Aims and Scope

Clinical and Experimental Reproductive Medicine (CERM) is an international peer-reviewed journal for the gynecologists, reproductive endocrinologists, urologists and basic scientists providing a recent advancement in our understanding of human and animal reproduction. CERM is an official journal of Pacific Society for Reproductive Medicine, the Korean Society for Reproductive Medicine and Korean Society for Assisted Reproduction. Abbreviated title is Clin Exp Reprod Med. The aims of CERM are to publish the high quality articles that facilitate the improvement of the current diagnosis and treatment in couples with reproductive abnormalities through human or relevant animal model research. Its scope is the infertility, reproductive endocrinology, urology, andrology, developmental biology of gametes and early embryos, basic reproductive science, reproductive physiology, reproductive immunology, genetics and biology of stem cell.

Background

CERM continues the Korean Journal of Reproductive Medicine (pISSN: 2093-8896, 2007-2010) that continues the Korean Journal of Fertility and Sterility (pISSN: 1226-2951) launched in 1974. It was initially published annually, biannually from 1983, tri-annually from 1994, and quarterly from 2000. It is published quarterly on the 1st day of March, June, September and December. This journal is supported by the Korean Academy of Medical Sciences and the Korean Federation of Science and Technology Societies (KOFST) Grant funded by the Korean Government. All or part of CERM is indexed/tacked/covered by PubMed, Emerging Sources Citation Index (ESCI), SCOPUS, KoreaMed, KoMCI, CrossRef and Google Scholar.

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Health concerns regarding the effect of the COVID-19 pandemic on male fertility

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel coronavirus found in China in 2019. The disease caused by SARS-CoV-2, coronavirus disease 2019 (COVID-19), has been found to be closely related to the cells that secrete angiotensin-converting enzyme 2 (ACE2). ACE2 is involved in the renin-angiotensin system and is widely secreted in several tissues, including the testis, which has raised concerns because organs with high expression of the ACE2 receptor are susceptible to infection. Analyses have shown that in testicular cells, such as spermatogonia, seminiferous duct cells, Sertoli cells, and Leydig cells, there is a high expression level of ACE2. Therefore, SARS-CoV-2 may damage male reproductive tissues and cause infertility. Since male infertility is an important problem, scientists are evaluating whether COVID-19 may influence male infertility through the ACE2 receptor.

Keywords: COVID-19; Male fertility; Pandemic

Introduction

In late 2019, a novel coronavirus, known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was found in China [1]. Shortly thereafter, the World Health Organization (WHO) has prolonged its classification of the coronavirus disease 2019 (COVID-19) outbreak as the highest level of a worldwide pandemic [2]. SARS-CoV-2 is a novel type of coronavirus that causes disease in humans. Severe acute respiratory syndrome (SARS-CoV, also now sometimes referred to as SARS-CoV-1) and Middle East respiratory syndrome coronavirus (MERS-CoV) are other members of this virus family, outbreaks of which occurred in 2003 and 2013, respectively [3].

Effects of COVID-19 on some systems other than the lungs (e.g., the gastrointestinal and cardiovascular systems) have been detected [4]. The potential impact of COVID-19 on the male reproductive system has also been discussed. Important data have shown that the reproductive organs in men are susceptible to attack by hepatitis B virus, hepatitis C virus, human immunodeficiency virus (HIV), and human papillomavirus [5]. Viruses can impact and gain entry to the male reproductive system both directly and indirectly [6]. Recent studies have documented the function of the angiotensin-converting enzyme 2 (ACE2) receptor as a gate that allows SARS-CoV-2 to enter into cells into the male reproductive tract [7]. ACE2 receptors facilitate infection by coronaviruses [8]. ACE2 is a zinc metalloprotease [9] that is expressed in some organs, such as the lung and testis [10]. Although previous studies have reported limited information on the relationship of coronavirus diseases with reproductive function, the latest research on the pathophysiological features of SARS-CoV-2 underscores the need to determine whether COVID-19 can influence male infertility [2].

Infertility represents the most serious reproductive disorder [11]. According to the definition of the WHO, infertility is a reproductive system disease that causes disability and affects more than 50 million couples throughout the world [12]. In light of concerns in the field of reproductive medicine, doctors and patients are examining the most appropriate methods to face the challenges that lie ahead [12]. COVID-19 has distinctive effects between the sexes, and the question of why men are more strongly affected by COVID-19 than...
women remains to be elucidated [13]. The numerous health concerns associated with COVID-19 pose an intrinsic danger to the reproductive system [14]. Since infertility in men is a global problem with potential implications for the survival of the human race, it is important to evaluate exactly how the COVID-19 pandemic affects male infertility.

**SARS-CoV-2 cellular pathways**

SARS-CoV-2 is genetically closer to bat-originated SARS-like coronaviruses, which include bat SARS-like coronavirus isolate (bat-SL-CoV-ZC-45) and bat-SL-CoV-ZXC-21 (approximately 88% genomic similarity, than to SARS-CoV-1 (approximately 79%) and MERS-CoV (approximately 50%). However, homology modeling has shown that SARS-CoV-2 has the same receptor binding field organization as that of SARS-CoV-1, indicating that SARS-CoV-2 could have a similar pathogenesis to that of SARS-CoV-1 [15].

The coronavirus family includes large enveloped viruses with single-stranded RNA genomes (roughly 32 kilobases) [16]. These RNA viruses are covered by a coating [17]. This coating includes three different proteins: an envelope protein, a membrane protein, which plays a role in viral assembly; and a spike protein, which facilitates entry of the virus into its target. The coronavirus spike protein includes two unique subunits (S1 and S2) that assist viral-host attachment (Figure 1). The S1 domain plays roles in adhering to the membrane of the target cell. Several receptors on the human cell membrane are involved in S1 attachment have been recognized, such as the ACE2 receptor. The function of the S2 domain is to merge the host and viral cell membranes, permitting the SARS-CoV-2 genome to enter the target cell [18].

**Role of ACE2 in COVID-19 Infection**

Early reports recognized that SARS-CoV-1and SARS-CoV-2 are associated with cells that express the ACE2 receptor on their outer surface, and that the ACE2 receptor is an efficient target for these two coronaviruses [2]. ACE2 is a zinc metalloprotease, the gene of which is located on Xp22.2. It is a protein with 805 amino acids that belongs to the category of dipeptidyl carboxypeptidases. ACE2 includes an N-terminal 17-amino-acid signal peptide and a C-terminal 22-amino-acid membrane anchor. Additionally, it has a preserved motif at the zinc metalloprotease consensus sequence (HEXXH) and a preserved glutamine residue at Glu402 that functions as the third zinc ligand [9].

ACE2 can hydrolyze angiotensin I to make angiotensin 1-9 and cleaves angiotensin II to form angiotensin 1-7 [9, 19]. Angiotensin II exerts damaging effects on tissues by angiotensin 1 (AT1) receptors, whereas the angiotensin 1-7–MAS receptor axis induces beneficial stability and favorable functions, including vasodilatory, antifibrotic, and anti-inflammatory effects, in various tissues. ACE2 is widely expressed in numerous organs, such as the lung, intestine, liver, heart, kidney, and testis [10]. It has been reported that ACE2 plays a relevant regulatory role, including the facilitation of cell death by 5-hydroxytryptamine receptor 3A through tumor necrosis factor alpha secretion as a result of increased serotonin expression. Furthermore, cholinergic receptor nicotinic alpha 1 is involved in inflammation induced by leukocytes. Therefore, it is expected that SARS-CoV-2, as a novel coronavirus, will have intrinsically negative effects on humans [20].

COVID-19 attaches to the ACE2 receptor more powerfully than other SARS viruses, enhancing its pathogenicity and capability for person-to-person transfer [10]. ACE2 receptor detection occurs in the initial phase of virus invasion; therefore, ACE2 receptor expression could be a main factor of tissue and host cell tropism and pathoge-
nicity following duplication of the virus [21]. The virus enters cells through the ACE2 receptor. In theory, tissues that overexpress ACE2 are less resistant to infection [22]. Paradoxically, compared with other organs, the lungs have very low expression of ACE2, which is involved in the renin-angiotensin system (RAS). ACE2 antagonizes the initiation of the typical RAS and defends against tissue injury [23]. Similarly to the circulating RAS, the local RAS plays a critical function in the physiology of numerous organs, including the testes [2].

ACE2 receptors have an important function in the pathogenesis of COVID-19; therefore, cells that express high levels of the ACE2 receptor can be inherently targeted and damaged by the virus [8]. In the study conducted by Fu et al. [24], the mRNA expression of the ACE2 gene in humans was investigated using RNA-sequencing. The number of reads per kilobase of transcript per million mapped reads counts of ACE2 release of the lung was 0.345, while it was 26.895 in the testes.

**Impact of coronaviruses on the male reproductive system via ACE2**

The most important part of the male reproductive system is the testis [25]. The blood-testis barrier and blood-epididymis barrier are critical physical blockades in the male reproductive system, but ACE2 receptors help SARS-CoV-2 infiltrate these barriers, leading to testicular dysfunction [26]. Immunohistochemical evaluations of ACE2 antibodies in healthy testes showed that ACE2 is present at extremely high levels in Leydig and seminiferous duct cells, but at very small (or nonexistent amounts) in other tissues in the male reproductive tract, such as the seminal vesicles, ductus deferens, epididymis, and prostate. Excessive ACE2 secretion in the testis shows that SARS-CoV-2 does not just infect the lung, but could also affect other organs such as the testis, potentially disrupting puberty in young boys and causing infertility in adult men [24]. In 2016, Xu et al. [27] detected orchitis in cadavers of the patients infected by SARS-CoV-1.

Several analyses discovered that in testicular cells, there is a high expression level of ACE2, mostly in spermatogonia, seminiferous duct cells, Sertoli, and Leydig cells. In comparison with other organs, the testis displays the highest level of ACE2 mRNA and protein expression; therefore, it may serve as an innate host for SARS-CoV-2 [8]. Wang and Xu [7] studied the ACE2 gene expression pattern in mature male testes at the level of single-cell transcriptomes. The outcomes showed that ACE2 expression was markedly enhanced in spermatogonia, Leydig cells, and Sertoli cells. A gene ontology study reported that genes related to transmission and reproduction of SARS-CoV-2 were very strongly enhanced in ACE2-positive spermatogonia; however, genes related to gamete generation had little appearance. Liu et al. [28] concluded that the somatic cells in the testis might be more susceptible than germ cells for attack by COVID-19; this was especially true for Sertoli cells, because they expressed ACE2 more than 90%. As an additional examination of the role of ACE2 in testis tissue, they evaluated highly expressed genes and then concentrated on the utility of these genes, which were found to be related to reactive oxygen species in metabolic processes, cellular responses to stress, cohesion to the host cell, activation of leukocytes in the immune response, cell growth arrest, and homeostasis [28].

Illiano et al. [23] made two hypotheses. The first hypothesis was that the virus could enter the testis tissue and alter its function. The second hypothesis was that virus-ACE2 receptor binding leads to increased expression of ACE2 and also increases inflammatory feedback. Inflammatory cells can affect Sertoli and Leydig cells functionally. As a result of damage to male genital tissues by the virus, spermatogenesis might be disturbed, which could pose a risk for male fertility [8].

The first role of Leydig cells is to make sex steroid hormones, especially testosterone. The existence of MAS receptors might imply that angiotensin (1-7) moderates the production of testosterone. In a recent study, SARS-CoV-2 RNA was not detected in the semen and tissue samples by testicular biopsy. These outcomes could show that the virus does not directly infect the testes or male genital tract even in the acute stage. Inflammatory cells could affect the role of Leydig cells, thereby inhibiting testosterone production and destroying the cells of seminiferous ducts [23]. Ma et al. [29] reported that in males with COVID-19, luteinizing hormone (LH) was significantly raised, but the ratio of follicle-stimulating hormone to LH and the ratio of testosterone to LH were dramatically reduced. It is very interesting that the testicular expression of ACE2 is associated with age. Older men are therefore at a lower risk of testicular attack by SARS-CoV-2 than younger men [20].

Clinical examinations found angiotensin 1-7–MAS receptors in the seminiferous ducts and interstitial region, mainly Leydig cells, in healthy men. However, neither mechanism of the RAS was detected in the seminiferous ducts of infertile men who had nonobstructive azoospermia [10]. Shen et al. [20] found that tests ACE2 expression was higher in infertile men than in healthy men, suggesting that SARS-CoV-2 may lead to reproductive disorders by irregular activation of the ACE2 pathway. They found that 30-year-old and 60-year-old men had the highest and lowest expression of ACE2 in the tests, respectively.

Recent research on nonobstructive azoospermia patients showed that MAS and ACE2 had low mRNA expression levels, which might furnish additional evidence that these genes are associated with infertility in men [30]. Furthermore, COVID-19 infection might increase sperm DNA fragmentation through the activation of pathogenetic pathways, which, in turn, can disturb fertilizing potential [31].
Conclusion

Theoretically, there is a possibility of damage to the male reproductive system and, consequently, infertility after COVID-19 infection. The pattern of SARS-CoV-2 infection within testicular tissue has yet to be clarified. However, outcomes from other members of the coronavirus family, especially SARS-CoV-1, provide useful insights into tissue-specific viral pathophysiology. To date, there is no evidence regarding whether SARS-CoV-2 uses ACE2 in the genital tissues and what, or any effect, it has in male infertility. Unfortunately, primary examinations have many limitations (e.g., examination techniques, small sample size, and the progress of the viral infection). More analyses are necessary to clarify the consequences of COVID-19 for male fertility and to investigate ways of preventing reproductive system damage and/or treating such damage. Additional research on this topic should be conducted as soon as possible.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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Introduction

Polycystic ovary syndrome (PCOS) is a common disorder in reproductive-age women. In 2018, an international evidence-based guideline announced recommendations spanning a wide range of issues on the assessment and management of PCOS. From the 166 recommendations, the present study reviews those that are of particular clinical relevance for daily practice and introduces other relevant studies that have been published since the global guideline. The 2018 guideline increased the antral follicle count cutoff for the diagnosis of PCOS from 12 to 20 when using a high-frequency probe. Hirsutism was defined as having a score of ≥4–6 based on a lower percentile of 85%–90% or cluster analysis, which was lower than the traditionally used 95th percentile-based cutoff. The diagnosis of PCOS in adolescents is challenging, and irregular menstruation was defined carefully according to years from menarche. The use of ultrasonography for the diagnosis of PCOS was restricted to those 8 years after menarche. As medication for non-fertility indications, combined oral contraceptives are the first-line drug. Metformin, in addition to lifestyle modifications, should be considered for adult patients with a body mass index ≥25 kg/m² for the management of weight and metabolic outcomes. An aromatase inhibitor is the recommended first-line medication for ovulation induction, a subsequent individual patient data meta-analysis also reported the same conclusion. Whether the new global guideline will be fully adopted by many specialists and change clinical practice is open to question. Further studies are needed to better understand and manage PCOS patients well.

Keywords: Hirsutism; Hyperandrogenism; Infertility; Ovulation induction; Polycystic ovary syndrome

Diagnosis

PCOS is usually diagnosed based on the Rotterdam criteria [2], according to which a diagnosis of PCOS is made if a woman meets two of the following three criteria: (1) oligo- and/or anovulation, (2) HA (clinical and/or biochemical), and (3) polycystic ovary morphology (PCOM) on ultrasonography (either 12 or more follicles measuring 2–9 mm in diameter and/or an increased ovarian volume > 10 cm³). Irreg-
ular menstruation (IM) is defined as <21 days or >35 days or <8 cycles per year. Clinical HA includes hirsutism, acne, or alopecia. Biochemical HA typically refers to an elevated serum testosterone level.

One of the notable recommendations in the 2018 international evidence-based guideline was the revision of the ultrasound criteria for the diagnosis of PCOM. Since the Rotterdam criteria, substantial improvements have been made in ultrasound resolution. A high-resolution probe facilitates the detection of more antral follicles, and when using transvaginal transducers including 8 MHz, the guideline development group recommended that the antral follicle count (AFC) threshold for PCOM should be ≥20 in adult women. Other recommended protocols such as the follicle size (2–9 mm) for the AFC, the ovarian volume criterion (>10 cm³), and ensuring the absence of corpus luteum, cysts, or dominant follicles (>10 mm) have not changed.

The AFC threshold change for PCOM is relevant because ultrasound criterion is the most commonly used parameter for the diagnosis of PCOS [3,4], which was observed in 96.5% of a sample of Korean PCOS patients [4]. Furthermore, among subgroups based on the Rotterdam criteria, the IM and PCOM phenotype and the HA and PCOM phenotype essentially require the presence of PCOM, and 38.0% of Korean PCOS patients were categorized as having an IM and PCOM phenotype [4]. In large Chinese studies, 36.5% and 52.2% of PCOS patients had the IM and PCOM phenotype, respectively [5,6]. As shown by those findings, the IM and PCOM phenotype constitutes a major subgroup in East Asian patients; thus, the AFC threshold change might have particular significance in these populations. Therefore, we investigated the impact of the AFC cutoff change in Korean women with PCOS [7]. In that study, about one-fifth of the total adult patients were excluded from the diagnosis of PCOS using the new AFC cutoff. However, the excluded subjects had worse metabolic profiles (body mass index [BMI] and diabetes status, and the prevalence of IR and metabolic syndrome) and were more androgenized than controls, and were indistinguishable from the remaining patients. Our study suggests that a substantial proportion of PCOS patients might be labeled as “not having PCOS” according to the new AFC cutoff, although these women visited a clinic for IM or hyperandrogenic symptoms. The impact of the AFC cutoff change needs to be consistently evaluated, especially in diverse ethnicities.

The major diagnostic tool for clinical HA is hirsutism. The modified Ferriman-Gallwey (mFG) score is widely used for the diagnosis of hirsutism, and the international evidence-based guideline defines hirsutism as an mFG score of ≥4–6 based on a lower percentile of 85%–90% or cluster analysis. Traditionally, using the 95th percentile of the population, a score of 6–8 represented hirsutism in women [8,9]. However, the guideline development group considered that the 95th percentile is not appropriate for defining hirsutism. In our Korean study, which defined a cutoff score of 6, 50.0% of women had a score of 0, 83.2% had a score of ≤3, and 89.9% had a score of ≤5 [9]. Thus, it is reasonable to suppose that a cutoff score of 4–6, which was equivalent to the lower percentile range of 85%–90% in our study, can also be used for Korean women.

Alopecia can be used as a marker of clinical HA in adult women. Since the publication of the international evidence-based guideline, the Androgen Excess and PCOS Society reported that the relationship between hair loss and HA in women is neither clear nor consistent, and the term “female pattern hair loss” (FPHL) should be used instead of the previously used terms, alopecia or androgenetic alopecia [10]. They stated that isolated FPHL should not be regarded as a sign of HA when androgen levels are normal, but in all women with FPHL, assessment of potential excess androgen level is mandatory. The diagnosis of PCOS in adolescents is always challenging. As in a previous guideline [11], the international evidence-based guideline also recommends that the diagnosis of PCOS in adolescents be made based on both the presence of HA and persistent oligomenorrhea. Ultrasound criteria should not be used for the diagnosis of PCOS in adolescent girls (more specifically, within 8 years after menarche). Adolescent girls who have HA or persistent oligomenorrhea, but do not meet the diagnostic criteria, can be labeled as being at “increased risk,” and reassessment is advised at or before full maturity.

Management

Screening for diabetes is important for the management of PCOS. The prevalence of type 2 diabetes in women with PCOS is significantly increased regardless of age (odds ratio, 2.87; 95% confidence interval, 1.44–5.72) compared to women without PCOS, and this relationship is independent of, yet exacerbated by, obesity [12]. The prevalence of type 2 diabetes in young Korean women with PCOS (mean age, 24.7±5.8 years) was 3.0% (27/899) in our recent study [13], whereas that of the young population (15,050 women aged 20–29 years) in the Korean National Health Insurance Database was 0.3% [14]. Moreover, the incidence rate of type 2 diabetes was 9.3 per 1,000 person-years in women with PCOS, which was significantly higher (p < 0.0001) than that of the overall population of women aged 20–29 years (0.9 per 1,000 person-years).

The optimal screening protocol for women with PCOS remains controversial, but baseline glycemic status should be assessed in all patients. Measurements of fasting glucose, hemoglobin A1c, and the 75-g oral glucose tolerance test (OGTT) can be used [15]. Although the 75-g OGTT is relatively inconvenient, it is recommended for high-risk women with PCOS (including a BMI >23 kg/m² in Asians, history of impaired fasting glucose, impaired glucose tolerance or gestational diabetes, family history of type 2 diabetes, hypertension, or high-
risk ethnicity). South Asian women with PCOS showed an increased degree of hirsutism, early onset of symptoms, severe IR, and metabolic risks compared with Caucasians [16], and thus they can be considered a high-risk ethnicity.

Meanwhile, the 2018 international evidence-based guideline recommends that an OGTT should be considered for all women with PCOS when they plan pregnancy or seek fertility treatment, given the high risk of gestational diabetes and associated complications in pregnancy. Although many obstetricians will not plan an OGTT purely based on PCOS alone, this recommendation may have a significant impact on future clinical practice. In a USA study, a high prevalence (19%) of gestational diabetes was reported in a cohort of 988 consecutive pregnant women with PCOS [17], which was 2–3 times higher than the prevalence of 6%–8% in the general population [18]. In contrast, a Korean study reported that gestational diabetes frequently developed in obese women, rather than being linked to PCOS itself, and that PCOS without obesity was not a risk factor for gestational diabetes [19]. Thus, the recommendation that advises routine OGTT in all women with PCOS who plan pregnancy or fertility treatment needs to be further evaluated, especially in non-obese women with PCOS.

In terms of pharmacological treatment for non-fertility indications, there is a clear recommendation to consider combined oral contraceptives as a first-line medication, including for adolescents. There is not enough evidence to choose the “best” oral contraceptives, despite the common approach of using the lowest effective estrogen dose (20–30 μg of ethinylestradiol).

There is a wealth of literature on metformin use in PCOS, and the international evidence-based guideline states that metformin, in addition to lifestyle modification, should be considered for adult women with PCOS with a BMI ≥ 25 kg/m² for the management of weight and metabolic outcomes. In this guideline, there are no Asian-specific recommendations, such as a BMI ≥ 23 kg/m² based on Asian criteria for being overweight [20]. The 2013 Endocrine Society guideline recommends metformin for PCOS patients who have type 2 diabetes or impaired glucose tolerance in whom lifestyle modification fails [11]. It also suggests metformin as a second-line medication for PCOS patients with menstrual irregularity who cannot take or tolerate combined oral contraceptives. The Endocrine Society guideline may be more specific than that of the international evidence-based guideline since the latter vaguely defines the indications as the “management of weight and metabolic outcomes.” The specific meaning of metabolic outcomes is not described in detail in the 2018 international guideline.

Inositol is a nutritional supplement that plays a role in insulin signaling, and has also been reported as playing a role in modifying metabolic and biochemical components of PCOS. Menstrual cyclicity and ovulation may also be improved. Although caution is needed due to limited data, the international evidence-based guideline suggests that inositol has only a few side effects and low costs. However, a recent Cochrane review could not make a recommendation on the benefits of inositol for subfertile women with PCOS [21].

In terms of pharmacological treatment for fertility indications, an aromatase inhibitor (letrozole) is the recommended first-line medication for ovulation induction in PCOS patients. However, clomiphene citrate (CC) is still an acceptable choice within this guideline. According to the international guideline, women with PCOS were significantly more likely to ovulate after the use of letrozole than after the use of CC. The likelihood of live birth has also been reported to be 40%–60% higher with letrozole than with CC. Multiple pregnancy rates appear to be lower with letrozole than with CC, but it needs to be further investigated whether this shift in favor of letrozole results in an obvious reduction in multiple pregnancy rates.

After the international evidence-based guideline, the International Ovulation Induction Collaboration group reported the results of an individual participant data meta-analysis [22]. In this meta-analysis, letrozole improved clinical pregnancy and the live birth rate and reduced time-to-pregnancy compared to CC. Thus, it can be considered as the preferred first-line ovulation induction medication for women with PCOS, which is consistent with the recommendation of the international evidence-based guideline. CC with metformin may increase clinical pregnancy and reduce time to pregnancy compared to CC alone. The treatment effects of letrozole are affected by baseline serum total testosterone levels, while those of CC with metformin are influenced by baseline serum insulin levels. These associations between treatment effects and markers of hyperandrogenemia or IR provide the basis for a personalized approach to ovulation induction related to PCOS.

In terms of in vitro fertilization, the gonadotropin-releasing hormone (GnRH) antagonist cycle is recognized as superior to the agonist cycle in reducing ovarian hyperstimulation syndrome (OHSS) with similar outcomes, and a GnRH agonist trigger might further eliminate the risk of OHSS. In the GnRH agonist cycle, adjunct metformin reduces the risk of OHSS. In vitro maturation has been performed over the years as a tool to eradicate OHSS.

Conclusion

The international evidence-based PCOS guideline summarizes evidence-based key points for all features of PCOS, and might provide an opportunity to appraise the literature about PCOS. Other relevant recommendations or studies have also been reported in the field of PCOS since the publication of the global guideline. However, controversies still exist, and further updates and collaborative studies are needed to better understand and manage women with PCOS.
Conflict of interest

No potential conflict of interest relevant to this article was reported.

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References

Management of endometrial polyps in infertile women: A mini-review

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Considerable disagreement exists regarding whether endometrial polyps should be removed before attempting natural pregnancy and before pregnancy via intrauterine insemination (IUI) or in vitro fertilization (IVF). Through a literature review, we obtained information on the impact of endometrial polyps and polypectomy on fertility outcomes. Several observational studies have suggested that women with unexplained infertility may benefit from endometrial polypectomy for a future natural pregnancy. A few studies reported benefits from endometrial polypectomy in infertile women who plan to undergo IUI. However, no strong evidence supports polypectomy as a way to improve the pregnancy rate in infertile women who plan to undergo IVF or polypectomy during controlled ovarian stimulation for IVF. Although no studies have defined criteria for the polyp size that should be removed in infertile women, clinicians should be aware that small endometrial polyps (<10 mm) sometimes regress spontaneously. Endometrial polypectomy is currently justified in patients with repeated IVF failure, but more studies are needed to verify that endometrial polypectomy itself will eventually increase the pregnancy rate. Although several mechanisms by which endometrial polyps exert a negative effect on fertility have emerged, there is no consensus about the proper management of endometrial polyps in infertile women. Therefore, the management of endometrial polyps should be individualized depending on the patient’s situation and clinician’s preference.

Keywords: Endometrial polyp; In vitro fertilization; Infertility; Intrauterine insemination; Polypectomy; Pregnancy

Introduction

Endometrial polyps are commonly seen in infertile women [1]. However, there is considerable disagreement regarding whether endometrial polyps should be removed before attempting natural pregnancy and pregnancy via intrauterine insemination (IUI) or in vitro fertilization (IVF) [2-4]. From a clinical point of view, the following questions are important: (1) should endometrial polyps be removed in infertile women who are attempting natural pregnancy and who are planning to undergo IUI or IVF? and (2) is there a standard for the size of endometrial polyps that should be removed?

In this mini-review, we provide an overview of the impact of endometrial polyps on fertility and the impact of endometrial polypectomy on natural pregnancy and pregnancy via IUI or IVF. We also suggest the most appropriate approach to the management of endometrial polyps in infertile women.

Endometrial polyps in infertile women who are attempting natural pregnancy

Varasteh et al. [5] reported a pregnancy rate of 78.3% after endometrial polypectomy in infertile women compared with 42.1% in those with a normal uterine cavity. Spiewankiewicz et al. [6] reported a pregnancy rate of 76% during 12 months after endometrial polypectomy in infertile women, and Shokeir et al. [7] reported a 50% pregnancy rate after endometrial polypectomy. These observational studies suggest that women with unexplained infertility may benefit from endometrial polypectomy for a future natural pregnancy.

The location of endometrial polyps may influence the pregnancy
rate. The pregnancy rate after endometrial polypectomy was highest for polyps located at the utero-tubal junction (57.4%), followed by 40.3% for multiple polyps, 28.5% for posterior uterine wall polyps, 18.8% for lateral uterine wall polyps, and 14.8% for anterior uterine wall polyps [8]. However, endometrial polypectomy may improve fertility irrespective of the size or number of polyps. No difference was found in the pregnancy or miscarriage rate after hysteroscopic polypectomy for small polyps (≤ 10 mm) compared to larger or multiple polyps [9].

**Endometrial polyps in infertile women undergoing IUI**

Perez-Medina et al. [10] performed a randomized controlled trial in women undergoing IUI. The inclusion criteria were women with at least 24 months of inability to conceive and a sonographic diagnosis of endometrial polyps, who were candidates for IUI. They compared the cumulative clinical pregnancy rate after four IUI cycles between women who underwent hysteroscopic polypectomy versus those who underwent diagnostic hysteroscopy and a polyp biopsy only. The size of the endometrial polyps was similar in both groups (mean, 16 mm; range, 3–24 mm). The first IUI was planned for three cycles after hysteroscopy in both groups. They reported a significantly higher cumulative pregnancy rate in the patients who underwent hysteroscopic polypectomy than in those who underwent diagnostic hysteroscopy and polyp biopsy (51.4% vs. 25.4%; relative risk [RR], 2.1; 95% confidence interval [CI], 1.5–2.9). In their report, endometrial polyps ≤ 10 mm accounted for 56% and 61% in the two groups, respectively. Unfortunately, the pregnancy rate was not presented according to the size of the endometrial polyps.

In women undergoing IUI, Kalampokas et al. [11] retrospectively compared the cumulative clinical pregnancy rate after three IUI cycles between a group of patients who underwent hysteroscopic polypectomy versus a group with remaining. The size of the endometrial polyps was similar in both groups (mean, 13.6 mm vs. 12.0 mm). Endometrial polyps ≤ 10 mm accounted for 54.6% and 58.8% in the two groups, respectively, and endometrial polyps > 20 mm accounted for 24.4% and 20% in the two groups, respectively. They reported a significantly higher cumulative pregnancy rate in the group with hysteroscopic polypectomy (40.7% vs. 22.3%).

**Endometrial polyps in infertile women undergoing IVF**

Lass et al. [12] retrospectively compared IVF cycle outcomes between 49 women who underwent fresh embryo transfer (ET) without polypectomy versus 34 women who received cryopreservation of all embryos and underwent polypectomy followed by frozen ET 3 months. The endometrial polyps were less than 20 mm and were diagnosed by transvaginal ultrasonography. The pregnancy rate was similar (22.4% vs. 23.4%), but the miscarriage rate was higher in the group with fresh ET without polypectomy (27.3% vs. 10.7%, p = 0.08). The authors concluded that endometrial polyps < 20 mm did not decrease the pregnancy rate, but there was a trend toward increased pregnancy loss. However, a histological diagnosis of the endometrial polyp was made in only 58.3% of the polypectomy group.

Isikoglu et al. [13] retrospectively compared intracytoplasmic sperm injection (ICSI) cycle outcomes among three groups: (1) 15 women with polyps remaining, (2) 40 women who underwent hysteroscopic polypectomy, and (3) 956 women with no polyps. All endometrial polyps were less than 15 mm and were discovered during controlled ovarian stimulation (COS). The clinical pregnancy rate and implantation rate were similar among the three groups. The authors concluded that endometrial polyps discovered during COS did not negatively affect pregnancy and implantation outcomes in ICSI cycles.

