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## 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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## REVIEW ARTICLE



## ORIGINAL ARTICLES



## CASE REPORT

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CERM

# Coenzyme Q10, oxidative stress, and male infertility: A review

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Male infertility has a complex etiopathology, which mostly remains elusive. Although research has claimed that oxidative stress (OS) is the most likely underlying mechanism of idiopathic male infertility, the specific treatment of OS-mediated male infertility requires further investigation. Coenzyme Q10 (CoQ10), a vitamin-like substance, has been found in measurable levels in human semen. It exhibits essential metabolic and antioxidant functions, as well as playing a vital role in mitochondrial bioenergetics. Thus, CoQ10 may be a key player in the maintenance of biological redox balance. CoQ10 concentrations in seminal plasma directly correlate with semen parameters, especially sperm count and sperm motility. Seminal CoQ10 concentrations have been shown to be altered in various male infertility states, such as varicocele, asthenozoospermia, and medical or surgical regimens used to treat male infertility. These observations imply that CoQ10 plays an important physiological role in the maintenance and amelioration of semen quality. The present article thereby aimed to review the possible mechanisms through which CoQ10 plays a role in the regulation of male reproductive function, and to concisely discuss its efficacy as an ameliorative agent in restoring semen parameters in male infertility, as well as its impact on OS markers, sperm DNA fragmentation, pregnancy, and assisted reproductive technology outcomes.

**Keywords:** Antioxidant; Coenzyme Q10; Male infertility; Oxidative stress

## Introduction

Infertility is defined as the failure to successfully achieve pregnancy after 12 months of regular unprotected sexual intercourse [1]. Worldwide, 15% of the world's population is affected by infertility [2]. The factors responsible for infertility have been grouped as male and female factors. Approximate 50% of cases are attributed to combined male and female factors, while 25% are attributed to male factors alone [3]. Infertility in males unambiguously reflects a complex of underlying causes [4,5], and more than 25% of cases of male infertility are idiopathic with no identifiable cause [6]. Oxidative stress (OS) and reactive oxygen species (ROS) are considered damaging to sperm and are responsible for 30%– 80% of cases of subfertility [7]. OS, caused by the disruption of the prooxidant-antioxidant balance [8], affects male fertility and sperm function [9-12].

Although low levels of ROS possess some physiological functions in sperm maturation and capacitation, an imbalance between ROS and seminal antioxidants may disrupt male reproductive function [13]. Similarly, the acrosome reaction and capacitation are boosted by superoxide anion radicals [14]. However, excessive ROS generation leads to OS and diminishes spermatozoa's antioxidant capacity [15-17]. The generation of seminal ROS could be attributed to genital tract infection, genital tract inflammation, varicocele, testicular torsion, and cryptorchidism [18,19]. Other factors include aging and various lifestyle factors, such as exposure to toxic chemicals, cigarette smoking, exposure to radiation, and alcohol abuse [18,20]. Excessive

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ROS generation has been associated with degradation of DNA via the induction of breakage of DNA strands, chromatin cross-linking, and base modifications [21], and lower potential of the mitochondrial membrane [22,23]. The plasma membrane of spermatozoa is composed of lipids, and a high level of polyunsaturated fatty acids and an excessive level of ROS makes the membrane susceptible to damage due to lipid peroxidation [10,24]. In sperm, mobility and decreased membrane fluidity caused by lipid peroxidation have been associated with a lower fertilization capacity [25].

Spermatozoa have a scavenger activity exerted by enzymatic and non-enzymatic antioxidants. The enzymatic antioxidants present in semen include catalase, superoxide dismutase, and glutathione peroxidase, whereas non-enzymatic antioxidants constitute coenzyme Q10 (CoQ10), glutathione peroxidase; vitamins A, B complex, C, and E; carnitines; and minerals (chromium, selenium, zinc, and copper) [21,26]. An imbalance between ROS production and antioxidant capacity results in increased sperm exposure to OS, which plays a critical role in the pathogenesis of male infertility and alters sperm function [9]. Despite the recognition of ROS and OS as a factor contributing to male infertility, antioxidant use for treatment is still debatable. To explore the *in vitro* effective role of different OS in various models, specific differences have been found in the effectiveness exerted by enzymatic and non-enzymatic molecules [27]. Antioxidant therapy has been considered for supplementation and has been introduced into routine clinical practice for the treatment of male infertility [28]. The antioxidants used for male infertility include CoQ10, vitamin A, carnitines, N-acetyl cysteine, vitamin C, vitamin E, pentoxifylline, and micronutrients such as selenium and zinc [28,29]. Antioxidants have been associated with beneficial effects on sperm concentration, motility, morphology, DNA fragmentation, assisted reproductive technology (ART) outcomes (both *in vitro* fertilization [IVF] and intracytoplasmic sperm injection [ICSI]), and seminal plasma antioxidant capacity [30]. However, there is insufficient agreement on the type, dosing, and use of single or combined antioxidants [30,31]. The potential role of CoQ10 in the management of male infertility has been widely investigated. Our attention in this narrative review will be focused on comprehensive and updated evidence on the impact of CoQ10 on the male reproductive system and its efficacy on sperm parameters, sperm DNA fragmentation (SDF), seminal markers of OS, pregnancy, and ART outcomes.

## CoQ10: biochemical properties and physiological functions

In humans, CoQ10 is synthesized from tyrosine. CoQ10 is a vital constituent of the inner mitochondrial membrane. It is involved in the inhibition of lipid peroxidation and DNA oxidation [7]. CoQ10 also plays an essential role in electron transport in the mitochondrial respiratory chain and oxidative phosphorylation, and functions as a lipid-soluble antioxidant in cell membranes and lipoproteins [32,33]. CoQ10 also participates in adenosine triphosphate production in aerobic respiration [21]. Moreover, CoQ10 therapy has been applied as a prospective intervention in the management of various pathological dysfunctions such as diabetes, cancer, Parkinson disease, Huntington disease, heart disorders, and infertility [34].

The introduction of CoQ10 therapy started in patients with heart failure; subsequently, it has been more widely recognized as a way to slow down age-related pathologies, improve bioenergetics in the cell, and counteract OS. Various studies have proven the effectiveness of CoQ10 supplementation in enhancing male fertility and cardiovascular function [9,34,35]. CoQ10 functions as an antioxidant by inhibiting lipid peroxidation of the sperm membrane [35]. There are three redox states of CoQ10 in the Q-cycle in organisms. These are ubiquinol (CoQ10-H2-reduced form), ubiquinone (oxidized form), and semiquinone (a radical) [36]. CoQ10 is concentrated in the mitochondria-containing midpiece of the sperm, where it takes part in all energy-regulated processes [35].

## Effects of CoQ10 on sperm parameters

Several clinical studies have reported beneficial effects of CoQ10 supplementation on sperm parameters of infertile patients [21,37,38]. In 287 patients with idiopathic oligoasthenoteratozoospermia (OAT), CoQ10 supplementation (600 mg/day) for 12 months significantly increased sperm concentration (+113.7%), sperm progressive motility (+104.8%) and normal sperm morphology (+78.9%) [39]. A systematic review and meta-analysis evaluating the effects of CoQ10 oral administration on CoQ10 seminal concentration, sperm concentration, and sperm motility was conducted on three trials with a total of 149 patients receiving CoQ10 and 147 control men. The results showed that CoQ10 supplementation led to a significant increase in all three endpoints taken into consideration (namely seminal concentration of CoQ10, sperm concentration, and sperm motility) [40]. Furthermore, our recent meta-analysis of three placebo-controlled randomized clinical trials (RCTs) involving 296 participants demonstrated a significant impact of CoQ10 on improving sperm total and progressive motility [32].

Gvozdjakova et al. [36] showed that the administration of CoQ10 (30 mg/day), L-carnitine fumarate (440 mg/day), vitamin E (75 IU/day), and vitamin C (12 mg/day) to infertile male patients improved sperm concentration and pregnancy rates. In one of our RCTs, 35 men with idiopathic OAT were treated for 3 months with CoQ10 at the dose of 200 mg/day and 30 patients with 400 mg/day. The results showed greater improvement in semen parameters in men


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who took 400 mg/day [38]. In another study of 70 men with idiopathic OAT, we also demonstrated that CoQ10 therapy (200 mg/day) was associated with improved sperm concentration and motility, as well as a reduction in OS markers [38].

In another study, patients with idiopathic infertility were supplemented with CoQ10 (200 mg/day) and D-Asp (2,660 mg/day) for 12 weeks. The concentrations of CoQ10 and D-Asp increased significantly in the spermatozoa and seminal plasma. In addition, sperm motility improved, whereas no effect was found on sperm concentration and morphology [36]. Similarly, the efficacy of CoQ10 supplementation has also been evaluated in infertile patients with varicocele. A significant improvement of sperm parameters and total antioxidant capacity (TAC) was reported in men treated with CoQ10 at a dose of 100 mg/day for 3 months [41]. Furthermore, in a study comparing the effects of two doses of CoQ10 on sperm parameters and TAC in patients with idiopathic OAT for 3 months, it was found that CoQ10 significantly increased sperm concentration, total motility, and progressive motility [3]. CoQ10 also increased TAC, superoxide dismutase, and catalase activities, with a stronger improvement found in patients taking the highest dose [21].

Overall, these studies show that supplementation with CoQ10 enhances sperm parameters such as sperm concentration, motility, and morphology, and improves OS markers in men with idiopathic infertility. However, there is no consensus on the dosage to prescribe. In an attempt to answer this question, evidence from clinical trials and meta-analyses on the impact of CoQ10 treatment in male infertility revealed that oral supplementation with CoQ10 raised seminal CoQ10 levels, sperm motility, and spermatozoa concentration [32,42].

## CoQ10 effects on OS markers

As previously discussed, ROS affect sperm quality, leading to DNA, protein, and lipid oxidation, and are involved in the pathogenesis of male infertility [43]. However, there is no general agreement on the validation, reproducibility, and standardization for the measurement of ROS-induced changes in DNA, lipids, and proteins; TAC in human body fluids; or enzymatic players involved in redox status [44,45].

There is evidence supporting the protective role of CoQ10 against ROS-induced sperm damage [37,38]. CoQ10 is known to inhibit superoxide production [46], and a strong negative association has been observed between CoQ10 levels and hydrogen peroxide [46]. In patients with idiopathic OAT, a significant increase in superoxide dismutase, TAC, and catalase activity after CoQ10 treatment has been reported [30]. We demonstrated that CoQ10 treatment (200 mg/day) could reduce or ameliorate OS markers such as ROS, TAC, catalase, superoxide dismutase, and glutathione peroxidase in infertile men with idiopathic OAT [21,38] and idiopathic oligoasthenozoospermia (OA) [30,37]. Overall, these studies have demonstrated beneficial effects of CoQ10 on improving both enzymatic and non-enzymatic antioxidant capacity among men with idiopathic infertility (Table 1).

## CoQ10 and SDF

SDF is one of the main underlying molecular-level disruptions that may explain idiopathic male infertility. OS is considered to be the key mechanism causing SDF [47]. An excess of ROS causes nicks and breaks in DNA, which need to be repaired [48]. Most DNA in human spermatozoa is transported in a condensed form of chromatin in the sperm head, making sperm DNA more resistant to injury during transit in both the male and female reproductive tracts; however, SDF may result from exposure to seminal OS in the epididymis or abnormal chromatin packaging [49]. Spermatozoa are susceptible to ROS due to their composition of high levels of polyunsaturated fatty acids in their cytoplasm and plasma membrane. DNA damage can be the result of decreased protamination, replication errors, environmental toxins, ultraviolet rays, endogenous endonuclease activation, and ionizing rays [47]. DNA fragmentation can lead to infertility by altering sperm function [50]. Males with a high SDF rate have a substantially lower likelihood of conceiving naturally or via ART procedures [51]. Accordingly, patients with a high percentage of spermatozoa affected by DNA fragmentation have high levels of seminal ROS and decreased antioxidant capacity [26].

Both *in vivo* and *in vitro* studies have shown that increased SDF could impair male reproductive functions via its impacts on fertilization, implantation, early embryo development, and pregnancy [49]. Studies have reported that deficiency of CoQ10 is associated with high sperm DNA damage and low sperm count and motility [32,40,52,53]. This may be explained by the fact that seminal CoQ10, with antioxidant and metabolic properties, plays a major role in mitochondrial bioenergetics and maintenance of seminal redox status [33].

Evidence has shown that antioxidant treatment reduces the prevalence of SDF in semen samples and seems to improve the outcomes of ICSI in patients with elevated SDF levels [49]. Gual-Frau et al. [54] administered antioxidants containing CoQ10 to 20 infertile patients with low-grade varicocele and high SDF levels. A significant decrease in SDF levels and a substantial rise in sperm concentration were observed following treatment . These findings are consistent with our recent randomized controlled study on 65 infertile men with idiopathic OA and 40 fertile men, which illustrated an improvement in semen parameters and a reduction in OS markers and SDF in infertile patients following CoQ10 therapy (200 mg/day for 3 months) (Table 1) [37].

**Table 1.** Effects of CoQ10 on male infertility, pregnancy outcomes, and assisted reproductive techniques

| Study | Participan | RCT | Intervention | Intervention period | Outcome |
|---|---|---|---|---|---|
| Alahmar et al. (2021) [37] | Infertile patients with idiopathic oligoasthenozoospermia; 65 patients | Yes | CoQ10 200 mg/day orally | 3 mo | Improved sperm concentration, progressive motility, total motility, seminal fluid CoQ10 concentration, TAC, ROS levels and SDF percentage, and glutathione peroxidase levels. |
| Alahmar and Sengupta (2021) [38] | Men with OAT; 70 patients | Yes | CoQ10 200 mg/day | 3 mo | Improved sperm concentration, motility, and antioxidant status. |
| Alahmar (2019) [21] | Men with idiopathic OAT 35 subjects treated with CoQ10 at the dose of 200 mg/day and 30 patients with 400 mg/day | Yes | CoQ10 200 mg/day, 400 mg/day | 3 mo | Idiopathic OAT with a greater improvement shown in men who took 400 mg/day than in those who took 200 mg/day |
| Cheng et al. (2018) [55] | Idiopathic oligoasthenozoospermia; 262 patients | Yes | L-carnitine 10 mg twice daily and CoQ10 20 mg thrice daily | 3 mo | Combination of L-carnitine and CoQ10 can improve the sperm motility and outcome of clinical pregnancy in idiopathic OAT patients.<br>Pretreatment with CoQ10 improves ovarian response to stimulation and embryological parameters in young women with poor ovarian reserve in IVF-ICSI cycles. |
| Tiseo et al. (2017) [35] | Subfertile couples; 211 subjects | No | CoQ10 19.2 mg/day (2.4–247.2 mg/day) | Not specified | Mean dietary intake of CoQ10 in this study was 10-fold lower than the supplemental dose used in clinical trials, showing improved sperm motility. |
| Giacone et al. (2017) [56] | 12 Normozoospermic men and 12 asthenozoospermic patients | No | Zinc, D-aspartic acid, CoQ10 12 mg | Not specified | Improved sperm motility and increased fertilization rate in IVF/ICSI. |
| Nadjarzadeh et al. (2014) [51] | Idiopathic OAT; 60 patients | Yes | CoQ10 200 mg/day or placebo | 3 mo | Enhanced semen quality and motility. |
| Gaby et al. (2013) [57] | Idiopathic OAT; 228 patients | Yes | CoQ10/200 mg/day | 26 wk | Increased sperm concentration and morphology. Decreased motility and follicle stimulating hormone activity. |
| Abad et al. (2013) [58] | Asthenteratozoospermic patients; 20 subjects | No | L-carnitine 1,500 mg, CoQ10 20 mg, vitamin C 60 mg, vitamin E 10 mg, vitamin B 9200 µg, vitamin B12 1 µg, zinc 10 mg, selenium 50 µg | 3 mo | DNA damage reduced from 28.5% to 20.12%. |
| Safarinejad (2012) [39] | Idiopathic OAT; 287 patients | No | CoQ10 300 mg twice daily | 12 mo | Increased sperm concentration, progressive motility, and normal morphology. |
| Nadjarzadeh et al. (2011) [49] | Infertile men with idiopathic OAT; 60 patients | Yes | CoQ10 200 mg once daily | 19 mo | Improved seminal parameters, lipid peroxidation. |
| Safarinejad et al. (2009) [59,60] | Infertile men with idiopathic OAT; 212 patients | Yes | CoQ10 300 mg once daily | 26 wk | Improved seminal parameters and testicular volume. |
| Balercia et al. (2009) [61] | Idiopathic asthenozoospermia; 60 patients | No | CoQ10 200 mg/day | 3 mo | Administration of CoQ10 increased CoQ10 levels in semen. It could be effective in enhancing sperm kinetic features in idiopathic asthenozoospermic patients. |

CoQ10, coenzyme Q10; RCT, randomized clinical trial; TAC, total antioxidant capacity; ROS, reactive oxygen species; SDF, sperm DNA fragmentation; OAT, oligoasthenoteratozoospermia; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection.

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In a study of 29 known asthenozoospermic males, a substantial decrease of SDF from 28.5% to 20.12% was reported after 3 months of CoQ10 plus L-carnitine administration [62]. In another study, Ghanbarzadeh et al. [33] showed that pretreatment with CoQ10 and L-carnitine significantly improved sperm parameters, sperm function, and reproductive hormone profile in male Wistar rats with high low-density lipoprotein (LDL) and oxidized LDL serum levels. In a recent study, our research group also observed that when idiopathic OA patients were received 3 months of CoQ10 supplementation, their semen parameters significantly improved, along with a significant reduction in seminal OS markers and SDF compared to the baseline [37]. These data suggest that CoQ10 plays a positive role in the amelioration of SDF, although limited studies are available so far. More unbiased and well-performed RCTs are needed to better clarify this issue.

## CoQ10 and pregnancy outcomes

Several studies have demonstrated improved pregnancy rates after CoQ10 administration [63,64]. Some studies suggested that the increased pregnancy rate is due to the beneficial effects of CoQ10 on sperm concentration and motility. In line with such findings, Gvozdjakova et al. [36] showed a significant improvement in the pregnancy rate after the administration of CoQ10 at a daily dose of 90 mg for 3 to 9 months in 40 infertile men with OA. The administration of Carni-Q-Nol (each soft gel containing 440 mg L-carnitine fumarate, 30 mg CoQ10, 75 IU vitamin E, and 12 mg vitamin C) was effective for improving the pregnancy rate, as 45% of the female partners of these patients achieved pregnancy. In the same group, three males (7.5%) achieved fatherhood after undergoing ART, and the other 12 women (30%) became pregnant 5–6 months after their partners began therapy [36].

Safarinejad [39] also reported an increase in the pregnancy rate after treatment with CoQ10 in 287 patients with idiopathic OAT who received supplementation of 300 mg of CoQ10 twice daily for 12 months. After treatment, the participants showed improved sperm quality. A positive impact was found on pregnancy rates, as 34.1% of couples achieved spontaneous clinical pregnancy after 9–12 months of treatment [40]. In a study aiming to assess the effects of CoQ10 administered in combination with L-carnitine in idiopathic OAT patients, sperm parameters were found to be improved, with a lower percentage of SDF and consequently a higher clinical pregnancy rate [45], showing that the combination of CoQ10 and L-carnitine improved pregnancy outcomes in patients with idiopathic male infertility.

