUNEL-positive cells are stained in red and nuclei in blue (DAPI), **B.** and **C.** Immunofluorescence staining to label connexin 37 (Cx37) and connexin 43 (Cx43) proteins; both Cx37 and Cx43 proteins are stained in green and nuclei in blue with DAPI (scale bars: 100 μ m).

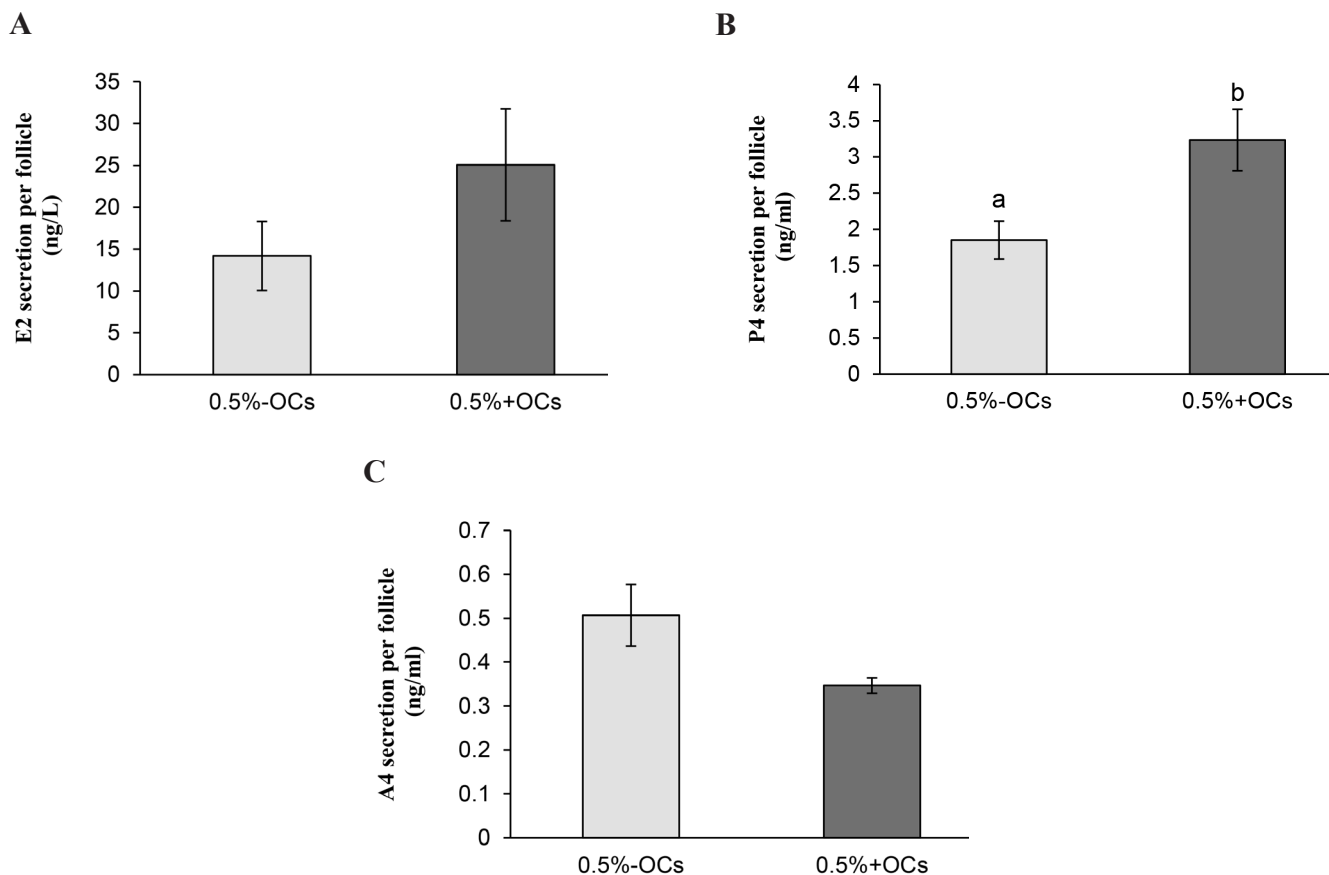


Fig.3: Secretion of hormones by antral follicles encapsulated and cultured in 0.5% alginate hydrogel in the absence or presence of ovarian cells (-OCs and +OCs, respectively). **A.** Estradiol (E2), **B.** Progesterone (P4), and **C.** Androstenedione (A4). Conditioned media were collected on day 13 of culture. Data are presented as mean $\pm$ standard error. Data points a and b are significantly different ($P < 0.05$).

The normal and abnormal MII oocytes in terms of cortical granule distribution, meiotic spindle organization, and chromosomal alignment are shown in Figure 4. Our data revealed that all *in vivo*-developed oocytes had a normal cortical granule distribution; whereas 100 and 40% of the oocytes that were developed in 0.5%-OCs and 0.5%+OCs groups, respectively, were abnormal (lack of a cortical distribution of cortical granules) ($P < 0.05$). Concerning meiotic spindle organization, under both *in vivo* and *in vitro* conditions, oocytes with abnormal spindle or chromosomal alignments were observed (disorganized spindle or misaligned chromosomes were considered as an indicator of spindle abnormality) (*in vivo*: 20%; 0.5%-OCs: 40%; 0.5%+OCs: 50%). Interestingly, the presence of OCs had no significant effect on the rate of abnormalities.