Furthermore, Check et al. [14] confirmed a similar clinical pregnancy rate and miscarriage rate after IVF cycles among three groups: (1) women who underwent polypectomy, (2) women with polyps remaining, and (3) women without polyps. Tiras et al. [15] retrospectively compared ICSI cycle outcomes among four groups: (1) 47 women diagnosed with endometrial polyps before the ICSI cycle who underwent hysteroscopic polypectomy, (2) 47 matched controls with no polyps, (3) 128 women diagnosed with endometrial polyps during COS, and (4) 128 matched controls with no polyps. The size of polyps was only identified in 98 women in group 3, and ranged from 4 mm to 14 mm. The clinical pregnancy rate (29.8% vs. 38.3%) and live birth rate (25.5% vs. 31.9%) were similar between groups 1 and 2, and the clinical pregnancy rate (45.3% vs. 46.9%) and live birth rate (40.6% vs. 39.8%) were also similar between groups 3 and 4. From their study, it is unknown whether hysteroscopic polypectomy increases the pregnancy rate in ICSI cycles, because the control group was not composed of women with polyps remaining. However, their study suggests that endometrial polyps diagnosed during COS do not decrease the pregnancy rate in ICSI cycles.

Elias et al. [16] retrospectively compared IVF cycle outcomes between 60 women with endometrial polyps diagnosed during COS versus 2,933 women without polyps. The clinical pregnancy, spontaneous miscarriage, and live birth rates were similar between the two groups. However, the biochemical pregnancy rate was significantly higher in the polyp group than in the non-polyp group (18.3% vs. 9.6%). The authors concluded that newly diagnosed endometrial polyps during COS were associated with increased biochemical pregnancy rate, but ultimately did not adversely impact the clinical pregnancy or live birth rates after fresh IVF.
If endometrial polyps are noticed during COS for a fresh IVF-ET cycle, two options are possible: (1) proceeding with fresh ET without polypectomy and (2) freezing all embryos and performing polypectomy with frozen ET later. However, a third option may be possible (i.e., polypectomy during COS and proceeding with fresh ET). Although the number of women was small in two studies, reasonable pregnancy rates (4/9 and 3/6) were obtained when hysteroscopic polypectomy was performed during COS followed by fresh ET [17,18].

The effects of the so-called “without cycle cancellation” policy (i.e., polypectomy during COS followed by fresh ET) were also reported in a subsequent non-randomized study [19]. The authors compared the IVF cycle outcomes between a study group (polypectomy during COS followed by fresh ET without cycle cancellation) versus a control group (without polypectomy and proceeding with fresh ET). Endometrial polyps were all diagnosed incidentally during COS for fresh IVF cycles (size range, 5–20 mm). Endometrial polyps were all confirmed pathologically in the study group. The average diameter of the polyps in the study group was higher than that of the polyps in the control group (10.0 ± 4.0 mm vs. 13.3 ± 5.7 mm, p = 0.001). The two groups were similar in terms of the clinical pregnancy rate, implantation rate, and live birth rate. In the study group, the interval between polypectomy and ET was 3–17 days, and this was the only significant predictor of the live birth rate. No pregnancies were achieved in women with an interval < 5 days. In the subgroup with an interval of 5–9 days, the pregnancy rate was 39.5%, and in the subgroup with an interval of > 10 days, the pregnancy rate was 40%. The authors suggested that polypectomy is unnecessary during fresh IVF-ET cycles because polypectomy did not improve the cycle outcomes.

The study by Ghaffari et al. [19] may provide insights into whether polypectomy should be performed in fresh IVF-ET cycles. Based on the aforementioned studies, endometrial polyps < 20 mm appear to have no impact on IVF outcomes. Although the real impact of endometrial polyps on fertility is uncertain, most of the aforementioned studies favor expectant management of endometrial polyps diagnosed in women planning to undergo IVF. If polyp removal is unnecessary in women planning to undergo IVF, we can reduce the number of unnecessary surgical procedures in many cases. In a prospective study of 1,000 women undergoing hysteroscopic evaluation of the uterine cavity prior to IVF, the prevalence of endometrial polyps was found to be 32% [1].

Size criteria for endometrial polyps that should be removed

No study has established criteria for the size of polyps that should be removed or could be observed in infertile women. However, small endometrial polyps (< 10 mm) sometimes regress spontaneously [20]. Therefore, the American Association of Gynecologic Laparoscopists guideline states that management of endometrial polyps may be conservative, with up to 25% of polyps regressing, particularly if they are less than 10 mm in size [21].

Messages from other systematic reviews

In two systematic reviews and 1 practice guideline, removal of endometrial polyps was recommended if they are identified in infertile women [2,3,21]. However, Affifi et al. [3] and Pereira et al. [4] advocated that the management of endometrial polyps observed during COS for IVF should be individualized considering the number of embryos formed, the woman’s previous reproductive history, and the individual clinic’s success with frozen ET.

In a recent systematic review, Zhang et al. [22] reviewed eight studies regarding hysteroscopic polypectomy and assisted reproductive technology outcomes compared with no treatment. They concluded that hysteroscopic polypectomy was associated with an increased rate of clinical pregnancy in patients who underwent IUI, but no clear benefit was observed for clinical pregnancy, live birth, miscarriage, or implantation rates in patients who underwent IVF.

Negative impact of endometrial polyps on fertility

If endometrial polypectomy is helpful in women trying natural pregnancy or IUI cycles, why are they not helpful in women trying IVF cycles? Why do endometrial polyps not have a negative effect on IVF pregnancies? It is believed that the high implantation potential of IVF can overcome the negative impact of endometrial polyps.

The mechanism by which endometrial polyps may interfere with implantation is not clear. Endometrial polyps may interfere with embryo implantation because endometrial polyps are space-occupying lesions. In a uterine flushing study, an increased concentration of glycodelin in the proliferative and preovulatory phase (cycle day 5–14) was found in women with endometrial polyps [23]. It is known that endometrial glycodelin levels are absent or low during the follicular phase and peri-ovulatory period, but increase during the implantation period [24]. Although glycodelin levels were not measured precisely during the peri-ovulatory period in the uterine flushing study, the authors speculated that endometrial receptivity might be altered by increased glycodelin production in the uterine cavity of patients with polyps.

It has been reported that the activated mast cell number increases more than seven-fold throughout the menstrual cycle in the endometrial cavity in women with endometrial polyps [25]. Mast cells
play a key role in inflammatory and allergic processes, and when activated, they release several substances such as histamine, prostaglandin, leukotrienes, and cytokines. The levels of cytokines, as well as matrix metalloproteinase-2 and matrix metalloproteinase-9, were also found to be elevated in the uterine cavity in women with endometrial polyps [26]. Thus, endometrial polyps may induce local inflammatory changes, which can interfere with normal embryo implantation. Moreover, endometrial polyps may affect endometrial receptivity via decreased mRNA levels of HOXA10 and HOXA11, which may impair normal embryo implantation [27].

Endometrial polyps in women with repeated IVF failure

In patients with repeated IVF failure (RIF), the principle is to identify intrauterine lesions and correct any abnormalities. Demirol and Gurgan [28] reported that intrauterine abnormalities were found in 26% of cases via repeated examinations, even though initial hysterosalpingography and diagnostic hysteroscopy were normal. They divided 421 RIF patients into two groups: 210 patients underwent hysteroscopy and 211 patients did not. In patients who underwent hysteroscopy, 56 patients were found to have abnormalities (such as endometrial hyperplasia, polyps, endometritis, synechia, or myoma) that were surgically corrected. The pregnancy rate of the surgically corrected group was 32.5%, which was significantly higher than the rate of 21.6% in the non-hysteroscopy group. Interestingly, the pregnancy rate of those who showed normal findings after hysteroscopy was also high (30.4%). This finding supports the idea that the hysteroscopy alone can increase the pregnancy rate.

According to a systematic review, the incidence of abnormal hysteroscopic findings in patients with RIF varied between 25% and 50%, and pooling data from randomized studies showed that hysteroscopy significantly increased the clinical pregnancy rate in the subsequent IVF cycle (pooled RR, 1.57; 95% CI, 1.29–1.92) [29]. It is not certain whether sole endometrial polypectomy will definitely increase pregnancy rate in patients with RIF, but polypectomy is at least justified in RIF patients.

Conflict of interest

Byung Chul Jee has been the editor-in-chief of Clinical and Experimental Reproductive Medicine since 2018; however, he was not involved in the peer reviewer selection, evaluation, or decision process of this article. No other potential conflict of interest relevant to this article was reported.

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Conceptualization, Data curation, Formal analysis, Methodology, Writing–original draft, Writing–review & editing: all authors.

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Efficacy of intralipid administration to improve *in vitro* fertilization outcomes: A systematic review and meta-analysis

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We performed a systematic review and meta-analysis to evaluate whether intralipid administration improved the outcomes of *in vitro* fertilization. Online databases (PubMed, Cochrane Library, Medline, and Embase) were searched until March 2020. Only randomized controlled trials (RCTs) that assessed the role of intralipid administration during *in vitro* fertilization were considered. We analyzed the rates of clinical pregnancy and live birth as primary outcomes. Secondary outcomes included the rates of chemical pregnancy, ongoing pregnancy, and missed abortion. We reviewed and assessed the eligibility of 180 studies. Five RCTs including 840 patients (3 RCTs: women with repeated implantation failure, 1 RCT: women who had experienced implantation failure more than once) met the selection criteria. When compared with the control group, intralipid administration significantly improved the clinical pregnancy rate (risk ratio [RR], 1.48; 95% confidence interval [CI], 1.23–1.79), ongoing pregnancy rate (RR, 1.82; 95% CI, 1.31–2.53), and live birth rate (RR, 1.85; 95% CI, 1.44–2.38). However, intralipid administration had no beneficial effect on the miscarriage rate (RR, 0.75; 95% CI, 0.48–1.17). A funnel plot analysis revealed no publication bias. Our findings suggest that intralipid administration may benefit women undergoing *in vitro* fertilization, especially those who have experienced repeated implantation failure or recurrent spontaneous abortion. However, larger, well-designed studies are needed to confirm these findings.

**Keywords:** *In vitro* fertilization; Infertility; Intralipid; Recurrent pregnancy loss; Repeated implantation failure

**Introduction**

Although *in vitro* fertilization (IVF) has come a long way, the success rate of IVF is still less than 40% [1]. Furthermore, approximately 10% of women who receive IVF with intracytoplasmic sperm injection experience repeated implantation failure (RIF) [2]. RIF is generally defined as three cycles of IVF that are unsuccessful even though 1–2 good-quality embryos are transferred in each cycle [3]. Impaired endometrial receptivity has been suggested as a major cause of RIF, and immune abnormalities reduce endometrial receptivity and consequently prevent implantation. Immune abnormalities have also been reported as the cause of recurrent spontaneous abortion (RSA) [4]. Therefore, many immunotherapies have been explored to improve endometrial receptivity and increase the pregnancy rate. Immunotherapy methods suggested for immune dysfunction include leukocyte immunization, intravenous immunoglobulin (IVIG), low-molecular-weight heparin, and intralipid [5,6].

Intralipid, which refers to a lipid emulsion comprising soybean oil, is an example of immunotherapy. Because intralipid is a source of fat, it has traditionally been used as a nutritional supplement for patients unable to eat orally. In addition to its nutritional role as an energy source, intralipid has biological functions, including immune function [7]. Although the immunological mechanism of intralipid is not...
fully understood, several studies have reported that its active component, soybean oil, inhibited the cytotoxic activity of natural killer (NK) cells [8,9]. Increased NK cell cytotoxicity has been associated with RSA and RIF [10,11]. In this context, many studies have explored the use of intralipid for women with/without RIF or RSA undergoing IVF [12-16]. However, the results of these studies have proven inconsistent and controversial. Therefore, the effectiveness of intralipid administration in infertile women undergoing IVF has not been conclusively established.

In view of the conflicting results of prior studies, we performed a systematic review and meta-analysis to evaluate the effect of intralipid administration on infertile women during IVF.

Methods

We followed the guidelines of the Cochrane Handbook for Systematic Reviews and preferred reporting items for systematic reviews and meta-analyses (PRISMA) checklist protocol. This study protocol was registered with PROSPERO (CRD42020201739).

1. Eligibility criteria

Only randomized controlled trials (RCTs) evaluating whether administration of intralipid plays a beneficial role in women undergoing IVF were considered. We included both published RCTs and unpublished RCTs and searched abstracts presented at major infertility conferences to identify any unpublished trials. Non-randomized studies were excluded from the current meta-analysis due to the high risk of bias. As the crossover design is invalid, these trials were also excluded. The target population was infertile women who had undergone IVF. We measured the rates of clinical pregnancy (CPR) and live birth (LBR) per randomized woman as the primary outcomes. Clinical pregnancy was determined based on the presence of a gestational sac and detectable fetal heartbeat. Live birth was defined as delivery of a live neonate after 24 weeks of gestational age. Secondary outcomes included the rates of ongoing pregnancy (defined when the pregnancy reached ≥ 12 weeks of gestation) and missed abortion (defined as the intrauterine death of a fetus occurring before 20 weeks of gestational age) per randomized woman. We also assessed adverse events, including adverse reactions to intralipid administration and congenital anomalies.

2. Search methodology for literature identification

We searched online databases including PubMed, Medline, Embase, and the Cochrane Library for all relevant papers through March 2020. The following Medical Subject Headings (MeSH) and text terms were used to retrieve all relevant literature: “intralipid” and (“in vitro fertility” or “IVF” or “assisted reproductive techniques” or “ART” or “repeated implantation failure” or “RIF” or “recurrent pregnancy loss” or “RPL” or “recurrent miscarriage” or “recurrent spontaneous abortion” or “RSA”). There was no language restriction. Studies were independently identified by EJH and SWL.

3. Study selection and data extraction

Initially, two review authors (EJH and SWL) identified potentially relevant trials, and the retrieved titles and abstracts were then screened. We retrieved the full texts of all potentially eligible articles, and both authors (EJH and SWL) independently read through the full-text articles to determine whether they met the inclusion criteria and selected studies for inclusion in the review. Any disagreements regarding study eligibility were resolved by discussion or arbitration by a third author (WSL). The selection process was presented in a PRISMA flow chart.

4. Assessment of risk of bias in the included studies

Two review authors (EJH and SWL) independently evaluated the risk of bias in individual articles using the Cochrane Risk of Bias tool presented in the Cochrane Handbook for Systematic Reviews of Intervention (www.training.cochrane.org/handbook). They evaluated the following seven sources of bias: (1) random sequence generation (selection bias); (2) allocation concealment (selection bias); (3) blinding of participants and personnel (performance bias); (4) blinding of outcome assessment (detection bias); (5) incomplete outcome data (attrition bias); (6) selective reporting (reporting bias); and (7) other sources of bias (other bias). We summarized the results of the risk of bias assessment for the included studies in graphs.

5. Statistical analysis

We conducted the meta-analysis using the RevMan 5.3 software package (Cochrane Collaboration, Oxford, UK). The risk ratios (RRs) with 95% confidence intervals (CIs) for binary data variables were calculated using the Mantel-Haenszel method. Using the $I^2$ statistic to assess the statistical heterogeneity across the included articles, we determined that statistical heterogeneity was absent when $I^2$ was < 50%. In selecting the effect models, $I^2$ was used as the standard (fixed model: $I^2 < 50\%$, random-effect model: $I^2 \geq 50\%$). We used a forest plot to graphically present the heterogeneity of the treatment effects. Publication bias was also estimated using funnel analysis and the Egger test. Forest plots were generated to present the results of the meta-analysis. When sufficient studies were available, we carried out further analyses within the following subgroups: (1) women without RIF or RSA versus women with RIF or RSA, and (2) women with previous implantation failure versus women with RSA. We also planned sensitivity analyses to evaluate whether our outcomes would have differed if (1) a random-effect model had been adopted.
or (2) we had restricted the analysis to only published studies.

Results

1. Study selection and characteristics

In total, 180 articles were collected from electronic databases. Of these, 149 studies were considered ineligible as screening of the titles and abstracts revealed they did not meet the inclusion criteria. After reviewing the full texts of the remaining 31 studies, 26 studies were excluded for the following reasons: 10 studies were non-RCTs, nine studies were reviews, and seven studies reported no relevant comparisons or outcomes. Finally, five RCTs involving a total of 840 patients were included in the present meta-analysis [12-14,17,18]. The search results were presented in a PRISMA flow chart (Figure 1).

Three of the included trials were published as full articles [12-14], and two were published as conference abstracts [17,18]. Three studies evaluated the effects of intralipid administration on RIF [12,17,18], one study investigated the influence of intralipid administration on RSA in patients with elevated NK cell levels (> 12%) [13], and one study assessed the efficacy of intralipid for infertile women who experienced implantation failure more than once [14]. Most studies excluded women with an abnormal uterine cavity (i.e., endometrial polyp, submucosal myoma, or arcuate uterus). The method of intralipid administration varied among the included trials. In most studies, 20% intralipid was administered twice intravenously. For the first dose, intralipid was infused on the fourth to ninth day of controlled ovarian hyperstimulation [17], the day of ovum pick-up [13,14], or the day of embryo transfer [12]. The second dose of intralipid was administered on the day of embryo transfer [14], the day of the pregnancy test [12], or within 1 week of a positive pregnancy test [13,17]. All five included trials reported the CPR and LBR as outcomes. The chemical pregnancy rate was reported in two trials [13,14], the ongoing pregnancy rate was reported in two trials [13,14] and the miscarriage rate was reported in two trials [12,13]. Table 1 shows the characteristics of the five included studies.

![Figure 1. Preferred reporting items for systematic reviews and meta-analyses flow diagram of study selection. RCT, randomized controlled trial.](www.eCERM.org)
Table 1. Characteristics of the included studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Study design</th>
<th>Participant characteristics</th>
<th>Sample size (T/C)</th>
<th>Intervention</th>
<th>Control</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al-Zebeidi et al. (2020)</td>
<td>Randomized controlled study</td>
<td>Unexplained recurrent implantation failure (≥ 3 cycles)</td>
<td>142 (71/71)</td>
<td>Intravenous infusion of intralipid (100 mL of 20% intralipid diluted in 500 mL of normal saline) on the day of embryo transfer and a second dose on the day of pregnancy test</td>
<td>No intervention</td>
<td>Clinical pregnancy rate, live birth rate, miscarriage rate</td>
</tr>
<tr>
<td>Singh et al. (2019)</td>
<td>Randomized controlled study</td>
<td>Previous implantation failure (≥ 1 cycle)</td>
<td>102 (52/50)</td>
<td>Intravenous infusion of intralipid (4 mL of 20% intralipid diluted in 250 mL of normal saline) on the day of oocyte retrieval (after retrieval) and a second dose on the day of embryo transfer, 1 hour prior to transfer</td>
<td>Intravenous saline injection</td>
<td>Chemical pregnancy rate, clinical pregnancy rate, miscarriage rate, ongoing pregnancy rate, live birth rate</td>
</tr>
<tr>
<td>Gamaleldin et al. (2018)</td>
<td>Randomized controlled study</td>
<td>Unexplained recurrent implantation</td>
<td>97 (48/49)</td>
<td>Intravenous infusion of intralipid (20%) starting 6–7 days before embryo transfer and a second dose in case of a positive pregnancy test</td>
<td>Intravenous saline injection</td>
<td>Clinical pregnancy rate, live birth rate</td>
</tr>
<tr>
<td>Dakhly et al. (2016)</td>
<td>Randomized controlled study</td>
<td>Recurrent spontaneous abortion (≥ 3) with elevated NK cell levels (&gt; 12%)</td>
<td>296 (144/152)</td>
<td>Intravenous infusion of intralipid (2 mL of 20% intralipid diluted in 250 mL of normal saline) on the day of oocyte retrieval and repeated within 1 week of a positive pregnancy test and every 2 weeks until the end of the first trimester</td>
<td>Intravenous saline injection</td>
<td>Chemical pregnancy rate, clinical pregnancy rate, miscarriage rate, ongoing pregnancy rate, live birth rate, miscarriage rate</td>
</tr>
<tr>
<td>El-Khayat et al. (2015)</td>
<td>Randomized controlled study</td>
<td>Unexplained recurrent implantation failure (2–6 cycles)</td>
<td>203 (101/102)</td>
<td>Intravenous infusion of intralipid (20%) between day 4 and 9 of ovarian stimulation and a second dose when participants became pregnant, within 1 week of the positive pregnancy test</td>
<td>Intravenous saline injection</td>
<td>Clinical pregnancy rate, implantation rate, live birth rate</td>
</tr>
</tbody>
</table>

No data were presented about the NK cell level except by Dakhly et al. [13]. T, trial group; C, control group; NK, natural killer.

2. Methodological quality of the included studies

Most of the included trials had a relatively low to moderate risk of bias according to the guidelines of the Cochrane Collaboration (Supplementary Table 1). We present the outcomes of the risk of bias assessments for the included studies in figures (Supplementary Figures 1 and 2).

3. Outcome measures

1) Clinical pregnancy rate

All five included RCTs (840 patients) reported the CPR. All five RCTs showed that the CPR was higher in the intralipid group than in the placebo group, but the results were statistically significant in only two RCTs [14,17]. Pooling of the data showed a significant improvement in the CPR in the intralipid group when compared with the control group (RR, 1.48; 95% CI, 1.23–1.79; p = 0.13). Heterogeneity was not found among the trials, as judged by the I² value (45%). Thus, we selected a fixed-effect model (Figure 2A).

2) Live birth rate

All five included RCTs (840 patients) were eligible for inclusion in the meta-analysis of the LBR. In three of the five RCTs, it was confirmed that intralipid significantly increased the LBR when compared to the placebo group [13,14,17]. The RR for the LBR was 1.85 (95% CI, 1.44–2.38) in favor of intralipid administration for infertile women undergoing IVF. Homogeneity was found among the five RCTs according to the I² value (0%). According to the result of I², we analyzed the data using a fixed-effect model (Figure 2B).

3) Ongoing pregnancy rate

Two trials (398 patients) assessed the ongoing pregnancy rate [13,14]. Both studies noted that the rate of ongoing pregnancy in the intralipid group was significantly higher than that in the placebo group. The results of the meta-analysis showed a higher ongoing pregnancy rate when intralipid was administered (RR, 1.82; 95% CI, 1.31–2.53; p = 0.38). When the I² statistic was calculated, no heterogeneity was identified (I² = 0%). We performed the meta-analysis using a fixed-effect model because I² was < 50% (Figure 3A).

4) Miscarriage rate

The miscarriage rate was reported in two RCTs with 438 patients [12,13]. One of them showed that intralipid significantly decreased the miscarriage rate when compared to the control group [13]. Pool-
The miscarriage rate (RR, 0.75; 95% CI, 0.48–1.17; p = 0.21). Homogeneity between the two RCTs was identified using the $I^2$ statistic (22%). Thus, we selected a fixed-effect model (Figure 3).

### 5) Adverse events

Four of the included studies assessed adverse events, which did not show a statistically significant difference between the intervention group and control group [12–14,18]. In two of these trials [14,18], one congenital diaphragmatic hernia and two congenital external ear anomalies were reported in the intralipid treatment groups. In the other trials [12,13], no adverse events of intralipid administration were reported.

### 4. Publication bias analysis

The five RCTs included in this meta-analysis were evenly distributed across the graph in the funnel plot analysis, showing no publication bias (Figure 4).

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**Figure 2.** Meta-analysis of the clinical pregnancy rate (A) and live birth rate (B). M-H, Mantel-Haenszel; CI, confidence interval.

**Figure 3.** Meta-analysis of the ongoing pregnancy rate (A) and miscarriage rate (B). M-H, Mantel-Haenszel; CI, confidence interval.
5. Subgroup analysis and sensitivity analysis

We did not conduct a subgroup analysis since it was not possible to extract data for populations without RIF or RSA. In a subgroup analysis of women with previous implantation failure versus women with RSA, we found higher CPR and LBR for women with previous implantation failure, and the pooled RRs were 1.74 (95% CI, 1.27–2.40) and 1.98 (95% CI, 1.39–2.80), respectively (Supplementary Figure 3). For women with RSA, only one study was reported, according to which LBR improved compared with placebo or no treatment (Supplementary Figure 3). Sensitivity analyses using a random-effect model or limited to only published studies did not result in different conclusions regarding the CPR, LBR, ongoing pregnancy rate, and miscarriage rate (Supplementary Tables 1 and 2).

Discussion

This meta-analysis was conducted to evaluate the effectiveness of intralipid administration for infertile women undergoing IVF. Five RCTs involving a total of 840 infertile women were included. The meta-analysis showed that IVF outcomes, including the CPR, ongoing pregnancy, and live birth, improved when intralipid was used as an adjunct treatment in women undergoing IVF, especially for RIF or RSA.

The current review showed that intralipid administration improved the LBR compared with the control group (RR, 1.85; 95% CI, 1.44–2.38) (Figure 2B). This finding is consistent with the preliminary results of a meta-analysis presented by Asif et al. [19] at the 34th Annual Meeting of the European Society of Human Reproduction and Embryology (RR, 2.13; 95% CI, 1.35–3.36). This review provided more reliable evidence because it analyzed more participants than the study of Asif et al. [19] (5 RCTs with 840 participants vs. 2 RCTs with 303 participants). Another difference relates to the inclusion criteria, as the authors [19] included only women with RIF, whereas this review included women with RSA as well as RIF, because intralipid is known to effective in suppressing NK cytotoxicity which has been suggested as a common cause of RIF and RSA.

Although not included in this meta-analysis, we did find non-RCTs assessing the effects of intralipid on IVF outcomes. Martini et al. [16] evaluated the effects of intralipid infusion on 127 women with RIF or RSA who had elevated NK cell levels (≥ 19%) and had undergone assisted reproductive technology (IVF or intrauterine insemination). They administered intralipid (4 mL of 20% intralipid diluted in 250 mL of normal saline) 7–10 days before embryo transfer or insemination, a second dose at a gestational age of 6 weeks and again at a gestational age of 10 weeks. In that study, intralipid failed to improve the CPR or LBR when compared with the baseline rates published by Tang et al. [20] (p = 0.12 and p = 0.80, respectively). The use of historical control data was a notable limitation of this study. Another non-RCTs assessing the utility of intralipid in infertile women with RIF or RSA was reported by Check and Check [15]. They analyzed the effect of intralipid in a more specific patient group (aged 40–42 years) compared to the patient groups of the studies included in our meta-analysis. Intralipid (4 mL of 20% intralipid diluted in 100 mL normal saline) was infused during the mid-follicular phase. The authors demonstrated that intralipid did not increase the LBR in infertile women undergoing IVF who experienced RIF or RSA (p = 0.087). The differences between these findings and those of our meta-analysis may be due to the different study populations. The efficacy of intralipid in women of advanced reproductive age with RIF or RSA should be evaluated in further studies.

The immune mechanism of intralipid has not been fully identified, but several previous studies have suggested that it has immunosuppressive properties that inhibit NK cytotoxic activity and production of pro-inflammatory cytokines such as tumor necrosis factor-alpha, interleukin-6, and interleukin-8 [8,9,21]. In particular, NK cytotoxicity has been suggested as a cause of RIF and RSA [10,11]. It has been shown that intralipid affects NK cells through receptors such as the G protein-coupled receptor and peroxisome proliferator-activated receptor (PPAR) [22–25]. The fatty acids in intralipid and its metabolites act as ligands that activate PPAR expressed in NK cells. PPAR activation reduces NK cytotoxicity and consequently enhances implantation and maintains pregnancy [26–29]. PPAR also plays an important role in implantation, invasion of cytotrophoblasts, embryo growth, and formation of the placenta [25,30].

Like intralipid, IVIG is also effective in suppressing NK cytotoxicity [29,31] and has been used successfully to treat RIF and RSA. However, IVIG is expensive and is associated with side effects such as transfusion-transmitted diseases because of a blood product. In recent years, some authors have suggested that intralipid and IVIG are equally effective in decreasing NK cell cytotoxicity. Coulam and Aca-
cio [32] compared the effects of intralipid and IVIG in infertile women who had experienced reproductive failure and increased NK cell levels (n = 442). There were no significant differences between the intralipid group (n = 200) and IVIG group (n = 242) in the LBR or ongoing pregnancy rate (61% vs. 56%, respectively). In 2016, Meng et al. [10] conducted an RCT to compare the effects of intralipid (n = 78) and IVIG (n = 78) in women with unexplained RSA and elevated NK cell levels (> 20%). The authors noted that the successful pregnancy rates of the two groups were similar (92.1% vs. 88.2%; p = 0.415). These data suggest that intralipid is sufficiently effective and may be used as an alternative to IVIG for the treatment of RSA or RIF. Furthermore, intralipid is less expensive than IVIG. However, RCTs with larger sample sizes are needed to confirm that intralipid and IVIG are both effective treatments for RIF or RSA.

To ensure quality, we included only RCTs in the meta-analysis. Furthermore, selection bias was reduced, as two reviewers independently selected the trials and extracted the data. There were also several limitations to our review. One was the heterogeneity of the included trials. There were differences among the study populations (infertile women with RIF, RSA, or a history of implantation failure). There was also variability among the intralipid administration protocols in terms of dose, frequency, and duration. Therefore, the optimal dose, frequency, and duration of intralipid were not confirmed. Another limitation is that only a small number of studies were included in the meta-analysis. Despite these limitations, the present meta-analysis provides clinicians with a meaningful summary of the existing studies on the effectiveness of intralipid for infertile women undergoing IVF with RIF or RSA.

In conclusion, the findings of the current meta-analysis and systematic review suggest that intralipid administration may improve IVF outcomes, especially in women with RIF or RSA. However, due to some limitations of this review, intralipid in women undergoing IVF should be used with caution and these findings need to be further evaluated in larger, well-designed studies. The mechanisms and safety of intralipid and the optimal protocol for intralipid administration should also be explored in future research.

Conflict of interest

Sang Woo Lyu is an editorial board member of the journal but was not involved in the peer reviewer selection, evaluation, or decision process of this article. No other potential conflicts of interest relevant to this article were reported.

Author contributions

Conceptualization: SWL. Data curation: EJH, SWL. Formal analysis: EJH, SWL. Funding acquisition: SWL. Methodology: EJH. Project administration: WSL. Visualization: HNL, MKK. Writing—original draft: EJH. Writing—review & editing: SWL.

Supplementary material

Supplementary material can be found via https://doi.org/10.5653/cerm.2020.04266.

References


Protective effects of curcumin against methotrexate-induced testicular damage in rats by suppression of the p38-MAPK and nuclear factor-kappa B pathways

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Objective: The present study aimed to investigate the possibility that curcumin (CMN) protects against methotrexate (MTX)-induced testicular damage by affecting the phospho-p38 (p-p38) mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB) signaling pathways.

Methods: Eighteen male Wistar albino rats were randomly divided into three groups. The control group was given an intragastric administration of dimethyl sulfoxide (DMSO) daily for 14 days, the MTX group was given a single intraperitoneal dose of MTX (20 mg/kg) on the 11th day, and the MTX+CMN group was given intragastric CMN (100 mg/kg/day, dissolved in DMSO) for 14 days and a single intraperitoneal dose of MTX (20 mg/kg) on the 11th day. At the end of the experiment, all animals were sacrificed and the testicular tissues were removed for morphometry, histology, and immunohistochemistry. Body and testicular weights were measured.

Results: Body weights, seminiferous tubule diameter, and germinal epithelium height significantly decreased in the MTX group compared to the control group. Whereas, the number of histologically damaged seminiferous tubules and interstitial space width significantly increased in the MTX group. In addition, the number of p-p38 MAPK immunopositive cells and the immunoreactivity of NF-κB also increased in the MTX group compared to the control group. CMN improved loss of body weight, morphometric values, and histological damage due to MTX. CMN also reduced the number of p-p38 MAPK immunopositive cells and the NF-κB immunoreactivity.