## CoQ10 and ART outcomes

OS significantly impacts the success rate of ART. Spermatozoa and oocytes, once removed from their microenvironments, can be exposed to excessive levels of ROS as a consequence of the lack of scavenger system systems present in the reproductive tract. For this reason, pretreatment with antioxidants could be useful to improve the quality of gametes [30]. According to Arhin et al. [65], evidence from many RCTs has shown that oral antioxidant supplementation leads to a significant increase in the pregnancy rate in couples undergoing ART cycles by enhancing male fertility. However, the results of some of these studies must be interpreted with the utmost care due to discrepancies in the treatment regimens. Thanks to its ability to improve sperm quality, CoQ10 could play a role in improving ART outcomes. An *in vitro* study showed that incubation of spermatozoa for 3 hours with an antioxidant formula containing zinc, D-Asp, and Co-Q10 had a beneficial effect on sperm motility, recovery of spermatozoa by swim-up, and lipid peroxidation. This suggests that these molecules may have a place in sperm preparation before ART [63]. In another study carried by Lewin and Lavon [66], the effects of oral CoQ10 administration on the outcomes of ICSI were investigated in seven patients with low fertilization rates after ICSI at a dose of 60 mg/day for an average of 103 days before undergoing subsequent ICSI cycles. The treatment significantly increased the fertilization rate, from 10.3% ± 10.5% in ICSI cycles without treatment to 26.3% ± 22.8% after CoQ10 intake. Lewin and Lavon [66] also examined the seminal fluid of 38 subjects (normozoospermic and asthenozoospermic) and noted that in patients with asthenozoospermia, there was an increase in motility after incubation with 50 μM CoQ10 for 24 hours. However, they did not test the increase in the fertility rate in ICSI.

In another retrospective study of 797 intrauterine insemination and 253 IVF cycles, women who received supplementation with 600 mg of CoQ10 along with dehydroepiandrosterone (DHEA) for over a month were found to have reduced levels of gonadotropins upon stimulation and a higher number of mature follicles than women taking DHEA alone [50]. In a recent meta-analysis of 61 RCTs including 6,264 infertile patients, antioxidant treatment was found to be correlated with an increase in clinical pregnancy rate and live birth rate [67].

## Conclusion

The present review shows that the antioxidant properties of CoQ10 and its vital role in mitochondrial bioenergetics form the basis of the ameliorative role of seminal CoQ10 in male fertility parameters. Evidence reveals that CoQ10 mainly improves sperm count and motility in infertile men, with most studies emphasizing its role in as-

thenozoospermia. It appears that CoQ10 also protects sperm from oxidative damage, thereby improving OS markers and SDF. Moreover, CoQ10 administration in couples resulted in improved ART outcomes, such as increased fertilization rates in IVF/ICSI. Further in-depth interventions are needed to reveal the exact mode of action of CoQ10 and to determine the appropriate standardized dose and duration of CoQ10 supplementation in the treatment of specific male infertility cases.

## Conflict of interest

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

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Conceptualization, Data curation, & Formal analysis: ATA. Methodology: ATA, AEC, RS, PS. Project administration: ATA, REC, RS, PS. Visualization: ATA, RS, RC, SD. Writing–original draft: ATA. Writing–review & editing: AEC, RS, RC, PS, SD.

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# The effect of lipopolysaccharide from uropathogenic *Escherichia coli* on the immune system, testis tissue, and spermatozoa of BALB/c mice

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**Objective:** Uropathogenic *Escherichia coli* is known to cause urinary tract infections, and the endotoxin (lipopolysaccharide [LPS]) of this bacterium may cause deficiencies of sperm quality and morphology. In the present study, the effects of LPS on mouse sperm were studied, and the levels of interleukin (IL)-17A and possible changes in testis tissue were evaluated.
**Methods:** LPS of uropathogenic *E. coli* was extracted using the methanol-chloroform method, followed confirmation using sodium dodecyl sulfate-polyacrylamide electrophoresis. Purified LPS (100 μg/kg) or phosphate-buffered saline was injected intraperitoneally into BALB/c mice for 7 days consecutively in the test and control groups, mice were sacrificed on days 3, 7, and 42 after the first injection. Blood was tested for levels of IL-17A using the enzyme-linked immunosorbent assay method. Testis tissue and sperm were collected from each mouse and were studied according to standard protocols.
**Results:** The mean sperm count and motility significantly decreased ($p$=0.03) at 3, 7, and 42 days after the injections. The level of IL-17A in the test groups increased, but not significantly ($p$=0.8, $p$=0.11, and $p$=0.15, respectively). Microscopic studies showed no obvious changes in the morphology of the testis tissue; however, significant changes were observed in the cellular parenchyma on day 42.
**Conclusion:** LPS can stimulate the immune system to produce proinflammatory cytokines, resulting in an immune response in the testis and ultimately leading to deficiency in sperm parameters and testis tissue damage. In addition, the presence of LPS could significantly impair sperm parameters, as shown by the finding of decreased motility.

**Keywords:** Interleukin-17; Lipopolysaccharide; Spermatozoa; Uropathogenic *Escherichia coli*

## Introduction

According to currently available data, 15% of cases of male infertility are due to sexually transmitted infections (STIs) [1,2]. Both Gram-positive and Gram-negative bacteria are associated with STIs, and these infections may cause men to become infertile [3]. Among these bacterial agents, uropathogenic *Escherichia coli* has been recognized as a Gram-negative bacterium that plays an important role in male infertility, and data have shown that the most common microbe isolated from semen was *E. coli* [4,5].

*E. coli* can damage spermatozoa through various mechanisms, including one-way direct interactions between bacteria and spermatozoa that result in immobilization of spermatozoa [6]. The tight ad-

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hesions between bacteria and sperm cause sperm to become immobile and to agglutinate, resulting in dramatic structural alterations and damage. Another mechanism is soluble factors produced and secreted by pathogenic bacteria [7,8]. For instance, the *E. coli* endotoxin (lipopolysaccharide [LPS]) can lead to the loss of sperm viability and sperm DNA fragmentation [9,10]. Furthermore, proinflammatory cytokines, which are usually released by leukocytes during the inflammatory response, impair sperm quality [11]. Interleukin (IL)-17 is a proinflammatory cytokine important for host immune modulation in infection and inflammatory diseases. IL-17A has been shown to play a critical role in bacterial infections [12,13]. Although IL-17A seems to be unnecessary for the generation of a protective response to uropathogenic *E. coli*, it may nonetheless cause adverse effects on spermatozoa. In this study, the effects of LPS extracted from *E. coli* and the subsequent production of IL-17A on both the quality and quantity of mouse spermatozoa and testis tissue were investigated in comparison with control mice.

## Methods

### 1. Ethics statement

The present study was approved by the Ethical Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran (No. IR.SSU.MEDICINE.REC.1394.263). All animal testing procedures were performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals [14].

### 2. Bacterial purification

Uropathogenic *E. coli* ATCC 7852 containing the eaeA gene was inoculated on an eosin methylene blue medium (Merck, Darmstadt, Germany), and following incubation at 37°C for 24 hours, the grown colonies were further mixed with 250 mL of Luria-Bertani broth medium (Merck). After overnight incubation at 37°C, the mixture was centrifuged (180 rpm). Then the supernatant was discarded and the sediment was mixed with 95% alcohol. The process followed the instructions in the manual published by Sezonov et al. [15].

### 3. LPS extraction by chloroform methanol

Dried bacteria were suspended in 1 mL of 10% ethylenediaminetetraacetic acid (EDTA) solution and then sonicated for 20 minutes. One milliliter of methanol/chloroform (1:2 ratio) in a saturated solution was added to the bacterium-EDTA solution and the tube lids were closed and shaken for 2 hours, then centrifuged at 2,000 rpm for 10 minutes. After the formation of three layers (methanol, cellular extract, and chloroform, respectively) the chloroform and methanol layers were separated, and after evaporation, the bacterial LPS was obtained [16].

### 4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out to confirm the extracted LPS in comparison to commercially available LPS. The extracted LPS was dissolved into 0.05 M Tris-HCl buffer with a pH of 6.8 containing 2% SDS, 10% sucrose, and 0.01% bromophenol blue, and incubated at 100°C for 5 minutes. Then, 0.5 μg of extracted LPS and commercial LPS (Sigma-Aldrich, St. Louis, MA, USA) were loaded into the polyacrylamide gels. For detection of the bands, the gel was visualized with the silver staining method [17,18].

### 5. Intraperitoneal injection of LPS extracted in mice (BALB/c)

Twenty-eight healthy adult male BALB/c mice with an average age of 7–8 weeks and homogeneous weight were obtained from the animal laboratory facilities at Shahid Sadoughi University of Medical Sciences. All experimental animals were housed in standard environmental conditions in cages maintained at an ambient temperature of 25°C ± 2°C under 12-hour light/dark conditions. The animals had free access to food and water during the experiment. The mice were randomly divided into four groups (group 1, control; group 2, test sample on day 3; group 3, test sample on day 7; and group 4, test sample on day 42). All mice received intraperitoneal injections for 7 days with phosphate-buffered saline (PBS) in the control group and 100 μg of isolated LPS per kilogram of body weight per day in the test group. After 3, 7, or 42 days, the mice were sacrificed by cervical dislocation. The cauda epididymis of each animal was dissected and placed in 1 mL of Ham's F10 medium and then incubated for 30 minutes [19].

Sperm parameters including total sperm count, normal morphology, and motility were evaluated for each mouse. For motility and count, a Makler chamber (Sefi Medical Co., Haifa, Israel) and phase contrast microscopy were used (Olympus, Tokyo, Japan) at × 20. The motility of sperm was graded as rapid linear motile (A), slow linear (B), nonprogressive (C), and immotile (D). Sperm morphology was evaluated by bright field microscopy and Giemsa staining. All analyses were done by an experienced technician according to World Health Organization criteria [20]. Blood samples were also collected from each mouse by cardiac puncture. The serum was then separated and the level of IL-17A was measured using a commercial enzyme-linked immunosorbent assay kit (eBioscience, San Diego, CA, USA) according to the kit's protocol.

### 6. Statistical analysis

Between-group comparisons were performed using the nonparametric Mann-Whitney *U*-test. All statistical analyses were performed using SPSS ver. 18.0 (SPSS Inc., Chicago, IL, USA) and *p*-values of less


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than 0.05 were considered to indicate statistical significance.

## Results

SDS-PAGE analysis and silver staining showed that the extracted LPS had a similar molecular weight to that of the commercially obtained LPS (Figure 1). LPS injections reduced sperm count on days 3, 7, and 42. Furthermore, sperm of grades A/B and D showed a significant decrease compared to the control group after day 7 ($p=0.03$).

As shown in Table 1, the mean serum level of IL-17A was higher in the test group injected with LPS than in the control group on day 3, but this difference was not significant ($p=0.8$). Furthermore, other factors related to sperm quality and morphology did not show significant differences between the two groups, although a significantly lower sperm count was observed in the test group than in the control group ($p=0.000$). As presented in Table 2, which shows data from day 7, sperm grades A/B and D, sperm count, and sperm morphology variables were significantly lower in the test group than in the control group ($p=0.03$, $p=0.03$, $p<0.001$, and $p<0.001$, respectively).

The mean serum level of IL-17A in the group injected with LPS was higher than that of the control group on day 42, but this difference was not significant ($p=0.1$). However, sperm grades of A/B and D, sperm count, and sperm morphology variables were significantly lower in the group injected with LPS at day 42 than in the control group ($p=0.005$, $p=0.004$, $p=0.000$, and $p=0.006$, respectively) (Table 3). Table 4 presents a comparison of concentrations of IL-17A; sperm grades of A/B, C, and D; sperm count; and sperm morphology on days 3, 7, and 42. Sperm grade A/B and morphology variables significantly decreased over time ($p<0.05$).

The microscopic studies of the tissues showed that the treatment of mice with 1 mg/kg/day of LPS did not give rise to a noticeable change in the morphology of the testis tissue on days 3 and 7, but significant changes in the cellular parenchyma and order were observed on day 42, including extensive necrosis of testicular paren-

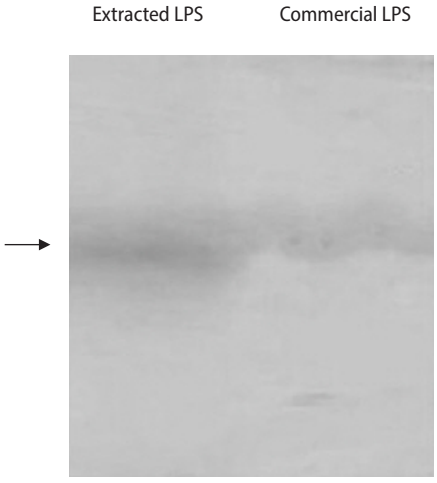


**Figure 1.** Extracted lipopolysaccharide (LPS) from uropathogenic *Escherichia coli* compared to commercially available LPS. The arrow indicated the LPS band on the sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

**Table 1.** Mean concentrations of IL-17A, sperm grades A/B, C, and D, sperm count, and abnormal sperm factors in the test and control groups on day 3

| Factor | Control group | Test group | *p*-value |
|---|---|---|---|
| IL-17A (pg/mL) | 13.8±1.1 | 14.12±2.3 | 0.8 |
| A[a]/B[b] (%) | 45.5±14.5 | 38.3±12.5 | 0.4 |
| C[c] (%) | 23.25±6.9 | 19.2±15.3 | 0.6 |
| D[d] (%) | 31.2±8.5 | 42.5±9.8 | 0.1 |
| Count (×10$^6$) | 46±2.9 | 17±2.5 | 0.000[e] |
| Abnormal sperm (%) | 55±5 | 70±10.4 | 0.6 |

Values are presented as mean±standard deviation.
IL, interleukin.
[a]Rapid linear motile; [b]Slow linear motile; [c]Nonprogressive motile; [d]Immotile; [e]Significant at $p<0.05$.

**Table 2.** Mean concentrations of IL-17A, sperm grades A/B, C, and D, sperm count, and abnormal sperm factors in the test and control groups on day 7

| Factor | Control group | Test group | *p*-value |
|---|---|---|---|
| IL-17A (pg/mL) | 13.8±1.1 | 15.8±1.3 | 0.11 |
| A[a]/B[b] (%) | 45.5±14.5 | 23.1±14.4 | 0.03 |
| C[c] (%) | 23.3±6.9 | 17.2±8.1 | 0.2 |
| D[d] (%) | 31.2±8.5 | 59.7±8.1 | 0.03 |
| Count (×10$^6$) | 46±2.9 | 15±1.6 | <0.001[e] |
| Abnormal sperm (%) | 55±5 | 85±11 | <0.001[e] |

Values are presented as mean±standard deviation.
IL, interleukin.
[a]Rapid linear motile; [b]Slow linear motile; [c]Nonprogressive motile; [d]Immotile; [e]Significant at $p<0.05$.

**Table 3.** Mean concentrations of IL-17A, sperm grades A/B, C, and D, sperm count, and abnormal sperm factors in the test and control groups on day 42

| Factor | Control group | Test group | *p*-value |
|---|---|---|---|
| IL-17A (pg/mL) | 13.8±1.1 | 17.7±3.7 | 0.15 |
| A[a]/B[b] (%) | 45.5±14.5 | 16.2±10.2 | 0.005[e] |
| C[c] (%) | 23.3±6.9 | 22.5±6.9 | 0.8 |
| D[d] (%) | 31.2±8.5 | 61.3±12.9 | 0.004[e] |
| Count (×10$^6$) | 46±2.9 | 13±4.7 | 0.000[e] |
| Abnormal sperm (%) | 55±5 | 90±6.3 | 0.006[e] |

Values are presented as mean±standard deviation.
IL, interleukin.
[a]Rapid linear motile; [b]Slow linear motile; [c]Nonprogressive motile; [d]Immotile; [e]Significant at $p<0.05$.

**Table 4.** Mean concentrations of IL-17A, sperm grades A/B, C, and D, sperm count, and abnormal sperm factors in the test and control groups on days 3, 7, and 42

| Factor | Day 3 | Day 7 | Day 42 | *p*-value |
|---|---|---|---|---|
| IL-17A (pg/mL) | 14.1±2.3 | 15.8±1.3 | 17.7±3.7 | 0.4 |
| A[a]/B[b] (%) | 38.3±12.5 | 23.1±14.4 | 16.2±10.2 | 0.02[e] |
| C[c] (%) | 19.16±15.3 | 17.2±8.1 | 22.5±6.9 | 0.7 |
| D[d] (%) | 42.5±9.8 | 59.7±8.1 | 61.3±12.9 | 0.1[e] |
| Count ($\times 10^6$) | 17±2.5 | 15±1.6 | 13±4.7 | 0.1 |
| Abnormal sperm (%) | 70±10.5 | 85±11 | 90±6.3 | 0.006[e] |

Values are presented as mean±standard deviation.
IL, interleukin.
[a]Rapid linear motile; [b]Slow linear motile; [c]Nonprogressive motile; [d]Immotile; [e]Significant at $p<0.05$.

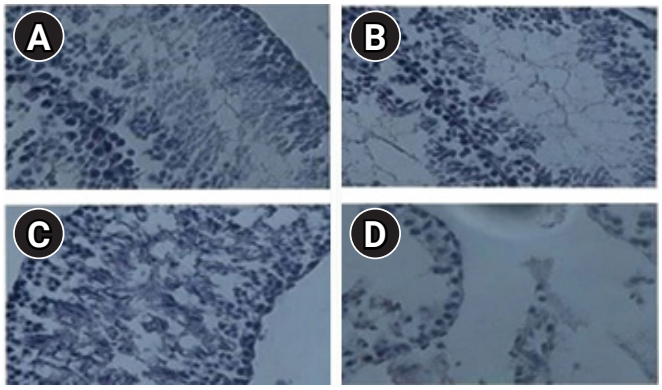


**Figure 2.** Optical micrograph of cell populations in seminiferous tubules stained with H&E at ×40 magnification. The control group with normal germinal epithelium (A). Histology of tubules after 3 (B), 7 (C), and 42 (D) days. The cells of the germinal epithelium were significantly reduced in group D.

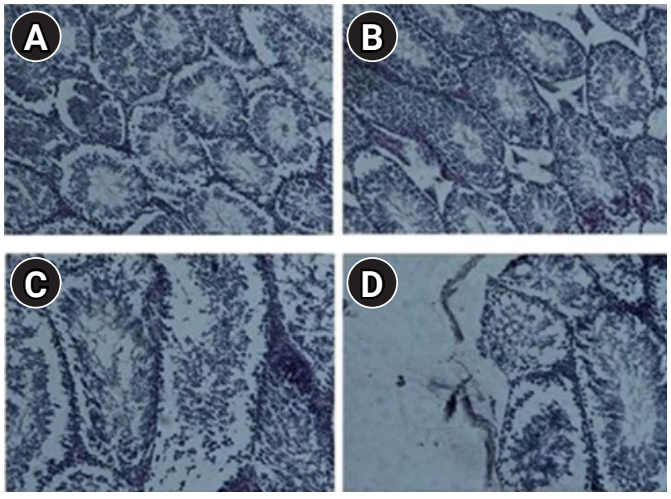


**Figure 3.** Optical micrograph, cross-section from testicular tissue stained with H&E at ×40 magnification. (A) The control group with normal cell populations. Histology of tubules after 3 (B), 7 (C), and 42 (D) days. It is clear that the lumen of tubules in lipopolysaccharide-treated mice showed extensive morphological changes, including loss of cell adhesions and necrosis after 42 days.

chyma, severe karyolysis of parenchymal cells, extensive degradation of tissue parenchyma in seminiferous tubules, degradation of Sertoli cells, fragmentation of cell communication, destruction of the tissue organization, a lack of inflammatory cells, damage of the testicular network cells and the epididymis. No metaplastic changes were found in the seminiferous cells. In the inflammatory process, the factor of hyperemia was observed. Necrosis in Leydig cells and the interstitial cell tissue of the testes was also evident Figures 2 and 3.