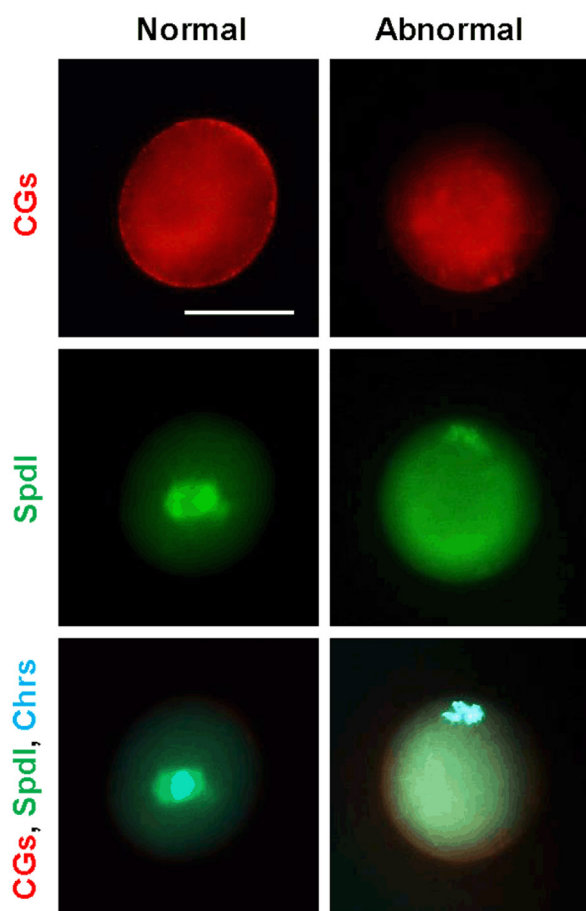


Fig. 4: Immunofluorescence staining and abnormality assessment of cortical granules (CGs; red), meiotic spindle (Spdl; green) and chromosomes (Chrs; blue) in MII oocytes. Oocytes with cortical distributed cortical granules, a well-organized spindle, and centrally aligned chromosomes were considered as normal oocytes (scale bar: 50 μ m).

Discussion

The aim of the present study was to simultaneously evaluate the effects of ALG concentration and OCs on the development and function of follicles.

To understand whether the proposed culture condition is ideal for follicle development, the morphological characteristics, diameter, survival and antrum formation rates

of the cultured follicles and meiotic resumption of their oocytes were assessed. Previous studies had shown that the rigidity of the matrix used for encapsulation and culture of follicles changes all the above parameters (10, 12, 25). In the studies that have tested mouse ovarian follicle encapsulation and culture in 0.125 to 3% ALG concentrations, it is shown that lower ALG concentrations are more favorable for mouse folliculogenesis (10-12, 25).

In our study, in the absence of OCs, the survival rate of the follicles cultured with 0.5% ALG was significantly higher than that with 0.75 and 1% ALG. The results were generally consistent with the study of Park et al. (25), which evaluated 0.125 and 0.25% concentrations of ALG hydrogel, where the lower ALG concentration resulted in a higher follicle survival rate. However, some other studies that have examined different ALG concentrations in the range of 0.25 to 3% have reported that ALG rigidity could not affect the follicle survival rate (10-12). It has been previously suggested that increasing the ALG concentration could possibly limit follicles' access to hormones such as FSH and other nutrients (26, 27). Also, it may increase the mechanical stress exerted on granulosa/theca cells around the exterior of the follicles, which subsequently influence the oocytes maturation via activating mechano-responsive pathways (28). Since in the present study, oocyte maturation was not significantly altered by increasing the ALG concentration, it could be proposed that the lower survival rate of 1% ALG-cultured follicles was likely due to the small size of the hydrogel's pores, which limits accessibility of follicular cells to the nutrients, leading to follicle degeneration. Surprisingly, the antrum formation rate in the hydrogel with the most rigidity (1% ALG), was higher than the other ones, while its oocytes degeneration rate was lower. Since it has been confirmed that increasing the matrix rigidity negatively influences the oocyte maturation (10-12), these results were unexpected. Therefore, further investigations are necessary to explain the reasons for these ironic results.

Interestingly, in the presence of OCs, we observed no remarkable difference between 0.5, 0.75 and 1% ALG-cultured follicles in terms of diameter, survival and antrum formation rates and oocyte maturation. Nonetheless, similar to the cultures without OCs, the rate of oocyte degeneration was lower in the group with 1% ALG. However, the comparison of -OCs and +OCs groups showed that the follicles in the +OCs groups had a more spherical shape, a relatively larger diameter, higher survival rate, better antrum formation, and higher GV to GVBD/MII transition rates. The applied OCs in this study comprised of a heterogeneous population of theca/interstitial cells, endothelial cells of the blood vessels, immune cells such as macrophages and smooth muscle cells, which produce high levels of androgens, growth factors and cytokines (16, 29-31). Presumably, these secreted factors affect the follicles via activating signaling pathways involve in both development and growth of the follicles (16, 32).

The cultured antral follicles in 0.5% ALG hydrogel (the

best-suited hydrogel for follicle growth and development), in the absence or presence of OCs, also were evaluated for DNA fragmentation, Cx37 and Cx43 protein expressions, hormonal secretions and the quality of their oocytes. Data showed that only a small percentage of the follicular cells were TUNEL-positive after 13 days of culture, either in the absence or presence of OCs. Since the percentage of the TUNEL-positive cells was less than 10%, according to the classification explained in the previous studies (33, 34), both evaluated groups are categorized as minimally damaged, showing an appropriate culture condition, which leads to the high survival rate of the follicular cells.

On the other hand, there was strong immunolabeling of both Cx37 and Cx43, which are two important gap junction proteins in the follicles, in both the absence and presence of OCs. Cx37 and Cx43 are responsible for transportation of nutrients and growth factors essential for the growth and development of the follicles (18-21). Therefore, their higher expression may provide further support for better follicle growth and development. Nonetheless, this increase in Cx37 and Cx43 expressions did not prevent oocyte damage.

Furthermore, the evaluation of hormonal secretion by 0.5% ALG-cultured antral follicles showed that the follicles that were co-cultured with OCs secreted more P4 than the non-co-cultured ones. Earlier studies have found that macrophages enhance progesterone production in the granulosa cells of follicles (35, 36). Therefore, it could be suggested that the higher progesterone secretion by antral follicles co-cultured with OCs might be due to the stimulatory effects of cytokines secreted by the macrophages present in the OCs population.

Finally, to assess the cytoplasmic and nuclear maturations of 0.5% ALG-developed oocytes, the distribution pattern of cortical granules, the formation of the meiotic spindle and the alignment of chromosomes were evaluated and compared with the *in vivo*-developed ones. As reported in previous studies, in a normal mature oocyte, cortical granules represent a uniform cortical distribution and the meiotic spindle is also well-assembled. Moreover, a normal mature oocyte contains the correct number and set of chromosomes (37, 38). We have shown that unlike the *in vivo*-developed oocytes, most of 0.5% ALG-developed oocytes did not show a uniform cortical distribution of cortical granules, neither in the absence nor presence of OCs. This observation is likely due to the clumping of cortical granules within the oocyte cytoplasm as reported in the study by Mainigi et al. (39). So, it could be proposed that an *in vitro* culture of follicles could increase the sensitivity of their oocytes to errors in cortical granule distribution during development. Surprisingly, the oocytes that were co-cultured with OCs, showed a lower percentage of cortical granule abnormalities compared to the non-co-cultured ones. This could represent an affirmative effect of the secretions of OCs on factors involved in the clumping of cortical granules, such as oocytes' Ca^{2+} concentration or the expression of proteins, which have important

roles in fusion of cortical granules (39, 40). Regarding the assessment of spindle and chromosomal abnormalities, in contrast to the results of Mainigi et al. (39), the *in vivo* and *in vitro* oocytes showed no significant difference. Therefore, it is assumed that our *in vitro* culture systems did not negatively affect spindle formation and chromosomal alignment. However, further evaluations are required to confirm the mentioned assumption.