Conclusion: CMN may reduce MTX-induced testicular damage by suppressing the p38 MAPK and NF-κB signaling pathways.

Keywords: Curcumin; Methotrexate; Nuclear factor-kappa; p38; Testis

Introduction

Methotrexate (MTX) is a folic acid antagonist used as a medication for the treatment of acute lymphoblastic leukemia; osteosarcoma; choriocarcinoma; lymphoma; breast, bladder, and head and neck cancers, as well as for the treatment of non-malignant diseases such as psoriasis and rheumatoid arthritis [1-3]. It is also effective for the termination of pregnancy [3]. Anticancer drugs such as MTX have lethal effects on cancer cells, but they also affect normal tissues that have a high proliferation rate, such as bone marrow, intestinal mucosa, and gonads [4]. In addition to its gonadotoxicity, MTX also has known mutagenic and teratogenic properties [5]. Therefore, the current recommendation is to cease MTX treatment in both female and male patients at least 3 months before a planned pregnancy [3]. A single dose of MTX administered to rats causes an increase in oxidative stress in the testes, thereby leading to infertility directly or for the treatment of acute lymphoblastic leukemia; osteosarcoma; choriocarcinoma; lymphoma; breast, bladder, and head and neck cancers, as well as for the treatment of non-malignant diseases such as psoriasis and rheumatoid arthritis [1-3]. It is also effective for the termination of pregnancy [3]. Anticancer drugs such as MTX have lethal effects on cancer cells, but they also affect normal tissues that have a high proliferation rate, such as bone marrow, intestinal mucosa, and gonads [4]. In addition to its gonadotoxicity, MTX also has known mutagenic and teratogenic properties [5]. Therefore, the current recommendation is to cease MTX treatment in both female and male patients at least 3 months before a planned pregnancy [3]. A single dose of MTX administered to rats causes an increase in oxidative stress in the testes, thereby leading to infertility directly or
through the toxic effects of MTX [6-8]. It has been reported that male individuals exposed to MTX exhibit oligozoospermia and structural chromosomal rearrangements [9].

Various signals related to cell proliferation, cell differentiation, and cell death are regulated by mitogen-activated protein kinases (MAPKs) [10]. In particular, p38 MAPK can be activated by a variety of cellular stresses, including oxidative stress, and is related to inflammation and programmed cell death (apoptosis) [10-13]. A cell culture study using the human bronchial cell line BEAS-2B confirmed that MTX increased p38 MAPK expression and dose-dependently increased p38 MAPK phosphorylation, whereas pre-treatment with the p38 MAPK inhibitor SB203580 decreased both p38 MAPK expression and phosphorylation [14]. An organ culture study using human nasal polyp cells showed that MTX caused apoptosis by increasing the levels of the phosphorylated forms of p38 MAPK in a dose-dependent manner [15]. These studies indicate a potential association between the p38 MAPK signaling pathway and MTX.

Another known effect of MTX is its ability to promote significantly increased activation and translocation of nuclear factor-kappa B (NF-kB) [16-19]. NF-kB is a protein complex containing specific transcription factors with known involvement in inflammatory and innate immune responses [20]. These responses are also considered to involve oxygen free radicals, so various antioxidant substances have been explored as agents for the prevention of the testicular damage caused by MTX [6-8,21-27].

An antioxidant compound that is receiving particular attention is curcumin (CMN), which has substantial anti-inflammatory, immunomodulatory, antitumoral, antipsoriatic, and wound-healing properties in addition to its antioxidant activity [28-35]. CMN is a delicious yellow-orange-colored spice with a wide range of pharmacological and biological activities. It is obtained from the rhizomes of a Curcuma longa, a plant belonging to the Zingiberaceae family [36,37]. CMN is widely used in Far Eastern and Asian countries, especially in India and China, in the food industry (as a sweetener, preservative, coloring, spice, etc.) and in traditional medicine as a treatment for inflammation and end of the experiment, and the weight of the testes was measured at the beginning and end of the experiment, and the weight of the testes was measured at the end of the experiment. The testes weight index (TWI) was calculated for each animal by taking into account the body weight and the sum of the weights of the right and left testes of the same animal, using the following formula: TWI: ([sum of the weights of right+left testes]/body weight) × 100.

3. Histological examinations

All testis tissues for histological and immunohistochemical examinations were fixed in 10% neutral-buffered formalin (Sigma-Aldrich), dehydrated in an increasing ethanol series, and embedded in paraffin. Then, 5-μm-thick sections were cut from the paraffin-embedded testis tissues with a microtome (RM-2245; Leica, Wetzlar, Germany). The sections were deparaffinized in toluene, rehydrated through a series of decreasing ethanol concentrations, and stained with hematoxylin and eosin (H&E). After staining, the slides were dehydrated through a se-
ries of increasing ethanol concentrations, immersed in toluene, and covered with Entellan (Merck). The slides were then examined and photographed under a light microscope (Olympus BX51, Tokyo, Japan) equipped with a DP 20 digital camera attachment.

The seminiferous tubule diameter, the germinal epithelium height, and interstitial space width in H&E stained testes sections were measured with a light microscope at \( \times 100 \) or \( \times 400 \) magnification using an Imaging Analysis System ver. 2.11.5.1 (Kameram-Argenit, Istanbul, Turkey). These measurements were performed by evaluating the transverse sections of a total of 30 tubules in five fields. Thirty tubules were chosen as round or near-round in three testes sections of each animal [25].

Germinal series cell changes were assessed histologically by light microscopy examination of six testis sections of each animal at \( \times 100 \) magnification according to the following criteria: detachment (detachment of cohorts of spermatocytes from the seminiferous epithelium), sloughing (release of clusters of germ cells into the lumen of the seminiferous tubule), and vacuolization (appearance of empty spaces in the seminiferous tubule). For each parameter, the average percentages of normal and damaged tubules were determined. Average percentages for each sample were obtained by dividing the number of histologically damaged (detachment, sloughing, and vacuolization) or normal round tubules by the total number of round tubules in the same field, and multiplying the result by 100. Three areas were evaluated for each section and their averages were analyzed [45].

4. Immunohistochemical Examination

For the immunohistochemistry procedure, testis sections were incubated overnight at 56°C and then deparaffinized in toluene. The sections were rehydrated in a decreasing ethanol series and then boiled in a microwave for 15 minutes in 10 mM citrate buffer (pH 6) for antigen retrieval. The sections were cooled for 20 minutes at room temperature and then incubated with hydrogen peroxide solution (Thermo Scientific/Lab Vision, Fremont, CA, USA) for 10 minutes to inhibit endogenous peroxidase activity. The sections were then washed with phosphate-buffered saline (PBS; Sigma-Aldrich), and blocking solution (Ultra V Block-Thermo Scientific/Lab Vision) was applied for 5 minutes in a humidified chamber to prevent non-specific binding. The sections were then washed with phosphate-buffered saline (PBS) for 1 hour at room temperature with NF-κB/p65 antibody (polyclonal rabbit, 1:100 dilution; Thermo Scientific/Lab Vision). The negative control consisted of PBS that replaced the primary antibody. The sections were washed with PBS, and biotinylated secondary antibody (Biotinylated Goat Anti-Polyvalent, Thermo Scientific/Lab Vision) was applied at room temperature for 10 minutes. After washing again with PBS, streptavidin-peroxidase (Thermo Scientific/Lab Vision) was applied for 10 minutes. After PBS washing, 3-amino-9-ethyl carbazole (AEC; Thermo Scientific/Lab Vision) was added as the chromogen. The sections were washed with distilled water for 5 minutes and then counterstained with hematoxylin. The sections were washed once again with tap water and then covered with an aqueous mounting medium (Vision Mount, Thermo Scientific/Lab Vision) [43,46].

The p-p38 MAPK immunopositive staining index was determined at \( \times 400 \) magnification by randomly selecting 10 seminiferous tubules in a testis section from each animal and assessing the sections under a light microscope (Olympus BX51). Cells with red-stained nuclei were evaluated as positive. Both stained and non-stained germ cells were counted, and the ratio of stained cells to the total number of germ cells, multiplied by 100, gave the p-p38 MAPK index for each seminiferous tubule (calculated as %). The average p-p38 MAPK index in each animal was determined by taking the average of the indices of tubules [45,47].

By contrast, NF-κB immunoreactivity was semi-quantitatively evaluated with the H-score method. Assessments were made under a light microscope (Olympus BX-51) at \( \times 400 \) magnification by randomly selecting five areas in transverse sections of the testis of each animal. The scores were generated by taking the percentage of cells with immunoreactivity in the sections (Pi) and the degree of staining (i). The degree of staining was assessed as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The average H-score for each section belonging to each animal was calculated with the following formula: H-score = \( \Sigma \) i × Pi [46]. All semiquantitative assessments by light microscopy were made by two independent observers and the averages were considered.

5. Statistical analysis

All statistical analyses were performed using the IBM SPSS ver. 20.0 (IBM Corp., Armonk, NY, USA; license no. 10240642). The results are expressed as mean ± standard deviation, and \( p < 0.05 \) were considered to indicate statistical significance. All data were assessed for normal distribution with the Kolmogorov-Smirnov test. The values showed a normal distribution, so one-way analysis of variance was carried out. Depending on the homogeneity of the groups, the Tukey or Tamhane multiple comparison test was used to determine the significance of differences between groups. The nonparametric Kruskal-Wallis test was used to determine the significance of changes in the body weight of the animals. The Bonferroni-corrected Mann-Whitney U-test was conducted to assess the significance of between-group differences.
Results

1. Body and testicular weight findings

The initial and final body weights of animals were measured at the beginning and end of the experiment, respectively. A significant reduction was observed in the body weight of MTX-treated animals compared to the control group \( (p < 0.05) \) (Table 1), whereas no change was observed in testes weight or in the testicular weight index \( (p > 0.05) \). Conversely, pretreatment with CMN before MTX treatment caused a significant increase in the body weight compared to the MTX group \( (p < 0.05) \) (Table 1).

2. Morphometric findings

The seminiferous tubule diameter and germinal epithelium height significantly decreased in the MTX group, while the interstitial space width significantly increased \( (p < 0.001) \) (Table 2), in comparison to the control group. The administration of CMN before MTX treatment significantly increased the seminiferous tubule diameter and the germinal epithelium height \( (p < 0.05) \) (Table 2), while the interstitial space width significantly decreased \( (p < 0.001) \) (Table 2).

3. Histological findings

Light microscopy examination of the testis tissue sections in the control group revealed active spermatogenesis and a regular and normal appearance of the seminiferous tubules. The incidence of detached, sloughed, or vacuolized seminiferous tubules were also very low. Eosinophilic Leydig cells appeared as clusters in the interstitial field located between the neighboring seminiferous tubules and had a normal histological structure (Figure 1A and B).

Examination of the testis tissue sections in the MTX group revealed degeneration and loss of germinal series cells, disorganization of the germ cell layers, and vacuolization in the seminiferous tubules. The number of damaged seminiferous tubules containing detached \( (p < 0.001) \), sloughed \( (p < 0.001) \), or vacuolized \( (p < 0.001) \) tubules was higher in the MTX group than in the control group, whereas the number of normal \( (p < 0.001) \) tubules was smaller. Sloughing of germinal cells that had not completed maturation in the lumen was also evident in the MTX group, and some seminiferous tubules showed irregularities and undulations of the basal membrane. No change was observed in Sertoli cells, but the interstitial space showed cell loss with edema (Figure 1C and D).

Spermatogenesis was markedly preserved in the MTX+CMN group. Moreover, the percentages of damaged seminiferous tubules with disorganization of the germ cell layers were markedly lower in this group. Irregularity and cell loss in the interstitial space width were also less common than in the MTX group, and edema was also reduced (Figure 1E and F). The scoring results for changes in the histological structure of the seminiferous tubules in all groups are summarized in Table 3.

4. Immunohistochemical findings

p-p38 MAPK immunopositive cells showed nuclear staining, mostly in primary spermatocytes, in the sections of seminiferous tubules. The control group showed low numbers of p-p38 MAPK immunopositive cells (Figure 2A). By contrast, sections of seminiferous tubules in the MTX group showed a significantly higher number of im-

Table 1. Comparison of changes in body weight, testis weight, and testis/body weight ratio \( ([\text{weight of both testes/body weight}] \times 100) \) in rats in the control, methotrexate-treated, and methotrexate+curcumin-treated groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Methotrexate</th>
<th>Methotrexate+curcumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in body weight (g)</td>
<td>13.17 ± 5.00</td>
<td>−21.50 ± 6.16\textsuperscript{a}</td>
<td>−9.33 ± 4.18\textsuperscript{a,b}</td>
</tr>
<tr>
<td>Weight of both testes (g)</td>
<td>2.52 ± 0.14</td>
<td>2.53 ± 0.12</td>
<td>2.52 ± 0.19</td>
</tr>
<tr>
<td>(Weight of both testes/body weight) \times 100</td>
<td>0.97 ± 0.04</td>
<td>0.94 ± 0.06</td>
<td>0.94 ± 0.03</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation for six rats in each group. \( \textsuperscript{a}p<0.05, \text{significant difference compared to the control group}; \textsuperscript{b}p<0.05, \text{significant difference compared to the methotrexate group}. \)

Table 2. Comparison of seminiferous tubule diameter, germinal epithelium height, and interstitial space width in testis tissues of rats in control, methotrexate-treated, and methotrexate+curcumin-treated groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Methotrexate</th>
<th>Methotrexate+curcumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seminiferous tubule diameter (µm)</td>
<td>280.20 ± 6.91</td>
<td>250.65 ± 5.55\textsuperscript{b}</td>
<td>267.67 ± 3.01\textsuperscript{b,c}</td>
</tr>
<tr>
<td>Germinal epithelium height (µm)</td>
<td>63.23 ± 0.84</td>
<td>48.80 ± 1.26\textsuperscript{b}</td>
<td>58.27 ± 2.33\textsuperscript{b,c}</td>
</tr>
<tr>
<td>Interstitial space width (µm)</td>
<td>22.16 ± 1.80</td>
<td>39.53 ± 2.20\textsuperscript{b}</td>
<td>30.22 ± 1.60\textsuperscript{b,c}</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation for six rats in each group. \( \textsuperscript{a}p<0.05, \text{significant difference compared to the control group}; \textsuperscript{b}p<0.001, \text{significant difference compared to the control group}; \textsuperscript{c}p<0.001, \text{significant difference compared to the methotrexate group}. \)
munopositive cells \( p < 0.001 \) (Figure 2C), whereas the tubules in the MTX+CMN group showed a significant suppression of this increase \( p < 0.001 \) (Figure 2E). No staining was observed in the negative control group (Figure 2G).

The testicular seminiferous tubules of the control group showed cytoplasmic NF-κB immunoreactivity with weak intensity in primary spermatocytes and very weak intensity in spermatids, with no immunoreactivity apparent in spermatogonia and Sertoli cells. Similar-

![Figure 1](https://www.eCERM.org)

**Figure 1.** Light microscopy of testicular tissue of rats in control (A, B), methotrexate-treated (C, D), and methotrexate+curcumin-treated (E, F) groups. (A, B) Regular seminiferous tubules (asterisks) and normal interstitial space (arrowheads). (C, D) Irregular, detached (de), sloughed (sl), and vacuolized (v) seminiferous tubules and interstitial edema (arrowheads). (E, F) Highly regular and restored testicular tissue showing only a few histologically damaged detached (de) or sloughed (sl) seminiferous tubules and mild interstitial edema (arrowheads). H&E (A, C, E: ×100; B, D, F: ×400).

![Figure 2](https://www.eCERM.org)

**Figure 2.** Phospho-p38 (p-p38) mitogen-activated protein kinase (MAPK) immunopositive cells and nuclear factor-kappa B (NF-κB) immunoreactivity in testicular tissues of control (A, B), methotrexate-treated (C, D), and methotrexate+curcumin-treated (E, F) groups. Negative controls for p-p38 MAPK and NF-κB, respectively (G, H). PS, primary spermatocyte; S, spermatid. H&E (×400).

**Table 3.** Comparison of the histologic structure of seminiferous tubules in testis tissues of rats in the control, methotrexate-treated, and methotrexate+curcumin-treated groups

<table>
<thead>
<tr>
<th>Percentage of seminiferous tubules</th>
<th>Control ( \pm SD )</th>
<th>Methotrexate ( \pm SD )</th>
<th>Methotrexate+curcumin ( \pm SD )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>95.01 ( \pm 1.04 )</td>
<td>31.08 ( \pm 6.17 )</td>
<td>67.14 ( \pm 4.83 )</td>
</tr>
<tr>
<td>Detached</td>
<td>3.03 ( \pm 0.87 )</td>
<td>27.84 ( \pm 4.13 )</td>
<td>14.26 ( \pm 1.82 )</td>
</tr>
<tr>
<td>Sloughed</td>
<td>0.80 ( \pm 0.32 )</td>
<td>25.28 ( \pm 6.24 )</td>
<td>11.17 ( \pm 1.89 )</td>
</tr>
<tr>
<td>Vacuolized</td>
<td>1.16 ( \pm 0.51 )</td>
<td>22.23 ( \pm 3.66 )</td>
<td>10.18 ( \pm 1.93 )</td>
</tr>
</tbody>
</table>

Values are presented as mean \( \pm \) standard deviation for six rats in each group.

\( ^{a}p < 0.001 \), significant difference compared to the control group; \( ^{b}p < 0.05 \), significant difference compared to the methotrexate group; \( ^{c}p < 0.001 \), significant difference compared to the methotrexate group.
ly, no immunoreactivity was observed in Leydig cells in the interstitial space (Figure 2B). Sections of the seminiferous tubules in the MTX group showed strong immunoreactivity in primary spermatocytes and moderately strong reactivity in spermatids, but only weak to moderate reactivity in spermatogonia. Immunoreactivity was weak in Sertoli cells and moderately weak in Leydig cells (Figure 2D).

The NF-κB immunoreactivity of the MTX group was statistically significantly higher than that of the control group ($p<0.001$). An examination of seminiferous tubule sections from the MTX+CMN group revealed significantly lower immunoreactivity for NF-κB than in the MTX group ($p<0.001$). Staining was still moderate in spermatocytes and was mostly weak in spermatids and spermatogonia. Weak staining was also seen in Leydig cells. No staining was encountered in Sertoli cells (Figure 2F). No staining was encountered in the negative control group (Figure 2H).

For all groups, the p-p38 MAPK index was determined by evaluating the p-p38 MAPK positive cell number (%) and NF-κB immunoreactivity was determined from the H-score in seminiferous tubules. These values are summarized in Table 4.

### Discussion

MTX, an anticancer drug commonly used in chemotherapy, is a folic acid antagonist that belongs to the group of drugs known as antimetabolites [25,48,49]. Folic acid is an important dietary factor that is converted to tetrahydrofolate, an important carbon source for the synthesis of DNA (thymidylate and purines) and RNA (purines) precursors by enzymatic reduction [50]. MTX inhibits dihydrofolate reductase (DHFR). It causes the depletion of dihydrofolate and inhibits DNA synthesis indirectly by affecting thymidine synthesis [51]. Inhibition of DHFR leads to partial depletion of tetrahydrofolate cofactors requisite for related thymidylate and purine synthesis [50].

Previous studies have shown that MTX affects spermatogenesis by causing damage to the male reproductive system [21,22,24,44,52]. Some studies have used various chemical agents to protect against the testicular damage caused by MTX [21,22,24,25,27,53]. Therefore, the present study aimed to analyze the protective effect of CMN against MTX-induced testicular damage by histological and immunohistochemical analyses of the p-p38 MAPK and NF-κB signaling pathways.

A comparison of the body weights of the animals at the beginning and the end of our study revealed a significant weight loss in the MTX group compared to the control group, but this weight loss was ameliorated in the MTX+CMN group. This observation of weight loss in the MTX group is supported by several studies [54-56]. However, a study conducted by Padmanabhan et al. [57] reported that two groups with different experimental periods (5 and 10 weeks) given 4 different weekly doses of MTX (5, 10, 20, and 40 mg/kg) did not show significant weight loss after a short treatment duration, but showed a significant reduction with a longer duration, in agreement with our study. Another study carried out in mice by Padmanabhan et al. [53] also showed a certain reduction in body weight in a group given MTX for 10 weeks at a dose of 20 mg/kg once in a week, but the decrease was not considered statistically significant.

When testicular weight (both testes) was examined, no significant difference was observed between groups. Similarly, the total TWI did not significantly differ between groups. Many other studies have also found no significant differences, in agreement with our results [23,53,54,56,57]. The lack of any difference in testicular weight could be explained by compensation for the reduction in the seminiferous tubule diameter caused by MTX through the possible formation of edema in the interstitial space in response to MTX. An evaluation of the testicular weight index (the ratio of total testis weight/total body weight) revealed no statistically significant differences among all three groups in our study. Padmanabhan et al. [53,57] also reported that MTX administration did not affect the testicular weight index, whereas El-Sheikh et al. [55] demonstrated a significant reduction in this ratio at the end of the ninth day.

In our study, the MTX group showed a significantly lower diameter of the seminiferous tubules than the control group. Other studies that used a single dose of 20 mg/kg of MTX, as in our study, also showed decreases in the diameters of the seminiferous tubules [22,24,25]. Furthermore, other studies that used low doses daily and weekly also reported decreases in the diameter of the seminiferous tubules and damage to the germinal epithelium [26,56,58].

The MTX-treated tissues in the present study also showed a de-

### Table 4. Comparison of the p-p38 MAPK index and NF-κB immunoreactivity in testis tissues of rats in the control, methotrexate-treated, and methotrexate+curcumin-treated groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Methotrexate</th>
<th>Methotrexate+curcumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-p38 MAPK index (%)</td>
<td>3.24 ± 1.11</td>
<td>12.55 ± 2.40$^d$</td>
<td>7.39 ± 1.35$^d$</td>
</tr>
<tr>
<td>NF-κB Immunoreactivity</td>
<td>86.67 ± 13.66</td>
<td>237.50 ± 18.37$^d$</td>
<td>150.00 ± 17.89$^d$</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviation for six rats in each group.
p-p38, phospho-p38; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B.
*p<0.05, significant difference compared to the control group; **p<0.001, significant difference compared to the control group; ***p<0.001, significant difference compared to the methotrexate group.
crease in germinal epithelium height. This reduction, as well as the decrease in tubule diameter, could be the result of the decreased diameter of the cells forming the germinal epithelium and/or the sloughing of immature germinal cells into the tubule lumen due to DNA damage caused by MTX. In parallel to our study, some studies have reported decreases in the height of the germinal epithelium as a result of MTX injection \[26,52,56\]. Other studies have also shown that the measured diameters of spermatocytes and spermatids found within the tubules were significantly lower than those in the control group \[58,59\]. No changes were found in a study that measured the diameters of Sertoli cells \[58\].

In the present study, the interstitial space was significantly wider in the MTX group than in the control group. A similar study carried out by Oufi and Al-Shawi. \[25\] also reported an increase in the width of the interstitial space in an MTX-treated group. Another study also found a significant increase in the width of the interstitial space in histological sections from the testes of rats given MTX \[58\]. Conversely, Nouri et al. \[56\] used two different experimental periods and found no significant change in a short period after MTX treatment, but observed a significant increase in the width of the interstitial space at the end of a long experimental period.

The changes in the histological structure of the seminiferous tubules examined in this study were classified as normal, detached, sloughed, or vacuolized. The MTX group showed significantly fewer normal seminiferous tubules than the control group, while significantly more detached, sloughed, or vacuolized seminiferous tubules were observed. The studies carried out by Padmanabhan et al. \[53,57\] support these results. In our study, histological damage in the testes caused by MTX was observed in the seminiferous tubules, as shown by disorganization of germ cell layers and sloughing of germinal cells that had not completed maturation into the tubule lumen. Degeneration and loss of germinal series cells, disorganization of germ cell layers, and vacuolization in the seminiferous tubules were also observed. Some seminiferous tubules also showed irregularities and undulations of the basal membrane. Our observations of histological damages are consistent with earlier reports \[8,22\]. No change was observed in Sertoli cells, but the interstitial space showed cell loss and edema.

In this study, no significant difference was observed in the testicular weight index among the groups. The diameter of the seminiferous tubules and the height of the epithelium were significantly larger and the width of the interstitial space was significantly smaller in the MTX + CMN group than in the MTX group. The structure of the seminiferous tubules in the MTX + CMN group showed fewer damaged tubules containing detached, sloughed, or vacuolized tubules than in the MTX group. The tubules also had a fairly normal structure compared to those of the MTX group.

In our study, the assessment of p-p38 MAPK immunopositive staining in the MTX group revealed staining in the primary spermatocytes in most seminiferous tubules. In the MTX group, particularly strong NF-κB immunoreactivity was observed in primary spermatocytes, while staining was moderately strong in spermatids and weak to moderate in spermatogonia. The Sertoli cells showed weak immunoreactivity in the MTX group, but CMN administration significantly decreased the numbers of p-p38 MAPK positive cells and NF-κB immunoreactivity. An MTX-induced increase in the NF-κB activation in testes has previously been reported \[55\], as has an increase in p38 MAPK protein levels in bronchial cell culture in response to MTX \[14\]. Increases in NF-κB and p38 MAPK signaling have also been reported in testicular tissues damaged by other agents \[43\].

In our study, MTX administration was found to damage the seminiferous tubules, as shown by abnormal histological and immunohistochemical findings. In connection with our study, a previous study using of MTX showed upregulation of NF-κB protein expression after testis injuries, while administration of melatonin (an anti-inflammatory and antioxidant agent) downregulated NF-κB protein expression \[60\]. In another study, male obesity disrupted the balance between oxidation and antioxidation in the testicular tissue, upregulated NF-κB, increased inflammation, and disrupted sperm quality, thereby negatively affecting male reproductive function \[61\]. If increased NF-κB signaling initiates the inflammatory process, lower sperm production and a decrease in the fertilization rate will be observed due to MTX-induced upregulation of NF-κB, as in our study.

Previous studies showed that MAPKs were linked with disruption of sperm production \[62,63\]. The mitochondrial apoptosis pathway induced by activated p38 MAPK resulted in a breakdown in spermatogenesis, defective sperm formation, and consequently infertility \[62,64\]. In our study, the number of p-p38 MAPK immunopositive cells increased in animals with MTX-induced testicular injuries. According to previous studies \[62-64\], increased p-p38 MAPK expression after MTX-induced testis injuries may cause a decrease in the number of sperm and the fertilization rate.

p38 MAPK is a kinase that can be activated by a variety of cellular stresses, including oxidative stress. This kinase is mainly related to inflammation and apoptosis. By contrast, NF-κB is a dimeric transcription factor composed of different members that can activate a diverse range of genes related to stress responses, inflammation, and apoptosis \[65\]. Several studies have shown that CMN decreases both p-p38 MAPK and NF-κB activation \[43,66-68\]. The results presented here also demonstrated that CMN could significantly suppress the MTX-induced increase in p-p38 MAPK and NF-κB expression in rat testis tissue.

CMN is known to possess antioxidant, anti-inflammatory, and anti-tumoral properties \[28,30,32,34\]. Several studies have shown that
CMN can reduce the side effects of MTX in non-testicular tissues [28,32,69]. CMN has also demonstrated effectiveness against testicular damage induced by other agents, as reported by several investigators [43,70].

Taken together, our results and previous findings indicate that CMN may reduce MTX-induced testicular damage by suppressing the p38 MAPK and NF-κB signaling pathways. Therefore, CMN may represent a promising candidate for the treatment of male infertility caused by MTX.

**Conflict of interest**

No potential conflict of interest relevant to this article was reported.

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Conceptualization: YHU. Data curation: LK. Formal analysis: all authors. Funding acquisition: LK. Methodology: all authors. Project administration: YHU. Visualization: all authors. Writing–original draft: LK. Writing–review & editing: all authors.

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Effects of crocin and metformin on methylglyoxal-induced reproductive system dysfunction in diabetic male mice

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Objective: This study investigated the effect of crocin in methylglyoxal (MGO)-induced diabetic male mice.

Methods: Seventy 1-month-old male NMRI mice weighing 20–25 g were divided into seven groups (n=10): sham, MGO (600 mg/kg/day), MGO+crocin (15, 30, and 60 mg/kg/day), MGO+metformin (150 mg/kg/day), and crocin (60 mg/kg/day). MGO was administered orally for 30 days. Starting on day 14, after confirming hyperglycemia, metformin and crocin were administered orally. On day 31, plasma and tissue samples were prepared for experimental assessments.

Results: Blood glucose and insulin levels in the MGO group were higher than those in the sham group (p<0.001), and decreased in response to metformin (p<0.001) and crocin treatment (not at all doses). Testis width and volume decreased in the MGO mice and improved in the crocin-treated mice (p<0.05), but not in the metformin group. Superoxide dismutase levels decreased in diabetic mice (p<0.05) and malondialdehyde levels increased (p<0.001). Crocin and metformin improved malondialdehyde and superoxide dismutase. Testosterone (p<0.001) and sperm count (p<0.05) decreased in the diabetic mice, and treatment with metformin and crocin recovered these variables. Luteinizing hormone levels increased in diabetic mice (p<0.001) and crocin treatment (but not metformin) attenuated this increase. Seminiferous diameter and height decreased in the diabetic mice and increased in the treatment groups. Vacuoles and ruptures were seen in diabetic testicular tissue, and crocin improved testicular morphology (p<0.01).

Conclusion: MGO increased oxidative stress, reduced sex hormones, and induced histological problems in male reproductive organs. Crocin and metformin improved the reproductive damage caused by MGO-induced diabetes.

Keywords: Crocin; Diabetes mellitus; Methylglyoxal; Oxidative stress; Reproduction

Introduction

Diabetes poses a serious problem throughout the world, and sexual and reproductive disorders are among the most important secondary complications in patients with diabetes [1]. As the main male reproductive organs of the male, the testes are subjected to various effects of diabetes including altered sperm quality, spermatogenesis, and testicular morphology; changes in Sertoli cell glucose metabolism; decreased Leydig cell number and function; testosterone depletion [2]; and damage of the spermatozoa cell membrane in the seminiferous tubules [3]. Ekhoye et al. [4] stated that testicular oxidative stress has been associated with physiornorphological function in rats’ reproductive systems. Other studies have reported reduced...
sperm motility and concentration, erectile dysfunction [5], and decreased fertility in men with diabetes [6]. In people with diabetes, more severe androgen deficiency has been reported than in those without diabetes [7]. Diabetes causes disorders in the precise molecular processes that are essential for sperm quality and function. Various mechanisms can explain diabetes-associated destruction of sperm and damage to the reproductive system, including endocrine disruptions and increased oxidative stress [8].

Methylglyoxal (MGO), the levels of which increase with age and hyperglycemia, is a highly reactive compound and a strong precursor to the creation of advanced glycation end products (AGEs), which are mainly derived from glucose and fructose metabolism and are detoxified by the glycemic system under physiological conditions [9]. Diabetes-induced vascular dysfunction [10] and oxidative stress-induced apoptosis in the vascular endothelium [11] have been linked to the presence of MGO. Since MGO is detoxified through two stages of enzymatic reactions by glyoxalase 1 and 2, using glutathione as a cofactor [12], glyoxalase inducers can be used to prevent and treat possible complications of diabetes [13]. MGO is a toxic byproduct of glycolysis and intracellular stress [14], and it has been noted that MGO can contribute to molecular damage in ovarian dysfunction in the female reproductive system [15].

Metformin treatment is the first line of diabetes mellitus management. Due to the high risk of lactic acidosis [16] and induction of acute kidney injury [17], metformin’s future was initially precarious, but the ability of metformin to counter insulin resistance and to reduce hyperglycemia without weight gain or an increased risk of hypoglycemia have enabled it to play a major role in diabetes treatment. Sixty years after its introduction in diabetes treatment, metformin has become the most prescribed glucose-lowering medicine worldwide [16].