## Discussion

Based on previous studies, bacterial infections and the subsequent immune response are one of the major causes of infertility in men [5]. Therefore, these infections at different stages of evolution, maturation, and transmission have adverse effects on sperm quality and thereby lead to reduced fertility or infertility. In order to explain the association of uropathogenic *E. coli* with fertility disorders caused by pathogens, the present study focused on the injection of LPS from this strain into BALB/c mice and evaluated its effects on serum and tissues. The results showed that injecting LPS of *E. coli* into healthy male mice during 42 days led to reductions in sperm count, poorer sperm quality, and defective sperm morphology compared to the control group. Seminiferous tubule damage was found, clearly due to the initial inflammation.

The findings of this study are consistent with those reported by Matsuura [21], who showed that in mice infected with Gram-negative bacteria, the presence of LPS stimulated the innate immune response and consequently the inflammatory response, both of which play vital roles in the elimination of pathogens. The common point in these studies was spermatogenic damage in response to Gram-negative bacteria, which account for a large proportion of genital infections. In the study of Bhatt et al. [22], 347 semen specimens were cultured and 62.9% of the isolates were Gram-negative. In the study of Sabra and Al-Harbi [23], more than 40% of sperm contamination was caused by Gram-negative bacteria. Pilatz et al. [24] found that *E. coli* was one of the most common symptomatic or asymptomatic infections of the urinary and genital tract and could change the sperm parameters such as motility. They also showed that the presence of *E. coli* caused the death of 80% of sperm *in vitro* [24]. Boguen et al. [25] reported that *E. coli* incubated with human sperm at a ratio of 1:2 gave rise to diminished sperm parameters, reduced motility, and increased levels of free reactive oxygen species. However, it should be noted that other bacteria such as *Helicobacter pylori*, as well as aerobic and anaerobic enteric bacteria, are directly and indirectly involved in male infertility [3].

The results showed that the injection of LPS led to changes in sperm parameters, such as a reduction in the sperm count, dimin-



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ished motility, and unusual morphology of the sperm on days 7 and 42. Although some researchers believe that the presence of bacteria does not affect the morphology of spermatozoa [26,27], other studies have shown that the morphology is severely damaged. It has been pointed out that the immobility factor produced by *E. coli* has a major impact on the semen of men with oligospermia [28].

Previous research has demonstrated that LPS exerts direct or indirect effects on the seminiferous epithelium. Toll-like receptor 4 on the surface of leukocytes detects the LPS from Gram-negative bacteria, and subsequent signaling induces the production of proinflammatory cytokines such as IL-17A [29]. These cytokines can cause pathological damage in the cells and parenchyma of testicular tissue. In the present study, IL-17A levels on days 3, 7, and 42 after LPS injection increased and could have been correlated with sperm damage. This result is consistent with the study conducted by Babinets et al. [30], who showed that increased Il-17A levels were correlated with male infertility. A study on urinary tract infections (UTIs) caused by uropathogenic *E. coli* reported that IL-17 was a key mediator of the innate immune response to UTIs [13]. As a result, IL-17 could affect the sperm and testis tissue and may play a role in male infertility. Qian et al. [31] showed that high levels of IL-17 in seminal fluid could cause deficiencies in sperm motility, resulting in male infertility related to sperm motility. Huo et al. [32] also determined that extracellular fluoride could stimulate the immune response and cause high expression of the IL-17 signaling pathway in mice. Therefore, infection with Gram-negative bacteria including uropathogenic *E. coli* could damage sperm and testis tissue through both mechanisms: directly through immobilization of sperm and indirectly through soluble factors.

In conclusion, the results showed that LPS from uropathogenic *E. coli* could cause defects in sperm parameters, resulting in decreased motility, and could stimulate the immune system as an antigen. The production of proinflammatory cytokines such as IL-17 gives rise to an immune response in testicular tissue, which results in reduced count and function of sperm, as well as necrosis and damage to the testicular tissue. While mechanisms such as apoptosis have been identified as induction pathways of the death of germ cells, more studies are needed to obtain a clear understanding of the mechanisms involved in sperm immunopathology.

## Conflict of interest

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

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CERM

# Influence of hydrogel encapsulation during cryopreservation of ovarian tissues and impact of post-thawing *in vitro* culture systems in a research animal model

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**Objective:** Using domestic cats as a biomedical research model for fertility preservation, the present study aimed to characterize the influences of ovarian tissue encapsulation in biodegradable hydrogel matrix (fibrinogen/thrombin) on resilience to cryopreservation, and static versus non-static culture systems following ovarian tissue encapsulation and cryopreservation on follicle quality.
**Methods:** In experiment I, ovarian tissues (n=21 animals; 567 ovarian fragments) were assigned to controls or hydrogel encapsulation with 5 or 10 mg/mL fibrinogen (5 or 10 FG). Following cryopreservation (slow freezing or vitrification), follicle viability, morphology, density, and key protein phosphorylation were assessed. In experiment II (based on the findings from experiment I), ovarian tissues (n=10 animals; 270 ovarian fragments) were encapsulated with 10 FG, cryopreserved, and *in vitro* cultured under static or non-static systems for 7 days followed by similar follicle quality assessments.
**Results:** In experiment I, the combination of 10 FG encapsulation/slow freezing led to greater post-thawed follicle quality than in the control group, as shown by follicle viability (66.9%±2.2% vs. 61.5%±3.1%), normal follicle morphology (62.2% ±2.1% vs. 55.2%±3.5%), and the relative band intensity of vascular endothelial growth factor protein phosphorylation (0.58±0.06 vs. 0.42±0.09). Experiment II demonstrated that hydrogel encapsulation promoted follicle survival and maintenance of follicle development regardless of the culture system when compared to fresh controls.
**Conclusion:** These results provide a better understanding of the role of hydrogel encapsulation and culture systems in ovarian tissue cryopreservation and follicle quality outcomes using an animal model, paving the way for optimized approaches to human fertility preservation.

**Keywords:** Cryopreservation; Fertility preservation; Hydrogel; *In vitro* culture; Ovarian tissue

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## Introduction

The survival rate of cancer patients has dramatically increased due to tremendous progress in diagnosis and medical treatments. Nonetheless, female cancer patients in prepubescence or reproductive age who have undergone gonadotoxic anticancer treatments, such as alkylating chemotherapy or ionizing radiotherapy in the pelvic or abdominal regions or total body radiation, may have a high risk of fertility impairment [1]. Over recent decades, ovarian tissue cryopres-

ervation and transplantation have been introduced as adequate options for female fertility preservation and restoration after patients recover from cancer [2]. So far, these technologies have resulted in more than 130 clinical cases of live births worldwide [3]. However, the risk of malignant cell reimplantation after ovarian tissue transplantation is a serious concern and should be addressed, especially in patients with leukemia [3]. In order to ensure the safety of fertility restoration without reseeding of cancer cells, the newly developed promising strategies of *in vitro* follicle activation (IVA) and *in vitro* follicle growth (IVG) have been extensively attempted as sequentially imperative steps prior to the construction of a cancer-free microenvironment for isolated follicle development [1].

Ideally, the *in vitro* activation of primordial follicle development to the pre-antral or secondary follicle stage mainly involves culturing cryopreserved cortical ovarian tissues [1,2,4]. Ovarian tissues are routinely cryopreserved by slow-freezing or vitrification methods using optimized freezing media and protocols that differ among laboratories [5]. Recently, a novel tissue engineering technology using biodegradable hydrogels or polymers (e.g., alginate, fibrin-collagen, fibrin-thrombin clot or three-dimensional (3D)-printed microporous hydrogel scaffolds) was introduced in reproductive medicine research, involving the encapsulation of immature testicular tissue fragments or prototype construction of scaffolds for follicle culture using fibrin-thrombin in various concentrations [4,6]. The porosity of this biodegradable matrix allows angiogenic factors and other essential nutrient components, such as epidermal growth factor and vascular endothelial growth factor (VEGF) [1,4], to be delivered into the gel network. Angiogenic factors and their protein phosphorylation (e.g., protein kinase B [Akt] and tyrosine kinase I [Tie1]) have been considered crucial keys for cell survival, graft neovascularization, and long-term *in vitro* tissue culture [6-8].

To date, very few tissue engineering technology studies related to ovarian tissue cryopreservation and *in vitro* culture have been conducted. The fundamental *in vitro* tissue culture protocol (static culture system) with sequential culture media changed every 1–2 days has been generally performed [1]. However, this culture system lacks medium circulation mimicking natural vascularization, which diminishes cell-to-cell interactions [9]. To address these issues, non-static culture systems using engineered fluid flow manipulation through the fine control of micro-fluid volume (known as microfluidics) have been actively explored in reproductive medicine laboratories [9,10]. A previous study in a mouse model demonstrated that a non-static culture system could potentially support follicle growth and granulosa cell luteinization after long-term culture for 14 days [9]. Although various attempts of ovarian tissue cryopreservation and transplantation have been successful, studies on ovarian tissue cryopreservation together with primordial follicle activation prior to IVG remain limited.

Due to defects in homologous genes resulting in similar molecular, pathological, and clinical phenotypes as in humans [11], domestic cats have been extensively studied as experimental models in a variety of research fields including genetic disorders, viral neuropathology, antiretroviral therapy, allergic asthma, type 2 diabetes mellitus, and assisted reproductive technology [12,13]. Thus, our goal was to address the lack of information on ovarian tissue cryopreservation in hydrogels and IVG using domestic cats as a biomedical research model. The main objectives of the present study were to characterize the influence of ovarian tissue encapsulation in a biodegradable matrix (with different concentrations of fibrinogen) on cryopreservation outcomes, and the influence of static versus non-static culture systems following encapsulation and cryopreservation on ovarian tissue integrity and follicle quality.

## Methods

### 1. Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and all antibodies from Cell Signaling Technology (Danvers, MA, USA), unless otherwise indicated.

### 2. Experimental design

#### 1) Experiment I: influence of ovarian tissue encapsulation on cryopreservation outcomes

A total of 27 ovarian cortical pieces (0.2 cm × 0.2 cm × 0.1 cm; width × length × thickness) from each animal (n = 21; total of ovarian fragments = 567) were retrieved and randomly allocated to different groups; fresh controls, encapsulation without or with 5 mg/mL fibrinogen, 50 IU/mL thrombin, and 0.1 ng/mL VEGF (5 FG) or encapsulation without or with 10 mg/mL fibrinogen, 50 IU/mL thrombin, and 0.1 ng/mL VEGF (10 FG) (Figure 1A). Ovarian cortical pieces from each group were then randomly selected for further equilibration and cryopreservation procedures (either slow freezing or vitrification) and evaluated for follicle viability (neutral red staining), normal morphology (histology and Hoechst 33342), follicle density (the number of follicles with normal morphology per 1 $mm^3$; histology). The evaluation of Tie1 and VEGF receptor (VEGF-R) phosphorylation (Western blot analysis) was performed after cryopreservation only.

#### 2) Experiment II: influence of static and non-static culture systems following encapsulation and cryopreservation on ovarian tissue integrity and survival

The design of experiment II was based on the findings from experiment I (Figure 1B). A total of 10 animals were included. ovarian cortical tissues (27 pieces per animal; total of ovarian tissue frag-


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ments = 270) were divided into three major groups; fresh control, slow freezing after encapsulation in 10 FG, or vitrification after encapsulation in 10 FG. Tissues then were cultured for 1 week in static or the non-static systems. Ovarian tissue quality was evaluated on day 0 and day 7 in terms of follicle viability (neutral red staining), follicle density and developmental stages (histology), and Akt phosphorylation (pAkt) (Western blot analysis).

### 3. Ovarian tissue retrieval

Ovaries from 31 female cats (age range, 1–3 years) without any pathological conditions (e.g., ovarian cysts, para-ovarian cysts, cystic endometrium hyperplasia, or pyometra) were included in the study. Tissues were collected from the routine ovariohysterectomy at the Veterinary Public Health Division of Bangkok Metropolitan Administration, Thailand. The collection and use of tissues were approved by Institutional Animal Care and Use Committee, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Ovaries were kept in isotonic saline solution supplemented with 1 IU/mL penicillin-streptomycin and transported to the laboratory at room temperature within 3 hours.

Cortical slices (~ 0.2 × 0.2 × 0.1 cm; width × length × thickness) were dissected from the ovaries using tissue scissors, scalpel blade and stored (38.5°C) in the maintenance medium (Eagle's Minimum Essential Medium [MEM] containing 2 mM L-glutamine, 10 IU penicillin G sodium, 10 µg/mL streptomycin sulfate, 1 mg/mL bovine serum albumin, and 25 mM [4-(2-hydroxyethyl)-1-piperazineethane-

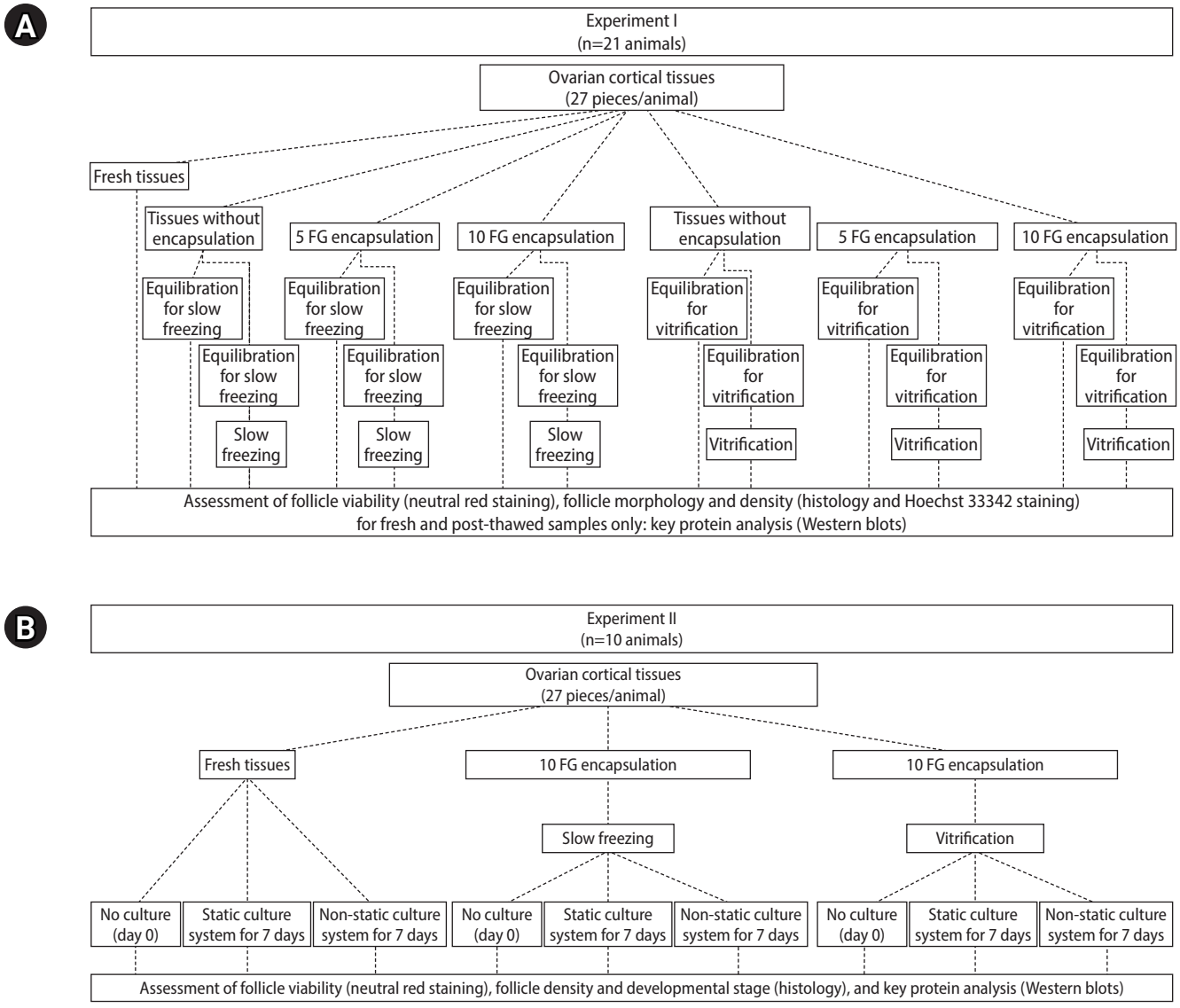


**Figure 1.** Designs of experiments I (A) and II (B). 5 FG, 5 mg/mL fibrinogen, 50 IU/mL thrombin, and 0.1 ng/mL VEGF; 10 FG, 10 mg/mL fibrinogen, 50 IU/mL thrombin, and 0.1 ng/mL VEGF; VEGF, vascular endothelial growth factor.

sulfonic acid; HEPES]) for less than 1 hour prior to encapsulation and cryopreservation procedures.

### 4. Ovarian tissue encapsulation

Ovarian tissue encapsulation by fibrin clot construction was performed according to previous studies [4,14]. Briefly, ovarian cortical pieces were directly placed in fibrin gel drops (fibrinogen/thrombin cocktail) composed of 5 mg/mL fibrinogen and 50 IU/mL thrombin (5 FG group) or 10 mg/mL fibrinogen and 50 IU/mL thrombin (10 FG group) supplemented with 0.1 ng/mL VEGF. To create the fibrinogen/thrombin cocktail, the fibrinogen was diluted in 25 mM HEPES-0.9% saline solution, whereas the thrombin was prepared in 40 mmol/L $CaCl_2$ solution. Ovarian tissues were placed in the hydrogel drops and then incubated at 37°C for 30 minutes to allow gel polymerization.

### 5. Ovarian tissue cryopreservation

Each encapsulated ovarian cortical piece was randomly allocated to slow freezing or vitrification procedures, as previously reported [15,16]. For slow freezing, ovarian tissues with each designed fibrin gel concentration, including the fresh control, were equilibrated in the freezing medium (MEM medium supplemented with 0.1 ng/mL VEGF, 1.5 M dimethyl sulfoxide [DMSO; Fluka Chemie, Buchs, Spain] and 0.1 M sucrose) at 4°C for 15 minutes, transferred to pre-cooled 2-mL cryovials (cryogenic vials; Corning Inc., Corning, NY, USA), and stored at 4°C for another 15 minutes. Cryovials containing equilibrated ovarian cortical slices were then kept in a freezing container (Mr. Frosty Freezing Container; Thermo Fisher Scientific, Waltham, MA, USA) at −80°C for 24 hours (cooling rate: −1°C/min) and manually dropped into liquid nitrogen (−196°C). For thawing, cryovial tubes were transferred to a water bath at 37°C for 5 minutes. The thawed ovarian tissues were then placed in freshly prepared media; first in MEM with 0.75 M DMSO and 0.25 M sucrose (room temperature for 10 minutes), then in MEM with 0.25 M sucrose (room temperature for 10 minutes), and finally in MEM alone as the maintenance medium.

For vitrification, control and encapsulated ovarian tissues were sequentially placed in (1) the equilibration medium (MEM with 20% fetal bovine serum [FBS] [v/v], 0.1 ng/mL VEGF, 0.96 M DMSO, and 1.21 M ethylene glycol [EG]; room temperature for 10 minutes); (2) the vitrification medium (MEM with 20% FBS, 0.1 ng/mL VEGF, 1.92 M DMSO, 2.42 M EG, and 0.5 M sucrose; 4°C for 30 minutes), and then transferred to pre-cooled cryovials and immediately plunged into liquid nitrogen. For warming, cryovials containing vitrified ovarian tissues were held in air at room temperature for 30 seconds. Ovarian tissues were placed in the warming medium (MEM with 1.0 M sucrose and 20% FBS; 37°C for 10 minutes), and in MEM alone as the maintenance medium.