Conclusion

In the present study, we showed that both rigidity and concentration of ALG hydrogel influenced the survival rate of the follicles. Indeed, there was a linear trend toward a better survival rate with the lower ALG concentration, in either absence or presence of OCs. Nonetheless, the concentration of ALG did not significantly affect the diameter, antrum formation and maturation rate of the follicles. However, it could be concluded that among 0.5, 0.75 and 1% ALG, the hydrogel with lower concentration is the most suitable for the mouse preantral follicle culture. Moreover, it seems that OCs positively influence follicle diameter, survival, antrum formation and maturation rate, and hormonal secretions. Hence, OCs could be successfully applied to the follicle culture systems in order to improve their culture conditions.

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Authors' Contributions

P.J., M.R.V., L.M., H.B.; Contributed to conception and design of the study. P.J.; Carried out all experimental work, contributed to data and statistical analysis, and interpretation of data. M.R.V., H.B.; Were responsible for overall supervision. P.J.; Drafted the manuscript, which was revised by M.R.V. and H.B. All authors read and approved the final draft of the manuscript.

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Optimization of The Cell Aggregates Method for Isolation and Purification of Human Granulosa Cells from Follicular Fluid

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Abstract

Background: Aspirated ovarian follicular fluids (FF) contain luteal granulosa cells (LGCs) and other contaminating cell types. Several strategies, such as the antibody binding methods, the flask method, the cell strainer and positive selection of granulosa aggregates after density gradient (DG) centrifugation, were tested as LGC purification methods. Each of these strategies has its own advantages and disadvantages. Positive selection of granulosa aggregates after DG centrifugation is simple, rapid and efficient in terms of LGC recovery. However, it results in a low purity. Here, we aimed to test whether modifying the traditional protocol by collecting the aggregates from the FF, before the DG centrifugation could decrease the percentage of contaminating cells.

Materials and Methods: In the present prospective study, 32 FF, from 32 women, were randomly assigned into one of the two purification techniques: positive selection of granulosa aggregates from the FF, after DG centrifugation (DG/Agg, n=16) or positive selection of granulosa aggregates from the FF, before DG centrifugation (Agg/DG, n=16). At the end of each procedure cell count, vitality, morphology and purity of the cell suspension were evaluated.

Results: No significant difference was detected in the total number of GCs between DG/Agg and Agg/DG ($P>0.05$). However, higher percentage of GCs with normal morphology was detected in Agg/DG compared to DG/Agg ($P<0.001$). Moreover, lower percentages of white blood cells ($P<0.01$), red blood cells ($P<0.001$) and epithelial cells ($P<0.01$) were identified in Agg/DG compared to DG/Agg.

Conclusion: Here we showed that positive selection of granulosa aggregates from the FF prior to DG technique had a higher purity compared to the traditional protocol. Thus, it could be a method of choice to prepare GCs for research purposes in clinical *in vitro* fertilization settings.

Keywords: Density Gradient, Follicular Fluid, Granulosa Cells, Isolation and Purification

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Introduction

Granulosa cells (GCs) are the somatic cells surrounding the oocyte in the ovary (1). A bi-directional communication is set between GCs and the oocyte via locally secreted factors (2, 3). This cross-talk plays an important role in the differentiation of the GCs and the oocyte (2). In addition, GCs secrete sex hormones (e.g. estrogen and progesterone) under the control of the endocrine system to regulate the function of several body organs (4). After ovulation, GCs become luteinized (LGCs) and secrete progesterone to support potential pregnancy (5). Altogether, these characteristics made from LGCs an interesting model to study the ovarian physiology (5, 6).

In assisted reproductive technology, GCs can be col-

lected from follicular fluid (FF) during oocyte retrieval, form women undergoing controlled ovarian stimulation (COS) (5). The GCs in FF may be present as free cells or as clearly visible aggregates (Aggs). In parallel, other cell types could also be detected in this fluid, such as white blood cells, red blood cells and epithelial cells (7). Therefore, different strategies are used to separate LGCs from other FF contaminants (8-10).

The efficiency of purification methods that are based on the differential physical properties of LGCs and contaminating cells were tested in several reports (5, 8, 10). Positive selection of granulosa Aggs after density gradient (DG) procedure, under a dissecting microscope, is among the tested strategies (5, 7). It is a rapid, simple and

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relatively inexpensive technique (7). In addition, it allows the recovery of high LGC percentage (5, 7). However, it retains a certain percentage of contaminating cells. This limits the reliability of the results of some subsequent techniques, such as quantitative polymerase chain reaction (qPCR) and RNA chains analysis (5).

Therefore, the aim of present study was to test whether isolating granulosa Aggs at the beginning of purification procedure would decrease the percentage of contaminating cells at the DG interface. In order to answer this biological question, we collected the granulosa Aggs (which are larger than other FF contaminants) directly from the FF and then subjected them to the DG centrifugation. Next, we compared the outcome of this modified protocol to that of traditional one. This comparison was performed in terms of the percentage of recovered LGC, vitality and purity.

Materials and Methods

Collection of luteal granulosa cells

FFs were collected from preovulatory follicles of young women (<38 years old) undergoing oocytes retrieval for intra-cytoplasmic sperm injection (ICSI), via transvaginal ultrasound-guided aspiration (n=32) (5). After cumulus-oocyte complex (COC) collection from the FF for ICSI, the remaining liquid was directly assigned for LGC collection (within no longer than 5 minutes) (11). Before proceeding with oocytes retrieval, these women underwent COS. It was made up of gonadotropin-releasing hormone (GnRH) antagonist for pituitary down-regulation and recombinant follicle stimulating hormone (FSH) for ovarian stimulation. When three follicles reached 16 mm in diameter, subcutaneous injection of recombinant human chorionic gonadotropin (hCG) was given for ovulation induction (12). It is important to note that we excluded all women with poor ovarian response from the study, according to Bologna criteria (13). Couples gave their written informed consent and the study protocol form was approved by Mount Lebanon hospital Ethical Committee (MLH code: OBS-2018-002).