Crocin, a water-soluble carotenoid, is an active ingredient of saffron. Pharmacological studies have shown that crocin has anti-inflammatory, anticoagulant, and antitumor activity. Crocin has also been proposed for use as a chemical preservative. Recent studies have shown that crocin exerts antioxidant activity through free radical scavenging [18]. Crocin has also been shown to have a protective effect on sperm parameters and seminiferous tubules in mice under oxidative stress induced by paraquat and in diabetic rats [19].

Diabetes results in increased levels of reactive oxygen species (ROS) and free radicals. ROS include hydroxyl radicals, superoxide anion, hydrogen peroxide, nitric oxide, and lipid peroxidation products such as malondialdehyde (MDA). All ROS are able to react with membrane lipids, nucleic acids, proteins, enzymes, and other small molecules and eventually lead to cellular damage. Antioxidant defense systems include free radical inhibitors including catalase, glutathione peroxidase, and superoxide dismutase (SOD). Whenever the balance between ROS production and antioxidant defense disappears as a result of oxidative stress, various pathological conditions arise that can impair diabetes-related reproductive function [20, 21]. Although it has been reported that metformin is the most commonly prescribed glucose-lowering medicine worldwide [16] and crocin is a well-known antioxidant component [18], no study has yet investigated the effects of crocin and metformin on MGO-induced diabetes. Therefore, this study was conducted to evaluate the effects of crocin and metformin on the male reproductive system in an MGO-induced diabetic mouse model.

Methods

1. Animals

The study was performed in 70 1-month-old mice (NMRI) weighing 20–25 g. One week before the study, they were transferred to the animal room and experienced a 12-hour light-dark period with free access to food and water. The mice used in this study were treated in accordance with institutional principles and guidelines on animal care (IR.AJUMS.ABHC.REC.1398.035).

2. Experimental design

The mice were randomly divided into seven groups with 10 mice in each group. The experiment lasted for 30 consecutive days. The groups included: group 1, Normal saline administered by gavage; group 2, MGO (600 mg/kg/day) administered by gavage [22]; group 3, MGO+metformin administered at a dose of 150 mg/kg/day; group 4, MGO+crocin administered at a dose of 15 mg/kg/day; group 5, MGO+crocin administered at a dose of 30 mg/kg/day; group 6, MGO+crocin administered at a dose of 60 mg/kg/day; group 7, crocin administered at an effective gavage dose based on a pilot study.

The duration of diabetes induction was 30 days, and all mice except the sham group received MGO at a dose of 600 mg/kg/day by gavage for 30 consecutive days. To test the induction of diabetes at the end of each week, the animals’ blood glucose was measured using a tail vein blood sample and a glucose level above 180 mg/dL considered as indicating diabetes [14]. On day 14, after confirming hyperglycemia, metformin and crocin were administered orally. On day 31 of the study, plasma and tissue samples were prepared for experimental assessments. After blood sampling and separation of the epididymis and testis, biochemical parameters were evaluated. Finally, anesthesia was performed 24 hours after the last dose of crocin and metformin with ketamine-xylazine, and after chest dissection, blood samples were taken from the left ventricle and centrifuged. The obtained plasma was stored at −70°C for subsequent experiments, including glucose, insulin, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone plasma levels, as well as...
oxidative stress parameters including MDA and SOD. For histological examinations, the testes were isolated and stained with Bouin’s solution and prepared for histological studies. Cauda epididymis isolation was also performed to measure the sperm count. Plasma levels of insulin, testosterone, FSH, LH, and oxidative stress-related factors including MDA and SOD were measured using an enzyme-linked immunosorbent assay (ELISA) kit. The sperm count was performed using a hemocytometer. The testes of the animals were quickly fixed in 10% formalin. After tissue processing, 5- to 7-micron paraffin sections were prepared. Sections were then stained with Bouin’s solution, and testicular tissue changes (including testicular weight, width, and length and vacuolation of epithelial cells) were evaluated. The volume of the testes was analyzed using the formula: volume = (D^2/4 × πL × K, where L = length, D = width, K = 0.9, and π = 3.14 [23]. The slides were finally examined by light microscopy.

3. Sperm assessment

The cauda epididymis of every mouse was separated and transferred into a petri dish containing normal saline (0.9%) and minced into small pieces. The spermatozoa were then vented into surrounding fluid after the pieces were squashed. A drop of petri dish solution was transferred into a Neubauer chamber (depth 0.100 mm and area 0.0025 mm^2). The sperm count was assayed manually in white blood cell chambers under light microscopy. Data were expressed as the number of sperm per milliliter [24].

4. Hormonal and antioxidant assessment

The plasma levels of insulin, FSH, LH, and testosterone levels were measured by ELISA kits (DRG Instruments, Marburg, Germany). The hormone detection sensitivity was 1.27 mIU/mL for FSH, 0.856 mIU/mL for LH, and 0.287 nmol/L for testosterone. Glucose assessment was performed using biochemical assay kits (Pars Azmoun, Tehran, Iran), with a detection limit of 0.182 μU/mL in each test tube. SOD (Randox Laboratories, Crumlin, UK) and MDA testing were performed using an ELISA assay kit (ZellBio, Lonsee, Germany). The assay detects the MDA level calorimetrically in a range of 0.78–50 μM with 0.1 μM sensitivity.

5. Histopathological assessment

After blood sampling, the left testes of each mouse separated and fixed in 10% formalin solution. After tissue processing, 5- to 7-micron paraffin sections were prepared and stained with hematoxylin and eosin. Seven microscopy slides were examined to assess the histological changes (e.g., vacuolation of epithelial cells) in each mouse in all groups. The diameters of the seminiferous tubules and the lumen diameter were measured using Motic Images Plus 3.0 software. The height of the seminiferous epithelium was calculated by subtracting the lumen diameter from the tubules’ diameter. For each animal, 100 tubules were analyzed [23].

6. Statistical assessment

Data were statistically analyzed using IBM SPSS ver. 25 (IBM Corp., Armonk, NY, USA) as mean ± standard error of mean. The Kolmogorov-Smirnov test was used to determine the normality of the distribution of the variables in the study. The variance was also tested for homogeneity. One-way analysis of variance and the post hoc least significant difference test were used to determine differences between groups. Differences were considered statistically significant at p < 0.05.

Results

1. Blood glucose, insulin, and antioxidant levels

Blood glucose levels were higher in the MGO group (p < 0.001) than in the sham group and decreased with crocin treatment (p < 0.01) and metformin (p < 0.001). MGO enhanced insulin levels (p < 0.001) in comparison with the sham group, while crocin (30 mg/kg/day, p < 0.05) and metformin (p < 0.001) significantly decreased insulin levels. A crocin dose of 60 mg/kg/day led to significantly higher insulin levels compared to metformin (p < 0.05). The MGO group had lower SOD levels (p < 0.05) than the sham group, and SOD levels increased with crocin treatment (MGO + crocin 30 mg/kg/day and crocin 60 mg/kg/day, p < 0.05) and metformin (p < 0.01). The levels of MDA in the MGO group were higher than in the sham group (p < 0.001) and decreased with crocin and metformin treatment (p < 0.001) (Table 1).

2. Animal weight and reproductive organ weight

The animal weight and the reproductive organ weight did not change significantly. Testicular width and testicular volume were significantly lower in the MGO group than in the sham group (p < 0.05), and the administration of 30 and 60 mg/kg of crocin increased these parameters (p < 0.05). Metformin treatment did not change these values (Table 2).

3. Sex hormones and sperm count

The sperm counts in the MGO and metformin groups were lower than in the sham group (p < 0.05) and increased with crocin treatment at all doses in comparison with metformin (Table 3). Testosterone levels were clearly lower in the MGO group than in the sham group (p < 0.001) and increased with crocin (p < 0.001) and metformin (p < 0.05) treatment. Crocin doses of 30 and 60 mg/kg/day led to significant increases in testosterone levels compared to metformin (Figure 1). LH levels (Figure 2) were significantly higher in the MGO group than in the sham group (p < 0.001) and decreased with
crocin treatment (15 and 30 mg/kg/day p < 0.001, and 60 mg/kg/day p < 0.01). Metformin treatment did not change this parameter. FSH levels did not show significant between-group differences (Figure 3).

4. Testicular histopathology and sperm count

The observations of testis histology were normal in the sham group. Many vacuoles and epithelial ruptures were observed in the seminiferous tubule epithelium of the MGO-treated diabetic male mice compared to the sham group (Figure 4). The number and size of these vacuoles and damage (Table 4, Figure 4) were lower in the crocin- and metformin-treated diabetic groups (especially at a dose of 30 mg/kg). Clear areas of tissue damage such as sloughing of seminiferous epithelium and atrophy were seen in the MGO group (p < 0.001), which demonstrated relative improvement in the MGO + crocin group at all doses (p < 0.001) and the MGO+metformin group (p < 0.001) (Table 4, Figure 4). It has been proven that the diameters

Table 1. Effects of MGO, MT, and crocin on blood glucose, plasma insulin, SOD, and MDA levels in normal and diabetic male mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Insulin (µU/mL)</th>
<th>Glucose (mg/dL)</th>
<th>SOD (U/mg)</th>
<th>MDA (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>4.1 ± 0.1</td>
<td>113.7 ± 15.8</td>
<td>94.96 ± 1.42</td>
<td>25.6 ± 3.3</td>
</tr>
<tr>
<td>MGO</td>
<td>7.7 ± 0.6</td>
<td>179.3 ± 15.6</td>
<td>81.2 ± 8.12</td>
<td>69.1 ± 3.4</td>
</tr>
<tr>
<td>MGO+MT</td>
<td>4.3 ± 0.4</td>
<td>127.5 ± 2.7</td>
<td>95.3 ± 1.5</td>
<td>28.6 ± 9.4</td>
</tr>
<tr>
<td>MGO+CRO15</td>
<td>6.5 ± 1.6</td>
<td>161.5 ± 15.5</td>
<td>92.6 ± 2.18</td>
<td>52.0 ± 3.0</td>
</tr>
<tr>
<td>MGO+CRO30</td>
<td>5.6 ± 0.9</td>
<td>139.0 ± 20.0</td>
<td>95.5 ± 1.2</td>
<td>38.7 ± 1.7</td>
</tr>
<tr>
<td>MGO+CRO60</td>
<td>7.2 ± 0.9</td>
<td>151.0 ± 3.0</td>
<td>92.6 ± 2.43</td>
<td>40.0 ± 0.58</td>
</tr>
<tr>
<td>CRO60</td>
<td>6.5 ± 0.8</td>
<td>138.2 ± 11.5</td>
<td>93.5 ± 0.4</td>
<td>24.6 ± 1.3</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard error of the mean.
MGO, methylglyoxal; MT, metformin; SOD, superoxide dismutase; MDA, malondialdehyde; CRO, crocin.

Table 2. Effects of MGO, MT, and crocin on animal weight and male reproductive organs

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Testis weight (mg)</th>
<th>Testis width (mm)</th>
<th>Testis length (mm)</th>
<th>Testis volume (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>27.0 ± 0.9</td>
<td>88.2 ± 6.6</td>
<td>4.8 ± 0.4</td>
<td>7.5 ± 0.2</td>
<td>129.7 ± 27.7</td>
</tr>
<tr>
<td>MGO</td>
<td>25.0 ± 0.7</td>
<td>85.75 ± 2.7</td>
<td>4.0 ± 0.0</td>
<td>7.03 ± 0.0</td>
<td>79.41 ± 3.3</td>
</tr>
<tr>
<td>MGO+MT</td>
<td>28.0 ± 1.1</td>
<td>91.8 ± 17.3</td>
<td>4.2 ± 0.7</td>
<td>7.16 ± 0.4</td>
<td>90.07 ± 13.2</td>
</tr>
<tr>
<td>MGO+CRO15</td>
<td>23.8 ± 0.7</td>
<td>89.3 ± 2.0</td>
<td>4.6 ± 0.2</td>
<td>7.0 ± 0.3</td>
<td>105.8 ± 10.9</td>
</tr>
<tr>
<td>MGO+CRO30</td>
<td>26.2 ± 1.8</td>
<td>85.6 ± 8.5</td>
<td>5.0 ± 0.3</td>
<td>7.0 ± 0.1</td>
<td>126.9 ± 17.8</td>
</tr>
<tr>
<td>MGO+CRO60</td>
<td>25.4 ± 2.7</td>
<td>96.4 ± 10.0</td>
<td>5.0 ± 0.0</td>
<td>7.5 ± 0.3</td>
<td>132.5 ± 5.1</td>
</tr>
<tr>
<td>CRO60</td>
<td>26.5 ± 3.6</td>
<td>98.7 ± 7.9</td>
<td>5.3 ± 0.3</td>
<td>7.4 ± 0.3</td>
<td>131.8 ± 5.1</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard error of the mean (n=10).
MGO, methylglyoxal; MT, metformin; CRO, crocin.

Table 3. Effects of MGO, MT, and crocin on seminiferous morphology and sperm count in normal and diabetic male mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Seminiferous diameter (µm)</th>
<th>Seminiferous height (µm)</th>
<th>Sperm count (×10⁶)/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>272.2 ± 23.3</td>
<td>159.6 ± 18.9</td>
<td>3.2 ± 0.7</td>
</tr>
<tr>
<td>MGO</td>
<td>211.1 ± 25.1</td>
<td>128.1 ± 12.6</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>MGO+MT</td>
<td>271.8 ± 24.6</td>
<td>159.1 ± 16.4</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>MGO+CRO15</td>
<td>253.2 ± 15.4</td>
<td>135.3 ± 12.8</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>MGO+CRO30</td>
<td>264.1 ± 20.2</td>
<td>146.1 ± 16.6</td>
<td>4.4 ± 0.8</td>
</tr>
<tr>
<td>MGO+CRO60</td>
<td>223.2 ± 11.7</td>
<td>129.3 ± 14.2</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>CRO60</td>
<td>273.4 ± 21.8</td>
<td>158.5 ± 22.5</td>
<td>6.5 ± 0.9</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard error of the mean.
MGO, methylglyoxal; MT, metformin; CRO, crocin.

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of seminiferous tubules and the epithelium height decreased in diabetic mice compared to the sham group (\(p < 0.01\) and \(p < 0.05\), respectively), and this damage improved in the crocin- and metformin-treated groups (Table 3). Sperm count decreased in the MGO-treated group (\(p < 0.05\)), while the administration of crocin improved the sperm count in diabetic and non-diabetic groups (15, 30, and 60 mg/kg and alone 60 mg/kg crocin) in comparison with metformin (\(p < 0.05\)).

**Discussion**

In the present study, the induction of diabetes by MGO was confirmed by measurements of plasma insulin and blood glucose levels. In agreement with this finding, Truong et al. [25] demonstrated that MGO destroyed pancreatic \(\beta\)-cells by accumulation of \(\text{AGEs}\) in the plasma insulin and blood glucose levels.
tissue and inflicted insulin resistance. Diabetes induced by MGO changed testis weight, volume, and morphology. High blood glucose levels might cause abnormalities in homeostatic regulation due to changes in glucose consumption by cells. The anterior pituitary gland may be affected by hyperglycemia, resulting in dysfunction of the pituitary-gonadal axis. This phenomenon can destroy germ cells and decrease the size of seminiferous tubules and seminal vesicles [26]. Abnormal glucose homeostasis and hyperglycemia have harmful outcomes on testicular function and spermatogenesis in males with diabetes [7]. Indeed, the findings of the present study revealed that MGO-induced diabetes resulted in male reproductive dysfunction by reducing plasma testosterone levels and sperm count. Furthermore, levels of the antioxidant (SOD) decreased, while those of MDA increased. Diabetic insulin intolerance and hyperglycemia have been associated with dysregulation of the testis, resulting in imbalances of sex steroid hormone levels, such as reduced testosterone secretion by Leydig cells and increased plasma levels of LH. Testosterone secretion from Leydig cells is necessary for spermatogenesis and fertility. This hormone, through its androgen receptors, acts directly on target cells. As a result of stress induced at the testicular level, insulin resistance in diabetes is associated with impairments of Leydig cell testosterone secretion and its receptors [27]. In diabetes mellitus, the whole-body metabolism is affected, including endocrine disruption, testicular energy consumption, and male reproductive function. Sertoli cells are protective cells that provide a nutritional supporting environment for somatic and germ cells in the testes, which could be affected by diabetes mellitus. The relationship between male infertility and diabetes has been discussed in some studies based on diabetic models. It has been reported that hyperglycemia leads to increased production of oxidant components like ROS and disruption of antioxidant defenses. Because of excessive ROS generation through hyperglycemia, oxidation of proteins and lipids occurs in the cell, which experiences damage to macromolecules such as protein, lipids, and DNA [28]. In addition, the membranes of spermatozoa are rich in polyunsaturated fatty acids, and this fact makes it susceptible to oxidative stress or damage caused by free radicals [28,29]. Butchi Akondi et al. [28] reported significant reductions in sperm parameters, such as sperm count and motility, in diabetic mice through an increase in oxidative stress and ROS generation. The findings of the present study indicate that diabetes causes oxidative stress, which might be a major mechanism of sperm count reduction; however, further studies are required to determine the exact mechanism.

The present study found that crocin and metformin treatment improved reproductive parameters in diabetic mice. In agreement with these findings, Sefidgar et al. [18] showed in pharmacological studies that crocin had anti-inflammatory, anticoagulant, and anti-tumor activity. Crocin, which is mainly extracted from the saffron plant, has
also been proposed as a chemical preservative. It has been shown that crocin exerts free radical scavenging activity and therefore has antioxidant activity. Roshankhah et al. [3] also demonstrated that crocin had a protective effect on sperm parameters and seminiferous tubule morphology. Of course, many studies have been investigated to obtain an accurate dose of crocin with the desired antidiabetic effects. Nikbakht-Jam et al. [30] suggested that crocin could behave as both an antioxidant and pro-oxidant, and affects pro-oxidant/anti-oxidant status depending on concentration. The results of this study showed the best effects of crocin at a dose of 30 mg/kg. Interestingly, metformin led to reduced sperm count and testicular volume; the mechanism of action of these side effects of metformin require further study.

In accordance with previous research, the present results indicate that MGO administration changed testis morphology. According to experimental observations, vacuolation in testis tissue is a major symptom of Sertoli cell injury. Consistent with these results, Sertoli cell injury can lead to degeneration of spermatocytes and reduction in sperm count and reproductive factors [31]. Moreover, the role of Sertoli cells is to provide an essential environment for spermatogenesis and germ cell survival. Diabetes mellitus increases the number and size of vacuoles in Sertoli cell cytoplasm in the testes, and decreases seminiferous tubule diameter and epithelium height. All of these changes can affect spermatogenesis [32]. In addition, the present histopathological results showed seminiferous tubule disruption, decreased sperm count, and Sertoli cell dysfunction in the diabetic mice. Any agent resulting in decreased testicular testosterone production or secretion causes narrowing of the seminiferous tubular diameter, luminal epithelium atrophy, or Sertoli cell vacuolization [33]. Based on the results of this study, treatment with crocin and metformin improved diabetes-related and reproductive parameters. However, interestingly, metformin did not improve MGO-induced damage to testicular volume, testosterone secretion, and sperm count as much as crocin. A limitation of this study is the lack of an examination of other sperm parameters such as sperm motility, morphology, and apoptotic changes, and it is suggested that future research should address this gap.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

Author contributions

Conceptualization: AA. Data curation: AA, MKK. Formal analysis: AA, MKK. Fund acquisition: all authors. Methodology: all authors. Project administration: AA, MKK. Visualization: AA, MKK. Writing—original draft: AA, MKK. Writing—review & editing: all authors.

References


Effects of cholesterol and Lactobacillus acidophilus on testicular function

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Objective: In this study, the effects of Lactobacillus acidophilus on testosterone (TES), follicle-stimulating hormone (FSH), luteinizing hormone (LH), androgen-binding protein (ABP), factor-associated apoptosis (FAS), and total cholesterol (TC), as well as histopathological changes, were investigated in male rats fed a high-cholesterol diet.

Methods: The study included three groups. The control (C) group was fed standard-diet for 8 weeks. The hypercholesterolemia (HC) group was fed a 2% cholesterol-diet for 8 weeks. The therapeutic group (HCL) was fed a 2% cholesterol-diet for 8 weeks and administered L. acidophilus for the last 4 weeks. FSH, TES, and FAS levels in testicular tissue were determined using an enzyme-linked immunosorbent assay (ELISA), while another sample was examined histopathologically. LH and ABP levels were determined using ELISA, and serum TC levels were assessed via an autoanalyzer.

Results: In the HC group, the TC levels were significantly higher and the LH levels were lower (p<0.05) than in the C group. The ABP levels were lower (p>0.05). In the HCL group, the LH and ABP levels were higher (p>0.05) and the TC level significantly lower (p<0.05) than in the HC group. The TES and FSH levels were lower, and the FAS levels were higher, in the HC than in the C group (p<0.05). In the HCL group, levels of all three resembled control levels. Histologically, in the testicular tissue of the HC group, the cells in the tubular wall exhibited atrophy, vacuolization, and reduced wall structure integrity. However, in the HCL group, these deteriorations were largely reversed.

Conclusion: Supplementary dietary administration of an L. acidophilus to hypercholesterolemic male rats positively impacted testicular tissue and male fertility hormone levels.

Keywords: Hypercholesterolemia; Lactobacillus acidophilus; Testicular function

Introduction

Cholesterol is a sterol biosynthesized in all animal cells due to its structural role in animal cell membranes [1]. In addition, cholesterol is a precursor to the biosynthesis of steroid hormones, bile acids, and vitamin D [2]. Most cholesterol synthesis takes place in the liver, although some occurs in the kidneys, intestines, and adrenal glands [3]. Hypercholesterolemia is associated with male reproductive dysfunction. For example, a high-cholesterol diet in male rats is associated with decreases in sperm quality and in the nuclear dimensions of Leydig cells, both of which lead to infertility [4,5].

Probiotics are live microbial food additives that have beneficial effects on host health by regulating the microbial balance of the intestinal tract. The most widely used probiotic microorganisms are Bifidobacterium and Lactobacillus species. Lactic acid bacteria cultures, especially dairy products containing Lactobacillus acidophilus, have been found to lower serum cholesterol and increase the quantity of fecal Lactobacilli [6].

Differing opinions exist regarding the mechanism of the serum cholesterol-lowering effects of probiotic bacteria. The most common belief is that probiotics are rapidly eliminated from the intestinal tract by breaking down bile salts into free acids. The synthesis of bile acids from cholesterol decreases the overall concentration of cholesterol in the body, as free bile salts are excreted from the body [7].

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Globally, hypercholesterolemia is a very common health issue. In this study, we investigated the addition of the probiotic *L. acidophilus* to male rats fed a high-cholesterol diet. We aimed to investigate the histopathological impact on testicular tissue along with the effects on testosterone (TES), follicle-stimulating hormone (FSH), luteinizing hormone (LH), androgen-binding protein (ABP), and apoptosis.

**Methods**

1. **Animal materials**
   Twenty-four male Sprague-Dawley rats 10–12 weeks of age and weighing 300–350 g were used in this study. The rats were obtained from Ondokuz Mayis University Experimental Animals Application and Research Center. The present study was conducted with the permission of the Ondokuz Mayis University Animal Experiments Local Ethics Committee (2016/27). During the study, an environment with a room temperature of 22°C ± 2°C, 60% humidity, and a 12/12 hour light/dark cycle was provided. The experimental animals were fed ad libitum throughout the study.

2. **Preparation of probiotic suspensions**
   Lyophilized *L. acidophilus* ATCC 4356 was supplied for use as a probiotic medication. The bacteria were inoculated with de Man, Rogosa, and Sharpe (MRS) agar for viability, and the purity was confirmed by diluting the lyophilized bacteria with MRS broth. One milliliter of the culture was suspended in the main culture, and a probiotic suspension was inoculated to MRS agar plates and incubated at 35°C for 48 hours. After incubation, the bacterial colonies were counted, and the concentration of bacteria (colony-forming units [CFU]/mL) in the main culture was calculated. Then, 10^8 CFU/mL bacteria were suspended in the main culture, and a probiotic suspension of *L. acidophilus* was prepared for use as treatment [8].

3. **Animal experiment**
   For the animal experiment, three groups of eight rats were established. (1) Group 1 (n = 8, control group [C]): the animals of the control group were fed standard rat food ad libitum for 8 weeks. (2) Group 2 (n = 8, 2% cholesterol [HC]): this group was fed standard rat food with 2% cholesterol ad libitum for 8 weeks [9,10]. (3) Group 3 (2% cholesterol + *L. acidophilus* [HCL]): this group was fed standard rat food with 2% cholesterol ad libitum for 8 weeks [9,10]. For the last 4 weeks of the experiment, *L. acidophilus* probiotic (2 × 10^8 CFU/mL/day) was administered via oral gavage [8].
   At the end of the study, the rats were individually weighed, and 10% ketamine (Ketasol; Richter Pharma Ag, Wels, Austria; 0.8–1.3 mL/kg) and 2% xylazine (Bavet, Istanbul, Turkey; 2–5 mg/kg) were applied via intraperitoneal injection. Each rat was decapitated after the blood was drained from the heart, and testicular tissue samples were taken after necropsy.

4. **Preparation of testicular tissue samples for enzyme-linked immunosorbent assay**
   The testicular tissue samples were weighed after washing with ice-cold phosphate-buffered saline (0.01 mol/L, pH 7.0–7.2) before homogenization. After the tissue was divided into small pieces, homogenization was performed in 5–10 mL phosphate buffer solution with an ice homogenizer. The cell membrane was subsequently broken down twice with ice ultrasonics, and ice cream thawing was applied twice for better cell disintegration. The homogenates were centrifuged at 5,000 × g (4°C) for 5 minutes. They were then divided into supernatant aliquots and stored at −80°C until the analyses were performed.

5. **Determination of testicular TES, FSH, and factor-associated apoptosis levels using enzyme-linked immunosorbent assay**
   Specific enzyme-linked immunosorbent assay (ELISA) kits were used to assess the levels of TES, FSH, and factor-associated apoptosis (FAS) in the testicular tissue supernatants of each rat. The ELISA procedures were performed according to the manufacturer’s instructions, and the absorbance levels (as optical density values) of the ELISA plates were measured with an ELISA reader.

6. **Determination of serum biochemical parameters**
   The LH and ABP levels of each rat were determined using specific ELISA kits. The ELISA procedures were performed according to the manufacturer’s instructions, and the absorbance levels (as optical density values) of the ELISA plates were measured with an ELISA reader. Serum TAC levels were measured spectrophotometrically with an autoanalyzer. For this analysis, appropriate quantities of the samples and reagents were mixed with standard solutions. The results of the analyses were calculated using optical reading at a specific time and temperature.

7. **Histopathological examination of testicular tissues**
   For the histopathological examination, the testicles of the rats were removed and cleaned from the surrounding tissues. The testicles were fixed in a 10% formaldehyde solution for histological examination. The tissues were subjected to routine histopathological follow-up procedures after the detection process and blocked with paraffin. From the resulting paraffin blocks, 5-μm sections were taken using a Leica RM2235 microtome (Leica Biosystems, Wetzlar, Germany). To analyze the complex histological structure of the testicles, the Crossmon triple staining technique was applied to the sections.
stained preparations were examined and photographed in detail with a Nikon Eclipse 50i research microscope (Nikon, Tokyo, Japan).

8. Statistical analysis

SPSS ver. 16.0 (SPSS Inc., Chicago, USA, USA) was used for statistical analysis. One-way analysis of variance, Duncan multiple range, and Pearson correlation tests were used to evaluate the differences and relationships among the groups.

Results

1. Determination of TES, FSH, and FAS levels using ELISA

The levels of FSH, FSH, and FAS in the testicular tissue supernatants are presented in Table 1 as mean ± standard error (SE). The average TES levels in the C, HC, and HCL groups were 172.34 ± 3.86, 121.75 ± 8.82, and 170.61 ± 6.59 (ng/mg tissue), respectively. The mean TES level in the testicular tissue was significantly lower in the HC group than in the C group, while the level was higher in the HCL group than in the HC group.

The FSH levels of the C, HC, and HCL groups were 129.41 ± 1.43, 115.47 ± 4.67, and 125.08 ± 1.28 IU/mg tissue, respectively. In the HC group, the mean FSH level was dramatically lower than in the C group; the level in the HCL group was higher than in the HC group and approached that of the C group. In the C, HC, and HCL groups, the FAS levels in the testicular tissues were 1.32 ± 0.06, 1.87 ± 0.08, and 1.51 ± 0.03 ng/mg tissue, respectively. The mean FAS level was significantly greater in the HC group than in the C and HCL groups.

2. Serum biochemical parameter levels in serum

The means and standard deviations of the serum LH, ABP, and total cholesterol (TC) levels of the C, HC, and HCL groups are presented in Table 2 as mean ± SE. The LH levels of the C, HC, and HCL groups were 12.15 ± 0.41, 7.77 ± 0.15, and 7.85 ± 0.43 (mIU/mL), respectively. The LH levels were lower in the HC and HCL groups than in the C group (p > 0.05). The ABP levels of the C, HC, and HCL groups were 46.74 ± 0.76, 43.91 ± 1.72, and 47.12 ± 0.56 (nmol/L), respectively. Relative to the control, the ABP level was slightly higher in the HCL group and lower in the HC group (p > 0.05). The TC levels of the C, HC, and HCL groups were 52.25 ± 1.75, 75.75 ± 1.98, and 60.95 ± 0.84 mg/dL, respectively. The TC levels were greater in the HCL and HC groups than in the C group.

3. Correlations

Correlations between groups with respect to TES, ABP, LH, TC, FSH, and FAS levels are presented in Table 3 as mean ± SE. The results included a significant negative correlation between TC and LH levels (r = −0.711, p < 0.01), a negative correlation between ABP and TC levels (r = −0.282), a significant positive correlation between FSH and TES levels (r = 0.535, p < 0.01), and a significant negative correlation between FSH and TC levels (r = −0.656, p < 0.01). A significant negative correlation (r = −0.723, p < 0.01) was found between TES and TC levels. FAS level was significantly negatively correlated with LH, FSH, and TES levels (r = −0.548, p < 0.01; r = −0.698, p < 0.01; r = 0.859, p < 0.01), while it was significantly positively correlated with TC level (r = 0.821, p < 0.01).

4. Results of the histopathological examination

The testicular tissues were found to be surrounded by tunica albuginea, an externally irregular tight connective tissue. Seminiferous tubules of different lengths and diameters were observed in these structures, where the tunica albuginea divides the organ into sep-

Table 1. The levels of FSH, TES, and FAS in the testicular tissue supernatants

<table>
<thead>
<tr>
<th>Variable</th>
<th>C (IU/mg tissue)</th>
<th>HC (IU/mg tissue)</th>
<th>HCL (IU/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH</td>
<td>129.41 ± 1.43</td>
<td>115.47 ± 4.67</td>
<td>125.08 ± 1.28</td>
</tr>
<tr>
<td>TES</td>
<td>172.34 ± 3.86</td>
<td>121.75 ± 8.82</td>
<td>170.61 ± 6.59</td>
</tr>
<tr>
<td>FAS</td>
<td>1.32 ± 0.06</td>
<td>1.87 ± 0.08</td>
<td>1.51 ± 0.03</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard error.