### 6. Ovarian tissue culture

Fresh and cryopreserved cortical slices were randomly allocated to either the static [17] or the non-static culture system. For the static culture system, ovarian tissues were cultured on top of 1.5% (w/v) agarose gel submerged in 500 µL of culture medium (MEM supplemented with 2 mM glutamine plus 10 IU/mL penicillin G sodium, 10 µg/mL streptomycin sulfate, 0.05 mM ascorbic acid, 0.5 µg/mL insulin, 0.4 µg/mL transferrin, 0.5 ng/mL selenium, 10 ng/mL follicle-stimulating hormone, and 0.1% [w/v] polyvinyl alcohol) at 38.5°C in humidified 5% $CO_2$ in air for up to 1 week. The culture medium was fully replaced every other day throughout the culture period. For the non-static culture system, a 12-well culture plate was modified by adding a 0.16-cm inlet tube connecting with the syringe pump (Chemyx, Stafford, TX, USA) and a 0.16-cm outlet port (Figure 2). A total of 20 mL of culture medium was loaded into the pump, which provided a constant flow rate (0.000173 mL/min) to the modified culture plate for 1 week. The constant flow rate was calculated from the volume of the culture medium in the static culture system that was refreshed every second day divided by 2,880 (48 hours multiplied by 60 minutes); the constant flow rate of 500 µL/2,880 min was therefore equivalent to 0.000173 mL/min.

### 7. Follicle viability and morphology assessments

The viability of follicles enclosed in the ovarian cortex was evaluated by neutral red staining [16]. Briefly, cortical slices were placed in the culture medium supplemented with 50 µg/mL neutral red and incubated in dark at 37°C for 1 hour. Follicular viability was assessed under the light microscope (Nikon Eclipse E100; Nikon, Tokyo, Japan) at ×100 magnification. All follicles within each cortical piece were counted and classified as viable (red color) or non-viable (non-staining) to calculate the percentage of viable follicles.

To estimate the developmental stage (percentage and ratio), the structural morphology and the density of follicles, ovarian tissues were fixed in 4% paraformaldehyde at room temperature and dehydrated in graded series of ethanol solutions prior to tissue sectioning [18]. Serial sections of each cortical piece (5 µm thickness each) were stained with hematoxylin and eosin. Two sections at 10-µm intervals of each cortical piece were evaluated under light microscopy at ×100 and ×400 magnification. The developmental stage was classified according to one of three categories: (1) primordial follicle (one layer of flattened granulosa cells circumscribing the oocyte); (2) transitional follicle (one layer of flattened and cuboidal granulosa cells surrounding the oocyte), or (3) primary follicle (one layer of cuboidal granulosa cells surrounding the oocyte). Follicles with intact oocytes and granulosa cells were categorized as having normal morphology


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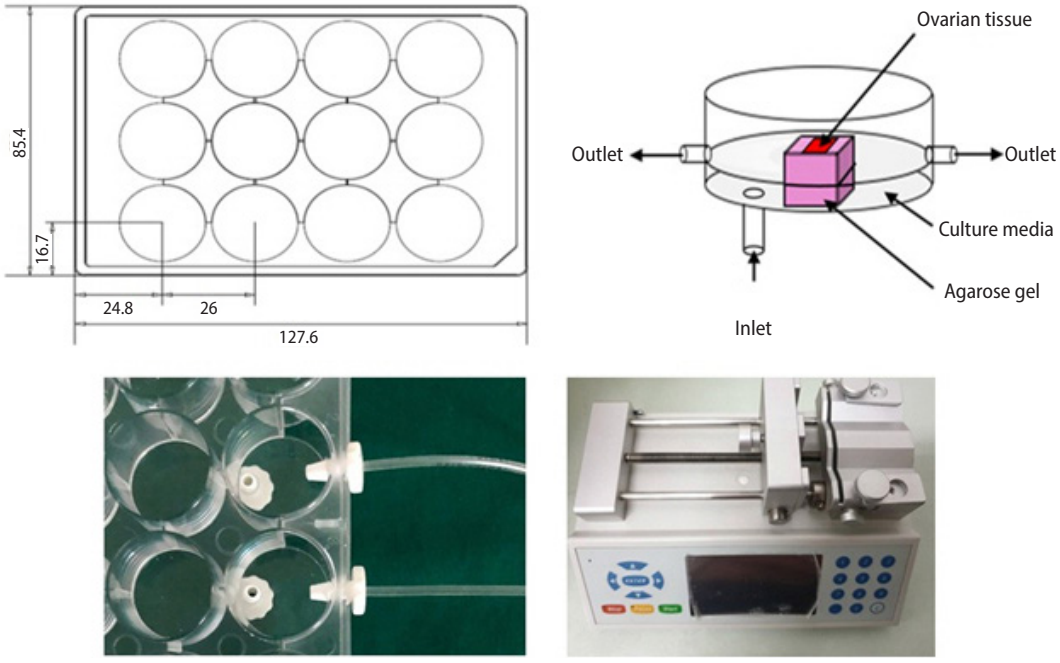


**Figure 2.** Modified non-static culture system using 12-well culture plate connected with a 0.16-cm inlet tube-syringe pump (constant flow rate) and a 0.16-cm outlet tube.

in contrast with their abnormal counterparts with fragmented, pyknotic nuclei or disruption of granulosa cells [17]. Furthermore, the structural morphology of isolated follicles from the cortical tissue (left from neutral red staining) was assessed using 2 μM Hoechst 33342 staining (Invitrogen, Thermo Fisher Scientific) under a fluorescence microscope at ×200 magnification (Nikon Eclipse TI fluorescence microscope). To measure the follicle density, the number of the intact follicles in 10 random microscopic reticule areas (0.0625 $mm^2$ per scale) were counted at ×100 magnification. The relative follicle density per 1 $mm^2$ was calculated from the microscopic reticule areas and recorded.

### 8. Evaluations of Akt, Tie1, and VEGF-R phosphorylation

The ovarian tissues were minced using a mortar and were incubated with a protein lysis buffer (CelLytic MT) supplemented with a protease inhibitor for 30 minutes on a shaker at room temperature (22°C). After centrifugation at 12,000 ×*g* at 4°C for 10 minutes, the supernatant was assessed for total protein concentration using NanoDrop One (Thermo Fisher Scientific) and adjusted to 15 μg/μL in phosphate-buffered saline before storage at –80°C. The Western blot analysis was performed as previously reported [19]. Briefly, post-thawed protein samples were mixed with SDS sample loading buffer (4× Laemmli sample buffer and β-mercaptoethanol; Bio-Rad, Hercules, CA, USA) and processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4%–15% Mini-protein TGX Precast Gels; Bio-Rad) for 1.5 hours. The precision Plus Protein Dual Color Standards (Bio-Rad) loaded into the first lane were used as the molecular weight standard. The complete SDS-PAGE run was transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Billerica, MA, USA) and blocked (non-specific binding protein) with 2.5% skim milk (Bio-Rad) diluted in washing buffer (Tris-buffered saline plus 0.1% [v/v] Tween) for 1 hour at room temperature (22°C). The transferred membrane was then incubated on the shaker in the dark at 4°C overnight with 1 of the following primary antibodies: (1) rabbit anti-β-actin (1:1,000); (2) rabbit anti-phospho-Akt (1:1,000; Ser473 pAkt); (3) rabbit anti-Tie1 (1:100; Tie1 D2K2T) or IV) rabbit anti-phospho-VEGF-R-2 (1:1,000; Tyr1175). The sample incubated with the normal rabbit IgG (1:1,000) served as the negative control. After overnight incubation with the primary antibody, the blot membrane was incubated with the secondary antibody (anti-rabbit IgG antibody conjugated with horseradish peroxidase, 1:1,000) for 2 hours at room temperature (22°C). Immunoreactivity was detected by the colorimetric method (Opti 4CNTM Substrate Kit, Bio-Rad). The intensity of each protein band and background was measured using the Axygen Gel Documentation System (Corning, Corning, NY, USA). The relative quantification was normalized by dividing the intensity of each primary antibody (intensity of each protein band minus background) by that of the β-actin band.

### 9. Statistical analysis

Data analyses were performed using SAS ver. 9.2 (SAS Institute, Cary, NC, USA). The normal distribution of residuals was tested using

the univariate procedure with the "normal" option. The dependent variables included follicle viability, normal morphology, follicle developmental stage, follicle density, and relative quantification of protein bands. When the data were normally distributed, differences in dependent variables among the experimental groups were compared using one-way analysis of variance followed by the Tukey-Kramer test. Non-normally distributed data were analyzed using the Wilcoxon signed rank test. Relative quantification of protein bands was evaluated using a mixed model (the "mixed" procedure). All results are presented as mean ± standard deviation (SD). The level of significance was set at $p \leq 0.05$.

## Results

### 1. Experiment I: influence of ovarian tissue encapsulation on cryopreservation outcomes

More than 80% of follicles were viable in the freshly-excised ovarian cortices. Although the values were similar ($p > 0.05$) during equilibration, viability decreased ($p \leq 0.05$) during cryopreservation (Table 1). Follicle viability was influenced by the cryopreservation procedure, and vitrification without tissue encapsulation yielded the lowest percentages ($p \leq 0.05$) (Table 1). Although not significantly different from 5 FG encapsulation, the samples processed with 10 FG encapsulation before slow freezing or vitrification had better percentages of viable follicles ($p \leq 0.05$) than their non-encapsulated counterparts. However, slow freezing led to the highest number of viable follicles ($p \leq 0.05$) (Table 1).

In accordance with the viability observations, the histology revealed a decrease in the percentage of structurally normal follicles after cryopreservation ($p \leq 0.05$) (Table 2). The proportion of normal follicle morphology in the cryopreserved non-encapsulated tissues (control) was lower ($p \leq 0.05$) than in the encapsulated samples (Table 2, Figure 3). Although not significantly different from 5 FG encapsulation, the samples processed with 10 FG encapsulation and slow freezing presented the highest proportions of normal follicles (Table 2). When the isolated follicles were stained using the fluorescence dye, follicles with abnormal structural morphology were present in the fresh samples and all freezing conditions (Table 2). In the post-thawing evaluation, the percentage of structurally normal follicles in the control was lower than in the encapsulated samples (slow freezing group) ($p \leq 0.05$). As with the previous evaluation criteria, cryopreservation procedures negatively affected follicle density ($p \leq 0.05$) (Table 3). However, encapsulation could not mitigate the loss (Table 3). The best follicle density after thawing tended to be from 10 FG encapsulation and slow freezing (Table 3).

The Western blot analysis revealed appreciable levels of Tie1 relative band intensity in the fresh controls (mean ± SD, 0.32 ± 0.09) and in all treatment groups (range, 0.24–0.32). In the 5 FG group, the Tie1 relative band intensity was not significantly different ($p > 0.05$) between fresh controls and all treatment groups (range, 0.24–0.27). In the 10 FG group, the Tie1 relative band intensity ranged from 0.25 to 0.31 and also was not significantly different between the controls and the treatment groups (Figure 4A). For the VEGF-R phosphorylation relative band intensity, the values in the fresh and treatment groups ranged from 0.35 to 0.58 (fresh sample: mean ± SD, 0.51 ± 0.1). In the 5 FG group, the relative band intensities were not significantly different ($p > 0.05$) between the fresh controls and all treatment groups (range, 0.37–0.41). In the 10 FG group, the VEGF-R phosphorylation relative band intensity in the encapsulated cortical tissues that were cryopreserved by slow freezing (mean ± SD, 0.58 ± 0.06) was significantly higher ($p \leq 0.05$) than in the non-capsulated samples (mean ± SD, 0.42 ± 0.09) and the ovarian tissues cryopreserved by vitrification (mean ± SD, 0.35 ± 0.08) (Figure 4B). The western blot bands revealed Tie1 and VEGF-R phosphorylation from selected animal were demonstrated in (Figure 4C).

Overall, the outcomes were better with 10 FG encapsulation than with 5 FG encapsulation. Therefore, this treatment was selected for further exploration in experiment II.

**Table 1.** Percentages of viable follicles in ovarian tissues that were encapsulated or not encapsulated before equilibration and cryopreservation (slow freezing vs. vitrification)

| Variable | Slow freezing | | | Vitrification | | |
|---|---|---|---|---|---|---|
| | No encapsulation | Fibrin encapsulation | | No encapsulation | Fibrin encapsulation | |
| | | 5 FG | 10 FG | | 5 FG | 10 FG |
| Fresh (%) | 85.7 ± 3.5[a)] | - | - | 85.7 ± 3.5[a)] | - | - |
| Equilibration (%) | 80.4 ± 4.7[a)] | 82.9 ± 2.9[a)] | 84.3 ± 2.4[a)] | 81.5 ± 4.0[a)] | 82.6 ± 4.8[a)] | 82.5 ± 3.2[a)] |
| Post-thawing (%) | 61.5 ± 3.1[b)] | 63.1 ± 2.4[b)] | 66.9 ± 2.2[b)] | 53.7 ± 3.7[b)] | 58.1 ± 4.2[b)] | 61.2 ± 3.6[b)] |

Values are presented as mean±standard deviation. n=21 animals.
5 FG, 5 mg/mL fibrinogen, 50 IU/mL thrombin, and 0.1 ng/mL VEGF; 10 FG, 10 mg/mL fibrinogen, 50 IU/mL thrombin, and 0.1 ng/mL VEGF; VEGF, vascular endothelial growth factor.
[a),b)]Different letters indicate statistically significant differences within the column ($p \leq 0.05$).



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**Table 2.** Percentages of morphologically normal follicles in ovarian tissues that were encapsulated or not encapsulated before equilibration and cryopreservation (slow freezing vs. vitrification)

| Evaluation technique | Slow freezing | | | Vitrification | | |
|---|---|---|---|---|---|---|
| | Control | Fibrin encapsulation | | Control | Fibrin encapsulation | |
| | | 5 FG | 10 FG | | 5 FG | 10 FG |
| Fresh (%) | | | | | | |
| Histology | 78.4 ± 3.2[a)] | - | - | 78.4 ± 3.2[a)] | - | - |
| Hoechst staining | 81.3 ± 5.1[a)] | - | - | 81.3 ± 5.1[a)] | - | - |
| Equilibration (%) | | | | | | |
| Histology | 75.2 ± 4.4[a)] | 77.7 ± 2.9[a)] | 76.8 ± 3.8[a)] | 74.1 ± 5.1[a)] | 75.7 ± 4.0[a)] | 76.2 ± 3.6[a)] |
| Hoechst staining | NE | NE | NE | NE | NE | NE |
| Post-thawing (%) | | | | | | |
| Histology | 55.2 ± 3.5[b)] | 59.1 ± 3.8[b)] | 62.2 ± 2.1[b)] | 54.2 ± 2.8[b)] | 56.7 ± 3.5[b)] | 57.5 ± 2.9[b)] |
| Hoechst staining | 58.9 ± 5.7[b)] | 64.4 ± 5.1[b)] | 65.7 ± 4.4[b)] | 56.1 ± 3.9[b)] | 59.7 ± 5.0[b)] | 58.8 ± 6.4[b)] |

Values are presented as mean±standard deviation. n=21 animals.
5 FG, 5 mg/mL fibrinogen, 50 IU/mL thrombin, and 0.1 ng/mL VEGF; 10 FG, 10 mg/mL fibrinogen, 50 IU/mL thrombin, and 0.1 ng/mL VEGF; NE, not evaluation; VEGF, vascular endothelial growth factor.
[a),b)]Different letters indicate statistically significant differences within the column ($p \leq 0.05$).

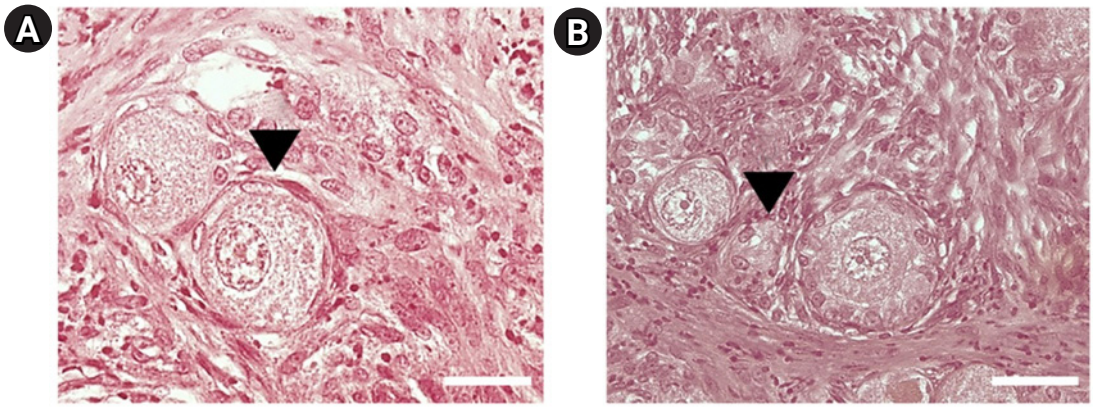


**Figure 3.** Normal follicle morphology (one layer of flattened granulosa cells circumscribing the oocyte; arrow) retrieved from encapsulated ovarian cortex (A) compared to abnormal follicle morphology (disruption of granulosa cells; arrow) from the non-encapsulated group (B). H&E, bar=50 μM.

**Table 3.** Follicle density (number of normal follicle morphology per 1 $mm^3$) in ovarian tissues that were encapsulated or not encapsulated before equilibration and cryopreservation (slow freezing vs. vitrification)

| Variable | Slow freezing | | | Vitrification | | |
|---|---|---|---|---|---|---|
| | Control | Fibrin encapsulation | | Control | Fibrin encapsulation | |
| | | 5 FG | 10 FG | | 5 FG | 10 FG |
| Fresh (%) | 19.7 ± 2.5[a)] | - | - | 19.7 ± 2.5[a)] | - | - |
| Equilibration (%) | 18.3 ± 3.5[a)] | 18.1 ± 4.8[a)] | 18.6 ± 3.1[a)] | 19.1 ± 4.1[a)] | 18.9 ± 4.0[a)] | 19.5 ± 4.7[a)] |
| Post-thawing (%) | 12.8 ± 3.3[b)] | 14.1 ± 2.4[b)] | 14.7 ± 3.7[b)] | 10.3 ± 2.6[b)] | 12.2 ± 2.6[b)] | 12.7 ± 2.1[b)] |

Values are presented as mean±standard deviation. n=21 animals.
5 FG, 5 mg/mL fibrinogen, 50 IU/mL thrombin, and 0.1 ng/mL VEGF; 10 FG, 10 mg/mL fibrinogen, 50 IU/mL thrombin, and 0.1 ng/mL VEGF; VEGF, vascular endothelial growth factor.
[a),b)]Different letters indicate statistically significant differences within the column ($p \leq 0.05$).

### 2. Experiment II: influence of static and non-static culture systems following encapsulation and cryopreservation on ovarian tissue integrity and survival

A decrease in follicle viability was detected after both slow freezing and vitrification ($p \leq 0.05$) while more than 50% of follicles remained viable (Table 4). The culture systems or the freezing protocols did not markedly influence follicle viability and density (Table 4). Importantly, the histological analysis demonstrated that encapsulation and culture systems were able to maintain proportions of follicular stages that were similar to those of the fresh controls (Figure 5). The ratio to primordial follicles (on day 0 [D0]) to transitional stage follicles (on day 7 [D7]) slightly varied depending on the condition (1.0:0.91 for the slow freezing and static culture system, 1.0:0.97 for the slow freezing and non-static culture system, 1.0:1.48 for the vitrification and static culture system, and 1.0:1.07 for the vitrification and non-static culture system). However, the average ratio of primordial follicles (D0) to primary follicles (D7) was 1.0:0.40 across all experimental groups (1.0:0.40 for the slow freezing and static culture system, 1.0:0.43 for the slow freezing and non-static culture system, 1:0.36 for the vitrification and static culture system, and 1.0:0.40 for the vitrification and non-static culture system). The phosphorylation of Akt was observed in all fresh control and treatment tissue samples (Figure 2). The relative quantification of pAkt band intensity did not significantly differ among groups (range, 0.19–0.25; $p \leq 0.05$) (Figure 6).