Experimental design

COC-free FFs (n=32) were randomly assigned to one of two IVF GC preparation methods. The first technique was positive selection of granulosa Aggs, after DG centrifugation (DG): DG/Agg (Fig.1A) (5, 7). The second technique was a positive selection of granulosa Aggs directly from FF, before DG centrifugation: Agg/DG (Fig.1B). Each technique was performed on 16 samples from 16 women. At the end of both preparation methods, total cell concentration was estimated, percentage of total cell vitality was established, and purity of the obtained cell suspension was evaluated (Fig.1) (5). It is important to note that all of the centrifugation steps were performed using an Eppendorf 5702 centrifuge (Eppendorf, Lebanon).

Tested luteal granulosa cell preparation methods

Positive selection of granulosa aggregates, after density gradient centrifugation

Each FF was pooled into a 14 ml falcon tube and centrifuged for 10 minutes at 2000 rpm. The collected pellet was gently pipetted onto a DG made up of two layers: 40% and 80% (Sperm Gradient Kit, Sydney IVF, COOK medical, EMEC Lebanon). After centrifugation for 10 minutes at 1200 rpm, the ring-like layer at the interface was transferred into a 60 mm petri dish. The Aggs were positively selected under a dissecting microscope and washed in human tubal fluid medium (HTF medium, Life Global, Ibra Haddad Lebanon). The wash consisted of a centrifugation for 10 minutes at 2000 rpm. Next, the pellet was resuspended in 1 ml HTF. Then, Aggs breaking up was performed mechanically, using a Pasteur pipette (Fig.1A) (5, 7).

Positive selection of granulosa aggregates from the follicular fluid, before density gradient centrifugation

Aggregates were collected from the COC-free FF, in HTF medium. Next, these Aggs were gently pipetted onto a DG made up of two layers: 40% and 80%. They were next centrifuged for 10 minutes at 1200 rpm. The ring-like layer in the interface was then transferred into a 5 ml round tube, mixed with 1 ml HTF and centrifuged for 10 minutes at 2000 rpm. At the end, the pellet was suspended in 1 ml HTF followed by up- and down-pipetting for 1 minute, to dissociate the Aggs (Fig.1B).

Estimation of total cell count and viability

The purified GCs were counted using a hemocytometer microscopic slide and cell viability was determined using Trypan Blue (0.4%) (14-18).

Evaluation of the cell suspension purity and luteal granulosa cells morphology using Wright-Giemsa stain

In order to compare purity of the cell suspension and the LGCs morphology derived from DG/Agg and Agg/DG techniques, a thin smear slide was prepared from each cell suspension (19). The smears were stained with Wright-Giemsa (8, 19). The interpretation of cytological slides was evaluated by a clinical pathologist who was blinded to the used technique. A cytologically normal GC is characterized by a large dark-stained nucleus, a foamy paler cytoplasm and intact cell having no cytoplasmic shrinkage (Fig.2) (19, 20). By contrast, the neutrophils were distinguished by their multi-lobed nucleus, lymphocytes had a nucleus occupying most of the cell volume, eosinophils contained a bilobed nucleus, and the monocytes had about 20 µm diameter with an irregular nucleus (11). Moreover, the total granulosa count was estimated by: (% of granulosa on Wright-Giemsa smear) X (total cell count on the hemocytometer microscopic slide).