Table 2. The levels of LH, ABP, and TC in the serum

<table>
<thead>
<tr>
<th>Variable</th>
<th>C (mIU/mL)</th>
<th>HC (mIU/mL)</th>
<th>HCL (mIU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH</td>
<td>12.15 ± 0.41</td>
<td>7.77 ± 0.15</td>
<td>7.85 ± 0.43</td>
</tr>
<tr>
<td>ABP</td>
<td>46.74 ± 0.76</td>
<td>43.91 ± 1.72</td>
<td>47.12 ± 0.56</td>
</tr>
<tr>
<td>TC</td>
<td>52.25 ± 1.75</td>
<td>75.75 ± 1.98</td>
<td>60.95 ± 0.84</td>
</tr>
</tbody>
</table>

Table 3. Correlation relationships among the groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>L (mIU/mL)</th>
<th>ABP (nmol/L)</th>
<th>FSH IU/mg tissue</th>
<th>TES ng/mg tissue</th>
<th>TC mg/dL</th>
<th>FAS ng/mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH</td>
<td>1</td>
<td>0.129</td>
<td>0.365</td>
<td>0.343</td>
<td>−0.711</td>
<td>−0.548</td>
</tr>
<tr>
<td>ABP</td>
<td>1</td>
<td>0.232</td>
<td>0.472</td>
<td>−0.282</td>
<td>−0.356</td>
<td></td>
</tr>
<tr>
<td>FSH</td>
<td>1</td>
<td>0.535</td>
<td>0.656</td>
<td>−0.698</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TES</td>
<td>1</td>
<td>−0.723</td>
<td>−0.859</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>1</td>
<td>0.82</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAS</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LH, luteinizing hormone; ABP, androgen-binding protein; TC, total cholesterol; C, control group; HC, hypercholesterolemia group; HCL, hypercholesterolemia + probiotic group.

G Çiftci et al. Ratlar testicular function
tum by sending arms inside the organ, termed the septula testis. Spermatocytes and Sertoli cells at different developmental stages were observed in the seminiferous tubules. In the loose connective tissue between the tubules, small blood vessels and Leydig cells were detected. The tubule walls of the groups were evaluated and compared. In the testicles of the HC group, atrophy, vacuolization, and decreased integrity of the wall structure were observed in the cells in the tubule wall. A significant difference was found relative to the C group. However, in the samples from the HCL group, degenerate tubule wall cells were almost nonexistent, and the wall structure was preserved (Figure 1).

Discussion

The effects of probiotics on living beings and their health benefits have been emphasized in several studies. This includes their effect on hypercholesterolemia, particularly their role in suppressing advanced cholesterol synthesis and preventing the absorption of excess cholesterol [11]. Cholesterol is essential for the body because it plays a very important role in daily life. It is a crucial component of the cell membrane, as well as bile acid synthesis, fat and fat-soluble vitamin absorption, and synthesis of steroid hormones (sex hormones, mineralocorticoids, and glucocorticoids) [12]. A high-cholesterol diet is the primary cause of hyperlipidemia, atherosclerosis, and other lipid metabolism disorders that lead to male reproductive system defects [13]. In hypercholesterolemia, erythrocytes and endothelial cells, as well as serum, increase the amount of cholesterol present. The increase in the quantity of oxidized free radical products in these cells has been reported to cause elevated cholesterol [14]. Hypercholesterolemia is currently a widespread and common health issue. Several studies have shown that beneficial bacterial additives can lower serum cholesterol levels in fermented milk products or milk products containing lactic acid bacteria [10]. The therapeutic value of fermented dairy products depends on the survival of these bacteria during development and storage [15]. In the present study, a rat model of hypercholesterolemia was developed, and L. acidophilus ATCC 4356—the lipid-lowering properties of which have been demonstrated in vitro and in vivo—was administered intragastrically [10,16]. The results are consistent with previous reports suggesting that L. acidophilus has a cholesterol-lowering effect [17-19]. Walker and Gilliland [20] posited that L. acidophilus secretes bile salt hydrolase to deconjugate bile salts, although no connection was found between the reduction of in vitro cholesterol and the degree of bile salt deconjugation [21]. These conflicting findings raise the possibility of other mechanisms that may be associated with the assimilation of cholesterol by probiotic bacteria during their development [22]. In the present study, the TC level was elevated in the HC and HCL groups (p < 0.05) relative to the control. Our analysis indicates that L. acidophilus plays a major role in cholesterol synthesis. The administration of this probiotic may result in increased catabolism of cholesterol in the liver, contributing to a hypocholesterolemic impact. Several clinical and epidemiological studies have shown an inverse association between serum TES levels and TC [23]. In addition, animal experiments in TES-deficient male mice have also shown dramatically elevated serum cholesterol levels [24,25]. Circulating TES levels are determined by the steroidogenic potential of Leydig cells and the to-

Figure 1. Rat testis tissue belonging to the control group (A), the hypercholesterolemia group (B), and the probiotic and hypercholesterolemia group (C). Arrow, spermatogenetic serial cells separated from each other in seminiferous tubules; asterisk, seminiferous lumen (H&E; triple, ×20).
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The number of Leydig cells per testicle. Stress-induced increases in serum glucocorticoid concentration have been reported to decrease the rate of TES release by inhibiting the activity of TES biosynthetic enzymes [26]. In another investigation of the impact of probiotics, larger testes and higher serum TES levels were observed in male mice that regularly ingested lactic acid bacteria than in age-matched control mice. Lactobacillus reuteri was administered as a probiotic in that study, and a thorough analysis with microscopy-assisted histomorphometry showed an increase in the extent of spermatogenesis and the number of Leydig cells per testis. Furthermore, gonadal aging symptoms were claimed to decrease [27]. Mega Acidophilus (a mixture of L. acidophilus, Bifidobacterium bifidum, and Lactobacillus helveticus) was administered to New Zealand rabbits for 1 month to determine how the probiotic would affect certain parameters. In that study, probiotic supplements were shown to improve antioxidant activity and substantially increase the level of TES. In our study, the TES level in the group given 2% cholesterol for 8 weeks decreased in the testicular tissue, and the level of TES increased in the group given the L. acidophilus probiotic. Therefore, TES deficiency in the liver in HC does not affect de novo cholesterol synthesis. A number of human studies have shown that hypercholesterolemia and low semen quality are associated with male infertility [29-31]. Research on animals fed a high-cholesterol diet has uncovered more information about this link. In animals with diet-induced hypercholesterolemia, adverse effects of hypercholesterolemia on testicular function, including spermatogenesis, were noted. Increased oxidative stress is of considerable research interest among the many pathways suggested for hypercholesterolemia-induced testicular injury [16]. When mice were fed a high-fat diet, decreased TES, decreased semen quality, seminiferous tubule atrophy, and degeneration were reported. The researchers stated that the addition of selenium-enriched probiotics to the high-fat diet decreased damage to testicular tissue and raised serum TES levels [36]. In another study, researchers examined the reproductive system structure of male rats fed a high-fat diet for 2 months. They found that sperm quality and count decreased, Leydig cell disturbances increased, spermatocyte and spermatid structures decreased, and significant narrowing of the seminiferous tubule occurred [37]. In our sample, relative to the control group, the testicles of the group given cholesterol feed (HC) showed atrophy, vacuolization, and damaged wall structure integrity in the tubule wall cells. However, in the HCL group, degenerate tubule wall cells were almost nonexistent, and the wall structure retained its integrity. According to recent research, high cholesterol levels caused by high-fat diets contribute to urological disorders (problems with penile erection, irregular spermatogenesis, benign enlargement of the prostate, cancer, etc.). They also disrupt the epithelial structure of the tissues and cause functional disability [11]. The secretion of FSH and TES is required for the successful completion of spermatogenesis. TES, the male sex hormone, is secreted by Leydig cells under LH stimulation and plays important roles in the differentiation of peripheral tissues and the promotion of spermatogenesis [38,39]. ABP binds to TES and estrogens and assists their transport into the seminiferous tubule, allowing for their use for spermatozoa maturation when required [40]. Testicular ABP synthesis has been reported to be increased by FSH and TES in rats [41]. In another study, researchers investigated the impact of Lactobacillus rhamnosus PB01 on sperm kinematic parameters and found that TES, LH, and FSH levels, as well as sperm motility rates, were all significantly elevated. L. rhamnosus has also been found to act as a positive regulatory agent on weight loss and reproductive hormones [42]. In our study, serum ABP and LH levels were lower in the HC than in the control group, and the amount of FSH in the testicular tissue was also lower. ABP and LH levels were greater in the HCL group than in the HC group (p > 0.05). In parallel to our results, when a 2% cholesterol diet was given to male Wistar rats for 21 days, non-significant changes were seen in TES, LH, and prolactin levels, while the FSH level was significantly decreased [43]. In another study, a high-cholesterol diet induced a significant increase in the TC level and significant decreases in the FSH, LH, and TES levels in the serum of male rats [44]. Hypercholesterolemia has also been suggested to cause reproductive and testicular damage through excessive free radical generation and increased oxidative stress, which is cytotoxic to spermatozoa [13,34,45]. In hypercholesterolemia, the administration of antioxidants and lipid-lowering agents has been shown to protect the testis and reproductive functions [13,34,46]. In the present study, the FAS level was significantly greater (p < 0.05) in the HC group than in the control group, while the FAS levels did not differ significantly between the HCL group and the control group (p > 0.05). The FAS level was significantly negatively correlated with LH, FSH, and TES levels, while it was significantly positively correlated with TC level.

This study revealed that probiotic treatment is beneficial for reducing the cell degeneration in testicular tissue caused by high cholesterol. Probiotic treatment increased LH, FSH, TSH, and ABP levels, while partially reversing the increase in factor-related apoptosis associated with a high-cholesterol diet. Probiotics have an important role as a supplementary treatment for degeneration and as an adjunct in the treatment of cell structure disorders, although they are not therapeutic in isolation.

Conflict of interest

No potential conflict of interest relevant to this article was reported.
Author contributions

Conceptualization: all authors. Data curation: all authors. Formal analysis: all authors. Methodology: all authors. Project administration: all authors. Visualization: all authors. Writing—original draft: GC. Writing—review & editing: GC.

References


The effect of genistein on insulin resistance, inflammatory factors, lipid profile, and histopathologic indices in rats with polycystic ovary syndrome

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Objective: Polycystic ovary syndrome (PCOS) is characterized by hyperandrogenism, irregular menstruation, ovulatory dysfunction, and insulin resistance. Recent studies have reported the possible role of phytoestrogens in PCOS. This animal study aimed to evaluate the effects of genistein on insulin resistance, inflammatory factors, lipid profile, and histopathologic indices on PCOS.

Methods: PCOS was induced by 1 mg/kg of letrozole in adult Sprague-Dawley rats. The rats then received normal saline (PCOS group), 150 mg/kg of metformin, or 20 mg/kg of genistein dissolved in 1% methylcellulose solution for 42 days. Body weight, the glycemic and lipid profile, and inflammatory, antioxidative, and histopathological parameters were assessed at the end of the intervention.

Results: Treatment with genistein significantly alleviated the increased level of fasting blood insulin (p=0.16) and the homeostatic model assessment of insulin resistance (p=0.012). In addition, the genistein group had significantly lower levels of serum malondialdehyde (p=0.039) and tumor necrosis factor-alpha (p=0.003), and higher superoxide dismutase enzyme activity (p<0.001). Furthermore, the histopathological analysis indicated that genistein administration led to an increase in luteinization and the development of fewer cysts (p<0.05).

Conclusion: Biochemical and histopathological analyses indicated that genistein administration to rats with PCOS induced significant remission in oxidative, inflammatory, and glycemic and histopathologic parameters.

Keywords: Genistein; Inflammation; Insulin resistance; Metformin; Phytoestrogen; Polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) is one of the most prevalent gynecological diseases in the world. It has been estimated that 5.6%–6.1% of fertile women around the world are affected by PCOS [1]. Women with PCOS suffer from hyperandrogenism, irregular menstruation, hirsutism, and insulin resistance (IR). IR in these patients is partly due to the mild systemic inflammation that frequently occurs in this syndrome [2]. An increase in proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), can lead to impaired insulin
PCOS is reducing IR, which can worsen hyperandrogenism and induce further metabolic abnormalities [4-6].

Several medication strategies have been proposed to reduce IR, for which metformin is one of the most popular recommendations. Metformin enhances glucose homeostasis by inhibiting glucose secretion from hepatocytes and increasing peripheral glucose uptake [7]. Despite the effectiveness of metformin in lowering IR, its side effects such as gastrointestinal distress and interactions with other drugs have raised concerns regarding long-term usage [8]. Some environmental factors (e.g., genetics and nutrition) are also considered as risk factors for PCOS [9-11].

Recent studies have focused on using natural ingredients to reduce the symptoms of PCOS. Active substances, such as isoflavones, which are naturally present in some plants and especially soybean, have been considered as potential alternatives. Genistein, daidzein, and glycitein are the most prominent isoflavones that have been recently studied. Most biological activities of isoflavones are related to their antioxidant and anti-inflammatory properties [12-15]. At the same time, they are known as phytoestrogens, meaning that these compounds have structural similarities with estrogen. Moreover, some beneficial effects may be associated with improvements in insulin sensitivity and amelioration of the lipid profile [16-18].

Studies of animal models have identified favorable results of pure genistein, which could improve the lipid profile and IR [19,20]. Moreover, these changes were accompanied by the promotion of oxidative status and secretion of proinflammatory cytokines [19,21,22]. Romuald et al. [23] found that phytoestrogens supplementation significantly reduced total cholesterol levels and low-density lipoprotein (LDL) in rats. Zhang and Chi [24] investigated the associations of genistein activity with follicle-stimulating hormone receptor and luteinizing hormone receptor expression in rats with PCOS. They found that genistein could improve ovarian function, with increased luteinizing hormone receptor protein expression and decreased follicle-stimulating hormone receptor protein expression.

Genistein supplementation may attenuate the metabolic symptoms of PCOS through its anti-inflammatory and anti-diabetic activities. Therefore, this study aimed to assess the effect of genistein supplementation in comparison to metformin therapy on glycemic indices; the inflammatory, oxidative, and lipid profile; and histopathologic factors in PCOS-induced rats.

### Methods

The study was conducted in accordance with the recommendations of the European Council Directive (86/609/EEC) on November 24, 1986, regarding the protection of animals used for experimental purposes (http://data.europa.eu/eli/dir/1986/609/oj). All procedures were approved by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (IR.SUMS.REC.1395.S932). Consent for publication was not applicable. Letrozole (Aburaihan Pharmaceutical, Tehran, Iran) was used to induce PCOS (1 mg/kg dissolved in normal saline).

#### 1. Experimental design

In this study, 40 adult (10–12 weeks) female Sprague-Dawley rats were obtained from Shiraz University of Medical Sciences and were housed in a controlled environment (temperature, 22° ± 2°C; relative humidity, 55± 3%; 12-hour light-dark cycle). All the rats were fed with a normal diet ad libitum. The selected rats had two or three regular estrous cycles during the 2 weeks of vaginal smear observation, and they were in the estrous phase of their reproductive cycle. PCOS was induced using 1 mg/kg of letrozole (Aburaihan Pharmaceutical) dissolved in normal saline. The controls and PCOS-induced rats were evaluated using vaginal smears to determine the irregularity of the estrous cycle and the appearance of persistent vaginal cornification as a symptom of follicular cysts in the ovary.

The rats were initially kept in steel cages for 1 week to acclimate to the new environment. Then, they were randomly divided into four groups (10 rats in each group) as follows: the control group comprised healthy rats, the PCOS group included PCOS-induced rats that received normal saline by gavage, the metformin group comprised PCOS-induced rats treated with 150 mg/kg of metformin (Shafapharma, Tehran, Iran) dissolved in normal saline by gavage [25], and the genistein group included PCOS-induced rats supplemented with 20 mg/kg of genistein (DSM Co., Heerlen, the Netherlands) dissolved in 1% methylcellulose solution. The genistein dosage was determined based on the effective dose of genistein supplementation reported in previous studies [26,27].

#### 2. Study design

This study was carried out for 63 days. The first 21 days were dedicated to induction of PCOS by letrozole and the remaining 42 days to the specific interventions. At the end of the study, the rats were kept fasting overnight and were prepared for sacrifice in the next morning. Ketamine (100 mg/kg) and xylazine (10 mg/kg) were used to anesthetize the rats before cardiocentesis, blood sampling, and ovary sampling. After centrifuging the blood samples at 4,000 rpm for 10 minutes, the serum was separated and stored at −80°C. The ovaries were cleaned of fat, weighed, and fixed in 10% formalin. All rats were weighed on days 1, 21 (after PCOS induction), and 63 (at the end of the study).
3. Assessment of serum biochemical parameters

Fasting blood sugar (FBS), high-density lipoprotein cholesterol, LDL cholesterol, total cholesterol, and triglyceride levels were measured by commercial assay kits (Pars Azmoon, Tehran, Iran) using an Auto-analyzer (BT 1500; Biotecnica, Roma, Italy). In order to estimate superoxide dismutase (SOD) activity and total antioxidant capacity (TAC), enzymatic and colorimetric methods were used based on commercial kit instructions (ZellBio, Lonsee, Germany). In addition, serum malondialdehyde (MDA) was measured using the modified thiobarbituric acid method, as reported by Satoh et al. [28]. Finally, circulating TNF-α levels were determined using the enzyme-linked immunosorbent assay (ELISA) method based on factory instructions of the commercial kit (Diaclone, Besançon, France).

4. Hormonal assay

Regarding to estimate IR, the fasting insulin level was measured using ELISA kits (Mercodia, Sweden). The homeostatic model assessment of insulin resistance (HOMA-IR) was then calculated using the following equation: (fasting insulin [mU/L] × fasting blood glucose [mg/dL])/405.

5. Histopathological examinations

The harvested ovaries were fixed in 10% formalin and firmly embedded in paraffin blocks before being sectioned at 5-μm thickness. Hematoxylin and eosin-stained slides were prepared using the method proposed by Manneras et al. [29]. The slides were then examined for histopathological alterations via a light microscope (Olympus CX31; Olympus, Tokyo, Japan). The histopathological slides were evaluated using different criteria including hyperplasia of theca cells, decreased number of corpora lutea, incomplete luteinization, capsular thickening, and numerous subcapsular follicular cysts, and they were scored as showing no changes, slight changes, or remarkable changes for each criterion.

6. Statistical analysis

The data were reported as mean ± standard deviation. The study groups were compared using one-way analysis of variance followed by the Tukey multiple comparison test. All analyses were done using IBM SPSS ver. 19 (IBM Corp., Armonk, NY, USA). The significance level was set at 0.05.

Results

1. Body weight, ovarian weight, and biochemical analysis

As presented in Figure 1, PCOS induction markedly increased the weight gain in comparison to the healthy group. Accordingly, the weight of the PCOS-induced rats (PCOS, metformin, and genistein groups) was significantly lower than that of the control group on day 21. At the end of the study, the body weight decreased in the metformin and genistein groups in comparison to the PCOS group, but the differences were not statistically significant. Ovarian weights in the PCOS and metformin groups were significantly higher than in the control group. The metformin and genistein groups had significantly lower ovarian weights than the PCOS groups (Figure 1).

Figure 1. Changes in body weight (A) and ovarian weight (B) in the experimental groups. PCOS, polycystic ovary syndrome; Met, metformin (150 mg/kg); Gen, genistein (20 mg/kg). Statistically significant differences: a) p<0.05 and b) p<0.001, respectively, comparison with the PCOS group; c) p<0.01 and d) p<0.001, respectively, compared to the control group.

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As shown in Table 1, FBS increased significantly in the PCOS group, but not in the rats treated with metformin and genistein ($p = 0.16$). Similarly, fasting serum insulin levels increased only in the PCOS group 43 days after PCOS induction. Thus, PCOS induction was accompanied by an increase in IR in the PCOS group ($p = 0.012$). However, no significant increase was observed in HOMA-IR in the rats receiving metformin and genistein in comparison to the control group (Table 1). The results revealed no significant changes in circulating lipids in the study groups, except for LDL cholesterol, which increased significantly in the PCOS group compared to the control group and significantly decreased in the metformin- and genistein-treated rats compared to the PCOS group (Figure 2).

2. Oxidative and inflammatory indices

The PCOS group had a similar degree of SOD activity to the control group (Figure 3). However, the rats treated with genistein showed a significant increase in SOD activity compared to the healthy rats ($p < 0.001$). Moreover, TAC significantly decreased in the PCOS group compared to the control group, whereas TAC was maintained in both the metformin and genistein groups. In addition, lipid peroxidation as determined by MDA level was significantly elevated in the PCOS group ($p = 0.01$). A similar change was observed in the rats treated with met-

![Figure 2](image-url)

**Table 1.** The effects of metformin and genistein on glycemic indices in letrozole-induced PCOS

<table>
<thead>
<tr>
<th>Group</th>
<th>Insulin (ng/mL)</th>
<th>Glucose (mg/dL)</th>
<th>HOMA-IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.87 ± 0.09</td>
<td>88.80 ± 2.60</td>
<td>3.45 ± 0.43</td>
</tr>
<tr>
<td>PCOS</td>
<td>1.75 ± 0.34$^a$</td>
<td>121.66 ± 5.51$^{a,b}$</td>
<td>9.34 ± 1.80$^{a,b}$</td>
</tr>
<tr>
<td>PCOS+Met</td>
<td>1.18 ± 0.20</td>
<td>99.50 ± 5.34$^{a,b}$</td>
<td>5.01 ± 0.68$^{a,b}$</td>
</tr>
<tr>
<td>PCOS+Gen</td>
<td>1.01 ± 0.14</td>
<td>104.6 ± 3.89$^{a,b}$</td>
<td>4.81 ± 0.79$^{a,b}$</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation.

HOMA-IR, homeostatic model assessment of insulin resistance; PCOS, polycystic ovary syndrome; Met, metformin (150 mg/kg); Gen, genistein (20 mg/kg).

$^a p < 0.05$, PCOS group vs. control group; $^b p < 0.05$, PCOS group vs. PCOS+Met and PCOS+Gen groups.
formin, while no change was observed in the genistein group. Furthermore, PCOS induction did not cause a higher degree of inflammation. Conversely, genistein supplementation reduced the levels of serum proinflammatory cytokines, such as TNF-α ($p = 0.003$), compared to the healthy rats.

### 3. Histopathological changes

Ovarian sections from the control group showed healthy follicles with oocytes at different developmental stages (Figure 4A and E). Letrozole-treated rats exhibited numerous sub-capsular cysts with very thin or no granulosa layers, and the volume of corpora lutea significantly decreased. In addition, there were few follicles in the ovaries of this group, which were mostly characterized by atresia (Figure 4B and F). Treatment with metformin (Figure 4C and G) and genistein (Figure 4D and H) led to a significant decrease in the number of cysts ($p < 0.05$) and the appearance of healthy follicles and corpora lutea. However, the number of atretic follicles was still significantly higher in these groups than in the control group.

### Discussion

The results of the present study identified a greater weight gain in the PCOS-induced groups, which could be attributed to fat accumulation induced by IR, hepatic lipogenesis [30], and the higher level of androgens in PCOS [2]. Treatment with metformin and genistein did not prevent PCOS-induced weight gain, which might be related to the insulin-sensitizing activity of metformin and genistein [31,32]. In the present study, dysregulation of glucose hemostasis and IR in

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**Figure 3.** The effects of metformin and genistein treatment on oxidative and inflammatory indices in letrozole-induced PCOS. (A) Total antioxidant capacity (TAC), (B) superoxidase dismutase (SOD), (C) malondialdehyde (MDA), (D) tumor necrosis factor-alpha (TNF-α). PCOS, polycystic ovary syndrome; Met, metformin (150 mg/kg); Gen, genistein (20 mg/kg). Statistically significant differences: $^a)p < 0.05$, $^b)p < 0.01$, and $^c)p < 0.001$, respectively, compared to the control group; $^d)p < 0.05$ and $^e)p < 0.001$ in comparison with the PCOS group.
the PCOS group was significantly alleviated after genistein supplementation. It was reported that genistein reduced HOMA-IR mainly by decreasing fasting insulin levels in patients with non-alcoholic fatty liver disease [33]. This effect of genistein has been reported in several diseases [18,33,34]. Genistein may increase glucose uptake by augmentation of shifting glucose transporter type 4 (GLUT4) toward the cell membrane. It has been stated that phosphatidylinositol 3-kinase, protein kinase C, and S'-adenosine monophosphate-activated protein kinase (AMPK) pathways are involved in the effects of genistein on IR [35].

The histopathological slides in the genistein-supplemented rats showed fewer cysts, and the presence of corpora lutea in the ovaries indicated follicle maturation and ovulation. This aligns with the results of a previous study by Rajan et al. [36], in which treatment of PCOS-induced rats with 100 mg/kg of soy isoflavones caused the development of antral follicles and thickening of granulosa cell layer to the normal state. Interestingly, the glycemic and histopathological changes in the genistein group were fully comparable to those in the metformin-treated group.

Based on oxidative and antioxidant indices, PCOS induction led to higher oxidative status compared to the healthy group. This may itself be a cause of IR induction, as previously reported [37-39]. In addition, folliculogenesis and oocyte maturation were unfavorably affected by oxidative stress [40]. Genistein supplementation in the present study reversed TAC to the normal level and prevented lipid peroxide production by reactive oxygen species, while the metformin group had increased MDA levels. The effect of metformin on increasing TAC may be due to improvements in glucose metabolism, as explained previously. Similarly, genistein can augment antioxidant defenses by upregulating SOD activity. Genistein antioxidant activity mostly relies on two properties: first, the ROS scavenging activity of hydroxyl groups on the 4', 5, and 7 positions [41,42] and second, upregulation of gene expression for antioxidant enzymes (SOD, catalase, and glutathione peroxidase) [43]. The results of the current study confirmed the proposed mechanism, as the genistein group had significantly higher SOD activity than the PCOS and control groups. The current study demonstrated that PCOS induction significantly elevated levels of circulating proinflammatory cytokines, such as TNF-α. This low-grade, but chronic status of inflammation potentially increases the risk of metabolic disorders [44]. It has been reported that increased levels proinflammatory cytokines such as TNF-α in PCOS are independent of body weight. However, the involved signaling pathways are similar, including increased serine phosphorylation, decreased insulin-stimulated IRS1 activation, and downregulation of GLUT4 expression [3,45]. The preservation of normal TNF-α levels in the genistein group may have been due to the anti-inflammatory function of genistein. A possible underlying mechanism could have been the effect of genistein in downregulation of nuclear factor kappa B (NF-κB) expression [46]. NF-κB is a transcription factor involved in the expression of several proinflammatory genes (e.g., TNF-α and interleukin-6) [47]. IR can substantially increase levels of circulating proinflammatory cytokines, which is similar to hyperinsulinemia/hyperandrogenism interaction in PCOS [48].

The present study revealed that no aspects of the lipid profile were
affected by letrozole, except for LDL cholesterol levels, which were increased significantly in the PCOS group. Metformin and genistein significantly suppressed this elevation to normal levels. The lipid-lowering action of metformin may be due to activation of hepatocytes via AMPK, which leads to the inhibition of β-Hydroxy β-methylglutaryl-CoA reductase [49]. However, the mechanism of genistein in LDL cholesterol reduction mostly relies on activation of peroxisome proliferator-activated receptor alpha, which subsequently decreases SREBP-2 expression and HMG-CoA reductase activity [50,51].

The promising results in the current study show that genistein may be a possible strategy to confront various complications of PCOS, including IR, dyslipidemia, and oxidative stress. However, further studies are required to investigate the effects of long-term usage of genistein in terms of favorable changes in biological features of PCOS such as hyperandrogenism and to identify its possible side effects. Changes in hormones, especially androgens, should also be examined in future studies.

This study demonstrated that genistein supplementation in rats with letrozole-induced PCOS mitigated IR. Moreover, the histopathological results indicated an increase in luteinization and the development of fewer cysts. These changes were comparable to those observed in the metformin-treated group. Moreover, both genistein and metformin therapy led to lower levels of LDL cholesterol, but only the rats receiving genistein exhibited improvements in antioxidant and anti-inflammatory status. Overall, the results revealed that genistein might be a safe and effective dietary component to ameliorate the complications of PCOS. In addition to acting as a phytoestrogen and regulating metabolic features, genistein exerts antioxidant and anti-inflammatory properties. If these possibilities are confirmed in future studies, genistein may be considered as a supplementary treatment in patients with PCOS.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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Alteration of $\text{TGFB1}$, $\text{GDF9}$, and $\text{BMPR2}$ gene expression in preantral follicles of an estradiol valerate-induced polycystic ovary mouse model can lead to anovulation, polycystic morphology, obesity, and absence of hyperandrogenism

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**Objective:** In humans, polycystic ovary syndrome (PCOS) is an androgen-dependent ovarian disorder. Aberrant gene expression in folliculogenesis can arrest the transition of preantral to antral follicles, leading to PCOS. We explored the possible role of altered gene expression in preantral follicles of estradiol valerate (EV) induced polycystic ovaries (PCO) in a mouse model.

**Methods:** Twenty female balb/c mice (8 weeks, 20.0±1.5 g) were grouped into control and PCO groups. PCO was induced by intramuscular EV injection. After 8 weeks, the animals were killed by cervical dislocation. Blood serum (for hormonal assessments using the enzyme-linked immunosorbent assay technique) was aspirated, and ovaries (the right ovary for histological examinations and the left for quantitative real-time polymerase) were dissected.

**Results:** Compared to the control group, the PCO group showed significantly lower values for the mean body weight, number of preantral and antral follicles, serum levels of estradiol, luteinizing hormone, testosterone, and follicle-stimulating hormone, and gene expression of $\text{TGFB1}$, $\text{GDF9}$, and $\text{BMPR2}$ ($p<0.05$). Serum progesterone levels were significantly higher in the PCO animals than in the control group ($p<0.05$). No significant between-group differences ($p>0.05$) were found in BMP6 or BMP15 expression.

**Conclusion:** In animals with EV-induced PCO, the preantral follicles did not develop into antral follicles. In this mouse model, the gene expression of $\text{TGFB1}$, $\text{GDF9}$, and $\text{BMPR2}$ was lower in preantral follicles, which is probably related to the pathologic conditions of PCO. Hypoandrogenism was also detected in this EV-induced murine PCO model.

**Keywords:** Bone morphogenetic protein 6; Bone morphogenetic protein 15; Bone morphogenetic protein receptor II; Folliculogenesis; Growth differentiation factor 9; Multicystic morphology of ovary; Polycystic ovary syndrome; Transforming growth factor-β1
Higher androgen levels can lead to hyperandrogenism, anovulation, follicular cysts, menstrual cycle irregularities, and eventually, female infertility [2,3]. PCOS has a primarily genetic basis. Ovarian follicles are the smallest affected unit in folliculogenesis. Each follicle contains a single central oocyte, granulosa cells, and an outer structure of connective tissue named the theca. A fluid-filled cavity formed adjacent to the oocyte is known as an antrum, and a follicle with an antrum is designated as an antral follicle. A follicle with no antrum is referred to as a preantral follicle. The transition of preantral to antral follicles is a normal and routine procedure known as folliculogenesis. Alterations of the genes involved in this process can arrest the transition from the preantral to antral stages, which is a defining feature of PCO morphologically. EV is an exogenous chemical agent that can completely mimic PCO morphologically. EV (Sigma Chemical Co., St. Louis, MO, USA) was injected intramuscularly on 2 consecutive days (each injection comprised 0.2 mL of EV dissolved in normal saline) to induce PCO in estrous cycle based on the morphological feature according to the routine protocol, the classification of follicles was applied based on the morphological features, as follows: primary ovarian follicles (with a single layer of cuboidal granulosa cells), pre-antral follicles (containing one or two small spaces filled by follicular fluid), antral follicles (consisting of a single large antral space), and preovulatory follicles (a rim of cumulus cells around the oocyte suspended in a large space) [13].