## Discussion

Using domestic cats as a model for human fertility preservation,

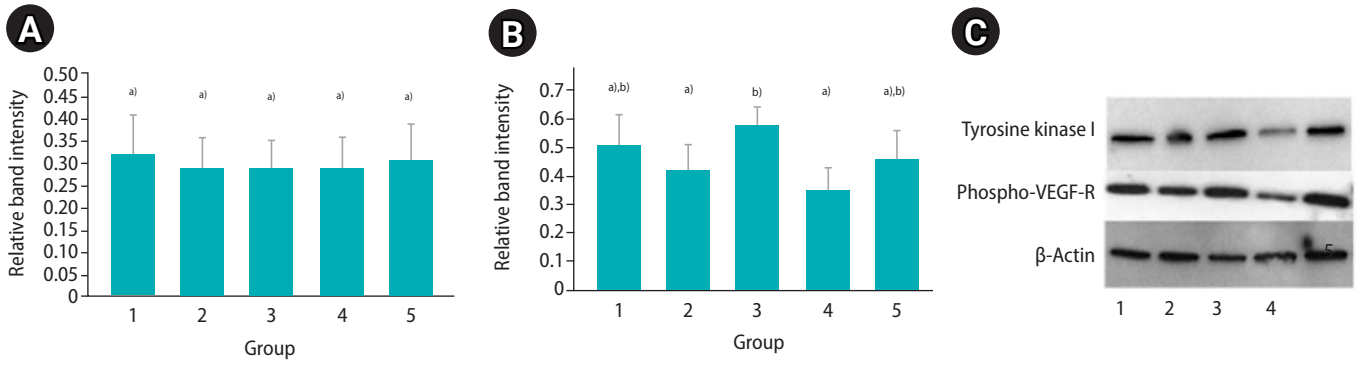


**Figure 4.** Relative band intensity of tyrosine kinase I (A) and vascular endothelial growth factor receptor (VEGF-R; B) phosphorylation in fresh (control), slow frozen, or vitrified cortical samples after encapsulation (with or without 10 mg/mL fibrinogen, 50 IU/mL thrombin, and 0.1 ng/mL VEGF; 10 FG) (mean±standard deviation; n=21 animals). Group 1, fresh ovarian tissue; group 2, ovarian tissue cryopreserved by slow freezing (without encapsulation); group 3, ovarian tissue cryopreserved by slow freezing (with encapsulation); group 4, ovarian tissue cryopreserved by vitrification (without encapsulation); group 5, ovarian tissue cryopreserved by vitrification (with encapsulation). (C) Western blot bands represent tyrosine kinase I and VEGF-R phosphorylation, signaling protein expression from a selected animal. [a),b)]Different letters indicate statistically significant differences between groups ($p \leq 0.05$).

**Table 4.** Percentages of viable follicles and follicle density (number of normal follicle morphology per 1 $mm^3$) in ovarian tissues that were encapsulated (10 FG), cryopreserved (slow freezing vs vitrification), and cultured (static and non-static culture systems) for 7 days

| Variable | Day 0 | Day 7 | Day 7 |
|---|---|---|---|
| | | Static culture system | Non-static culture system |
| Follicle viability | | | |
| Fresh control (%) | 81.7±4.9[a)] | 71.3±2.8[a)] | 74.1±4.4[a)] |
| Slow freezing (%) | 65.2±5.1[b)] | 52.7±4.1[b)] | 55.9±3.7[b)] |
| Vitrification (%) | 61.2±2.2[b)] | 50.1±5.3[b)] | 53.7±2.9[b)] |
| Follicle density per 1 $mm^3$ | | | |
| Fresh control (%) | 19.4±3.9[a)] | 15.1±4.8[a)] | 16.2±2.1[a)] |
| Slow freezing (%) | 14.6±2.7[a),b)] | 8.9±3.2[b)] | 9.5±4.4[b)] |
| Vitrification (%) | 13.1±4.2[b)] | 7.8±2.5[b)] | 8.6±3.1[b)] |

Values are presented as mean±standard deviation. n=10 animals.
10 FG, 10 mg/mL fibrinogen, 50 IU/mL thrombin, and 0.1 ng/mL VEGF; VEGF, vascular endothelial growth factor.
[a),b)]Different letters indicate statistically significant differences within the column ($p \leq 0.05$).


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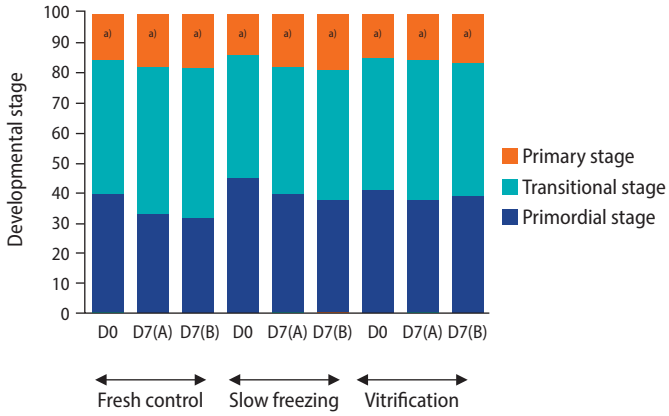


**Figure 5.** Mean percentage of follicles at each developmental stage in ovarian tissues that were freshly-excised (fresh control) or encapsulated (10 mg/mL fibrinogen, 50 IU/mL thrombin, and 0.1 ng/mL vascular endothelial growth factor), cryopreserved by slow freezing or vitrification procedures, and cultured for 7 days. D0, day 0; D7(A), static culture system for 7 days; D7(B), non-static culture system for 7 days (n=10 animals). [a)]Different letters indicate statistically significant differences in primary follicle development between groups ($p \leq 0.05$).

the combination of 10 FG encapsulation and slow freezing appeared to be the best combination to preserve follicle viability, normal morphology, and VEGF protein phosphorylation. Fibrin encapsulation also promoted post-thawing survival and the maintenance of different follicular stages during 7 days of culture (regardless of the culture system).

In 2017, a meta-analysis of 14 studies related to human ovarian tissue cryopreservation protocols reported non-significant differences between slow freezing and vitrification based on the proportion of intact primordial follicles [5]. However, ovarian tissue cryopreservation by slow freezing remains the most frequent technique in humans compared to vitrification methods [20]. Furthermore, slow freezing of ovarian tissue combined with transplantation has enabled more live births than vitrification [20]. Following human ovarian tissue cryopreservation and xenotransplantation, recent research data also revealed better outcomes with slow freezing than vitrification in terms of primordial follicle survival, primordial follicle counts, follicle growth, proliferation and angiogenesis markers (Ki-67 and CD31) [21]. In addition, the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and transmission electron microscopy showed higher levels of DNA damage and follicle deformation in the vitrification group [21]. Our findings are consistent with this previous study [21] since the percentages of the post-thawed follicle viability were lowest in the non-encapsulated vitrified cortical samples. Detrimental factors, such as an improper cooling rate and/or adverse effects of cryoprotective agents (CPA), have been shown to lead to follicle deformation, oocyte shrinkage, or granulosa cell disruption during ovarian tissue cryopreservation [22]. In the present study, the ovarian tissues were processed at a 2-fold higher concentration of CPA (15% DMSO) during vitrification protocol than during slow freezing (7.5% DMSO). The most up-to-date evidence as of 2019 markedly revealed the toxicity of DMSO when the DMSO concentration gradually increased (10% to 50%) during ovarian tissue cryopreservation by vitrification [23]. The resultant cellular membrane disintegration, chromatin thickening, and cell apoptosis induction likely led to follicle death [23].

Due to their unique properties, soft biodegradable materials or hydrogels have recently been explored and used in cell and tissue research [24]. In reproductive medicine, studies focused on isolated follicles encapsulated under the hydrogel micro-environment have been well documented [25,26]. However, reports on ovarian tissue enclosed in those novel materials are still limited. Our results notably demonstrated that fibrin encapsulation promoted favorable follicle morphology and survival after slow-freezing and thawing. Due to its typical 3D network structure and density, a porous hydrogel is typically adjustable, which allows it to have a proper affinity in the aqueous environment. It also mitigates changes of the osmotic stress caused by CPA exposure [6]. In addition, hydrogels can bind to the surface of very small ice crystals and prevent their detrimental expansion [6,27]. Therefore, hydrogel-based encapsulation has been considered an effective approach to preserve cells during cryopreservation [24]. This was confirmed in mouse immature testicular tissues encapsulated in a fibrin hydrogel [28]. Fibrin gel could serve as an integral component of the cryopreservation medium, which effectively contributed to the preservation of the average density of germinative cells and their metabolic activity (total lactate dehydrogenase activity) [28]. In addition to vitrification, fibrin encapsulation supported post-thawed follicle viability compared to non-encapsulated samples. Nonetheless, the observed results were lower than in the slow freezing group. In addition to the results related to the effects of CPA concentration, as discussed above, a previous study in c3h10t1/2 mesenchymal stem cells demonstrated that alginate encapsulation could significantly augment post-thawed cell viability when a cooling rate of more than 50,000°C/min was applied during the vitrification procedure by using a funnel-shaped quartz microcapillary as a freezing device [29]. Furthermore, the aqueous volume of the confined space around the cell should be less than 0.1 µL, which leads to the effective regulation of ice nucleation and water transport properties during vitrification [30]. Due to the complexity of the ovarian tissue compartment, the obtained results might be different from single-cell investigations. In addition, neither the cooling rate nor aqueous volume was measured in the present study. These might be among the principal factors that could detrimentally affect post-thawed follicle quality in ovarian tissue vitrification, as

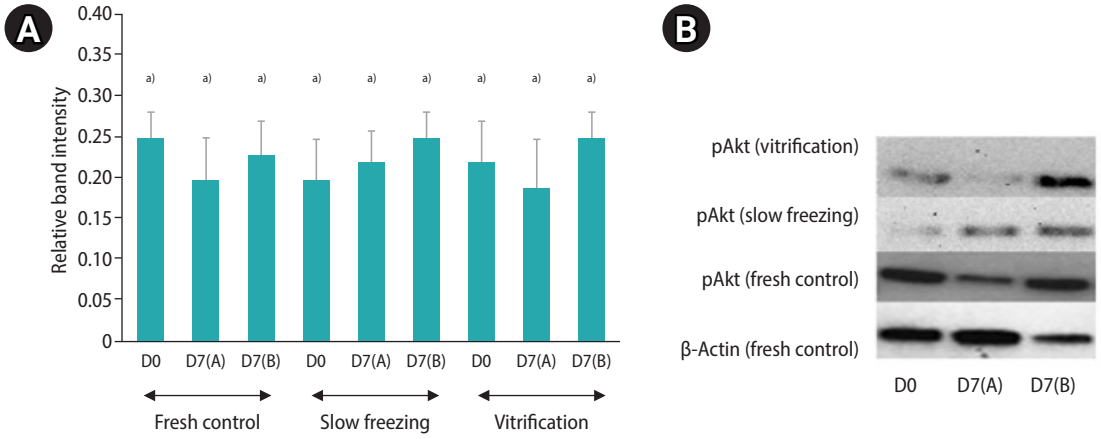


**Figure 6.** (A) Relative band intensity of protein kinase B phosphorylation (pAkt) expression in fresh (control), slow freezing and vitrification cortical samples (with or without 10 mg/mL fibrinogen, 50 IU/mL thrombin, 0.1 ng/mL vascular endothelial growth factor) and cultured in various conditions; D0, day 0; D7(A), static culture system for 7 days; D7(B), non-static culture system for 7 days (mean±standard deviation; n=10 animals).[a)]Different letters indicate statistically significant differences between each group ($p \leq 0.05$). (B) Western blot bands represent pAkt signaling protein (60 kDa) expression from a selected animal.

observed in our study.

Regarding protein phosphorylation results, the highest VEGF-R phosphorylation was observed in samples that underwent 10 FG encapsulation and slow freezing, while Tie1 phosphorylation did not differ among treatment groups. Along with their influential effects on cell integrity preservation, another crucial feature of fibrin hydrogels at gradually increasing concentrations (i.e. 12.5 or 25 mg/mL) is their support of angiogenic activity (i.e., VEGF-R) [31,32]. This is related to the presence of arginine-glycine-aspartate peptides, which can effectively promote crosslinking with various types of exogenous angiogenic or growth factors [31,32]. Furthermore, the fibrin scaffold could potentially mimic physiological angiogenesis, immobilize VEGF in the matrix, and prolong and activate its release, which might lead to VEGF-R activation [33,34]. Supporting data were found in a mouse ovarian tissue transplantation study, in which the post-thawed cortical piece encapsulated with the fibrin hydrogel modified with heparin-binding peptide (HBP) and VEGF had the greatest number of viable primordial follicle count per tissue section (follicle density) compared to non-encapsulated or fibrin-HBP samples [34].

For Tie1, this receptor is structurally related to tyrosine kinase receptors, which are selectively expressed on vascular endothelial cells, promote vascular integrity, and are considered to be an endothelial cell activation marker [8]. However, data about VEGF-Tie1 activity related to ovarian function remain limited. A study of human umbilical vein endothelial cells noted that VEGF could stimulate the formation of the Tie1 endodomain during a 30-minute VEGF treatment, whereas it did not increase in response to 24-hour VEGF stimulation [35]. In addition, tumor necrosis factor-alpha (TNFα), a relevant marker of apoptotic cell death that increases during mammalian cell cryopreservation, could stimulate Tie1 protein cleavage and decrease the cellular Tie1 activity over 24 hours [35]. In our study, the imbalance between VEFG and TNFα activities might primarily explain the absence of a difference in Tie1 protein phosphorylation among all frozen groups due to the short exposure of VEGF to the ovarian samples prior to the cryopreservation process. To obtain stronger evidence, the dynamic regulation of TNFα during encapsulated ovarian tissue cryopreservation should be further investigated.

In recent research, dynamic microenvironment circulation in non-static culture systems using precise media flow rates (microfluidics) was employed and offered an innovative technology for single or multiple cells/tissues in long-term culture [9]. In our study, the best follicle quality was observed in the ovarian cortices cultured in the non-static system for 1 week. However, the data did not differ statistically from the control group. Previous studies reported the efficacy of dynamic culture media flow for maintaining gamete cell development and hormone production [9,10]. For example, neonatal mouse testicular tissues cultured in a microfluidic platform presented the distribution of green fluorescence protein (GFP) throughout the tissues, whereas GFP was absent in the agarose gel block tissue cultured under the static system [36]. Additionally, in observations after a 39-day dynamic culture system, round and elongated spermatids were perceived with GFP cap-like the arched structures, providing evidence of spermatid development [36]. In female reproductive biology research, a study in 2020 performed follicle activation in cat ovarian tissue culture under a microfluidic chamber of soft-lithography polydimethylsiloxane (PDMS) [37]. The results showed that viable and normal structural follicles could be maintained in the microfluidic device after 4 days, unlike samples on an agarose gel block [37]. Our contradictory findings may have resulted from differences in the tissue culture system (with or without an agarose gel block),


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culture media flow rate (0.002 mL/min [37] vs. 0.00017 mL/min in our study), or the type of device fabrication (3D printing-PDMS or a modified conventional cell culture plate). Although gonadal tissue culture on agarose gel blocks has been attempted [17,38], recent evidence showed a diminished distribution of GFP protein markers related to spermatid development during long-term mouse testicular tissue culture using agarose gel [36]. With more than a 10-times lower flow rate [37], the oxygen and nutrients diffused through the agarose gel and the hydrogel encapsulation might have been insufficient [35]. Consequently, precise microfluidic platform construction also requires expert-level device fabrication [10,35]. In the present study, the basic design of the non-static culture platform was preliminary and manually adjusted based on the conventional cell culture plate with a flexible size of inlet/outlet ports. Hence, less precise tissue culture compartments were conceivably made compared to the microfluidic device fabricated by 3D printing technology. Finally, the present findings did not reveal a difference in pAkt relative band intensity among groups, supporting the non-significant results for follicle stage and development. Although pAkt can trigger the phosphorylation of the transcription factor forkhead box O3, which is an important cell proliferation and differentiation regulator, the phosphorylation of Akt can be diminished by various factors, such as epigenetic regulation [39]. Recent data in 2018 demonstrated that the overexpression of non-coding RNA (microRNA), such as miRNA-195, activated interleukin and TNFα, while suppressing protein expression (VEGF, PI3K, and pAkt) [40]. Additionally, alterations of microRNA have been observed during cell culture and cryopreservation procedures [41]. Further expanded studies on genetic/epigenetic regulation and protein expression should be considered to augment the missing data in the present study.

In conclusion, our findings demonstrated the benefits of novel technologies focused on ovarian tissue encapsulation, cryopreservation, and *in vitro* culture by a non-static system using a cat model. Our study examined follicle viability, the percentage of normal morphology, and protein phosphorylation during tissue encapsulation and freezing procedures. These findings could potentially be translated to fertility preservation in cancer patients along with other potential modernized steps of ovarian tissue cryopreservation and culture strategies. Additionally, the present data may contribute to the achievement of IVA, leading to IVG and retransplantation of follicles without malignant cancer cell contamination.

## Conflict of interest

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

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## Author contributions

Conceptualization: PT (Paweena Thuwanut), AP, WS (Weerayut Srituravanich). Data curation: PT (Paweena Thuwanut), WS (Weerayut Srituravanich). Formal analysis & Funding acquisition: PT (Paweena Thuwanut). Methodology: PT (Paweena Thuwanut), WS (Weerayut Srituravanich). Project administration: PT (Paweena Thuwanut), KP. Visualization: WS (Wisan Sereepapong), CT, CS, PT (Punkavee Tuntiviriyapun). Writing–original draft: PT (Paweena Thuwanut). Writing–review & editing: PC, PS.

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CERM

# Transforming growth factor-beta and liver injury in an arginine vasopressin-induced pregnant rat model

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**Objective:** Approximately 30% of preeclamptic pregnancies exhibit abnormal liver function tests. We assessed liver injury-associated enzyme levels and circulating transforming growth factor beta (TGF-β) levels in an arginine vasopressin (AVP)-induced pregnant Sprague-Dawley rat model.
**Methods:** Pregnant and non-pregnant Sprague-Dawley rats (n=24) received AVP (150 ng/hr) subcutaneously via mini-osmotic pumps for 18 days. Blood pressure was measured, urine samples were collected, and all animals were euthanized via isoflurane. Blood was collected to measure circulating levels of TGF-β1-3 isomers and liver injury enzymes in pregnant AVP (PAVP), pregnant saline (PS), non-pregnant AVP (NAVP), and non-pregnant saline (NS) rats.
**Results:** The PAVP group showed significantly higher systolic and diastolic blood pressure than both saline-treated groups. The weight per pup was significantly lower in the AVP-treated group than in the saline group ($p<0.05$). Circulating TGF-β1-3 isomer levels were significantly higher in the PAVP rats than in the NS rats. However, similar TGF-β1 and TGF-β3 levels were noted in the PS and PAVP rats, while TGF-β2 levels were significantly higher in the PAVP rats. Circulating liver-type arginase-1 and 5′-nucleotidase levels were higher in the PAVP rats than in the saline group.
**Conclusion:** This is the first study to demonstrate higher levels of TGF-β2, arginase, and 5′-nucleotidase activity in PAVP than in PS rats. AVP may cause vasoconstriction and increase peripheral resistance and blood pressure, thereby elevating TGF-β and inducing the preeclampsia-associated inflammatory response. Future studies should explore the mechanisms through which AVP dysregulates liver injury enzymes and TGF-β in pregnant rats.