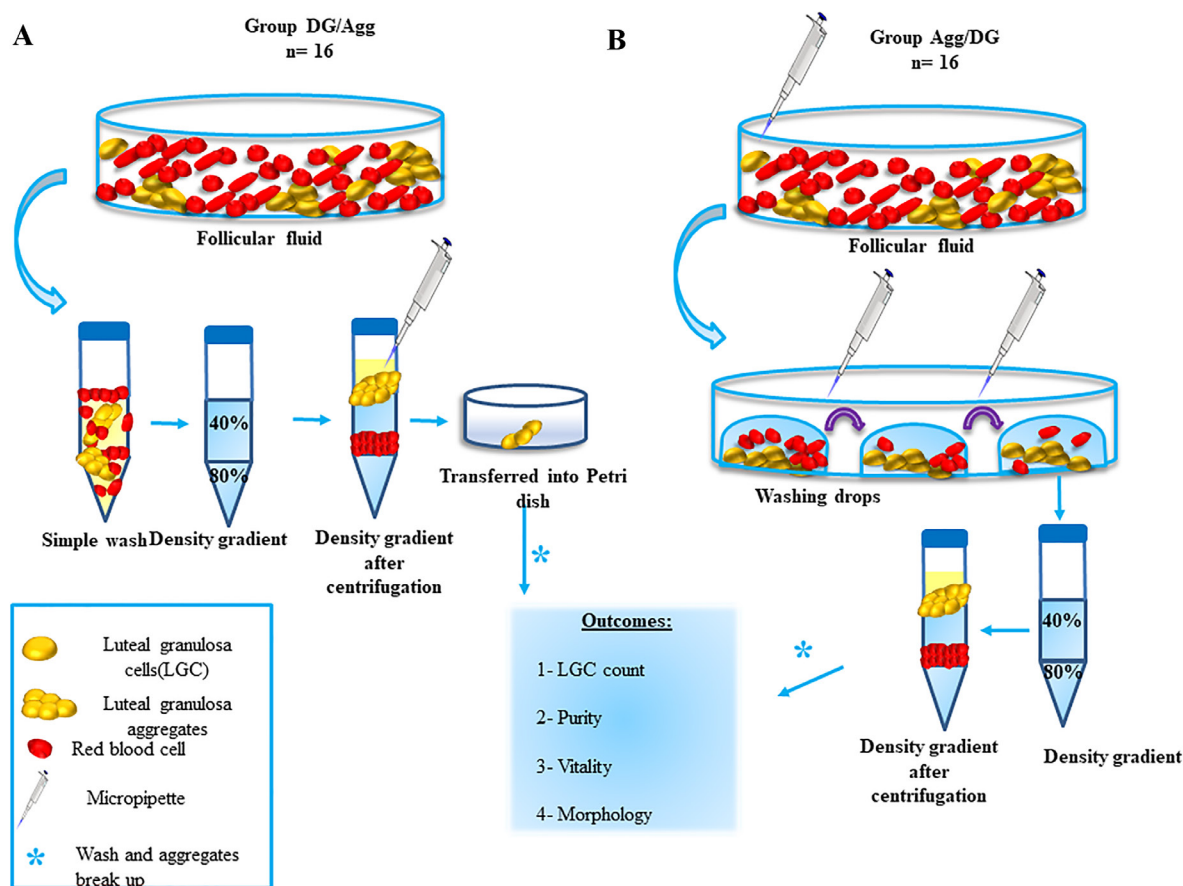


Fig.1: The schematic of luteal GCs purification procedures. **A.** Follicular fluid samples (n=16) were subjected to a simple wash, then subjected to a two layered (40-80%) density gradient centrifugation. After that, the luteal Aggs were transferred into a petri dish using a micropipette, washed and dissociated. Finally, LGC count, purity, vitality and morphology were assessed. **B.** Using a micropipette, luteal Aggs were collected from the FF samples (n=16), moved into washing drops to reduce contamination and then subjected to a two layered (40-80%) density gradient centrifugation. After that, the Aggs were washed, dissociated and analysed in terms of LGC count, purity, vitality and morphology. GCs; Granulosa cells, Aggs; Granulosa cell aggregates, FF; Follicular fluids, LGCs; Luteal GCs, and DG; Density gradient.

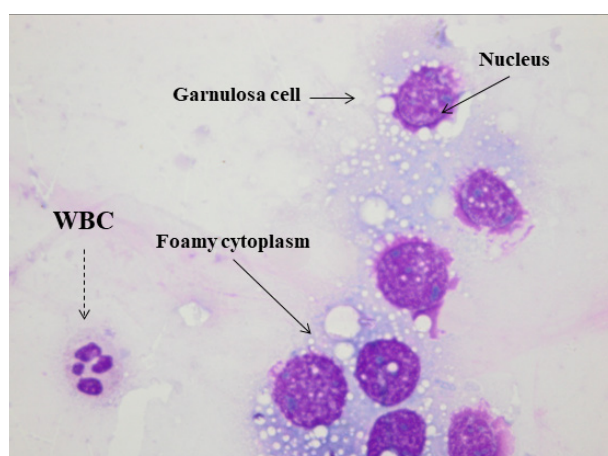


Fig.2: A cluster of granulosa cells stained with Wright stain. A cluster of granulosa cells (GCs) stained with Wright stain. The GC is made up of a central nucleus (purple) and foamy cytoplasm (clear purple): solid arrows. A white blood cell (WBC, multi-lobed nucleus) was observed: dashed arrow (magnification: $\times 100$ under immersion oil).

Statistical analysis

Statistical analysis was performed using IBM SPSS 23 software (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp). Data with normal distribution were then compared using independent samples t test. Data with non-normal distribution were compared using the Mann-Whitney non-parametric test. All data were presented as median [interquartile range (IQR)]. Categorical data were compared using Chi-square test. Results were considered statistically significant for a $P < 0.05$.

Results

Population characteristics

There was no statistically significant difference in the female age ($P=0.5$), number of retrieved oocytes ($P=0.2$) and infertility etiology ($P=0.8$) between these two techniques (Table 1).

Table 1: Comparison of the population characteristics in DG/Agg and Agg/DG groups

Population characteristics		DG/Agg technique	Agg/DG technique	P value
Infertility etiology	Female age (Y)	32.25 ± 5.4	31.06 ± 5.35	0.5
	Number of retrieved oocytes	9 ± 6.23	12 ± 6.10	0.2
	Male factor (%)	6/16 (37.5)	5/16 (31.3)	0.8
	Female factor (%)	4/16 (25)	6/16 (37.5)	
	Male and female factors (%)	2/16 (12.5)	1/16 (6.3)	
	Unexplained infertility (%)	4/16 (25)	4/16 (25)	

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

Results are expressed as mean ± standard deviation (SD) for normally distributed continuous variables and percentage for categorical data. Continuous variables were compared using the independent samples t test. Categorical data were compared using the Chi-square statistical test. There were no statistically significant difference between the groups of female age ($P=0.5$), number of retrieved oocytes ($P=0.2$) and infertility etiology ($P=0.8$). $P>0.05$ indicates that there is no statistically significant difference between two groups.

Assessment of cell concentration and vitality between DG/Agg and Agg/DG techniques

Hemocytometer slide and trypan blue staining were used to assess total cell concentration and vitality after two purification techniques. In one hand, a significantly lower concentration of cells was obtained after Agg/DG

compared to DG/Agg ($P<0.001$, Fig.3A). On the other hand, no significant difference was detected in the vitality percentage between DG/Agg and Agg/DG ($P>0.05$, Fig.3B).

Evaluation of the granulosa percentage, total count and morphology between the two techniques

A thin smear was prepared from each cell suspension, after processing and they were stained using Wright-Giemsa stain (Fig.2). A significant higher percentage of granulosa was identified in the cell suspension after Agg/DG compared to DG/Agg ($P<0.001$, Fig.4A). Moreover, no significant difference was detected in the total granulosa count between the two techniques ($P>0.05$, Fig.4B). Of particular interest, the percentage of granulosa with normal morphology was significantly higher post-Agg/DG compared to DG/Agg ($P<0.001$, Fig.4C).

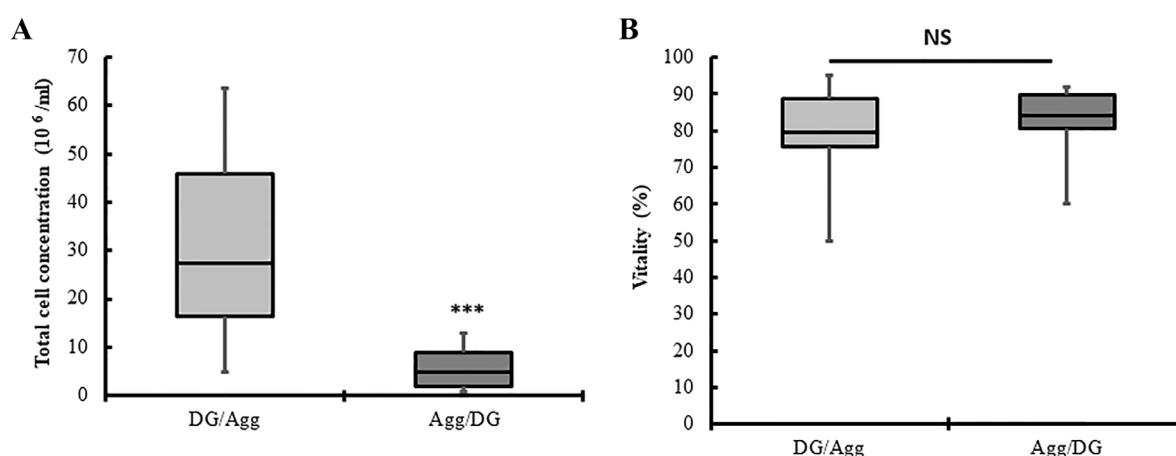


Fig.3: Boxplots show the total cell concentration and vitality percentage in DG/Agg and Agg/DG techniques. Boxes indicate the interquartile range. Horizontal bars within the boxes indicate the median. Whiskers indicate the range of data. Data were compared using the Mann-Whitney non-parametric test. **A.** Boxplot shows a lower concentration of all cell types after Agg/DG technique compared to DG/Agg technique (***, $P<0.001$). **B.** Boxplots show no statistically significant difference in the vitality percentage of cells after two techniques. NS; No significant difference.