2. Ethical considerations

All animals were treated in accordance with guidelines of the National Institute of Health for the Care and Use of Laboratory Animals approved by the Research Deputy based on the WMA Declaration of Helsinki (ethics approval No. IR.TBZMED.REC. 1396.346).

3. Study groups

The animals were randomly divided into control and PCO groups (n = 10 each). In the control group, the animals received an intramuscular injection of sesame oil (0.1 mL), and the animals in the PCO group received PCO induction by intramuscular injections of estradiol valerate (EV) [12].

4. Follicular classification

According to the routine protocol, the classification of follicles was

5. PCOS induction

EV is an exogenous chemical agent that can completely mimic PCO morphologically. EV (Sigma Chemical Co., St. Louis, MO, USA) was injected intramuscularly on 2 consecutive days (each injection comprised 0.2 mL of EV dissolved in normal saline) to induce PCO in estrous cycle based on the morphological feature according to the routine protocol, the classification of follicles was

6. Animal dissection and tissue sampling

Prior to the study and on the last day of the experiment, the animals were weighed. At 56 days (8 weeks) after PCO induction, the animals were killed by cervical dislocation. The subxiphoid approach was used for blood aspiration for future hormonal assays. Fifteen minutes later, the blood clot was centrifuged (4,000 rpm, 10 minutes). Midline laparotomy was then performed, and the ovaries were dissected from both sides of the body. The surrounding adipose tissue was separated, and the ovaries were quickly washed in normal saline. The right ovary was fixed in 10% formalin for 3 days for morphological assessments, and the left ovary was used for gene expression analyses.

Methods

1. Animals

Twenty balb/c female mice (20.0 ± 1.5 g, 8 weeks old) were obtained from the university’s home. All standard conditions for animal keeping were provided, including food pellets (Livestock and Poultry Feed Company, Tehran, Iran), free access to water, transparent and separated cages, equilibrium of the environmental temperature (22°C), humidity of 55% ± 3%, and a 12-hour light/12-hour dark photoperiod.
7. Separation of preantral and antral follicles
Two microliters of alpha-minimum essential medium containing 3% fetal bovine serum was used as a suspension for mechanical tissue dissection of the ovaries. One G-needle tip attached to an insulin syringe was used for mechanical dissection using a stereomicroscope (Olympus, Tokyo, Japan). The studied follicles were morphologically multilayered preantral (1–8 μm in diameter) and antral follicles. The isolated follicles were located inside a micro-tube and stored at –70°C.

8. Histological preparation and hematoxylin and eosin staining
The right fixed ovary underwent routine tissue processing for hematoxylin and eosin (H&E) staining. After tissue fixation (using 10% formalin), dehydration (by different alcohol concentrations), and clarification (with xylol), the ovaries were embedded in paraffin. The slides were cut into 5-µm sections and stained with H&E for morphological assessments of the follicles (Table 2).

9. Hormone measurements
To assess the levels of serum hormones, the aspirated blood samples were centrifuged (4,000 × g, 10 minutes), and isolated serum was stored in a freezer (–70°C) for biochemical analysis of luteinizing hormone (LH), FSH, progesterone, estradiol, and testosterone using enzyme-linked immunosorbent assays (Cayman Chemicals; Ann Arbor, MI, USA). In this test, specific monoclonal antibodies were used against an antigenic marker of the target hormone. One monoclonal antibody was used for solid-phase stabilization, and another anti-hormone antibody was used for conjugation with horseradish peroxidase. Upon adding a serum sample, the hormone reacted simultaneously with the above two antibodies and was sandwiched between the antibody bound to the solid phase and the conjugated antibody. After 4 minutes of incubation at room temperature to remove free labeled antibodies, the wells were rinsed with washing solution. After adding the substrate-dye solution (4 minutes of incubation), a blue color appeared. Color production was stopped by adding the stop solution and the blue color turned yellow. Finally, the color intensity was measured (at specific wavelengths, expressed in nanometers). The intensity of color was directly related to the concentration of hormones in serum.

10. RNA extraction and cDNA formation
Total RNA was extracted from the ovaries using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) based on the manufacturer’s instructions (Cat No.11667165001; Roche, Basel, Switzerland). The quality and quantity of each sample were assessed by a NanoDrop ND-2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) according to the optical density. The RNA integrity was confirmed by electrophoresis on 1% agarose gel (Ultra-Pure TM Agarose, Invitrogen) stained with the Gel Red RNA stain detected under ultraviolet light. High-quality RNA samples with no degradation were stored at –80°C. Then, complementary DNA (cDNA) synthesis was performed using the Takara cDNA kit (Takara Cat No. 6130; Takara, Mountain View, CA, USA). The first-strand cDNA was synthesized using a cDNA synthesis kit (Thermo Fisher). Reverse transcription of 500 ng of the total RNA extracted from all samples was performed using both random hexamer and oligo-dT primers.

11. Quantitative real-time polymerase chain reaction
Sense and antisense primers for the target and reference genes were designed using primer design software (National Center for Biotechnology Information, Primer-BLAST, https://www.ncbi.nlm.nih.gov/tools/primer-blast/). cRPL13A and GAPDH were used as reference genes for normalization. The primers were: GDF9 (forward [F]: CAGTCCACCTGGAGGCCTTTA, reverse [R]: GAGCGGATGGCTTTCTGCG-
CCT), BMP15 (F: TGGGCCTCTCTCCTCGGTTA, R: AATCTTCTGGGC-CAAAGCGAG), TGFβ1 (F: ACTTCTTGGCACTGCGCTGTC, R: GAACGCCGGGTAGCGATCGAG), BMPR2 (F: AGGAGCTGTGAACCTGAGGGA, R: TCACCTGGGAAGAGGTCTGTA), and BMP6 (F: GGCGCTCTCTACCGAGGAGG, R: TAGTCTGAAAGACCGGAGCCC). The Quantitative polymerase chain reaction was performed using the Ampliqon Kit by Rotor-Gene 6000 system (Corbett Research, Sydney, Australia). The cycling conditions were 95°C (15 minutes), 40 cycles of amplification (95°C, 60°C, and 72°C; 40 seconds each), followed by a final extension of 72°C (5 minutes). Finally, melting was carried out for 5 seconds at 60°C–95°C (with 0.5°C increments for each step). Relative quantification was applied using the Livak method and the samples’ crossing point, the number of cycles completed, the efficiency of the reaction, and other values were used in the analysis for comparison and creating the ratios. Finally, the results were reported as normalized ratios (Table 3).

12. Data analysis

Data were analyzed using SPSS ver. 16 (SPSS Inc., Chicago, IL, USA). The normal distribution of the data was confirmed by the Kolmogorov-Smirnov test. The independent t-test and Tukey post-hoc test were used to compare differences among the groups. All data were presented as mean ± standard deviation, and \( p < 0.05 \) was considered as the significance level.

Results

1. Total animal weight

On day 56, the PCO group showed a significantly lower total weight (26.3 ± 1.2 g) than the control group (30.4 ± 0.9 g) \( (p < 0.05) \).

2. Number of preantral and antral follicles

According to the histological data, the PCO group showed significantly lower mean numbers of antral follicles (6.5 in the control group vs. 5.7 in the PCO group) and preantral follicles (5.1 in the control group vs. 4.4 in the PCO group) \( (p < 0.05) \). However, no significant differences were detected in the number of primary follicles (6.5 in the control group vs. 5.7 in the PCO group) and pre-ovulatory follicles (0.26 in the control group vs. 0.08 in the PCO group).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDF9</td>
<td>CAGGTACACCTGGAGGCTTCTTA</td>
<td>GAGGCCATGGCTTCTGCCCCT</td>
</tr>
<tr>
<td>BMP15</td>
<td>TGGGCCTCTCTCTCGGCTATTA</td>
<td>AATCTTCTGGGCAAGCGAG</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>ACTTCTGGCACTGCGCTGTC</td>
<td>GAACGCCGGGTAGCGATCGAG</td>
</tr>
<tr>
<td>BMPR2</td>
<td>AGGAGCTGTGAACCTGAGGGA</td>
<td>TCACCTGGGAAGAGGTCTGTA</td>
</tr>
<tr>
<td>BMP6</td>
<td>GGCGCTCTCTACCGAGGAGG</td>
<td>TAGTCTGAAAGACCGGAGCCC</td>
</tr>
</tbody>
</table>

3. Hormone assay

In the PCO group, the levels of testosterone (14.45 ng/mL in the control group vs. 5.13 ng/mL in the PCO group), estradiol (156.32 ng/mL in the control group vs. 52.14 ng/mL in the PCO group), LH (13.24 ng/mL in the control group vs. 7.63 ng/mL in the PCO group), and FSH (15.68 ng/mL in the control group vs. 8.85 ng/mL in the PCO group) were significantly lower \( (p < 0.05) \), whereas the level of progesterone (19.46 ng/mL in the control group vs. 42.52 ng/mL in the PCO group) was significantly higher \( (p < 0.05) \) than in the control group (Figure 1).

4. Histological assessment

In the PCO group, the oocytes were wrinkled compared to those in the control group, and large cystic follicles with a narrow granulosa layer (1–3 cellular layers) along with a significant decreased number of preantral follicles were discovered. Furthermore, no corpus luteum was observed in the PCO group (Figure 2). In addition, a morphological assessment of the ovaries showed that the ovaries of EV-treated mice were smaller than those of the control group, with large cystic follicles and non-stromal hypertrophy.

5. Gene expression

The preantral follicles of the PCO group showed significantly lower expression of the following genes than the control group: GDF9 \( (0.0068 \text{ vs. } 0.0042 \text{ in the control and PCO groups, respectively}) \), TGFβ1 \( (0.0045 \text{ vs. } 0.0020 \text{ in the control and PCO groups, respectively}) \), and BMPR2 \( (0.0075 \text{ vs. } 0.0040 \text{ in the control and PCO groups, re-} \))

![Figure 1. Serum concentration of hormones (ng/mL). Values are presented as mean ± standard deviation. PCO, polycystic ovaries; LH, luteinizing hormone; FSH, follicle-stimulating hormone. *a*Significant differences \( (p<0.05) \).](https://doi.org/10.5653/cerm.2020.04112)
Follicles. Thus, this drug was used as an exogenous chemical to model PCO in mice in the present study [12]. Furthermore, the ovulation processes in humans and rodents are mono-ovulation and poly-ovulation, respectively, suggesting that the FSH-dependent follicle selection process in rodents differs from that in humans [16,17].

There are various types of PCO models in animals with different features and limitations. It is important to state that no single method of PCO induction in animal models exactly resembles human follicles. Thus, this drug was used as an exogenous chemical to model PCO in mice in the present study [12]. Furthermore, the ovulation processes in humans and rodents are mono-ovulation and poly-ovulation, respectively, suggesting that the FSH-dependent follicle selection process in rodents differs from that in humans [16,17].

Discussion

In adult women, folliculogenesis is a monthly process that can eventually lead to ovulation. A crucial stage in ovulation is the transition from preantral to antral follicles, a process that is regulated by the expression of important genes. Thus, any disruption of the preantral to antral transition can potentially arrest ovulation. In patients with PCOS, preantral follicles do not develop into the antral stage. Thus, in the present study, the role of the expression of genes (TGFB1, GDF9, BMP6, BMP15, and BMPR2) involved in the transition of preantral to antral follicles was investigated in an EV-induced PCO mouse model, and relationships with serum levels of sex hormones were explored biochemically, histologically, and genetically.

In PCOS-affected humans, hormonal and metabolic alterations in the ovaries are most likely associated with upregulation of PI3K-Akt [14] and increased levels of ERK activity [15] as intracellular and extracellular pathways, respectively. In animals, the morphological features of PCO are induced by EV injection, which cannot precisely mimic the clinical and molecular conditions of PCOS in humans in terms of metabolic parameters and hyperandrogenemia. Although there are crucial differences between PCO induction in animals (through EV injection) and humans morphologically, EV causes anovulation and multicystic ovaries with less stroma and more cystic follicles. Thus, this drug was used as an exogenous chemical to model PCO in mice in the present study [12]. Furthermore, the ovulation processes in humans and rodents are mono-ovulation and poly-ovulation, respectively, suggesting that the FSH-dependent follicle selection process in rodents differs from that in humans [16,17].

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![Figure 2](image2.png)

**Figure 2.** Histological photographs of the polycystic ovaries and control groups (H&E, ×400). (A) Preantral (red arrow) and antral (black row) follicles (scale bar=200 μm). (B) Corpus luteum (black arrow) in the control group (scale bar=100 μm).

![Figure 3](image3.png)

**Figure 3.** Comparison of gene expression levels in control preantral follicles and the polycystic ovaries (PCO) group. Values are presented as mean±standard deviation. *a*Significant differences (p<0.05).
PCOS [18]. PCOS can be induced in different stages of animal life (prenatal, postnatal, prepubertal, and adolescence) with many inducing factors (environmental factors, genetic factors, and hormonal models); thus, this pathologic condition is known to be a multifactorial disease. Animal models of PCO are induced by various environmental factors, of which endocrine-disrupting chemicals (EDC) are the most common. Endocrine disrupting chemicals interfere with the hormonal balance, leading to PCO induction. Bisphenol-A (BPA), as a synthetic xenoestrogen, is an important EDC [18]. Neonatal exposure to BPA (500 μg) caused an increased testosterone concentration in serum, which led to the induction of PCO-like features in the adulthood of animals, such as the formation of ovarian cysts [19]. BPA also induces PCOS-associated metabolic syndrome, which stimulates pancreatic beta-cells to produce insulin and acts as an obesogenic agent to release adiponectin from adipose tissue, which is known to be an agent for animal PCO induction [20]. Di-(2-ethylhexyl) phthalate (DEHP), another EDC, is one of the major environmental estrogens. DEHP administration (2 g/kg body weight, 12 days) caused symptoms resembling PCO, including irregular cyclicity, anovulation, decreased serum estradiol, and polycystic follicle formation [21].

Tributyltin (TBT) is another endocrine disruptor with obesogenic properties. Since, obesity is associated with insulin resistance, which is one of the features of PCO, studies have focused on the development of PCO caused by TBT administration [22]. Since TBT causes irregular estrous cycles, hyperandrogenism, and obesity, this model exhibits most PCO metabolic features [23]. Hormonal interventions are another method to induce PCO in animal models. There are 3 main types of PCO animal models depending on the specific hormonal manipulation: androgen-induced, estrogen-related, and anti-progestosterone-induced. Testosterone, dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), and esters of testosterone (testosterone propionate [TP]) are used to induce hyperandrogenism in animal PCO conditions. Some papers stated that hyperandrogenism could disrupt normal reproductive physiology, leading to PCO induction [24]. Postnatal intraperitoneal exposure of TP (10 mg/kg bw) showed the characteristic features of PCOS, such as irregular estrous cyclicity, elevated testosterone and insulin levels, the presence of cystic follicles with unaltered estradiol levels, reduced progesterone levels, arrested ovulation, and the absence of corpora lutea [25]. Various studies have reported that the administration of TP did not lead to an ovarian phenotype exactly resembling that observed in PCOS [18]. DHEA was the first agent used by scientists to induce PCO in animals due to the elevated levels of DHEA found in women with PCOS [26]. Subcutaneous injections of DHEA (60 mg/kg bw in 42-day-old rats for 20–30 days) led to an improved PCO model with increased body weight, irregular estrous cyclicity, and an increased number of ovarian cystic follicles [27]. A DHEA-induced PCO rodent model exhibited all key features similar to women with PCOS. DHT is a nonaromatizable androgen that cannot be converted into estrogen by the action of aromatase; thus, it enhances androgen potency. Exposure of prepubertal rats (7.5 mg) [28] and mice (2.5 mg) [29] to DHT for 90 days was found to be a precise model with features similar to those of women with PCOS, such as increased body weight and insulin resistance [30]. In women with PCOS, serum estrogen levels are elevated. Based on this evidence, estrogen or other forms of estrogen (e.g., EV or estradiol benzoate [EB]) are used to induce PCO in animal models. One-day-old neonatal rats treated with EB showed acyclicity and anovulation. However, the serum LH levels, as well as the ovarian weight, decreased, and elevated serum FSH levels were observed, unlike human PCOS conditions [31]. Rats exposed to EV (2 mg) failed to exhibit key features of PCOS, representing dissimilarities with human PCOS [32]. Prior studies suggested that, in estrogenic conditions, ovarian alterations similar to those observed in PCOS would not happen, and hormonal and metabolic disruptions were not parallel with PCOS women under exposure to estrogenic conditions. Among the animal models of PCO, there are some important metabolic features and morphological characteristics, such as acyclicity, anovulation, hyperandrogenism, cystic follicles, insulin resistance, and obesity. Generally, DHEA is a chemical used to induce PCO with various serological, morphological, and metabolic alterations exactly resembling those found in human women with PCOS. Additionally, EV administration can induce acyclicity, anovulation, formation of cystic follicles, and obesity, with an approximate similarity to human PCOS. However, in EV-induced PCO animal models, there are no metabolic symptoms such as hyperandrogenism and insulin resistance [18]. It has finally been concluded that androgenized PCO mouse models are a better choice in terms of resembling human PCOS than models using estrogenic agents. Although we assessed preantral follicles in the present study, the EV-induced PCO mouse model did not exactly mimic human PCOS. The absence of hyperandrogenism and insulin resistance following the induction of PCO by EV constitute limitations of the present study. Due to these limitations of EV-induced PCO, DHEA is recommended for PCO induction in mice.

As shown by the data in the present study, weight loss occurred 56 days after PCO induction using intramuscular injections of EV. Although it is scientifically accepted that weight gain is common in PCOS patients [33], this study found weight loss in EV-induced PCO mice. This finding was also reported by Ikeda et al. [34] following DHEA administration to animals. They found that a 1-month administration of DHEA led to decreased total weight in PCO animals. Reduced levels of total body weight were also reported by Mesbah et al. [35] following EV administration. There are three main reasons underly-
ing the biological process of weight loss in animals following intramuscular injections of EV: (1) the lipolytic characteristics of increased adrenal glucocorticoid levels; (2) neurological modifications in the hypothalamic paraventricular nucleus interfering with food intake; and (3) the activation of peripheral sympathetic neurons leading to metabolic activity promotion, increased fat consumption, and finally weight loss [32].

In morphological assessments, the numbers of preantral and antral follicles were lower in the PCO group. This phenomenon probably relates to the effects of PCO on inhibiting follicular development. Overall, although the ovaries in EV-treated mice were lower, the majority of the ovarian tissue comprised abnormal cystic follicles with large and filled follicles. Since the entirety of the ovaries included cystic follicles, the stromal tissue was smaller in PCO-affected animals.

In this study, changes in three major hormones secreted by PCO-affected ovaries (estradiol, progesterone, and testosterone) and two hormones secreted by the pituitary gland (LH and FSH) were analyzed. The results showed significantly lower LH, FSH, testosterone, and estradiol levels in the PCO group than in the control group, but progesterone showed significantly higher levels. In humans, PCOS is characterized by hypersecretion of LH. The LH surge in the primary stage can lead to ovulation, but chronic hypersecretion of LH can arrest follicular growth, leading to anovulation [36]. As we found in this investigation, PCO was characterized by a decreased number of preantral, antral, and pre-ovulatory follicles. As PCO has no effect on primary follicles, the number of primary follicles did not show significant differences. In an experimental study, Brawer et al. [37] assessed the development of PCOS in EV-injected rats, and reported decreased levels of LH and FSH. FSH stimulates the growth and recruitment of immature ovarian follicles in the ovary. Thus, reduced levels of FSH can eventually lead to arrest of the development of ovarian follicles [38]. Miri et al. [39] also assessed the effects of exercise intensity on sex hormones in female rats with EV-induced PCOS. They found that the PCOS animals showed decreased levels of testosterone in comparison with the control group. This finding is in line with our results, as we found hypoadrogenism. Parallel to our findings, Brawer et al. [37] also stated that the basal levels of LH and FSH decreased in EV-treated PCOS animals. An experimental study conducted by Pournaderi et al. [40] found the same results, indicating that after EV-induced PCOS, the animals presented decreased levels of LH, FSH, and estradiol and also found increased levels of progesterone in comparison with the control group. Venegas et al. [41] similarly found that increased serum levels of progesterone. Laird et al. [42], in an in vitro study, examined the role of testosterone on the transition of preantral follicles to the antral form. They concluded that testosterone could stimulate the growth of preantral follicles. However, in the present study, testosterone levels were lower in the PCOS group, indicating arrest of the preantral to antral transition.

Among the genes affecting the ovulation process, TGFβ1, GDF9, BMP15, BMP6, and their receptor, BMPR2, were assessed in the present study. TGFβ1 is a factor that leads to cell division and differentiation [43]. Thus, based on scientific evidence, overexpression of TGFβ1 can lead to increased amounts of stromal tissue or tissue fibrosis. As we found in the present study, the gene expression level of TGFβ1 was decreased in PCO model animals. Histological assessments also showed that the stromal tissue surrounding cystic follicles accounted for the smaller size of ovaries in PCO animals. Recent evidence has indicated that expression of TGFβ1 and subsequent alterations in the extracellular matrix might contribute to the pathogenesis of multicystic ovaries [44]. As the results showed, the gene expression of TGFβ1 was significantly lower in the preantral follicles of the PCO group. However, on the contrary, Miao et al. [45] concluded that the TGFβ1 pathway affected the expression of fibrillin 3 in stromal compartments of fetal ovaries, in which the ovarian stroma was larger. In some studies, elevated levels of TGFβ1 have been seen in PCO, unlike the results of the present study. In the analysis of TGFβ1 gene expression in various PCO-affected preantral follicles, this difference can serve as a turning point. BMP signaling influences female fertility of different species in different ways. In this study, gene expression levels of BMP6 and BMP15, as well as their receptor (BMPR2), were measured. BMP-6 is required for normal female fertility [46]. TGFβ1 and BMP-6 are primary mediators of ovarian follicular growth [47]. However, although no major variations in BMP6 expression levels have been observed in the present sample, decreased levels of TGFβ1 were found in preantral PCO follicles. The gene expression of GDF9 and BMP15 in PCO depends on the follicular stage [11]. We also found no major variations in BMP15 gene expression in preantral PCO follicles. BMP15 causes the transition from primordial follicles to primary and secondary follicles. Along with the results of our study, another study also suggested that BMP15 has no effects on the follicular transition into preovulatory follicles [48]. There is evidence that the BMPR2 signaling pathway is impaired in the case of PCOS, most likely due to hyperaldosteronism [49]. An analysis of gene expression patterns showed that BMPR2 levels were significantly reduced. These results imply that PCO does not have any major effect on the expression of BMP6 or BMP15, but their receptor is significantly affected. Thus, the probable incomplete function of BMP6 and BMP15 is related to their receptor. In many mammalian species, GDF9 is important for early follicular development through its direct action on granulosa cells, allowing proliferation and differentiation [50]. GDF9 is important for folliculogenesis, oogenesis, and ovulation and therefore plays a major role in women’s fertility [51]. In this study, the expression level of GDF9 was
lower in preantral PCOS follicles. Karagul et al. [6] also found reduced levels of GDF9 expression in primary follicles. Teixeira Filho et al. [52] also reported the aberrant expression of GDF9 in oocytes of women with PCOS. Overall, PCO disrupts the expression of key genes in folliculogenesis.

We concluded that the expression of the GDF9, TGFβ1, and BMPR2 genes in the preantral follicles of the PCO group was significantly reduced, resulting in impaired folliculogenesis and no ovulation. The expression of the BMP6 and BMP15 genes in both normal antral follicles and antral follicles in the PCO group was normal, with no significant changes. All sexual hormones were decreased, except for progesterone, which showed increased levels in the PCO group. Thus, unlike the hyperandrogenism that has been reported in other published papers, we found hyperprogesteronism in PCO-affected animals following EV injections. Although gene expression, histopathological alterations, and serum hormone levels were measured in the present study in an EV-induced PCO mouse model, it is recommended to design an experimental study on DHEA-induced PCO animals to observe molecular alterations.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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References

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Predictive value of sperm motility before and after preparation for the pregnancy outcomes of intrauterine insemination

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Objective: This study aimed to investigate sperm motility and its changes after preparation as predictors of pregnancy in intrauterine insemination (IUI) cycles.

Methods: In total, 297 IUI cycles from January 2012 to December 2017 at a single tertiary hospital were retrospectively analyzed. Patient and cycle characteristics, and sperm motility characteristics before and after processing were compared according to clinical pregnancy or live birth as outcomes.

Results: The overall clinical pregnancy rate per cycle was 14.5% (43/297) and the live birth rate was 10.4% (30/289). Patient and cycle characteristics were similar between pregnant and non-pregnant groups. Sperm motility after preparation and the total motile sperm count before and after processing were comparable in terms of pregnancy outcomes. Pre-preparation sperm motility was significantly higher in groups with clinical pregnancy and live birth than in cycles not resulting in pregnancy (71.4%±10.9% vs. 67.2%±11.7%, p=0.020 and 71.6%±12.6% vs. 67.3%±11.7%, p=0.030, respectively). The change in sperm motility after processing was significantly fewer in the non-pregnant cycles, both when the comparison was conducted by subtraction (post–pre) and division (post/pre). These relationships remained significant after adjusting for the female partner’s age, anti-Müllerian hormone level, and number of pre-ovulatory follicles. According to a receiver operating characteristic curve analysis, an initial sperm motility of ≥72.5% was the optimal threshold value for predicting live birth after IUI.

Conclusion: Initial sperm motility, rather than the motility of processed sperm or the degree of change after preparation, predicted live birth after IUI procedures.

Keywords: Intrauterine insemination; Pregnancy; Semen analysis; Sperm motility

Introduction

Intrauterine insemination (IUI) is considered as a treatment option in patients with unexplained infertility or mild to moderate male factor infertility. Compared with in vitro fertilization (IVF) and intracytoplasmic sperm injection, IUI is known to be a more simple, less invasive, and inexpensive procedure [1]. Despite its widespread use in infertility practice, there is still controversy regarding the effectiveness of IUI and its use as a first-line treatment due to its relatively low success rate [2].

Prognostic factors for IUI success have been examined in a number of studies. Factors such as the female partner’s age, duration or etiology of infertility, ovarian stimulation method, number of preovulatory follicles, and sperm parameters have been suggested to determine the outcome of IUI [3-5]. Many studies have investigated semen characteristics that can predict the prognosis of IUI procedures, but they have shown conflicting results. Among semen parameters, the effects of sperm morphology by strict criteria, sperm motility, and initial total motile sperm count (TMSC) on IUI cycles have mostly...
been studied, and cut-off values were suggested in some studies [6-
11]. In addition, post-wash TMSC was also reported as a prognostic
factor for pregnancy after IUI [9,11-13]. However, some studies re-
ported that most semen parameters could not properly predict the
IUI outcome [14,15].

Semen preparation techniques have been developed to separate
morphologically normal motile spermatozoa from seminal plasma. It
is known that preparation improves sperm quality, and the pregnan-
city rate can be improved accordingly [16-18]. Indeed, while motil-
y of sperm initially collected on the insemination day varies from pa-
tient to patient, the percentage of motile sperm left after preparation
generally increases and becomes relatively homogeneous. Although
many studies have investigated the effect of initial and post-prepara-
tion semen parameters separately, few studies have examined the
impact of changes after semen preparation on the outcomes of IUI.
The purpose of this study was to examine whether changes in sperm
motility characteristics during the preparation process could predict
pregnancy outcomes in IUI.

Methods

1. Study population

This study included infertile couples who underwent IUI cycles at
Seoul National University Hospital from January 2012 to December
2017. Cases were included only if the IUI procedure was completed
and the pregnancy outcome was confirmed during this period. This
study was approved by the Institutional Review Board of Seoul Na-
tional University Hospital (IRB No. 2101-189-1194). This was an ob-
servation-only study using medical chart review and the need of in-
formed consent was waived. The medical records of the subjects
were reviewed retrospectively by a clinician.

The indications for IUI were mostly unexplained infertility, inferti-
ility with combined factors, and mild male factor or ovulatory factor
infertility. Couples were considered eligible if the female partner had
at least one patent fallopian tube and had a normal endometrial cav-
it. Cases with severe endometriosis (stage III or IV) were excluded
from the analysis. Patients with severe male factor infertility whose
TMSC was lower than 5 × 10
6
more was confirmed.
Pregnancy test was done by measuring serum hCG 14 days after
IUI and transvaginal ultrasound was performed one week later if the
test was positive. Confirmation of an intrauterine gestational sac on
transvaginal ultrasonography was considered as indicating clinical
pregnancy. Abortion was defined as fetal demise or loss of fetal heart
tones before the 20th week of pregnancy. Live birth was defined
when delivery of a live fetus at a gestational age of 24th week or
more was confirmed.

2. Semen analysis and preparation

After at least 2 days of ejaculatory abstinence, semen samples were
obtained by masturbation and collected in sterile containers. After liq-
uefaction for 30 minutes at room temperature, semen samples were
processed using the density gradient technique with SpermGrad (Vit-
rolife, Gothenburg, Sweden), and the total volume was adjusted to 0.3
mL using a wash medium. Before and after semen preparation, each
sample was analyzed for volume, concentration, and motility accord-
ing to the World Health Organization criteria [17]. The semen evalu-
tation was performed manually by one of three IVF laboratory research-
ers, each of whom had more than 10 years of experience.

3. Ovarian stimulation and IUI

Ovarian stimulation was conducted using a single agent or combi-
nation among clomiphene citrate, letrozole, and human menopausal
gonadotropin (IVF-M; LG Life Sciences, Seoul, Korea). In most cases,
cloviphene citrate (100–150 mg/day) was administered for 5 days
beginning on menstrual cycle day 3, and gonadotropin (75–150 IU/
day) injections were started thereafter. The gonadotropin dosage was
determined based on ovarian reserve markers and the female part-
tner’s age. All patients were monitored using serial transvaginal ultra-
sonography. When at least one follicle diameter reached 18 mm or
more, ovulation was triggered using human chorionic gonadotropin
(hCG), via either 250 μg of recombinant hCG (Ovidrel; Merck Serono,
Darmstadt, Germany) or 10,000 IU of hCG (IVF-C; LG Life Sciences).
IUI was performed 36 hours after hCG administration with a soft
IUI catheter. A single IUI procedure was performed for all cycles. The
luteal phase was supported vaginally using either Crinone (Merck
Serono) 8% vaginal gel or Lutinus (Ferring, Saint-Prex, Switzerland)
100 mg vaginal tablets beginning on the insemination day. After
confirmation of pregnancy, luteal phase support was continued until
the 10th week of gestational age.

Pregnancy test was done by measuring serum hCG 14 days after
IUI and transvaginal ultrasound was performed one week later if the
test was positive. Confirmation of an intrauterine gestational sac on
transvaginal ultrasonography was considered as indicating clinical
pregnancy. Abortion was defined as fetal demise or loss of fetal heart
tones before the 20th week of pregnancy. Live birth was defined
when delivery of a live fetus at a gestational age of 24th week or
more was confirmed.

4. Outcome measures and statistical analysis

The main outcome measures were clinical pregnancy and live
birth. Each sperm parameter, especially motility, and its change during processing were examined. The effect of sperm motility characteristics before and after preparation on pregnancy outcomes was analyzed. All statistical analyses were performed with IBM SPSS ver. 21.0 (IBM Corp., Armonk, NY, USA). Continuous variables were analyzed using either the Student t-test or the Mann-Whitney U-test. Categorical variables were analyzed using the chi-square test. Receiver operating characteristic (ROC) curve analysis was conducted to calculate clinically acceptable cut-off values for sperm motility characteristics to predict IUI outcomes. Multivariable logistic regression analysis was performed to control for possible confounding factors that could affect the pregnancy outcomes of IUI. Numerical data are presented as mean ± standard deviation, and categorical variables are expressed as numbers or percentages. A p-value < 0.05 was considered to indicate statistical significance.