**Keywords:** Arginase 1; Arginine vasopressin; Aspartate transaminase 1; Liver injury; Transforming growth factor beta

## Introduction

Modulation of maternal immunity is challenging as it requires the maintenance of the homeostatic balance between acceptance of the semi-allograft fetus and immunological protection of the mother [1]. Immune anomalies within a compromised maternal-fetal system are linked to liver injury in cases of pregnancy complications such as preeclampsia (PE) [1,2]. Notably, 3% of pregnancies worldwide are affected by various liver disorders [3], a third (35%) of which are a consequence of PE development, which is associated with considerable maternal and neonatal morbidity and mortality [4]. Liver damage in preeclamptic pregnancies includes hepatic arterial vasospasm and fibrin precipitation, eventually leading to lobular ischemia and hepatocyte necrosis [5]. It is believed that hepatic injury elevates transforming growth factor beta (TGF-β) gene expression [6].

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PE is characterized by high blood pressure with or without proteinuria at 20 weeks of gestation [7]. Despite extensive ongoing research surrounding its development, the mechanisms responsible for its progression remain uncertain. Extensive research has focused on the role of anti-angiogenic factors such as endoglin and soluble vascular endothelial growth factor receptor (sFt-1) in favor of proangiogenic factors, such as vascular endothelial growth factor and placental growth factor (PlGF) in the development and progression of PE [8,9]. More specifically, prior to the onset of maternal symptoms of PE, sFt-1 elevation is accompanied by a concurrent decline in circulating PlGF levels [10].

TGF-β is a profibrogenic, multifunctional cytokine that has 3 isoforms (TGF-β1-3). It binds to transmembrane type I and type II receptors, and this binding may be enhanced by connective tissue growth factors [11]. Its co-receptor, endoglin, is a transmembrane glycoprotein expressed on endothelial and syncytiotrophoblastic cells [12], and its expression has been reported to increase during early-onset PE rather than late-onset PE compared to gestational age-matched controls [13]. This elevation does not hinder TGF-β signaling, however; when interconnected with both onset types, it synergistically prevents the signaling of TGF-β1 and TGF-β2. The soluble form of endoglin decreases endothelial nitric oxide (NO) signaling by inhibiting TGF-β1 signaling [14], resulting in the endothelial dysfunction characteristic of early-onset PE [12]. The latent form of TGF-β is a key constituent of the extracellular matrix (ECM), an obligatory target for integrins [15]. TGF-β1 regulates cell growth, differentiation, ECM production, and proteolytic turnover; therefore, it plays a profound role in trophoblast cell migration and invasiveness [16]. The overexpression of placental TGF-β3 leads to deficient trophoblast invasion with consequential nonphysiological transformation of myometrial spiral arteries, a feature characteristic of PE development [17]. Moreover, its upregulation may be implicated in angiotensin II-induced target organ damage in hypertension [18].

Common pregnancy-initiated liver diseases include hemolysis, elevated liver enzymes, low platelet count syndrome (HELLP) syndrome and acute fatty liver of pregnancy. Earlier studies have suggested a role of TGF-β in every stage of the progression of chronic liver disease, including the initial stages of liver injury that lead to inflammation and fibrosis [19]. In light of the fact that abnormal liver function tests occur in 20% to 30% of pregnancies complicated by hypertensive disorders of pregnancy such as PE [20], this study assessed liver injury enzymes with concomitant circulating levels of TGF-β in an arginine vasopressin (AVP)-induced pregnant Sprague-Dawley rat model. It also attempted to correlate serum TGF-β levels with liver injuries.

## Methods

### 1. Ethical considerations and animal welfare

This study was approved by the Institutional Animal Research Ethics Committee (AREC/046/017) of University of KwaZulu-Natal (UKZN), South Africa. All procedures were conducted as described in the Approved Standard Protocols of the Animal Research Ethics Committee. Female Sprague-Dawley rats aged 10–12 weeks (weighing 160–180 g) were obtained from the Biomedical Research Unit of UKZN. All animals were housed in polycarbonate cages under standard laboratory conditions of temperature (22°C–24°C), humidity (60%) and illumination (12-hr light/dark cycles). They had ad libitum access to standard rat chow (Meadows Feeds, Pietermaritzburg, South Africa) and normal drinking water.

### 2. Mating

Daily vaginal smears were done to determine the progression of the estrous cycle. Following the confirmation of the estrous phase, two female rats were housed with a single male in polycarbonate cages for 24 hours, followed by vaginal smears 24 hours later to detect sperm and confirm fertilization.

### 3. Experimental study

Twenty-four pregnant Sprague-Dawley rats aged 10–12 weeks (160–180 g), were surgically implanted on gestational day (GD) 1 with ALZET mini-osmotic pumps (model 2004; Durect Corp., Cupertino, CA, USA) to subcutaneously deliver AVP at 150 ng/hr. All rats (saline and AVP delivery groups) were anaesthetized with isoflurane and maintained on gaseous anesthesia for the implantation of the subcutaneous mini-osmotic pumps, which remained implanted until sacrifice. Physiological parameters (weight, systolic and diastolic blood pressure) were measured at GD 8, 14, and 18 using an mouse rat blood pressure tail-cuff blood pressure monitor (IITC Life Science Inc., Woodland Hills, CA, USA). The animals were categorized into four groups containing six rats each: group 1, non-pregnant with saline delivery (NS); group 2, non-pregnant with AVP delivery (NAVP); group 3, pregnant with saline delivery (PS); and group 4, pregnant with AVP delivery (PAVP). Urinary protein levels were measured by collecting 24-hour urine samples (GD 8, 14, and 18) using the M-TP Microprotein Kit (Beckman Coulter, San Jose, CA, USA). All animals were euthanized on GD 18, via anesthesia inhalation using isoflurane (Safeline Pharmaceuticals, Johannesburg, South Africa). Blood samples were collected at sacrifice via cardiac puncture and centrifuged for 15 minutes at 3,500 rpm at 4°C. Serum was stored at –80°C to determine the circulating levels of TGF-β and liver injury enzymes.

### 4. TGF-β immunoassay protocol

The transforming growth factor magnetic bead kit (catalogue No. TGFBMAG-64K-03; Merck Millipore, Darmstadt, Germany) was used to quantify the levels of TGF-β1, TGF-β2 and TGF-β3, according to the manufacturer's instructions (Merck, Darmstadt, Germany). Magnetic antibody-conjugated beads were prepared by sonicating for 30 seconds, followed vortexing for 1 minute to reduce the aggregation of beads. All samples, including the quality control samples and standards, were prepared as recommended in the Milliplex map assay kit protocols. First, 200 µL of assay buffer was added to each well. The plate was then allowed to shake on a plate shaker for 10 minutes at room temperature. The assay buffer was thereafter decanted, and 25 µL of each standard/control was added to the appropriate wells. Subsequently, 25 µL of treated sample and prepared beads were added to the appropriate wells along with buffering solutions. Each plate was subsequently sealed and incubated overnight at 4°C. The plates were washed 2 times, followed by the addition of 25 µL of detection antibodies to each well. After 1 hour of incubation at room temperature, 25 µL of streptavidin-phycoerythrin was added to each well and incubated at room temperature for 30 minutes. The plates were washed twice and finally resuspended in 100 µL of sheath fluid in each well.

### 5. Liver injury immunoassay protocol

The rat liver injury magnetic bead panel (catalogue #RLI1MAG-92K) was used to determine the serum expression of 5'-nucleotidase/CD73 (5'-NT), aspartate transaminase 1 (AST) and glutamate oxaloacetate transaminase (GOT-1) and liver-type arginase 1 (ARG-1), according to the manufacturer's instructions (Merck). Standards, control, and background and diluted serum samples (25 µL each) were added to each well, followed by an assay buffer and mixed antibody-immobilized beads (25 µL). The plates were incubated with agitation for 2 hours at room temperature, followed by washing with wash buffer and the addition of 25 µL of detection antibodies. The plate was incubated for 1 hour at room temperature. Streptavidin-phycoerythrin (25 µL) was added to each well, incubated for 30 minutes at room temperature, and washed three times and resuspended in sheath fluid (150 µL).

### 6. Plate analysis

Both assay plates were then analyzed with the Bio-Plex MAGPIX Multiplex reader (Bio-Rad Laboratories, Pleasanton, CA, USA) with xPONENT v.3.2 software and further analyzed with BelysaTM Immunoassay Curve Fitting-Software (v1) (Merck).

### 7. Statistical analysis

All statistical analyses were carried out using Stata ver. 10 (StataCorp., College Station, TX, USA). Non-parametric data (blood pressure and proteinuria) are summarized as medians and interquartile ranges, and parametric data are presented as mean and standard deviation. The Kruskal-Wallis and Dunn post hoc tests were used to compare the medians between groups and to determine statistical significance. One-way analysis of variance followed by a pair-wise comparison of means was used to determine whether significant differences existed among the groups. Pearson correlation coefficients were also used to assess the relationship between TGF-β and liver injury enzymes, and to estimate whether TGF levels depended on the expression of liver enzymes. A $p$-value $< 0.05$ was considered to indicate statistical significance.

## Results

Changes in systolic (Figure 1A) and diastolic (Figure 1B) blood pressure, urinary protein levels (Figure 1C), liver and placental weight (Figure 1D-F) and birth outcomes (Figure 1G and H) are shown. Significant elevations were noted in both the systolic and diastolic blood pressure in the PAVP group in comparison to the NS and PS groups at GD8 ($p < 0.05$), GD14 ($p < 0.001$), and GD18 ($p < 0.001$) (Figure 1A and B). Liver weight was significantly higher in the PAVP group than in the NAVP group ($p < 0.001$) (Figure 1D). Placental weight (individual and total) was significantly lower in the PAVP group than in the saline groups ($p < 0.05$) (Figure 1E and F). Additionally, weight per pup was significantly lower in the AVP-treated group than in the saline group ($p < 0.05$) (Figure 1G); however, the PAVP rats demonstrated significantly higher pup numbers than the NAVP group ($p < 0.05$) (Figure 1H).

The mean serum levels of TGF-β (1, 2 and 3) and liver toxicity indicators (ARG-1, GOT-1/AST, and 5'-NT) for all groups are shown in Figure 2. Notably, TGF-β2 and TGF-β3 levels were significantly downregulated in the non-pregnant rats (saline and AVP) versus the pregnant rats (Figure 2B and C). However, only TGF-β2 was significantly different between the PAVP rats and the PS rats ($p < 0.001$) (Figure 2B). In contrast, no observable difference was noted between these pregnant groups for TGF-β1 and TGF-β3, regardless of AVP treatment (Figure 2A and C). Serum GOT-1 (AST) levels were significantly lower in the PAVP rats than in the non-pregnant controls (Figure 2E). Despite the lack of statistical significance, GOT-1 (AST) levels were lower in the PAVP rats than in the PS group (Figure 2E). Circulating ARG-1 and 5'-NT levels were significantly higher in the PAVP rats than in the PS rats ($p < 0.05$) (Figure 2D and F). In the PAVP group, ARG-1 levels were significantly higher than the NAVP group (Figure 2D), whereas 5'-NT levels were higher in the NAVP group than in the PAVP group (Figure 2F).

The Pearson correlation analysis revealed a negative association


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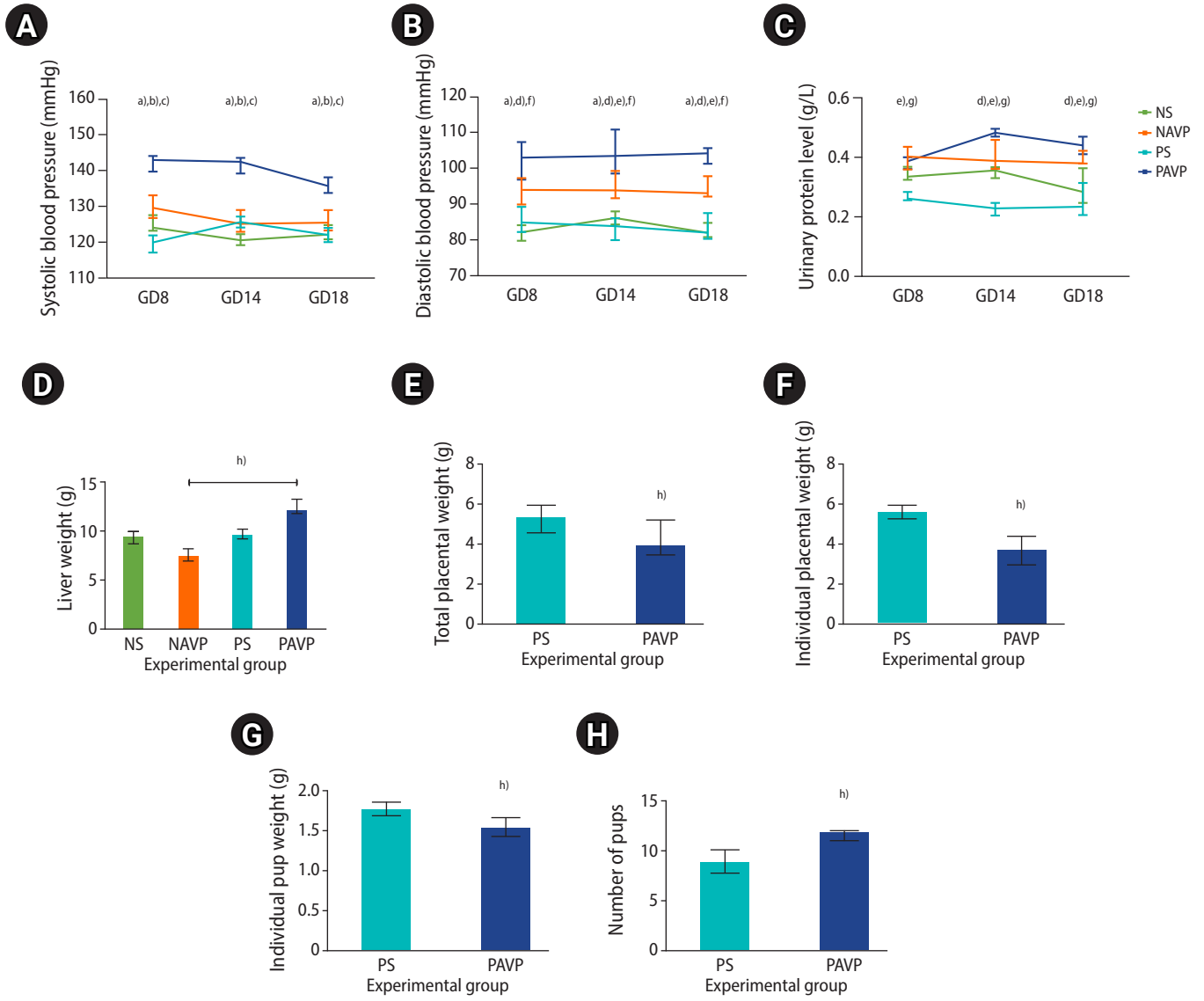


**Figure 1.** (A) Systolic blood pressure. (B) Diastolic blood pressure. (C) Urinary protein levels. (D) Liver weight (GD18). (E, F) Placental weight. (G, H) Pup weight. Summary statistics are presented as median and interquartile range (A-C) and mean±standard deviation (D-H). GD, gestational day; NS, non-pregnant saline control; NAVP, nonpregnant arginine vasopressin; PS, pregnant saline control; PAVP, pregnant arginine vasopressin. [a)] $p<0.001$: PAVP vs. PS; [b)] $p<0.05$: PAVP vs. NS; [C)] $p<0.05$: PAVP vs. NAVP; [d)] $p<0.001$: PAVP vs. NS; [e)] $p<0.05$: NAVP vs. PS; [f)] $p<0.05$: NAVP vs. NS; [g)] $p<0.05$: PAVP vs. PS; [h)] $p<0.05$.

between TGF-β1 and 5'-NT in the PAVP group ($r=-0.85$, $p=0.15$) and between TGF-β1 and 5'-NT in the NAVP group ($r=-0.75$, $p=0.25$), as well as a positive association between TGF-β2 and ARG-1 in the PS ($r=0.18$, $p=0.78$) and PAVP rats ($r=0.44$, $p=0.56$), albeit non-significant.

## Discussion

This novel study demonstrates an apparently paradoxical synergy between TGF-β dysregulation and liver injury in pregnant rats treated with AVP. Similar to the report by Santillan et al. [21], the experimental use of AVP elevated blood pressure in treated groups, and animals displayed the characteristic features of PE. Our data showed mild elevation of blood pressure (systolic and diastolic) and proteinuria in the PAVP group compared to the other study groups. This elevation is linked to the physiological role of AVP in promoting water reabsorption, which in excess will elevate blood pressure [22]. The AVP-induced elevation in blood pressure indicates low levels of circulating renin-angiotensin system activity, which decreases blood flow through its vasoconstrictive action on V1a receptors and V2 receptors [23]. This reduced blood flow may have resulted in low pup weights, mirroring the placental-related fetal growth restriction ob-

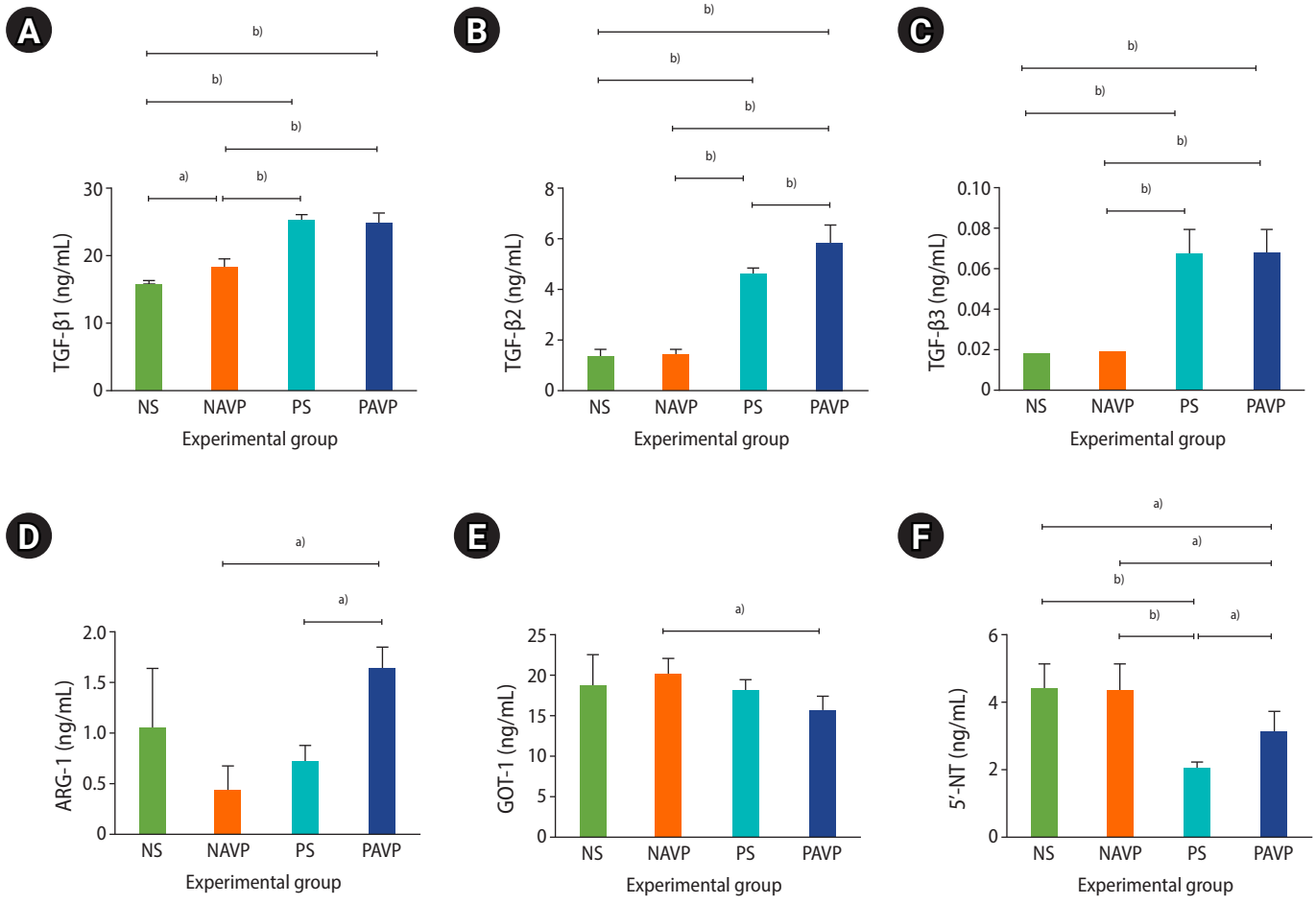


**Figure 2.** Serum expression of TGF-β (A: 1, B: 2, and C: 3) and liver toxicity indicators (D: ARG-1, E: GOT-1/AST, and F: 5′-NT). Summary statistics are presented as mean±standard deviation. TGF-β, transforming growth factor beta; ARG-1, arginase 1; GOT-1, glutamate oxaloacetate transaminase; 5'-NT, 5'-nucleotidase/CD73; NS, non-pregnant saline control; NAVP, non-pregnant arginine vasopressin; PS, pregnant saline control; PAVP, pregnant arginine vasopressin; AST, aspartate transaminase. [a)]$p<0.05$; [b)]$p<0.01$.

served in human PE [24]. Moreover, uncontrolled high blood pressure leads to glomerular damage and resulting protein leakage [25].