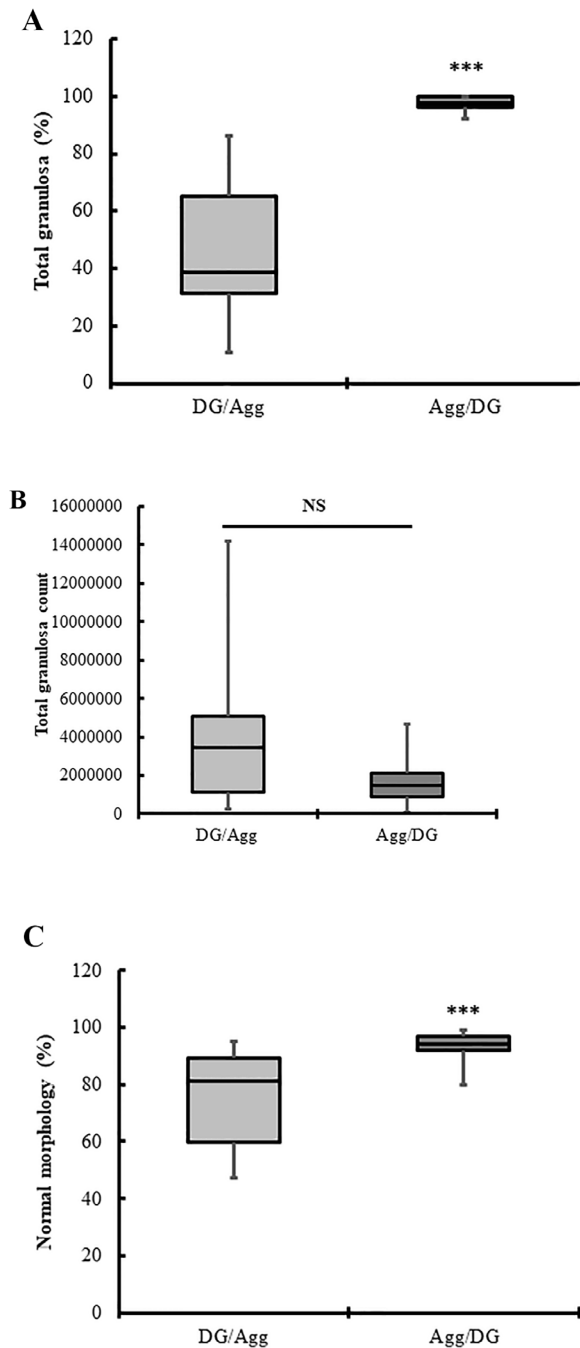


Fig.4: Boxplots show total recovered granulosa and percentage of GCs with normal morphology in DG/Agg and Agg/DG techniques. Boxes indicate the interquartile range. Horizontal bars within boxes indicate the median. Whiskers indicate the range of data. Data were compared using the Mann-Whitney non-parametric test. **A.** Boxplot shows a higher percentage of total GCs after Agg/DG compared to DG/Agg (***; $P<0.01$). **B.** Boxplots show that there was no statistically significant difference in the total granulosa count after DG/Agg and Agg/DG techniques. **C.** Boxplots show a higher percentage of GCs with normal morphology in the Agg/DG group compared to the DG/Agg group. NS; No significant difference.

Estimation of cell suspension purity in the two purification techniques

Purity of each preparation was estimated on Wright-Giemsa stained smears (Fig.2). Compared to DG/Agg, significantly lower percentages of white blood cells

($P<0.01$, Fig.5A), red blood cells ($P<0.001$, Fig.5B) and epithelial cells ($P<0.01$, Fig.5C) were detected in Agg/DG technique.