Results

In total, 184 infertile couples who underwent 297 IUI cycles were included in the study. The overall clinical pregnancy rate was 14.5% (43/297) per completed cycle. Of these, eight cases were lost to follow-up until delivery or termination of pregnancy, so the live birth outcome could not be confirmed. In the other 289 cycles, the abortion rate was 4.2% (12/289) and the live birth rate per cycle was 10.4% (30/289). Basal clinical and cycle-specific characteristics were compared between the two groups according to clinical pregnancy or live birth outcome (Table 1). Basal AMH levels and the number of follicles 17 mm or more in size on the hCG trigger day were significantly higher in cycles with clinical pregnancy than in those without clinical pregnancy, and the same tendency was observed for live birth. Other characteristics were not significantly different between pregnant and non-pregnant groups.

Sperm motility characteristics including initial and inseminated sperm motility or TMSC, along with the change in sperm motility after processing, were compared according to clinical pregnancy outcome (Table 2). Initial TMSC, inseminated TMSC, and post-preparation sperm motility were comparable according to clinical pregnancy outcome. Sperm motility before preparation was significantly higher in cycles that resulted in clinical pregnancy than in those that did not (71.4% ± 10.9% vs. 67.2% ± 11.7%, p = 0.020). Cycles that resulted in clinical pregnancy showed significantly fewer changes in sperm motility throughout preparation, as assessed using both subtraction (post-pre) and division (post/pre) (19.9% ± 11.5% vs. 23.4% ± 11.9%, p = 0.043 and 1.3 ± 0.2 vs. 1.4 ± 0.3, p = 0.039, respectively). These re-

| Table 1. Clinical and cycle characteristics according to the pregnancy outcome of cycles |
|-----------------------------------------------|-----------------|-----------------|---|-----------------|-----------------|
| **Variable** | **Clinical pregnancy** | **Live birth** | **p-value** | **Clinical pregnancy** | **Live birth** | **p-value** |
| | **Yes (n = 43)** | **No (n = 254)** | | **Yes (n = 30)** | **No (n = 259)** | |
| Female partner’s age (yr) | 33.4 ± 3.4 | 34.5 ± 3.9 | 0.131 | 33.2 ± 3.6 | 34.5 ± 3.9 | 0.127 |
| Male partner’s age (yr) | 36.2 ± 4.9 | 36.9 ± 4.5 | 0.204 | 36.1 ± 5.2 | 36.9 ± 4.5 | 0.150 |
| BMI (kg/m²) | 22.8 ± 3.4 | 22.6 ± 4.0 | 0.452 | 22.9 ± 3.4 | 22.6 ± 4.0 | 0.485 |
| AMH (ng/mL) | 7.0 ± 4.4 | 5.3 ± 4.3 | 0.004 | 7.7 ± 4.4 | 5.3 ± 4.3 | 0.001 |
| No. of IUI cycles | 1.6 ± 0.8 | 1.6 ± 0.8 | 0.917 | 1.6 ± 0.8 | 1.6 ± 0.8 | 0.666 |
| Primary infertility | 33 (76.7) | 182 (71.7) | 0.582 | 23 (76.7) | 185 (71.4) | 0.670 |
| Infertility diagnosis | | | 0.543 | | 0.982 |
| Unexplained | 14 (32.6) | 103 (40.6) | 7 (23.3) | 105 (40.5) | 0.204 |
| Male factor | 5 (11.6) | 24 (9.4) | 4 (13.3) | 25 (9.7) | |
| Ovulatory factor | 7 (16.3) | 26 (10.2) | 7 (23.3) | 26 (10.0) | |
| Tubal factor | 3 (7.0) | 21 (8.3) | 1 (3.3) | 22 (8.5) | |
| Combined and others | 14 (32.6) | 80 (31.5) | 11 (36.7) | 81 (31.3) | |
| Ovulation induction | | | 0.705 | 0.982 |
| Natural cycle | 2 (4.7) | 7 (2.8) | 2 (6.7) | 7 (2.7) | |
| CC only | 2 (4.7) | 26 (10.2) | 0 | 27 (10.4) | |
| Letrozole only | 1 (2.3) | 10 (3.9) | 1 (3.3) | 10 (3.9) | |
| Gn only | 5 (11.6) | 13 (5.1) | 5 (16.7) | 13 (5.0) | |
| CC+Gn | 30 (69.8) | 190 (74.8) | 20 (66.7) | 193 (74.5) | |
| Letrozole+Gn | 3 (7.0) | 8 (3.1) | 2 (6.7) | 9 (3.5) | |
| No. of follicles ≥ 17 mm | 1.6 ± 0.9 | 1.3 ± 0.6 | 0.048 | 1.6 ± 0.9 | 1.3 ± 0.7 | 0.039 |

Values are presented as mean±standard deviation or number (%). BMI, body mass index; AMH, anti-Müllerian hormone; IUI, intrauterine insemination; CC, clomiphene citrate; Gn, gonadotropin.
relationships remained significant after adjusting for the female partner’s age, basal AMH level, and number of pre-ovulatory follicles larger than 17 mm.

Similar results were obtained when examining sperm motility parameters and the live birth outcome (Table 3). Pre-preparation sperm motility was higher in cycles with live birth than in cycles without live birth (71.6% ± 12.6% vs. 67.3% ± 11.7%, p = 0.030), and this remained significant after controlling for the female partner’s age, AMH level, and number of follicles ≥ 17 mm. Cycles that resulted in live birth showed significantly fewer changes in sperm motility throughout preparation, as assessed using both subtraction (post-pre) and division (post/pre) (18.9% ± 13.1% vs. 23.4% ± 11.9%, p = 0.043 and 1.3 ± 0.3 vs. 1.4 ± 0.3, p = 0.025, respectively), but marginal significance was found for the latter measure of change after adjusting for confounding factors.

In ROC curve analysis, sperm motility after preparation and the change in sperm motility showed poor predictive power for the pregnancy outcome. The optimal threshold value of initial sperm motility to predict live birth was ≥ 72.5% with a sensitivity of 53.3% and a specificity of 70.3% (area under the curve, 0.619; p = 0.032) (Table 4). A dichotomous variable was then created by dividing the initial sperm motility into two groups (≥ 72.5% or < 72.5%) and multivariable analysis was done including this variable and other confounding factors. As a result, the predicted odds of live birth were significantly higher in cases where the pre-preparation sperm motility was ≥ 72.5% than in cycles where the initial sperm motility was < 72.5% (adjusted odds ratio, 2.741; 95% confidence interval, 1.241–6.057).

Table 2. Sperm motility characteristics according to the clinical pregnancy outcome

<table>
<thead>
<tr>
<th>Variable</th>
<th>Clinical pregnancy</th>
<th>aORa (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes (n = 43)</td>
<td>No (n = 254)</td>
<td>p-value</td>
</tr>
<tr>
<td>TMSC before preparation (× 10⁶)</td>
<td>282.0 ± 205.5</td>
<td>285.6 ± 193.2</td>
<td>0.646</td>
</tr>
<tr>
<td>TMSC after preparation (× 10⁶)</td>
<td>51.4 ± 22.9</td>
<td>51.2 ± 19.4</td>
<td>0.849</td>
</tr>
<tr>
<td>Sperm motility before preparation (%)</td>
<td>71.4 ± 10.9</td>
<td>67.2 ± 11.7</td>
<td>0.020</td>
</tr>
<tr>
<td>Sperm motility after preparation (%)</td>
<td>91.3 ± 5.9</td>
<td>90.6 ± 7.5</td>
<td>0.929</td>
</tr>
<tr>
<td>∆ Sperm motility (post-pre) (%)</td>
<td>19.9 ± 11.5</td>
<td>23.4 ± 11.9</td>
<td>0.043</td>
</tr>
<tr>
<td>∆ Sperm motility (post/pre)</td>
<td>1.3 ± 0.2</td>
<td>1.4 ± 0.3</td>
<td>0.039</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviation.
aOR, adjusted odds ratio; CI, confidence interval; TMSC, total motile sperm count.
a) Multiple logistic regression after adjusting for confounding factors (female partner’s age, anti-Müllerian hormone, and number of follicles ≥ 17 mm).

Table 3. Sperm motility characteristics according to the live birth outcome

<table>
<thead>
<tr>
<th>Variable</th>
<th>Live birth</th>
<th>aORa (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes (n = 30)</td>
<td>No (n = 259)</td>
<td>p-value</td>
</tr>
<tr>
<td>TMSC before preparation (× 10⁶)</td>
<td>256.7 ± 210.0</td>
<td>288.7 ± 194.6</td>
<td>0.143</td>
</tr>
<tr>
<td>TMSC after preparation (× 10⁶)</td>
<td>45.2 ± 21.0</td>
<td>51.4 ± 19.3</td>
<td>0.065</td>
</tr>
<tr>
<td>Sperm motility before preparation (%)</td>
<td>71.6 ± 12.6</td>
<td>67.3 ± 11.7</td>
<td>0.030</td>
</tr>
<tr>
<td>Sperm motility after preparation (%)</td>
<td>90.5 ± 6.6</td>
<td>90.6 ± 7.5</td>
<td>0.512</td>
</tr>
<tr>
<td>∆ Sperm motility (post-pre) (%)</td>
<td>18.9 ± 13.1</td>
<td>23.3 ± 11.8</td>
<td>0.025</td>
</tr>
<tr>
<td>∆ Sperm motility (post/pre)</td>
<td>1.3 ± 0.3</td>
<td>1.4 ± 0.3</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviation.
aOR, adjusted odds ratio; CI, confidence interval; TMSC, total motile sperm count.
a) Multiple logistic regression after adjusting for confounding factors (female partner’s age, anti-Müllerian hormone, and number of follicles ≥ 17 mm).

Table 4. Predictive power of sperm motility characteristics regarding live birth using ROC curve analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cut-off</th>
<th>AUC</th>
<th>95% CI</th>
<th>p-value</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm motility before preparation (%)</td>
<td>72.5</td>
<td>0.619</td>
<td>0.506–0.733</td>
<td>0.032</td>
<td>0.533</td>
<td>0.703</td>
</tr>
<tr>
<td>Sperm motility after preparation (%)</td>
<td>93.8</td>
<td>0.467</td>
<td>0.362–0.572</td>
<td>0.553</td>
<td>0.467</td>
<td>0.448</td>
</tr>
<tr>
<td>∆ Sperm motility (post-pre) (%)</td>
<td>21.5</td>
<td>0.376</td>
<td>0.266–0.485</td>
<td>0.026</td>
<td>0.333</td>
<td>0.452</td>
</tr>
<tr>
<td>∆ Sperm motility (post/pre)</td>
<td>1.3</td>
<td>0.375</td>
<td>0.265–0.485</td>
<td>0.025</td>
<td>0.333</td>
<td>0.425</td>
</tr>
</tbody>
</table>

ROC, receiver operating characteristic; AUC, area under the curve; CI, confidence interval.

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Discussion

This study revealed that initial sperm motility was the best sperm motility parameter for predicting pregnancy as an outcome of IUI compared with other parameters such as TMSC or the change in sperm motility after preparation. Pre-preparation sperm motility was higher in the pregnant group, and perhaps in this context, the change in sperm motility throughout preparation was fewer in the pregnant group. The optimal cut-off value of initial sperm motility to predict live birth was confirmed to be ≥ 72.5%.

Concerns have been raised that the risk of multiple pregnancy may increase in IUI procedures, especially in cycles with superovulation. We analyzed whether sperm characteristics were associated with multifetal pregnancy, and found that sperm motility parameters were not significantly different according to the outcome of multiple pregnancy (data not shown).

The sperm preparation process can yield as many motile spermatozoa as possible. Sperm motility characteristics before and after preparation have been evaluated as predictors of pregnancy after IUI. A retrospective study that investigated 1,007 IUI cycles reported that initial sperm motility and forward progression of processed sperm were independently associated with clinical pregnancy [19]. However, another retrospective study of 383 IUI cycles stated that initial sperm concentration, motility, and the percentage of rapid sperm were significantly different according to whether pregnancy was achieved, but no significant relationship was found for the sperm parameters after preparation [20].

As described above, several studies have investigated various sperm motility characteristics before and after processing, but we could only discover one study that analyzed the impact of changes in parameters after sperm preparation. Freour et al. [15] reported that computer-assisted sperm analysis (CASA) parameters and their changes after processing were comparable according to the pregnancy outcome, whereas improvement in the amplitude of lateral head displacement (ALH) of spermatozoa during preparation predicted clinical pregnancy well in IUI cycles with frozen-thawed donor semen.

In the present study, we found that couples with higher pre-preparation sperm motility showed better pregnancy outcomes in IUI cycles. This finding is consistent with the results of previous studies [19-22]. The change in motility during sperm preparation, which we initially tried to study, was found to be fewer in cycles that resulted in pregnancy than in those that did not. A reasonable interpretation of these results is that higher initial motility, rather than insufficient improvement in sperm motility through processing, can better predict pregnancy in IUI cycles.

Regarding the threshold of initial sperm motility for predicting conception after IUI, previous studies mostly suggested a threshold of 30%. In our study, the optimal cut-off value for pre-preparation sperm motility was 72.5%, which is considerably higher than that of existing studies. This may be related to differences in patient characteristics. This study was conducted at one of the largest university-based hospitals in Korea, and patients with a relatively old age or long duration of infertility tend to visit. Indeed, in two papers that previously proposed a 30% cut-off for sperm motility, the female participants were much younger than those in the current study, and thus they were probably patients with a better prognosis [21,22]. Zhao et al. [19] suggested 80% as a threshold of initial sperm motility, similar to our study, and the average age of female subjects in their study was 35.2 ± 4.5 years, also similar to our study.

In the current study, neither pre- nor post-preparation TMSC was correlated with the pregnancy outcomes of IUI. Initial or post-wash TMSC was previously reported to predict pregnancy after IUI in some studies. According to a systematic review, the most commonly suggested cut-off values for initial TMSC were 5–10 × 10⁶, and 0.8–5 × 10⁶ for processed TMSC in predicting IUI success [1]. The mean initial and post-processed TMSC of our subjects were approximately 285 × 10⁶ and 51 × 10⁶, respectively, both much higher than the previously suggested thresholds. Zhao et al. [19] stated that inseminated specimens with TMSC values ranging from 11–100 × 10⁶ resulted in the highest pregnancy rate after IUI, and the conception rate fell when TMSC was higher than 100 × 10⁶. Hansen et al. [23] also reported that the final TMSC available for IUI was significantly higher in the live birth group, with TMSC up to 20 × 10⁶, but that the live birth rate did not increase after the TMSC exceeded 20 × 10⁶. Among our study subjects, only 10% had male factor infertility, and even only mild male factors that exceeded threshold values were included, so which might explain why TMSC did not significantly differ according to the pregnancy outcome. Furthermore, the overall low live birth rate (and thus, the small number of cycles with live births) could have limited our ability to detect significant differences. Additional large-scale prospective studies with appropriate subjects are needed.

Two existing studies reported that post-wash sperm motility was related to pregnancy after IUI [24,25]. In our study, post-preparation sperm motility was similar in cycles regardless of whether pregnancy was achieved. These observed differences may have resulted from different patient populations and sperm preparation methods. Two previous studies used the sperm wash technique and our study used the density gradient method for sperm preparation. Moreover, rather than improving sperm motility, a small number of progressively motile spermatozoa are filtered out and sperm may become hyperactivated during preparation. Therefore, it may be hyperactivated sperm, not final sperm motility itself, that affects the pregnancy outcomes of IUI.

Hyperactivation of sperm is known to be critical to achieve fertilization because it assists sperm in reaching oocytes and penetrating...
the zona pellucida. CASA can identify hyperactivated sperm using threshold values for curvilinear velocity and path linearity. As an indirect measure of flagellar bend amplitude, ALH can also be used to assess hyperactivation [26]. As mentioned above, Freour et al. [15] reported that ALH progression during sperm preparation was a good predictor of IUI success. This might indirectly confirm the importance of sperm hyperactivation through processing. It is necessary to evaluate various CASA parameters, especially regarding hyperactivation, and to further study the effect of their changes in the preparation process on pregnancy outcomes.

To the best of our knowledge, this is the first study to explore the effects of changes in motility during sperm preparation on IUI outcomes using autologous semen. This study was performed at a single center, so the overall IUI regimen (specifically the methods of ovulation induction or luteal phase support) was relatively consistent. In addition, while the majority of the aforementioned studies investigated clinical pregnancy as a pregnancy outcome, it is meaningful that we examined both the clinical pregnancy rate and the live birth rate as outcomes in the present study.

However, this study has the inherent limitations of a retrospective design. The relatively small sample size and inability to confirm the cumulative live birth rates are also limitations of this study. Furthermore, previous studies that highlighted the effects of sperm motility on IUI outcomes mostly emphasized the importance of rapid progressive and linear motility [20,27,28]. Unfortunately, since our center performed semen analysis manually on the IUI day, we could not properly evaluate a full variety of sperm motility parameters.

In conclusion, initial sperm motility better predicted live birth after IUI cycles than the change in motility after preparation or the final sperm motility. According to our study, even though sperm motility is substantially improved by the preparation process, pregnancy results may not be favorable if the initial sperm motility is relatively low. With this in mind, we can provide appropriate consultations to infertile couples undergoing IUI procedures. Further well-designed prospective studies should be conducted to confirm these findings.

Conflict of interest

Byung Chul Jee is an Editor-in-Chief and Seul Ki Kim is an Associate Editor of the journal, but they were not involved in the peer reviewer selection, evaluation, or decision process of this article. No other potential conflicts of interest relevant to this article were reported.

Acknowledgments

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References


Author contributions

Conceptualization: MJ. Data curation: MJ. Formal analysis: MJ, HK. Methodology: MJ, SKK, JRL. Project administration: JRL, BCJ, SHK. Writing–original draft: MJ. Writing–review & editing: all authors.


Subcutaneous progesterone versus vaginal progesterone for luteal phase support in *in vitro* fertilization: A retrospective analysis from daily clinical practice

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**Objective:** Progesterone application for luteal phase support is a well-established concept in *in vitro* fertilization (IVF) treatment. Water-soluble subcutaneous progesterone injections have shown pregnancy rates equivalent to those observed in patients receiving vaginal administration in randomized controlled trials. Our study aimed to investigate whether the results from those pivotal trials could be reproduced in daily clinical practice in an unselected patient population.

**Methods:** In this retrospective cohort study in non-standardized daily clinical practice, we compared 273 IVF cycles from 195 women undergoing IVF at our center for luteal phase support with vaginal administration of 200 mg of micronized progesterone three times daily or subcutaneous injection of 25 mg of progesterone per day.

**Results:** Various patient characteristics including age, weight, height, number of oocytes, and body mass index were similar between both groups. We observed no significant differences in the clinical pregnancy rate (CPR) per treatment cycle between the subcutaneous (39.9%) and vaginal group (36.5%) ($p=0.630$). Covariate analysis showed significant correlations of the number of transferred embryos and the total dosage of stimulation medication with the CPR. However, after adjustment of the CPR for these covariates using a regression model, no significant difference was observed between the two groups (odds ratio, 0.956; 95% confidence interval, 0.512–1.786; $p=0.888$).

**Conclusion:** In agreement with randomized controlled trials in study populations with strict selection criteria, our study determined that subcutaneous progesterone was equally effective as vaginally applied progesterone in daily clinical practice in an unselected patient population.

**Keywords:** Fertilization *in vitro*; Human; Intravaginal administration; Luteal phase; Pregnancy rate; Progesterone; Reproduction; Retrospective studies; Subcutaneous injections

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**Introduction**

Luteal phase defects (LPDs) are a common issue in stimulated *in vitro* fertilization (IVF) cycles. The main causes of LPDs include supra-physiological concentrations of steroids, inhibition of luteinizing hormone (LH) release, premature luteolysis, and loss of progesterone synthesis [1]. Therefore, it is essential to supply women undergoing IVF with exogenous progesterone or human chorionic gonadotropin (hCG) [2]. As hCG injections are equally effective, but are associated with a higher risk of ovarian hyperstimulation syndrome, progester-
one has become the treatment of choice for luteal phase support in assisted reproduction [3,4].

Progesterone can be applied orally, vaginally, intramuscularly, or subcutaneously, and each route has certain advantages and disadvantages. Subcutaneous progesterone injection was introduced as a water-soluble compound at a daily dose of 25 mg in 2014 [5]. Clinical trials revealed that a daily dose of 25 mg of subcutaneous progesterone is sufficient to reach endometrial receptivity, even in the absence of endogenous progesterone [6]. A prospective study on patients’ opinions demonstrated a higher level of satisfaction in patients undergoing subcutaneous progesterone injections than among those undergoing administration through the vaginal route [7]. Although vaginal administration is widely used in the majority of IVF centers globally, a reasonable number of patients prefer injections [8-10]. Subcutaneous progesterone has been studied and approved based on two large, randomized, controlled phase III trials in Europe and the USA during 2013, establishing that patients undergoing IVF treated with subcutaneous progesterone injections exhibited similar pregnancy rates to those of patients using a vaginal gel or tablets [11,12].

At the time of the study, our center administered either vaginal micronized progesterone at 600 mg per day or, at patients’ discretion, daily subcutaneous progesterone. Our study was intended to investigate whether the results from earlier pivotal trials could be reproduced in daily clinical practice in an unselected patient population to establish whether subcutaneous progesterone is as effective as vaginal progesterone and results in the same pregnancy rates.

Methods

1. Study design and participants

This retrospective study was based on a cohort undergoing all stimulation cycles for IVF or intracytoplasmic sperm injection (ICSI) treatment between July 23, 2015, and February 11, 2017, with at least one embryo transfer at a university hospital in Marburg, Germany. Patients were included irrespective of previous treatment cycles, underlying diagnoses, current stimulation regimes, or dosing. Frozen embryo transfer cycles and patients with an endometrial thickness < 6 mm were not included.

Appropriate, individualized stimulation procedures were selected based on patient-specific characteristics, including anti-Müllerian hormone level, weight, and antral follicle count at the beginning of the stimulation, as well as preliminary results from previous stimulation cycles. Patients received for luteal phase support either vaginal administration of 200 mg of micronized progesterone three times daily using Progestan (Dr. KADE/BESINS Pharma, Berlin, Germany), Utrogestan (Kohlpharma, Merzig, Germany) and Famenita (Exeltis Germany, Ismaning, Germany) or 25 mg of a water-soluble and subcutaneous injectable complex of progesterone (Prolutex; Marckyl Pharma, Papenburg, Germany) once daily. The clinical pregnancy rate (CPR) was calculated based on successful pregnancies, which were determined by sonographically verified evidence of a gestational sac per transfer.

The data used in this study were collected as part of clinical treatment processes using the internal management and documentation program Meditex IVF (Critex, Regensburg, Germany). Patient baseline data, sterility-related factors, and any known previous treatments were recorded during clinical routines and analyzed anonymously according to local and European ethics and data protection regulations. As part of a routine clinical follow-up, pregnancies and births were documented according to Germany’s IVF registry and quality management obligations.

2. Stimulation protocol

In the short protocol, the ovarian stimulation started on the second or third day of the cycle using stimulation pens and injection accessories. The stimulation drugs used in this study were recombinant follicle-stimulating hormone (rFSH; Gonalf, Merck Serono, Darmstadt, Germany or Ovapeal, Teva, Ulm, Germany or Puregon, MSD Sharp & Dohme, Haar, Germany) and/or human menopausal gonadotropin (Menogon; Ferring, Kiel, Germany). Some of the patients also received rFSH and recombinant LH (Pergoveris, Merck Serono).

In the long protocol, a GnRH analogue was administered in the month preceding the stimulation from the middle of the luteal phase. The active ingredient nafarelin (Synarel; Pfizer, New York, NY, USA) was used in a nasal application form at 0.4 mg/day. The subgroup of female patients who underwent a natural cycle received either no stimulation or oral stimulation using clomiphene citrate, a selective estrogen receptor modulator (Clomifén, Ferring).

3. Statistical analysis

The two-tailed t-test was used to analyze and compare the two groups’ baseline characteristics. A mixed logistic regression model was used to compare the per-cycle pregnancy rates between the subcutaneous and vaginal groups while accounting for intra-patient correlations between multiple cycles in the same patients. The regression model was expanded to include each of the potential predictors: age, body mass index (BMI), transfer day, number of embryos transferred, medications used (stimulation), total stimulation medication dosage, and the stimulation protocol. We used a model including all of the predictors listed to account for confounding in comparisons of pregnancy between the subcutaneous and vaginal groups. The two-tailed t-test was conducted using IBM SPSS ver. 27 (IBM Corp., Armonk, NY, USA). The R programming environment was
applied for the mixed logistic regression model.

**Results**

During the study period, 195 women met the inclusion criteria of the study. The women underwent 273 IVF cycles. In 197 cycles, the women received vaginal micronized progesterone in soft capsules (200 mg of Progestan, Utrogestan, Famenita) three times daily, and in 76 cycles, they received daily subcutaneous progesterone through injections of an aqueous solution (Prolutex, 25 mg). The treatments started on the day of oocyte aspiration and lasted until 14 days after transfer or, if there was a positive pregnancy test, up to the 12th week of gestation.

Patients' demographic and baseline characteristics are presented in Table 1. No significant differences were observed between the treatment groups in age, weight, height, number of oocytes, number of inseminated oocytes, or the rate of blastocyst transfer. The mean BMI values were normal ($< 25 \text{ kg/m}^2$) and comparable between the two groups (24.4 ± 4.48 kg/m$^2$ in the vaginal group and 24.8 ± 4.94 kg/m$^2$ in the subcutaneous group) (Table 1).

In the subcutaneous progesterone group, 31.6% of embryos were transferred at the cleavage stage and 68.4% at the blastocyst stage, while these proportions in the vaginal progesterone group were 55.8% at the cleavage stage and 44.2% at the blastocyst stage. The stimulation characteristics (medication and protocol) varied between the treatment groups. However, the total doses of FSH stimulation medication used in the two groups were similar (2,217.4 ± 989.3 IU/mL in the vaginal group vs. 2,297 ± 692.9 IU/mL in the subcutaneous group) (Table 1).

There was no significant difference in the CPR per treatment cycle between the subcutaneous (39.9%) and vaginal groups (36.5%, $p = 0.630$) (Table 2). Moreover, the rates of live births per embryo transfer (28.9% in the vaginal group vs. 30.2% in the subcutaneous group, $p = 0.887$), implantation (25.8% in the vaginal group vs. 32.2% in the subcutaneous group, $p = 0.261$) and early spontaneous abortion (35% in the vaginal group vs. 39.5% in the subcutaneous group, $p = 0.386$) were also similar between the two groups (Table 2).

Investigating the influence of clinical covariates on pregnancy outcomes showed that the number of transferred embryos, embryo transfer day, and total dosage of stimulation medication correlated significantly with the CPR (Table 3). However, when the CPR was adjusted for these covariates using the regression model, no statistically

---

**Table 1.** Demographic characteristics and fertility treatment parameters in the study population

<table>
<thead>
<tr>
<th>Variable</th>
<th>Vaginal progesterone (n = 197)</th>
<th>Subcutaneous progesterone (n = 76)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>36.2 ± 4.21</td>
<td>35.2 ± 3.63</td>
<td>0.211</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68.1 ± 14.2</td>
<td>69.8 ± 13.6</td>
<td>0.308</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>167 ± 6.59</td>
<td>168 ± 6.85</td>
<td>0.243</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>24.4 ± 4.48</td>
<td>24.8 ± 4.94</td>
<td>0.440</td>
</tr>
<tr>
<td>Number of oocytes</td>
<td>7.71 ± 4.69</td>
<td>8.58 ± 4.14</td>
<td>0.154</td>
</tr>
<tr>
<td>Number of inseminated oocytes</td>
<td>6.09 ± 3.90</td>
<td>6.70 ± 3.56</td>
<td>0.253</td>
</tr>
<tr>
<td>Transfer</td>
<td></td>
<td></td>
<td>0.057</td>
</tr>
<tr>
<td>Early transfer</td>
<td>87 (44.2)</td>
<td>24 (31.6)</td>
<td></td>
</tr>
<tr>
<td>Blastocyst culture</td>
<td>110 (55.8)</td>
<td>52 (68.4)</td>
<td></td>
</tr>
<tr>
<td>Number of transferred embryos</td>
<td>1.6 ± 0.5</td>
<td>1.70 ± 0.52</td>
<td>0.169</td>
</tr>
<tr>
<td>Number of embryos</td>
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<td>0.169</td>
</tr>
<tr>
<td>1</td>
<td>79 (40.1)</td>
<td>27 (35.5)</td>
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<tr>
<td>2</td>
<td>118 (59.9)</td>
<td>49 (64.5)</td>
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<td>Stimulation medication</td>
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<td>0.054</td>
</tr>
<tr>
<td>Recombinant FSH</td>
<td>146 (74.9)</td>
<td>64 (84.2)</td>
<td></td>
</tr>
<tr>
<td>FSH+LH</td>
<td>30 (15.4)</td>
<td>12 (15.8)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>19 (9.74)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Stimulation protocol</td>
<td></td>
<td></td>
<td>0.872</td>
</tr>
<tr>
<td>Short</td>
<td>130 (66.0)</td>
<td>46 (60.5)</td>
<td></td>
</tr>
<tr>
<td>Long</td>
<td>37 (18.8)</td>
<td>24 (31.6)</td>
<td></td>
</tr>
<tr>
<td>Flare up</td>
<td>18 (9.14)</td>
<td>6 (7.89)</td>
<td></td>
</tr>
<tr>
<td>Natural cycle</td>
<td>12 (6.09)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total stimulation dosage (IU/mL)</td>
<td>2,217.4 ± 989.3</td>
<td>2,297 ± 692.9</td>
<td>0.078</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation or number of patients (%).

BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone.
significant difference was found between the subcutaneous and vaginal groups (odds ratio, 0.956; 95% confidence interval, 0.512–1.786; \( p = 0.888 \)) (Table 4).

**Discussion**

The results of our study showed that subcutaneous injections of progesterone were as effective as vaginal application in terms of the CPR in daily clinical practice in an unselected patient population. This study was intended to evaluate differences in the CPR between groups of unselected patients receiving either subcutaneous injections or transvaginal insertion of progesterone for luteal phase support in daily clinical practice. The groups were comparable in terms of their baseline characteristics. As expected from the pivotal studies, the CPR did not differ significantly in simple comparisons (Table 2). After adjustment for confounding factors, the slightly higher rate in the subcutaneous group remained nonsignificant (Table 4).

In 2014, Lockwood et al. [11] reported the first phase III randomized study in Europe to determine the safety, tolerability, and efficacy of the subcutaneous progesterone formulation compared with standard vaginal progesterone gel for luteal phase support in women undergoing IVF/ICSI cycles. In that study, the ongoing pregnancy rate per retrieval was similar between groups receiving 25 mg of subcutaneous Prolutex (27.4%) and 90 mg of vaginal Crinone 8% gel (30.5%). In the same year, Baker et al. [12] recruited 800 women in the United States undergoing IVF for the second phase III randomized multicenter trial, comparing 25 mg of subcutaneous progesterone (Prolutex) to 100 mg of vaginal micronized progesterone (Endometrin) twice daily. Despite differences in the vaginal preparations between the two studies, there were no significant differences in clinical outcomes, including the pregnancy rate per retrieval, between subcutaneous (41.6%) and vaginal (44.4%) groups [12], indicating that subcutaneous application is not inferior to vaginal progesterone for luteal phase support.