TGF-β isoforms regulate placentation and cell growth, survival, and death during pregnancy [26,27], as well as enhance the differentiation and invasiveness of trophoblast cells in rat pregnancies [28]. Serum TGF-β levels are higher in women with PE than in those with normotensive pregnancies [13,29,30]. However, TGF-β1 expression is variable in PE [31,32], which may reflect population-level differences in ethnicity, as well as variation in PE severity [33]. We highlight similar TGF-β1 and TGF-β3 levels in the PS and PAVP rats, whereas TGF-β2 levels were significantly higher in the PAVP rats. Our results corroborate those of Shaarawy et al. [30], who demonstrated significantly higher maternal serum TGF-β2 levels in PE cases compared to controls. The disproportionate expression of TGF-β2 in our study may be associated with reduced blood flow, which accounted for the reduced placental weight in AVP-treated pregnancies. It is possible that AVP-associated vasoconstriction alters placentation via elevated apoptosis of invasive trophoblast cells. Additionally, these elevations may be linked to a high placental affinity for TGF-β receptor, indicative of its role as a prime target for TGF-β action [34].

On the contrary, AVP infusion was found to result in a pro-inflammatory environment and a reduction in plasma TGF-β levels [35], contradicting the findings of our study, possibly due to the lack of specification of individual isoforms in that study. However, this pro-inflammatory environment potentially leads to poor placental development [21], and supposedly enhances the reaction of the renin-angiotensin system [36]. However, Sandgren et al. [37] highlighted the inability of AVP to create a hypoxic environment. This likely interrupts placental development, resulting in the similar TGF-β1 and TGF-β3 expression observed in our study. Moreover, the lack of a hypoxic environment may have also counteracted the placental secretion of the other TGF-β isoforms, as well as contributed to significantly higher pup numbers than in the saline groups.

Additionally, to verify whether the AVP infusion altered the liver


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function in this rat model, the circulating levels of liver injury enzymes were measured. Our data indicate that AVP-infused pregnant rats expressed higher ARG-1 and 5'-NT levels than their saline-treated counterparts. This elevation suggest some level of liver injury induced by AVP, but the liver injury was insufficient to model the hepatic dysfunction linked to HELLP syndrome, corroborating the findings of Sandgren's group [37]. Since TGF-β isoforms are profibrogenic in nature, they may also have an inhibitory action on the progression of liver injury [19]. Although pregnancy-initiated liver diseases such as HELLP syndrome and acute fatty liver disease are infrequent, their diagnosis should not be ruled out due to the possibility of acute liver failure, which contributes to maternal and neonatal mortality [38]. The cytosolic enzyme arginase I is significantly expressed in vascular endothelial and smooth muscle cells and responsible for urea metabolism in the liver [39]. It reciprocally regulates the synthesis of NO through L-arginine as the NO synthase substrate [40] and its reduced bioavailability is associated with endothelial dysfunction and the etiology of hypertension [41,42].

We report significantly higher ARG-1 and 5'-NT in the PAVP group than in the control groups. It is possible that the elevation in ARG-1 may be linked to its role as a protagonist in fibrosis, immunosuppression, and inflammatory-derived immune anomalies [43]. Moreover, its increased expression in the PAVP rats is indicative of its role in inducing an inflammatory response. Earlier in vivo reports also suggested that activation of murine macrophages by interleukins (4, 10, and 13) was associated with elevated arginase expression [44-46]. Bagnost et al. [39] also demonstrated raised arginase activity in the larger blood vessels, heart, and lungs of spontaneously hypertensive rats (SHR), however, they were unable to demonstrate any significant difference in tissue arginase activity between prehypertensive SHRs and Wistar-Kyoto rats.

The membrane glycoprotein 5'-NT is increased in the serum of patients with hepatobiliary disease, hepatitis, intrinsic liver damage, liver malignancy, and biliary cirrhosis [47], indicating that 5'-NT may be clinically relevant as a risk indicator for identifying those vulnerable to liver disease. Hence, the AVP-induced elevations in 5'-NT observed in the PAVP rats in contrast to the PS rats may be associated with hepatobiliary disease, as previously reported [48]. An increase in 5'-NT in AVP-induced pregnancies is suggestive of some level of hepatotoxicity, supporting the role of 5'-NT as a possible predictor of hepatobiliary lesions and hepatotoxicity [49]. Gowda et al. [50] linked elevated 5'-NT levels to obstructive jaundice and parenchymal liver disease, with the potential for it to be a biomarker of premature hepatic tumors. More recently, Hyde et al. [51] demonstrated a 3-fold increase in the circulating levels of 5'-NT in patients with viral hepatitis and a two-fold increase in patients with liver cirrhosis in comparison to control participants. The elevated levels we observed may be linked to liver inflammation, which occurred in response to the AVP-induced high blood pressure and associated reduced blood flow that lowered placental and individual pup weight, characteristic of mild PE development. With regard to AST/GOT-1, our data showed little or no difference in their serum expression in the PS group versus the non-pregnant groups, corroborating several other studies [52,53], where levels of this enzyme remained within normal limits.

The onset of fibrosis occurs due to elevated collagen deposition and insufficient ECM associated with the shift from fibroblasts to myofibroblasts [11]. We also report various positive associations between TGF-β and liver enzymes, supporting their role in hepatocyte injury and wound healing via transdifferentiation of hepatic stellate cells into myofibroblasts [19]. This transition is believed to be facilitated through the increased production of ECM collagen fibers and α-smooth muscle actin (α-SMA) [11,54]. Nonetheless, fibrosis may also occur due to an endothelial-to-mesenchymal shift [55], since α-SMA, which is typically expressed in vascular smooth muscle cells, stimulates collagen type I formation through autocrine stimulation of TGF-β1 [56]. The negative associations between TGF-β1 and ARG-1 in the PS and PAVP groups may be suggestive of fibroblast differentiation into myofibroblasts, which negatively regulates ARG-1 synthesis.

This is the first study to demonstrate AVP-induced elevations in TGF-β2, ARG-1, and 5'-NT activity in pregnant rats treated with AVP in contrast to untreated pregnant rats. AVP likely causes vasoconstriction, which increases peripheral resistance and blood pressure, potentially inducing TGF-β elevation and the inflammatory response associated with PE development. This suggests its potential diagnostic use in PE development; however, its prognostic effect remains to be elucidated. Future studies should explore the mechanisms through which AVP dysregulates liver injury enzymes and TGF-β in pregnant rats.

## Conflict of interest

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

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# Detrimental effects of lipopolysaccharide on the attachment and outgrowth of various trophoblastic spheroids on human endometrial epithelial cells

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**Objective:** Lipopolysaccharide (LPS) from Gram-negative bacteria causes poor uterine receptivity by inducing excessive inflammation at the maternal-fetal interface. This study aimed to investigate the detrimental effects of LPS on the attachment and outgrowth of various types of trophoblastic spheroids on endometrial epithelial cells (ECC-1 cells) in an *in vitro* model of implantation.
**Methods:** Three types of spheroids with JAr, JEG-3, and JAr mixed JEG-3 (JmJ) cells were used to evaluate the effect of LPS on early implantation events. ECC-1 cells were treated with LPS to mimic endometrial infection, and the expression of inflammatory cytokines and adhesion molecules was analyzed by quantitative real-time polymerase chain reaction and western blotting. The attachment rates and outgrowth areas were evaluated in the various trophoblastic spheroids and ECC-1 cells treated with LPS.
**Results:** LPS treatment significantly increased the mRNA expression of inflammatory cytokines (*CXCL1*, *IL-8*, and *IL-33*) and decreased the protein expression of adhesion molecules (ITGβ3 and ITGβ5) in ECC-1 cells. The attachment rates of JAr and JmJ spheroids on ECC-1 cells significantly decreased after treating the ECC-1 cells with 1 and 10 μg/mL LPS. In the outgrowth assay, JAr spheroids did not show any outgrowth areas. However, the outgrowth areas of JEG-3 spheroids were similar regardless of LPS treatment. LPS treatment of JmJ spheroids significantly decreased the outgrowth area after 72 hours of coincubation.
**Conclusion:** An *in vitro* implantation model using novel JmJ spheroids was established, and the inhibitory effects of LPS on ECC-1 endometrial epithelial cells were confirmed in the early implantation process.

**Keywords:** Attachment; Implantation; *In vitro*; Lipopolysaccharide; Outgrowth; Spheroids

## Introduction

Successful implantation depends on communication between the trophoblast of the blastocyst and the epithelial cells of the uterine endometrium. This communication is regulated by several inflammatory cytokines and adhesion molecules. Collectively, the process of implantation poses an immune challenge between the embryo (as a semi-allogenic) body and the uterus [1-3]. The maternal immune system promotes immune tolerance toward the embryo to maintain a normal pregnancy while defending against infection. The maternal immune response involves maintaining uterine receptivity, mediated by endometrial epithelial attachment molecules, trophoblast invasion, and extracellular matrix (ECM) breakdown [4,5].

Hormones, growth factors, and cytokines are crucial for the maintenance of uterine receptivity and implantation stages. Various cytokines, such as interleukin, leukemia inhibitory factor, and transforming growth factor-beta, are sensitive to local and systemic changes.

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They need to be appropriately regulated for successful implantation [6,7]. These cytokines are mostly mediated by the toll-like receptor (TLR) family, which is the main regulator of the immune response. Human endometrial tissue and trophoblasts express the TLR family [8]. The 10 membrane-spanning members of the TLR family play a critical role in modulating the inflammatory responses in humans. Each receptor responds to a specific ligand. Lipopolysaccharide (LPS) is a well-known endotoxin that consists of a lipid and a polysaccharide composed of O-antigen, outer core, and inner core joined by a covalent bond. LPS is a component of the cell wall of Gram-negative bacteria (e.g., *Escherichia coli*, *Ureaplasma urealyticum*, and *Gardnerella vaginalis*) [9,10]. LPS is an antigen that induces immune responses in uterine endometrial cells by TLR4 activation. LPS can trigger an imbalance in cytokines in the uterine endometrium [11,12].

Endometrial epithelial cells secrete hormones (estrogen and progesterone), growth factors, and various types of cytokines to enable successful implantation [13]. Adhesion molecules secreted by endometrial epithelial cells are an important factor in the embryo attachment stage [6,14]. Interactions between cells and the ECM are mediated by adhesion molecules [15], which consist of four subtypes: immunoglobulins, cadherins, integrins (ITGs), and selectins. ITGs are a major class of receptors within the ECM that mediate cell–ECM interactions with collagen, fibrinogen, fibronectin, and vitronectin. ITGs provide essential links between the extracellular environment and intracellular signaling pathways, and are transmembrane receptors that mediate cell adhesion [16,17]. ITGs consist of two subunits: alpha (α) and beta (β). In humans, ITGs have 18 α subunits and 8 β subunits [18]. In this study, we analyzed ITGαV, ITGβ3, and *ITGβ5* to understand the effect of LPS on the attachment of trophoblastic spheroids to uterine endometrial epithelial cells.

Many *in vitro* models have provided insights into the implantation process [19,20]. In a recent study, *in vitro* implantation models were designed using the spheroid form of trophoblastic cells to surrogate embryos [21]. Trophoblast cell lines, including JAr, JEG-3, and human endometrial epithelial cells of ECC-1 were used as *in vitro* implantation models in previous studies [22-24]. This study was performed to investigate the detrimental effects of LPS on the attachment and outgrowth of various types of trophoblastic spheroids and endometrial epithelial cells as an *in vitro* model of implantation.

## Methods

### 1. Culture of human trophoblastic and endometrial epithelial cells

The human trophoblastic JEG-3 cell line was cultured in DMEM (Welgene, Gyeongsan, Korea) and supplemented with 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA) and 1% penicillin-streptomycin (P/S; Lonza, Morristown, NJ, USA). The human trophoblastic JAr cell line and human endometrial epithelial cells (ECC-1 cells) were cultured in RPMI 1640 (Welgene) with 10% FBS and 1% P/S. Cells were cultured under standard conditions (37°C, 5% $CO_2$), and the culture medium was replaced with fresh medium every 48 hours. LPS from *E. coli* O111: B4 (Sigma-Aldrich, St. Louis, MO, USA) was used to treat the ECC-1 cells to mimic endometrial infections caused by Gram-negative bacteria.

### 2. Preparation of various trophoblastic spheroids using the hanging drop method and the Organoid 3D culture kit

Spheroids of JAr, JEG-3, and JAr mixed JEG-3 (JmJ) were prepared using the hanging drop method and the Organoid 3D culture kit (Cell Smith, Seoul, Korea). JAr and JEG-3 cells were mixed at a 1:1 ratio to prepare JmJ spheroids. Using the hanging drop method, trophoblast cells ($2 \times 10^4$/20 μL drop) were plated onto the lid of a 100-mm dish in a regular array (20 drops/lid). The lid was inverted over the bottom, which was filled with Dulbecco's phosphate-buffered saline (DPBS; Biowest, Riverside, MO, USA), and cultured under standard conditions for 72 hours. Using the Organoid 3D culture kit, cells were seeded at a density of $1 \times 10^5$ cells in 3 mL of the medium. The cells were incubated for 72 hours in the Organoid 3D culture kit. Spheroids sized between 200 and 300 μm were selected and used for subsequent experiments. The morphology of spheroids cultured by the different methods was observed using an EVOS XL Core Cell Imaging System (Thermo-Fisher, Waltham, MA, USA).

### 3. Histology of various types of trophoblastic spheroids

Three types of trophoblastic spheroids were fixed with 4% paraformaldehyde (Biosesang, Seongnam, Korea), and over 50 spheroids were suspended in 20 μL of 2% agarose gel solution. The pre-embedded agarose gel blocks were embedded in paraffin, and sections of paraffin blocks with a thickness of 6 μm were cut. The sections were placed on glass slides and stained with hematoxylin and eosin (H&E). The spheroids were observed under a Nikon Eclipse 80i microscope.

### 4. Quantitative analysis of mRNA expression in ECC-1 cells treated with LPS

Total RNA from the ECC-1 endometrial epithelial cells was isolated using TRIzol reagent (Ambion, Austin, TX, USA). Complementary DNA was synthesized using a cDNA reverse transcription kit (Takara Bio, Shiga, Japan). All quantitative SYBR-based quantitative real-time polymerase chain reaction (qRT-PCR) assays were performed in a 20 μL reaction volume using the StepOne software ver. 2.3 using the SYBR Green master mix, SensiFAST SYBR Hi-ROX Kit (Bioline, London, England), and 10 pM of each specific primer and 1 μL of cDNA per re-

action (Table 1). Each qRT-PCR involved an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at a specific temperature of each primer for 30 seconds, and extension at 72°C for 30 seconds. Finally, quantitative analysis was performed using the $2^{-\Delta\Delta ct}$ method with β-actin as an internal control [25].

### 5. Western blot analysis of ECC-1 cells treated with LPS

Equal amounts of total protein (20 μg) from ECC-1 cells treated with LPS were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim-milk/TBS-T (Tris-buffered saline-Tween 20) solution (Bio-Rad, Contra Costa, CA, USA) and incubated with anti-ITGαV (ab179475), ITGβ3 (ab197662), ITGβ5 (ab31327; Abcam, Cambridge, England), and anti-GAPDH (SC-32233; Santa Cruz Biotechnology, Dallas, TX, USA) antibodies. After the reaction with appropriate secondary antibodies linked to horseradish peroxide (Abcam), the signals were visualized using the ChemiDoc MP Imaging System (Bio-Rad). Densitometry was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Relative optical density was calculated by dividing the optical density of ITG protein by that of the internal control (GAPDH).

### 6. Evaluation of the attachment rate and outgrowth of trophoblastic spheroids on ECC-1

Endometrial epithelial cells of ECC-1 were cultured until confluence in T75 flasks and then detached using 0.25% trypsin/EDTA. Approximately $1 \times 10^5$ endometrial cells were seeded in 12-well plate culture dishes and cultured at 37°C until 100% confluency. After LPS treatment in ECC-1 (0, 1, and 10 μg/mL of LPS, incubated for 24 hours), trophoblastic spheroids were added to ECC-1 and co-cultured for 6 hours. The attachment rate of trophoblastic spheroids to endometrial epithelial cells was evaluated at 0, 1, 2, 4, and 6 hours. The number of attached spheroids was counted by tapping by hand 3–5 times. After 72 hours of co-culture, the outgrowth area and spheroid area were analyzed using ImageJ software (National Institutes of Health). The areas of outgrowth of the spheroids were measured in pixel units. The ratio of the outgrowth area (outgrowth area/spheroid area) was calculated and analyzed.

### 7. Statistical analysis

All experiments were performed at least in triplicate, and more than 250 spheroids were used in each group. Data are presented as mean ± standard error of the mean. The statistical significance of differences between groups was analyzed using one-way analysis of variance and the Tukey test. Data were analyzed using Prism GraphPad software ver. 5.0 (GraphPad, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

## Results

### 1. Morphology and histology of trophoblastic spheroids using the hanging drop method and Organoid 3D culture kit

Microscopic analysis of the various types of spheroids was performed to obtain more detailed observations of their morphology. Spheroid formation and cell distribution were observed histologically by H&E staining. The spheroids cultured by both methods (hanging drop and the Organoid 3D culture kit) were well prepared for the *in vitro* implantation model. All trophoblastic spheroids were maintained at a high cell density (Figures 1 and 2).