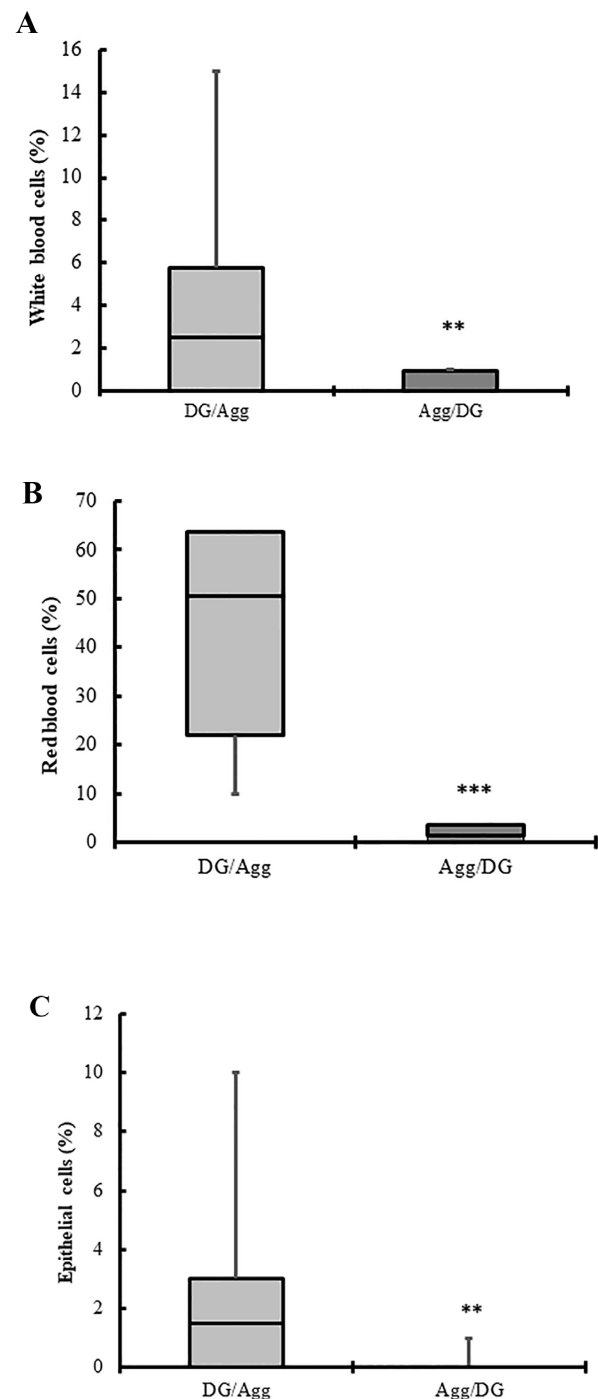


Fig.5: Boxplots show the percentage of contaminant cell types presented after DG/Agg and Agg/DG techniques. Boxes indicate the interquartile range. Horizontal bars within boxes indicate the median. Whiskers indicate the range of data. Data were compared using the Mann-Whitney non-parametric test. **A.** Boxplots show a lower percentage of white blood cells in the Agg/DG technique compared to the DG/Agg technique (**; $P<0.01$). **B.** Boxplots show a lower percentage of red blood cells in the Agg/DG technique compared to DG/Agg technique (***; $P<0.001$). **C.** Boxplots show a lower percentage of epithelial cells in the Agg/DG technique compared to the DG/Agg technique (**; $P<0.01$).

Discussion

The aim of present report was to decrease percentages of contaminating cells in the suspension obtained from the positive selection of granulosa Aggs after DG procedure. Here we showed that collecting Aggs from the FF prior to DG centrifugation significantly decreased the percentages of contaminating cells.

In assisted reproductive technology, aspirated human FF contains heterogeneous population of cells (10). For instance, it contains LGCs that could be collected for research purposes (8). It also comprises white blood cells which play an important role in the process of ovulation (21, 22). In addition, it could be contaminated by red blood cells and epithelial cells originating from the invasive trans-vaginal guided-aspiration (5, 10).

Studying quality, quantity and gene expression of the GCs may improve the information given about ovarian function and oocyte physiology (23-25). Therefore, scientists have tested several strategies to extract LGCs from the FF.

The purification strategies that are based on the immunorecognition of specific cell markers, such as fluorescence activated cell sorting (FACS), magnetic activating cell sorting (MACS), and Dynabeads, are considered to be the most efficient in terms of purity and less efficient in LGCs recovery (5, 10, 26-28). However, besides achieving a certain level of purity, it is of paramount importance to maintain cell count, vitality and morphology during purification procedures in order to perform subsequent investigations (10, 29). Here, comes the positive selection of granulosa Aggs after DG centrifugation, which is characterized by its simplicity, affordability, high speed of operation and efficiency to recover a high number of LGCs (5, 7). This technique depends on the large size of Aggs allowing the ease of their identification in FF (7). Another purification method -the flask method- takes advantage of the ability of immune cells to adhere to plastic ware, while GCs remain in suspension. However, selecting granulosa Aggs from FF after DG or collecting them using the flask method could not efficiently isolate GCs from other FF contaminants.

Interestingly, our proposed protocol (positive selection of granulosa Aggs before DG centrifugation) led to a lower contamination by red blood cells, white blood cells and epithelial cells. Actually isolating granulosa Aggs from the heterogeneous cell population at the beginning of the purification procedure could explain the lower level of contamination obtained using this modified protocol. It is essential to mention that application of a cell strainer to collect GCs can also reduce the level of white blood cells despite being more expensive than our proposed procedure (5).

In addition, our modified protocol resulted in a vitality percentage as high as the original procedure (positive selection of granulosa Aggs after density gradient). Strikingly however, the percentage of granulosa with normal

morphology was higher in the suspension obtained from our modified procedure compared to the original procedure. In fact, GCs are very sensitive to reactive oxygen species (ROS) (30). ROS production could be increased due to the activation of leukocytes during centrifugation (31-33). The positive selection of granulosa Aggs from FF after DG centrifugation comprises two centrifugation steps during which ROS producing leukocytes are still in contact with GCs. This could have affected the GCs thus resulting in a lower percentage cells with normal morphology.

Conclusion

The positive selection of GCs before subjecting them to a DG centrifugation surpassed the original procedure in terms of purity and recovery of granulosa with normal morphology. It resulted in a relatively high number of recovered LGCs less contaminated by other cell types. In addition to its efficiency, the modified protocol is simple, inexpensive and rapidly operated. That being the case, it could be a method of choice to prepare GCs for research purposes in clinical settings *in vitro*.

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Authors' Contributions

G.R.; Conception and design of the manuscript. C.F., Joa.A., Jos.A.; Patients selection, ovarian stimulation and oocytes pick-up. G.R., J.T., M.B.; Granulosa cells purifications. Y.M.; Interpretation of cytologic slides. J.T., M.B., G.R.; Statistical analysis. G.R., J.T., M.B., J.A., Y.M.; Drafting and revising the manuscript. All authors read and approved the final manuscript.

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Is There any Mean to Postpone The Menopausal Ovarian Senescence?

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Abstract

The ovarian reserve (OR) gradually decreases throughout the female fertile life. This continuous depletion in OR is irreversible. This occurs through a programmed cell death, known as apoptosis. Some factors hasten such depletion, such as chemo- and radio-therapy. Others have been investigated in trials to preserve the OR including gonadotropins, cytokines, growth hormones, nitric oxide and reorganization of the actin cytoskeleton. Loss of OR occurs normally at the menopausal age, a stage called menopausal ovarian senescence. At some periods, there are other sources for ovarian hormones that are away from the ovary, like during use of contraceptive pills and at pregnancy after formation of placenta. Future trials to preserve ovarian follicles at these periods might postpone the onset of menopause and hence lengthen the fertile female age.

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Ovary is the primary sex organ in females. It is the only abdominal organ that is not covered by peritoneum (1). It contains hundreds of thousands (about 400000) primary follicles at puberty. These follicles support fertile period of female life. At the mid-30s years, there is an increase in the pace of oocyte depletion to reach about 25000 by the age of forty years. This means that the ovarian reserve (OR) decreases gradually till ends at the menopause. This continuous depletion in OR, as an irreversible process, is variable from a woman to the other and depends on many factors such as genetics, age, drugs, irradiations and other environmental variables. For example, hormonal suppression as well as chemo- and radio-therapy might hasten follicle depletion causing deleterious effects on the OR and ovarian function (2). On the other hand, there are environmental factors, including nutrition which might improve condition of ovarian aging (3).