These studies were the basis of the approval of subcutaneous progesterone for treatment. Randomized clinical trials are needed to evaluate medical outcomes, but those studies need to be done with highly specific patient populations. To our knowledge, no publications to date have confirmed the reproductive results of a randomized controlled trial in daily clinical practice. Of note, Baker et al. [12]

Table 2. Pregnancy, live birth, implantation, and early spontaneous abortion rates by treatment group

<table>
<thead>
<tr>
<th>Variable</th>
<th>Vaginal progesterone (n = 197)</th>
<th>Subcutaneous progesterone (n = 76)</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnancy rate per cycle (%)</td>
<td>36.5</td>
<td>39.9</td>
<td>0.630</td>
</tr>
<tr>
<td>Live birth per embryo transfer rate (%)</td>
<td>28.9</td>
<td>30.2</td>
<td>0.887</td>
</tr>
<tr>
<td>Implantation rate (%)</td>
<td>25.8</td>
<td>32.2</td>
<td>0.261</td>
</tr>
<tr>
<td>Early spontaneous abortion rate (%)</td>
<td>35.0</td>
<td>39.5</td>
<td>0.386</td>
</tr>
</tbody>
</table>

Table 3. Odds ratio and 95% CI for contrasts according to each of the covariates included in the model of the pregnancy rate per cycle

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Contrast</th>
<th>OR (95% CI vs. pregnancy rate per cycle)</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Per year</td>
<td>0.929 (0.868–0.994)</td>
<td>0.034</td>
</tr>
<tr>
<td>BMI</td>
<td>Per kg/m(^2)</td>
<td>0.996 (0.940–1.055)</td>
<td>0.887</td>
</tr>
<tr>
<td>Number of embryos</td>
<td>2 vs. 1</td>
<td>3.525 (1.881–6.606)</td>
<td>0.000</td>
</tr>
<tr>
<td>Transfer day</td>
<td>Blastocyst culture vs. early transfer</td>
<td>2.614 (1.471–4.646)</td>
<td>0.001</td>
</tr>
<tr>
<td>Total dosage</td>
<td>Per 100 IU</td>
<td>0.961 (0.931–0.990)</td>
<td>0.010</td>
</tr>
<tr>
<td>Stimulation protocol</td>
<td>Flare up vs. short</td>
<td>0.393 (0.105–1.470)</td>
<td>0.091</td>
</tr>
<tr>
<td></td>
<td>Natural cycle vs. short</td>
<td>0.120 (0.009–1.578)</td>
<td>0.050</td>
</tr>
<tr>
<td></td>
<td>Long vs. short</td>
<td>0.769 (0.348–1.697)</td>
<td>0.428</td>
</tr>
</tbody>
</table>

Table 4. Probability of pregnancy rate per cycle in treatment groups together with the odds ratio and 95% CI for the simple group comparison and after adjustment for the following covariates: age, BMI, number of embryos, transfer day, total dosage, and stimulation protocol

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Vaginal</th>
<th>Subcutaneous</th>
<th>Vaginal vs. subcutaneous</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnancy adjusted</td>
<td>0.365 (0.296–0.440)</td>
<td>0.399 (0.287–0.523)</td>
<td>1.155 (0.642–2.080)</td>
<td>0.630</td>
</tr>
<tr>
<td>Pregnancy adjusted</td>
<td>0.956 (0.512–1.786)</td>
<td>0.888</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as odds ratio (95% CI).

CI, confidence interval; OR, odds ratio; BMI, body mass index.
used 200 mg of Endometrin daily in the US trial, whereas the recommended dosage of vaginal micronized progesterone is 600 mg daily. This may be due to the fact that the method and dosage of vaginal application appear to be irrelevant in luteal phase supplementation [13]; however, as micronized vaginal progesterone is widely used, it is of interest to compare the recommended 600 mg daily dosage of vaginal micronized progesterone to subcutaneous application. Therefore, our study is the first of its kind to demonstrate no significant difference in the CPR between unselected patient cohorts receiving 25 mg subcutaneous injections (39.9%) or 600 mg of micronized vaginal progesterone (36.5%) in daily clinical practice. BMI, as a critical factor in assisted reproductive technology (a BMI ≥ 25 kg/m² leads to statistically significantly lower live birth rates) [14], was normal in our cohort (24.4 kg/m² in the vaginal group and 24.8 kg/m² in the subcutaneous group) (Table 1). The BMI values were also normal and similar between groups in both the European and U.S. trials [11,12], so there is limited knowledge of the possible impacts of being overweight on pregnancy outcomes with subcutaneous progesterone. Further studies could be designed with a broader range of BMI to investigate possible differences between progesterone types used for luteal phase support in IVF for overweight patients [15,16].

This study faces several limitations. First of all, the retrospective design does not allow any causal conclusions, and the control of bias was limited. After adjustment for confounding factors, patients on subcutaneous progesterone showed a nonsignificantly different CPR (Table 4). The nonsignificant difference in CPR might well point in a certain direction, and further studies on subgroups such as older women or obese patients might show relevant differences in pregnancy outcomes. This study was obviously not randomized. Patients were offered both options and given neutral explanations of the advantages and disadvantages of each treatment, but a subtle selection bias cannot be eliminated. However, the comparison of baseline data showed no major differences between the groups. Subcutaneously treated patients were slightly younger (35.2 ± 3.63 vs. 36.2 ± 4.21 years), received a few more oocytes (8.58 ± 4.14 vs. 7.71 ± 4.69), and had slightly more blastocyst transfers (68.4% vs. 55.8%). These might well explain the somewhat higher CPR in the unadjusted comparison (Table 2). After adjustment for these critical factors, a nonsignificant difference in the CPR was shown. In addition, the live birth rate per embryo transfer (28.9% vs. 30.2%) and the implantation rate (25.8% vs. 32.2%) did not differ significantly between the two groups. This should encourage further research, as there might be a particular population that could benefit from one or the other treatment.

In conclusion, our study confirms the results of pivotal RCTs in clinical practice through a comparison of subcutaneous progesterone to vaginal micronized progesterone (200 mg of Progestan, Utrogestan, Famenita, three times daily). Recent reviews of multiple clinical trials have shown that using different progesterone formulations for luteal support did not affect the pregnancy rates [17]. Therefore, women undergoing IVF have multiple choices for an appropriate progesterone administration route, and subcutaneous application appears to be an effective choice. Still, several questions concerning luteal phase support remain unanswered and require further evaluation.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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References


Birth of a healthy infant after bone marrow-derived cell therapy


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Bone marrow-derived cell (BMDC) therapy has numerous applications as potential biological cells for use in regenerative medicine. Here, we present an original case of endometrial atrophy associated with genital tuberculosis in a woman who achieved a live birth with BMDC. This 27-year-old woman came to our center with endometrial atrophy and primary infertility. She had a past history of genital tuberculosis and amenorrhea. Her husband's semen quality was normal. The patient was counseled for hysteroscopy due to thin endometrium and advised in vitro fertilization (IVF) with donor eggs in lieu of poor ovarian reserve. Several attempts of IVF with hormone replacement therapy (HRT) were made, but the desired thickness of the endometrium was not achieved. Uterine artery injection of BMDC through interventional radiology was given, followed by HRT for three months, which resulted in improved endometrium. This was subsequently followed by IVF with donor egg. The treatment resulted in the conception and delivery of a 3.1-kg baby boy through lower segment caesarean section with no antenatal, intranatal or postnatal complications. Recently, there has been massive interest in stem cells as a novel treatment method for regenerative medicine, and more specifically for the regeneration of human endometrium disorders like Asherman syndrome and thin endometrium, which was the reason behind using this strategy for treatment.

Keywords: Asherman's syndrome; Bone marrow-derived cell therapy; Endometrial atrophy; Regenerative medicine; Stem cell therapy

Introduction

The human endometrium is a dynamic remodeling tissue that undergoes more than 400 cycles of growth, differentiation, and shedding of endometrial cells during the reproductive period [1]. Asherman syndrome (AS) is an uncommon gynecological disorder caused by the destruction of the endometrium due to repeated or aggressive curettages and/or endometritis. As a result, there is a loss of functional endometrium in many areas, and the uterine cavity is obliterated by intrauterine adhesions, leading to amenorrhea, hypomenorrhea, infertility, recurrent pregnancy loss, and/or abnormal placentation, including placenta previa and accrete [2,3].

Endometrial atrophy (EA) is another rare condition in which the endometrium is too thin and never grows more than 5 mm thick. Factors that can cause EA include prolonged use of oral contraceptives and Tamoxifen. Genital tuberculosis (TB) affects endometrial receptivity leading to defective endometrial markers and EA. The prevalence of this pathology is 0.5% in infertile women undergoing assisted reproductive treatments (ARTs) [2].

Successful implantation in ART requires a high-quality embryo, receptive endometrium, and perfect embryo transfer technique. Current treatment strategies for AS aim to break up the synechiae and induce endometrial proliferation in order to restore functionality to the uterine cavity. Unfortunately, the risk of treatment failure is high. These fibrotic synechiae often lack endometrial lining entirely, or, if present, the lining is thin and largely nonfunctional [4].

Evidence suggests that adult stem cell populations exist in the human endometrium. Hence it might be possible to activate endogenous endometrial stem/progenitor cells in cases of atrophic or thin endometrium or to transplant bone marrow-derived cells (BMDCs) in the uterine lining for endometrial regeneration in AS or severe cases of intrauterine adhesions. Low levels of circulating BM-derived hematopoietic stem cells, mesenchymal stem cells, and endothelial progeni-
tor cells integrate into damaged tissues and trans-differentiate into host tissues, including the endometrium. Evidence is available where BMDC has been used to treat AS and EA in an attempt to achieve normal reproductive functioning of the endometrium [1,5,6].

Case report

A 27-year-old female presented to our center on May 15, 2017. Her husband’s age was 29 years, and they had been married for the last 4 years. The patient gave a history of secondary amenorrhea for 2 years and she informed that her menstrual cycle was very irregular (just drops during her periods) but denied any abdominal pain or cramps during this time. Her last menstrual period (LMP) was April 8, 2017.

Previous medical history revealed that she had genital TB 2 years before presentation to our clinic. TB was diagnosed by laparohysteroscopy done at another center, which revealed tubercles in the whole abdomen. Her husband’s semen analysis was found to be normal at 46 million counts, and motility was 54%. Transvaginal sonography was performed, which revealed that the endometrial lining was very thin with fluid (<5 mm) (Figure 1). She was given estradiol 2-mg tablet for 10 days but there was no improvement in the lining of the endometrium (Figure 2). On May 29, 2017, hysteroscopy was performed on the 14th day of the cycle, which showed that the cervix was stenosed and the uterus was acutely anteverted. Bands of adhesions were seen on the fundus and lateral walls (Figure 3). The fibrotic bands of adhesions were cut, and lateral metroplasty was done to enlarge the cavity. Hormone replacement therapy (HRT) was continued, and progesterone (10 mg/day) was added on June 18 for 5 days. The patient got withdrawal bleeding (very scanty on July 8).

Second attempt to prepare the endometrium was made in July with HRT, which comprised of Estradiol 2 mg, Norethisterone along with the addition of Estrogel three times a day (subcutaneous estrogen supplementation) and Aspirin 75 mg three times a day. Endometrial thickness was measured on the 8th day, which was 4.7 mm. On the 15th day, it was observed that the endometrium was patchy, and endometrial thickness was 5.5 mm. Progesterone was given to induce bleeding. She had scanty bleeding on the 14th of August.

Endometrial preparation was again tried with HRT plus adjuvants such as Arginitic sachet and Sildenafil. On August 30, 2017, the ultrasound showed an endometrial thickness of 4.3 mm with endometrial fluid. So, on September 5, 2017, a relook hysteroscopy was done, which revealed no adhesion in the cavity, and the endometrium was...
found to be pink (Figure 4). The patient did not come to the clinic for 3 months after this visit.

The patient reported to the center again on December 25, 2017, with LMP on December 20, 2017 (scanty bleeding). Endometrial preparation was tried with gonadotropin stimulation (injection HMG 75 mg) plus adjuvants such as Arginitric sachet and Sildenafil. On the 13th day, the endometrial thickness was 5.5 mm, and one follicle was seen on the left side of size 20 mm. Human chorionic gonadotropin (HCG) trigger was given on the 15th day. On the day of embryo transfer, endometrial thickness was 5.8 mm with a patchy endometrium. Embryo transfer was done, but with negative results.

The patient stopped all treatment after that visit and came back after 1 year. It was observed on studying the records that despite giving HRT, gonadotropin and adjuvants multiple times, the endometrial thickness was not achieved. So, the patient was counseled about the BMDC therapy for which she gave her consent.

1. Pre-procedure workup for BMDC

The patient was admitted on January 16, 2019, early morning and pre-procedure workup was commenced with injection Ceftriaxone 1g IV statim, O2 @ 2–4 L/min for 30 minutes, injection vitamin C150 mg (diluted in 100 mL normal saline [NS]), and injection glutathione 600 mg (diluted in 100 mL NS).

2. Collection of bone marrow aspirate

The patient was made to lie down in the horizontal position. The procedure began 1 hour later on the same day with sedation of the patient with intravenous Midazolam and Fentanyl. Approximately, 60ml of bone marrow aspirate (BMA) was collected over acid citrate dextrose-anticoagulant (5 mL) from the patient’s anterior superior spine of the iliac crest. The position of the Jamshidi needle was confirmed by loss of resistance after piercing the compact bone. BMA was collected in a 60 mL syringe in a series of discrete pulls on the plunger (targeting a collection of 5–10 mL per pull) with repositioning of the needle tip between pulls based on the reported enrichment of progenitor cells.

3. Preparation of BMDC

About 10 mL of diluted BMA (1:2 with sterile phosphate buffer saline [PBS]) was aseptically transferred to a 15.0 mL sterile centrifuge on top of a 3.0 mL density gradient solution. The tube was then centrifuged at 2000 RPM for 30 minutes at room temperature (15°C–25°C). This centrifugation step eliminated erythrocytes and poly leukocytes from the mixture of cells and a sharp band appeared containing mononuclear cells (BMDC). BMDC was then collected in an aseptic tube, washed with PBS twice, and counted using a hemocytometer. Cell viability was analyzed using trypan blue assay, and then cells (15 million cells/mL) were prepared for administration to the patient without any further manipulations.

4. Intra-arterial injection

Two hours after BMA and proper consent, the patient was taken for intra-arterial injection into the catheterization laboratory. Under aseptic precautions, bilateral femoral artery access was taken with 6-Fr sheath via the Seldinger technique under local anesthesia. Six-Fr diagnostic JR catheter was used to reach the contralateral internal iliac artery over a 0.035-inch Terumo guide wire. It was then exchanged for 6-Fr JR 3.5 guiding catheter over 0.035-inch stiff wire. The uterine artery was identified by contrast dye injection in the internal iliac artery. Super selective cannulation of the uterine artery and subsequently spiral arterioles was done with a microcatheter over 0.014-inch PTCA workhorse wire. On January 16, 2019, uterine artery spiral arterioles BMDC treatment was performed. Patency of artery post-injection was confirmed by contrast dye injection from guide catheter through the internal iliac artery. Post-procedure femoral sheaths were removed in the catheterization laboratory with manual compression, and lower limb immobilization was done in the supine position for 6 hours in the recovery room. The patient was slowly mobilized under observation and discharged the next day.

5. Post-procedure follow-up

The patient was put on HRT for 3 months consisting of sequential estrogen and progesterone therapy. In April, we checked her uterine

![Figure 4. Relook hysteroscopy showing endometrium without adhesion.](https://doi.org/10.5653/cerm.2020.04252)
lining with HRT and the serial scan showed good triple line and improved thickness (Figure 5). The endometrium was monitored every month until the month of frozen embryo transfer (FET). The patient showed an increase in the volume of menstrual blood flow for three cycles. Description of menstrual flow after the therapy is described in Table 1.

6. Frozen embryo transfer

Formation of embryos with donor eggs was performed. A total of 10 eggs were retrieved from a healthy donor of 31 years of age, fertilized with the patient's sperm sample. Four blastocysts were formed for prospective embryo transfer. FET was performed in May, endometrial preparation was done by HRT comprising of tablet Estradiol valerate 2 mg, Estrogel thrice a day, Arginitric sachet thrice a day and Aspirin 75 mg. On the 8th day of HRT, endometrial thickness was 5.4 mm, and on day 11, it was found to be 7.1 mm. Post-bone marrow-derived stem cells (BMDSC) in the endometrium showed improved response to HRT in terms of the thickness and echo texture both (Figure 6). Color doppler was favorable. Injection progesterone for 5 days was added for blastocyst transfer. The blastocyst was frozen under Kitazato vitrification and warming protocol before transfer.

On May 30, 2019, the embryo transfer was performed smoothly without any difficulty. Only one blastocyst of grade 3AA was transferred. Luteal phase support was given, and beta HCG was tested on June 14, 2019, which turned out to be 1,212 mg/dL. Doubling titer repeated on July 16, 2019, was 3,286.6, confirming a positive result. The first scan was performed at around 7 weeks that showed single live intrauterine pregnancy (Figure 7). The antenatal period was uneventful, except for an episode of preterm pain at around 24 weeks. Planned lower segment caesarean section (LSCS) was performed after 38 weeks of gestation. A 3.1-kg baby boy was delivered, and LSCS was uneventful.

**Table 1.** Description of menstrual flow after the therapy

<table>
<thead>
<tr>
<th>Date</th>
<th>No. of pads used</th>
</tr>
</thead>
<tbody>
<tr>
<td>January 28, 2019</td>
<td>2–3</td>
</tr>
<tr>
<td>February 26, 2019</td>
<td>3–4</td>
</tr>
<tr>
<td>March 27, 2019</td>
<td>4</td>
</tr>
<tr>
<td>April 24, 2019</td>
<td>4</td>
</tr>
<tr>
<td>May 13, 2019</td>
<td>3–4</td>
</tr>
</tbody>
</table>

**Figure 5.** Post-bone marrow-derived stem cells: the response to hormone replacement therapy showed drastic improvement in lining, thickness as well as echo texture in the April 2019.

**Figure 6.** Post-bone marrow-derived stem cells: the response to hormone replacement therapy showed drastic improvement in lining, thickness as well as echo texture in May 2019 when frozen embryo transfer was done.

**Figure 7.** Scan showing single live intrauterine pregnancy.
Discussion

Adult bone marrow is a reservoir of stem and progenitor cells. BMDCs can trans differentiate into multiple non-hematopoietic cell lineages, and play a role in the reconstitution of the human endometrium. Clinical studies suggest that ischemic/reperfusion injury provides a strong stimulus for homing and engraftment of BMDCs into the uterus and it has been suggested that one of the mechanisms by which uterine injury may improve endometrial receptivity is via increasing recruitment of BMDCs to the endometrium. Systemic administration of BMDCs can improve uterinescar healing and fertility in AS [7]. Santamaria et al. [2] conducted a clinical study to analyze the effect of BMDSC in patients with refractory AS and/or EA. BMDSC mobilization was performed by granulocyte colony-stimulating factor injection in a total of 16 patients. Endometrial thickness increased from an average of 4.3 mm to 6.7 mm. Similarly, four of the five EA patients experienced an improved endometrial cavity, and endometrial thickness increased from 4.2 mm to 5.7 mm [2].

Another study was conducted by Cervello et al. [8] using the engraftment of BMDSC predominantly around the endometrial blood vessels of traumatized endometrium. Results revealed that BMDSCs induces proliferation of the neighboring endometrial cells in the damaged endometrium, mainly at the epithelial component [8]. Azizi et al. [9] investigated whether BMDC treatment could improve the restoration of the endometrium and increase the endometrial receptivity. A thin endometrium model was designed, and BMDC was transplanted by tail vein injection. Results revealed that BMDC has a valuable effect on the thin endometrium, and may play a role through migration and immunomodulatory effect of BMDC [9].

To the best of our knowledge, this was one of the first successful cases of treating endometrial atrophy with BMDC leading to a living birth. This rare and novel form of treatment paves a way for future research in the treatment of endometrial factors of infertility by regenerative medicine.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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References

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When the journal faces suspected cases of research and publication misconduct such as redundant (duplicate) publication, plagiarism, fraudulent or fabricated data, changes in authorship, an undisclosed conflict of interest, ethical problems with a submitted manuscript, a reviewer who has appropriated an author’s idea or data, complaints against editors, and so on, the resolution process will be completed following the procedures outlined in the flowchart provided by the COPE (http://publicationethics.org/resources/flowcharts). The discussion and decision on the suspected cases will be carried out by the Editorial Board.

6. Conflict-of-interest statement

The corresponding author must inform the editor of any potential conflicts of interest that could influence the authors’ interpretation of the data. Examples of potential conflicts of interest are financial support from or connections to pharmaceutical companies, political pressure from interest groups, and academically related issues. In particular, all sources of funding applicable to the study should be explicitly stated.

7. Process for handling cases requiring corrections, retractions, and editorial expressions of concern

Cases that require editorial expressions of concern or retraction shall follow the COPE flowcharts (http://publicationethics.org/resources/flowcharts). If a correction is required, the procedure to provide the correction will follow the ICMJE Recommendation (http://www.icmje.org/recommendations/browse/publishing-and-editorialissues/corrections-and-version-control.html).

8. Editorial responsibilities

The Editorial Board will continuously work to monitor and safeguard publication ethics: guidelines for retracting articles; maintenance of the integrity of the academic record; preclusion of business needs from compromising intellectual and ethical standards; publishing corrections, clarifications, retractions, and apologies when needed; and excluding plagiarism and fraudulent data. The editors maintain the following responsibilities: responsibility and authority to reject and accept articles; avoiding any conflict of interest with respect to articles they reject or accept; promoting publication of corrections or retractions when errors are found; and the preservation of the anonymity of reviewers. Submitted manuscripts are screened for possible plagiarism or duplicate publication by the use of Similarity Check powered by iThenticate (https://www.crossref.org/services/similaritycheck/), a plagiarism-screening tool upon arrival. If plagiarism or duplicate publication related to the papers of this journal is detected, the manuscripts may be rejected, the authors will be announced in the journal, and their institutions will be informed of this situation. There will also be penalties that will be assessed and applied for the authors if this incident occurs.

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For clarification on result accuracy and reproducibility of the results, raw data or analysis data will be deposited to a public repository or CERM homepage after acceptance of the manuscript. Therefore, submission of the raw data or analysis data is mandatory. If the data is already a public one, its URL site or sources should be disclosed. If data cannot be publicized, it can be negotiated with the editor. If there are any inquiries on depositing data, authors should contact the Editorial Office for more information.

5. Clinical data sharing policy

This journal follows the data sharing policy described in “Data Sharing Statements for Clinical Trials: A Requirement of the International Committee of Medical Journal Editors” (https://doi.org/10.3346/jkms.2017.32.7.1051). As of January 1, 2019 manuscripts submitted to CERM that report the results of clinical trials must contain a data sharing statement. Clinical trials that begin enrolling participants on or after January 1, 2019 must include a data sharing plan in the trial’s registration. The ICMJE’s policy regarding trial registration is explained at https://www.icmje.org/recommendations/browse/publishing-andeditorial-issues/clinical-trial-registration.html. If the data sharing plan changes after registration this information should be reflected in the statement submitted and published with the manuscript, as well as being updated in the registry record.

IV. MANUSCRIPT SUBMISSION

Manuscripts for submission to CERM should be prepared according to the following instructions. CERM follows ICMJE Recommendations, if not otherwise described below. Any physicians or researchers throughout the world can submit a manuscript if the scope of the manuscript is appropriate. Manuscripts can be submitted either in English. Only those manuscripts which are original, have not been published elsewhere, and are not currently being considered for inclusion in another publication will be considered for publication in CERM. All manuscripts should be submitted online via the journal’s website (http://submit.ecerm.org/) by the corresponding author. Submission instructions are available at the website. All articles submitted to the journal must comply with these instructions. Failure to do so will result in return of the manuscript and possible delay in publication. Send all correspondence regarding submitted manuscripts to:

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V. CATEGORIES OF PUBLICATIONS

CERM publishes invited review articles, original articles, case reports, brief communications, and letter to editor.

• Invited review articles provide a concise review of a subject of importance to researchers written by an invited expert in reproductive medical science.
• Original articles are papers reporting the results of basic and clinical investigations that are sufficiently well documented to be acceptable to critical readers.
• Case reports deal with clinical cases of medical interest or innovation.
• Brief communications are short original research articles on issues important to medical and biological researchers.
• Letter to editor includes a reader’s comment on an article published in CERM and a reply from the authors.

VI. PREPARATION OF MANUSCRIPTS

1. General guideline

• The main document with manuscript text and tables should be prepared with an MS-word or RTF format. The manuscript should be written in 11-point font with double-line spacing on A4 (21.0 × 29.7 cm) or letter (8.5 × 11.0 in) sized paper with 2.5 cm (1.0 in) margins.
• All manuscript pages are to be numbered at the upper right corner consecutively, beginning with the title page as page 1.
• Submission items include a manuscript, table (s), and figure (s). Send also Author’s Signature Form and Copyright Transfer Form (These files can be found at the journal’s website) as jpg or pdf files. Revised manuscripts should also be accompanied by a response note.
• Submit each figure as individual files separate from the manuscript. Do not insert figures into the text document. Figures should be in tiff, tif, jpg, jpeg files. Do not submit your manuscript or figures as pdf files.
• For specific study designs, such as randomized control studies, studies of diagnostic accuracy, meta-analyses, observational studies, and non-randomized studies, authors are encouraged to also consult the reporting guidelines relevant to their specific research design. A good source of reporting guidelines is the EQUATOR Network (https://www.equator-network.org/) and the NLM (https://www.nlm.nih.gov/services/research_report_guide.html).
• Drug and chemical names should be stated in standard chemical or generic nomenclature.
• Description of genes or related structures in a manuscript should include the names and official symbols provided by the US National Center for Biotechnology Information (NCBI) or the HUGO Gene Nomenclature Committee.
• Standard metric units are used for describing length, height, weight, and volume. The unit of temperature is given in degree Celsius (°C). Specifically, use ‘sec’, ‘min’, ‘hr’, ‘day’, ‘wk’, ‘mo’, and ‘yr’ for time units. All others units of measure should be presented according to the International System (SI) of Units. All units must be preceded by one space except percentage (%), temperature (°C), and angle (°).
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2. Original article

Manuscripts will not be acceptable for publication unless they meet the following editorial requirements. Manuscripts includes (1) Title page, (2) Structured abstract and Keywords, (3) Introduction, (4) Methods, (5) Results, (6) Discussion, (7) Acknowledgments, (8) References, (9) Tables, and (10) Figure legends. Each component should begin on a new page in the following sequence. Manuscripts should be no longer than 5,000 words and the combined numbers of tables and figures should be no more than 10 items.

1) Title page
• Provide running title (a maximum of 50 spaces and letters), manuscript title, the full name of author and the author’s institutional affiliation(s). For different institution, use the sequential Arabic number (1, 2, 3…) in superscript ahead of institution.
• All persons designated as authors should be qualified for authorship (See the part of ETHICS IN PUBLISHING). Each author should have participated sufficiently in the work to take public responsibility for the content.
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• All funding, other financial support, and material support for the work, if it exists, should be clearly identified in the conflict of interest statement. If no conflicts of interest exist for any of the authors, this should be noted.
• Include presentation history at a meeting.

2) Structured abstract and keywords: The abstract should present the Objective, Methods, Results, and Conclusion. The abstract should also emphasize new and important aspects of the study or observation and tract may not exceed 250 words. Below the abstract, provide up to 10 keywords that will assist indexers in crossindexing the article. For selecting keywords, refer to the MeSH database (https://www.ncbi.nlm.nih.gov/mesh).

3) Introduction: Briefly describe the purpose of the investigation, including relevant background information.

4) Methods: Describe the research plan, the materials (or subjects), and the methods used, in that order. Explain in detail how the disease was confirmed and how subjectivity in observations was controlled. When
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5) Results: The results should be presented in logical sequence in the
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Examples of references

(1) Journal article
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quent development and clinical outcomes of fragmented human em-

(2) Website
American Society for Reproductive Medicine. Headlines in reproductive
medicine [Internet]. Birmingham: American Society for Reproductive
org/headlines/.
(3) Book

(4) In press

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12) Figure legends: Place figure legends on a separate page at the end of your manuscript.

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Review article will be requested by the editors. Review articles are generally prepared in the same format as original articles, but the details of manuscript format may be flexible according to the contents. The manuscripts are limited to 5,000 words of text and includes 250-word summary in the place of unstructured abstract.

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Case reports should be succinct, informative, and limited to 2,000 words of text (including Title page, 150-word Case report summary, Introduction, Case, Discussion, References, Table, and Figure legend).

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Brief communication submissions should be limited to 2,000 words of text and a maximum of one figure or one table. Include a two-sentence narrative abstract in place of a structured abstract and do not include section headings.

6. Letter to the editor

This section of the journal is set aside for critical comments directed to a specific article that has recently been published in the journal. Letters should be brief (500 words), double-spaced, and limited to a maximum of five citations. The letters and replies should be prepared according to journal format. These will only be published in the online (blog) version of the journal for 6 months and then stored in the archives which are accessible to readers on-line. Illustrative material is accepted only with permission of the Editor. Please include your complete mailing address, telephone and fax numbers, and e-mail address with your correspondence. The Editor reserves the right to shorten letters, delete objectionable comments, and make other changes to comply with the style of the journal.

VII. AUTHOR’S MANUSCRIPT CHECKLIST

1. Double-spaced typing with 11-point font using MS-Word or RTF format.
2. Sequence of Title page, Structured abstract and keywords, Introduction, Methods, Results, Discussion, Acknowledgments, References, Tables, and Figure legends. All pages should be numbered consecutively starting from the title page.
3. Title page with running title, manuscript title, author’s full name, and institution, address for correspondence.
4. Abstract in format within 250 words, and keywords as in MeSH.
5. References listed in proper format. Check that all references listed in the references section are cited in the text and vice versa.
6. Send also Author’s Signature Form and Copyright Transfer Form as jpg or pdf files.

VIII. PEER REVIEW PROCESS

All manuscripts will be evaluated by two peer reviewers who are selected by the editors. The acceptance criteria for all papers are based on the quality and originality of the research and its clinical and scientific significance. An initial decision will normally be made within 4 weeks of receipt of a manuscript, and the reviewers’ comments are sent to the cor-
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IX. MANUSCRIPT ACCEPTED FOR PUBLICATION

1. Final version

After the paper has been accepted for publication, the author(s) should submit the final version of the manuscript for review. The names and affiliations of the authors should be double-checked to omit any spelling errors, and if the originally submitted image files were of poor resolution, higher resolution image files should be submitted at this time. Color images must be created as CMYK files. The electronic original should be sent for review with appropriate labeling and arrows. The EPS, TIFF, Adobe Photoshop (PSD), JPEG, and PPT formats are preferred for submission of digital files of photographic images. Symbols (e.g., circles, triangles, squares), letters (e.g., words, abbreviations), and numbers should be large enough to be legible on reduction to the journal’s column widths. All of the symbols that are used must be defined in the figure caption. If the symbols are too complex to appear in the caption, they should appear on the illustration itself, within the area of the graph or diagram, not to the side of the illustration. If references, tables, or figures are moved, added, or deleted during the revision process, they should be renumbered to reflect such changes in order that all tables, references, and figures are cited in numeric order.

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Before publication, the manuscript editor may correct the manuscript in order that it meets the standard publication format. The author(s) must respond within 2 days when the manuscript editor contacts the author for revisions. If the response is delayed, the manuscript's publication may be postponed to the next issue to be considered for publication.

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2. Complaints and appeals

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