OR which represents the female potentials for fertility could be tested by measuring anti-Mullerian hormone (AMH). Women with low AMH levels should be counseled regarding the option of fertility preservation or tendency to attempt pregnancy at early stage of their fertility life (4). Moreover, low response of ovaries to ovulation induction by gonadotrophins is an indication of reduced OR and frequently associated with occurrence of early menopause (5).

At each ovarian cycle, some of the follicles begin to enlarge under hormonal control, till one of them reaches the stage of mature Graafian follicle. This ripe follicle approximates the ovarian surface epithelium (OSE) and releases its oocyte into the abdominal "peritoneal" cavity, a process that is known as ovulation (6). At the same time, other follicles degenerate throughout their journey of maturation.

Therefore, during each cycle, many follicles "approximately 1000 in terms of quantity" are lost (7).

At pregnancy, functions of ovaries become stationary. By that means, no hormone is produced from ovary after formation of the placenta taking over the role of ovary in production of the hormones supporting pregnancy and enhancing enlargement of breasts and uterus. Removal of ovaries after the first trimester does not affect the progress of pregnancy (7, 8). It has been found that many follicles are enlarged during pregnancy but no ovulation occurs, while it is stopped after fertilization. This might be because of the proliferation of OSE cells and increase in the thickening (9). Hormonal changes that are responsible for prevention of ovulation at pregnancy might be similar to that found with use of contraceptive pills. However, Cooper and Adigun (10) stated that the progesterone is the main hormone in the pills responsible for prevention of ovulation, by inhibiting follicle development.

Menopause is a females' stage of life representing about one third of their lives. Such stage is characterized by depletion of ovarian follicles and hence cessation of menstruation and loss of fertility. There is a loss of cyclic production of steroid hormones. The most noticed hormonal change corresponding to female reproductive aging in menopause is the subtle rise of follicle stimulating hormone (FSH) (1).

Degeneration of follicles or their atresia occurs through a programmed cell death, known as apoptosis (11). Several agents have been tested to rescue these cells from apoptosis. These include gonadotropins, estrogens, cytokines, growth hormones, nitric oxide and reorganization of the actin cytoskeleton (11-13). Wu et al. (14) noticed that use

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of resveratrol, which is a natural plant derivative, in mice could reduce cell apoptosis of oogonia induced by chemotherapy. They suggested that resveratrol might be used as a potential therapeutic drug against induced ovarian aging. Although such trials are in their infancy, our hope is enlarging to find a mean to preserve the ovarian follicles *in vivo* or to slow the natural rate of atretic loss, especially when two ovaries are in a state of stagnation such as occurring at pregnancy after the first trimester as well as in cases of use of contraceptive pills. This could lengthens the fertile female age and postpone the menopausal onset for additional years.

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Guide for Authors

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

Aims and scope

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

1. Types of articles

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

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

B. Review articles

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

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

D. Short communications

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

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

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

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

H. Letter to editors

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

I. Debate

2. Submission process

It is recommended to see the guidelines for reporting different kinds of manuscripts here. This guide

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

A. Author contributions statements Sample

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

B. Cover letter And Copyright Sample

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

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Authors whose first language is not English, encouraged to consult a native English speaker in order to confirm his manuscripts to American or British (not a mixture) English usage and grammar. It is necessary to mention that we will check the plagiarism of your manuscript **by iThenticate Software**. Manuscript should be prepared in accordance with the "International Committee of Medical Journal Editors (ICMJE)". Please send your manuscript in two formats Word and Pdf (including: title, name of all the authors with their degree, abstract, full text, references, tables and figures) and Also send tables and figures separately in the site. The abstract and text pages should have consecutive line numbers in the left margin beginning with title page and continuing through the last page of the written text. Each abbreviation must be defined in the abstract and text when they are mentioned for the first time. Avoid using abbreviation in the title. Please use the international and standard abbreviations and symbols.

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

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

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

1. Hardy-Weinberg Equilibrium (HWE) calculations must be carried out and reported along with the P-values if applicable [see Namipashaki et al. 2015 (Cell J, Vol 17, N 2, Pages: 187-192) for a discussion].
2. Linkage disequilibrium (LD) structure between SNPs (if multiple SNPs are reported) must be presented.
3. Appropriate multiple testing correction (if multiple independent SNPs are reported) must be included.

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

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

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

Changes to authorship such as addition, deletion or rearrangement of author names must be made only before the manuscript has been accepted in the case of approving by the journal editor. In this case, the corresponding author must explain the reason of changing and confirm them (which has been signed by all authors of the manuscript). If the manuscript has already been published in an online issue, an erratum is needed.

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

Running title is providing a maximum of 7 words (no more than 50 characters).

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

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

The following components should be identified after the abstract:

Introduction:

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

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Type of study and statistical methods should be mentioned and specified by any general computer program used.

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All of the Clinical Trials performing in Iran must be registered in Iranian Registry of Clinical Trials (www.irct.ir). The clinical trials performed abroad, could be considered for publication if they register in a registration site approved by WHO or www.clinicaltrials.gov. If you are reporting phase II or phase III randomized controlled trials, you must refer to the CONSORT Statement for recommendations to facilitate the complete and transparent reporting of trial findings. Reports that do not conform to the CONSORT guidelines may need to be revised before peer-reviewing.

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They must be presented in the form of text, tables and figures. Take care that the text does not repeat data that are presented in tables and/or figures. Only emphasize and summarize the essential features of the main results. Tables and figures must be numbered consecutively as appeared in the text and should be organized in separate pages at the end of the manuscript while their location should be mentioned in the main text.

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Supplementary materials would be published on the online version of the journal. This material is important to the understanding and interpretation of the report and should not repeat material within the print article. The amount of supplementary material should be limited. Supplementary material should be original and not previously published and will undergo editorial and peer review with the main manuscript. Also, they

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Discussion:

It should emphasize the present findings and the variations or similarities with other researches done by other researchers. The detailed results should not be repeated in the discussion again. It must emphasize the new and important aspects of the study.

Conclusion:

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

Acknowledgements:

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

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Article:

Surname(s) and first letter of name & middle name(s) of author(s). Manuscript title. Journal title (abbr). publication date (year); Volume) Issue(: Page number.

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

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Abstract book:

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

Thesis:

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

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Internet references

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Surname(s) and first letter of name & middle name(s) of author(s). Manuscript title. Journal title (abbr). publication date (year); Volume (Issue): Page number. Available from: URL link. (Observation date).

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Book:

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

Law:

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

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The Final Checklist

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

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