**Table 1.** Primer sequences of inflammatory cytokines, adhesion molecules, and internal controls

| Gene | Primer sequence | Product size | GenBank accession number | Annealing temperature (°C) |
|---|---|---|---|---|
| *CXCL1* | F : CACCTGGATTGTGCCTAATGT | 273 bp | NM_001511.4 | 60 |
| | R : TTGCAGGCTCCTCAGAAATA | | | |
| *IL-8* | F : GGCACAAACTTTCAGAGACAG | 153 bp | NG_029889.1 | 60 |
| | R : ACACAGAGCTGCAGAAATCAGG | | | |
| *IL-33* | F : GTGACGGTGTTGATGGTAAGA | 349 bp | NM_001314044.2 | 60 |
| | R : CCTTCTCCAGTGGTAGCATTT | | | |
| *ITGαV* | F : AATCTTCCAATTGAGGATATCAC | 140 bp | NM_001145000.3 | 61 |
| | R : AAAACAGCCAGTAGCAACAAT | | | |
| *ITGβ3* | F : AGTCAGGGAGAGCTGAACTA | 294 bp | NM_000212.3 | 60 |
| | R : GGGTGTGGAATTAGGAGGTAAA | | | |
| *ITGβ5* | F : TAGGTAGGCACCACAGAGAA | 219 bp | NM_002213.5 | 60 |
| | R : CAGCCCAGCATCTCAGTATTT | | | |
| *β-actin* | F : CATGTACGTTGCTATCCAGGC | 250 bp | NM_001101.5 | 60 |
| | R : CTCCTTAATGTCACGCACGAT | | | |

*CXCL1*, CXC motif ligand 1; *IL*, interleukin; *ITG*, integrin; F, forward; R, reverse.



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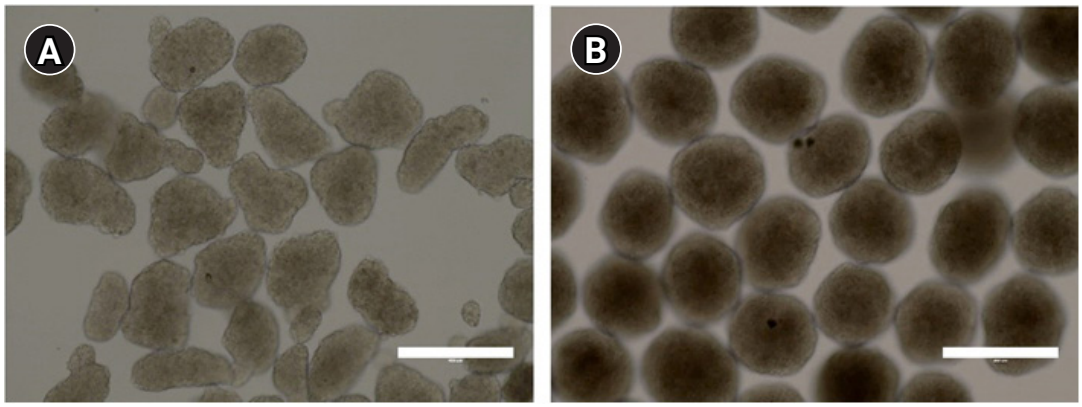


**Figure 1.** Microscopic morphology of JAr mixed JEG-3 trophoblastic spheroids generated by the hanging drop and organoid kit method. Spheroids were observed by EVOS XL Core Cell Imaging System (Thermo-Fisher). Representative spheroids generated (A) by the hanging drop method and (B) by the Organoid 3D cell culture kit method. Scale bar=400 μm.

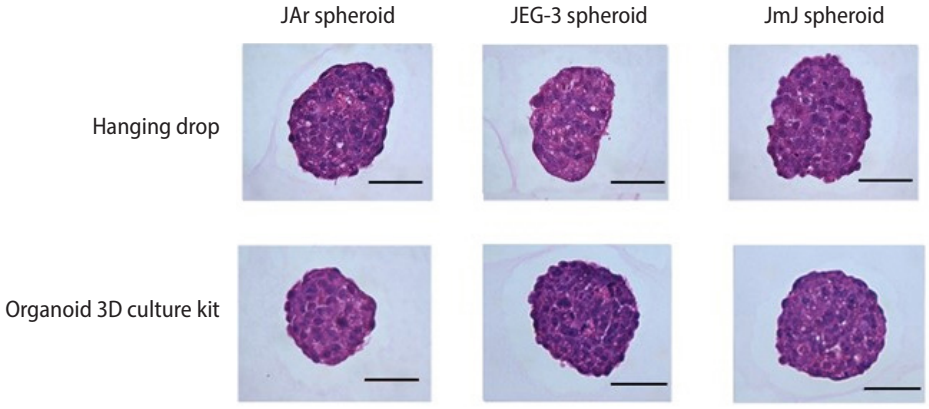


**Figure 2.** Histological characteristics of the various types of trophoblastic spheroids (JAr, JEG-3, and JAr mixed JEG-3 [JmJ]) in different culture methods. Histological characteristics of various spheroids by H&E staining observed with a Nikon Eclipse 80i microscope. The upper images of trophoblastic spheroids were formed by the hanging drop method, and the lower image by the Organoid 3D culture kit method. Scale bar=200 μm.

### 2. Effect of LPS on mRNA expression of adhesion molecules and inflammatory cytokines in ECC-1 cells

To evaluate the effect of LPS on ECC-1 cells, the expression of inflammatory cytokines and adhesion molecules was analyzed by qRT-PCR. LPS treatment significantly increased the mRNA expression of *CXCL1*, *IL-8*, and *IL-33* and decreased *ITGβ3* and *ITGβ5* expression in ECC-1 cells ($p < 0.05$). However, the mRNA expression of ITGαV in endometrial epithelial cells was not changed by LPS treatment (Figure 3).

### 3. Effect of LPS on protein expression of adhesion molecules in ECC-1

To evaluate the effect of LPS on ECC-1, the expression of adhesion molecules (ITGαV, ITGβ3, and ITGβ5) was analyzed by western blotting. ITGβ3 and ITGβ5 expression decreased in response to LPS treatment. However, ITGαV expression was not significantly different between the control and LPS-treated groups (Figure 4).

### 4. Effect of LPS on the attachment of various trophoblastic spheroids on ECC-1

The attachment rate between trophoblastic spheroids and the ECC-1 endometrial epithelial cells was evaluated in a time-dependent manner. The attachment rate was evaluated under 1% FBS or without FBS. LPS treatment did not affect the attachment rate of JEG-3 spheroids (Table 2). However, the attachment rates of JAr spheroids to ECC-1 cells were significantly decreased by LPS treatment ($p < 0.05$). The attachment rates of JAr spheroids on ECC-1 were similar in the LPS-treated and control groups after 6 hours of co-culture (Table 3). In the absence of FBS supplementation, the attachment rate was significantly decreased by LPS treatment compared to the condition with 1% FBS supplementation ($p < 0.05$). In the following experiments, the attachment rate of JmJ spheroids was analyzed in the absence of FBS supplementation. In JmJ spheroids, the attachment rate of LPS treatment was significantly lower than that of the

control after 2 hours of co-culture, as shown in Table 4 ($p < 0.05$).

### 5. Effect of LPS on the outgrowth of various trophoblastic spheroids on ECC-1 cells

The outgrowth of trophoblastic spheroids on the ECC-1 endometrial epithelial cells was evaluated after 72 hours of co-culture. The JAr spheroids did not show any outgrowth area, whereas the JEG-3 and JmJ spheroids showed outgrowth areas on ECC-1. Nevertheless, LPS treatment did not affect the outgrowth area of JEG-3 spheroids. Interestingly, LPS treatment significantly decreased the outgrowth area of JmJ on ECC-1 cells after 72 hours of co-culture, as shown in Figure 5 (control, 1.90 ± 0.06; 1 μg/mL LPS, 1.62 ± 0.06; and 10 μg/mL LPS, 1.64 ± 0.06; $p < 0.05$).

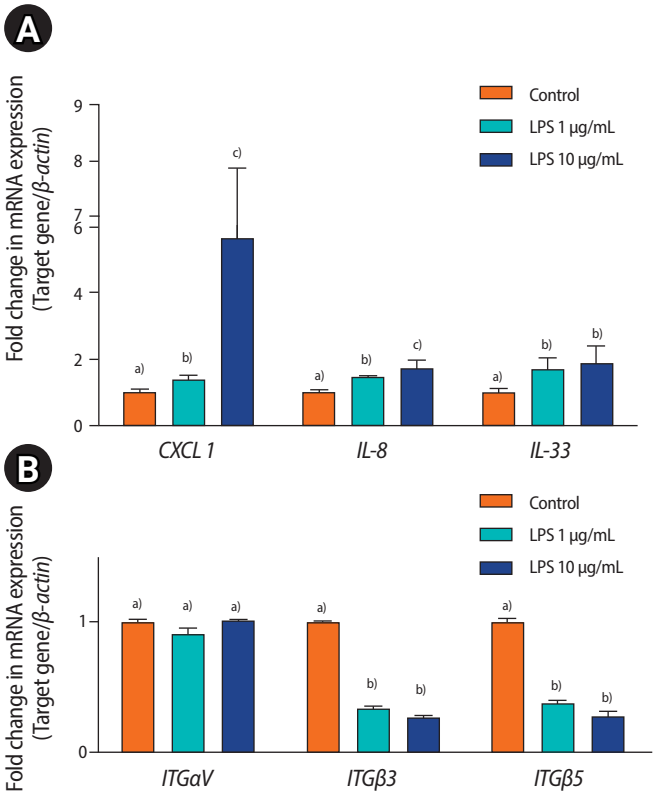


**Figure 3.** Quantitative analysis of mRNA expression in ECC-1 cells treated with lipopolysaccharide (LPS). Quantitative real-time polymerase chain reaction analysis of inflammatory cytokines (A) and adhesion molecules (B) in ECC-1 cells treated by LPS. Values are presented as mean±standard error of the mean. *CXCL1*, CXC motif ligand 1; *IL*, interleukin; *ITG*, integrin. [a),b),c)]Different letters indicate significant differences (one-way analysis of variance and the Tukey test, $p<0.05$).

## Discussion

Implantation is a complex immunological process. The inflammatory environment of the uterus changes from a pro-inflammatory to an anti-inflammatory state throughout implantation and pregnancy. A strong inflammatory response is necessary during implantation [26-30]. However, an imbalance in the immune status of the uterus can cause serious problems such as implantation failure and pregnancy loss [31,32]. In this study, activation of TLR4 in ECC-1) by LPS significantly reduced the attachment rate and outgrowth area between trophoblastic spheroids and endometrial epithelial cells.

It has been shown that implantation failure is induced by LPS through stimulation of the innate immune system and activation of the TLR4 pathway in the early stages of pregnancy [11,33]. A similar study suggested that TLR3 stimulated by a synthetic ligand, poly I: C (which is a double-stranded RNA molecule) in CBA/J female mice increased fetal losses [34] and reduced actin polymerization and adhesion molecule expression in endometrial cells [35]. In another study, activation of TLR5 in a human telomerase immortalized endometrial epithelial cell line (hTERT-EECs) by bacterial flagellin significantly decreased the attachment rate between JAr spheroids and underlying endometrial cells [36]. This study suggested that activation of the TLR family by various antigens at early stages of pregnancy could induce detrimental effects on implantation.

An endometrial epithelial cell line, ECC-1, was treated with LPS to

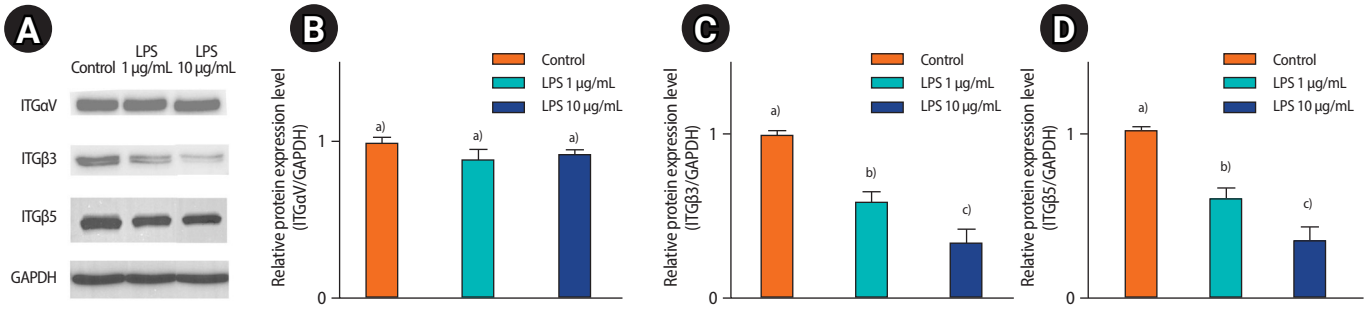


**Figure 4.** (A) Western blot analysis of ITGαV, ITGβ3, and ITGβ5 in ECC-1 cells treated with lipopolysaccharide (LPS). Western blot analysis of ITGαV, ITGβ3, ITGβ5, and GAPDH. Densitometry data of (B) ITGαV, (C) ITGβ3, and (D) ITGβ5. Values are presented as mean±standard error of the mean. [a),b),c)]Different letters indicate significant differences (one-way analysis of variance and the Tukey test, $p<0.05$).


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**Table 2.** Attachment rate of JEG-3 spheroids on ECC-1 treated by LPS

| Variable | Control (%) | LPS (1 μg/mL) (%) | LPS (10 μg/mL) (%) |
|---|---|---|---|
| With FBS (1%) | | | |
| 1 hr | 50.9±3.4 | 52.1±1.8 | 49.8±6.0 |
| 2 hr | 69.3±0.7 | 73.8±5.1 | 73.9±7.3 |
| 4 hr | 97.1±2.9 | 92.5±6.1 | 96.3±3.1 |
| 6 hr | 100.0 | 100.0 | 100.0 |
| Without FBS | | | |
| 1 hr | 54.8±2.9 | 45.6±4.4 | 62.9±7.6 |
| 2 hr | 69.2±0.4 | 66.4±7.5 | 72.9±8.4 |
| 4 hr | 93.1±3.8 | 96.6±2.2 | 99.1±1.0 |
| 6 hr | 96.7±4.1 | 98.3±1.7 | 100.0 |

Values are presented as mean±standard error of the mean (%). The attachment rate was evaluated in the condition with 1% FBS or without FBS supplementation.
LPS, lipopolysaccharide; FBS, fetal bovine serum.

**Table 3.** Attachment rate of JAr spheroids on ECC-1 treated by LPS

| Variable | Control (%) | LPS (1 μg/mL) (%) | LPS (10 μg/mL) (%) |
|---|---|---|---|
| With FBS (1%) | | | |
| 1 hr | 55.7±2.7[a)] | 37.4±2.9[b)] | 36.0±2.1[b)] |
| 2 hr | 81.7±4.5[a)] | 61.7±5.9[b)] | 56.0±7.3[b)] |
| 4 hr | 90.3±2.9[a)] | 90.9±1.9[a)] | 81.3±2.1[b)] |
| 6 hr | 97.7±1.4[a)] | 98.1±1.3[a)] | 84.1±6.1[b)] |
| Without FBS | | | |
| 1 hr | 43.2±3.0[a)] | 33.0±3.5[b)] | 28.2±4.0[b)] |
| 2 hr | 69.2±3.5[a)] | 55.4±3.4[b)] | 50.1±4.2[c)] |
| 4 hr | 90.1±2.0[a)] | 78.4±1.1[b)] | 75.0±3.2[b)] |
| 6 hr | 98.3±1.4[a)] | 93.9±2.1[a)] | 89.2±4.9[a)] |

Values are presented as mean ± standard error of the mean (%). The attachment rate was evaluated in the condition with 1% FBS or without FBS supplementation.
LPS, lipopolysaccharide; FBS, fetal bovine serum.
[a),b),c)]Different superscript letters indicate statistically significant differences ($p$<0.05) by one-way analysis of variance and Tukey test.

**Table 4.** Attachment rate of JmJ spheroids on ECC-1 treated by LPS

| Without FBS | Control (%) | LPS (1 μg/mL) (%) | LPS (10 μg/mL) (%) |
|---|---|---|---|
| 1 hr | 31.2±2.7[a)] | 19.5±2.8[b)] | 13.6±1.8[b)] |
| 2 hr | 48.5±1.7[a)] | 38.4±1.3[b)] | 36.4±1.1[b)] |
| 4 hr | 77.3±2.2[a)] | 71.5±3.6[a)] | 73.3±5.5[a)] |
| 6 hr | 97.2±0.8[a)] | 96.6±1.1[a)] | 98.4±0.9[a)] |

Values are presented as mean±standard error of the mean (%). The attachment rate was evaluated without FBS.
JmJ, JAr mixed JEG-3; LPS, lipopolysaccharide; FBS, fetal bovine serum.
[a),b)]Different superscript letters indicate statistically significant differences ($p$<0.05) by one-way analysis of variance and Tukey test.

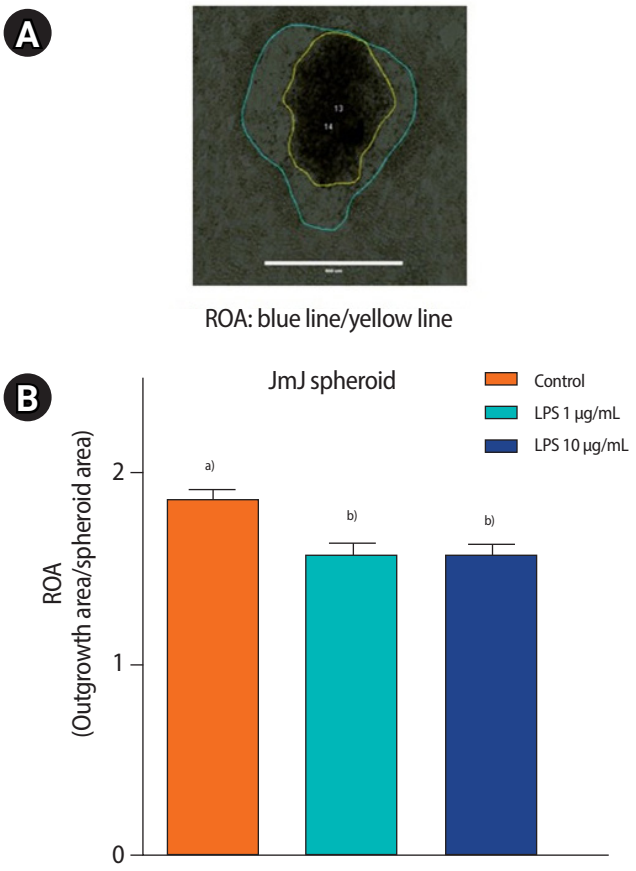


**Figure 5.** Ratio of the outgrowth area (ROA) of JAr mixed JEG-3 (JmJ) spheroids on ECC-1 cells treated with lipopolysaccharide (LPS). Outgrowth areas of spheroids were measured after 72 hours of co-culture. (A) The ROA (outgrowth area [blue line]/spheroid area [yellow line]) was calculated with ImageJ. (B) Mean ROA of three groups were represented. Each experiment was performed at least three times, and more than 110 spheroids were used in each group. Values are presented as mean±standard error of the mean. [a),b)]Different letters indicate significant differences (one-way analysis of variance and the Tukey test, $p$<0.05).

mimic endometrial infections caused by Gram-negative bacteria. In the implantation process, LPS affects not only endometrial epithelial cells, but also competent blastocysts [37]. LPS stimulates the TLR4 pathway to activate the inflammatory response [38]. TLR4 is essential for LPS activation. However, the JAr and JEG-3 human trophoblast cell lines do not express TLR4 on the membrane [39]. Other regulated pathways could potentially affect trophoblasts, resulting in the detrimental effect of LPS. Human endometrial epithelial cell lines (Ishikawa and ECC-1 cells) expressing TLR4 were used to evaluate the attachment rate of trophoblastic spheroids by LPS treatment [40]. In a pilot study, endometrial epithelial cell lines (Ishikawa and ECC-1 cells) were used in the implantation model to investigate the effects of LPS. LPS treatment did not affect the attachment rate between Ishikawa cells and trophoblastic spheroids. However, the attachment rate between ECC-1 cells and trophoblastic spheroids of JAr decreased with LPS treatment. Thus, we used EEC-1 endometrial epithelial cells in subsequent experiments of the implantation model.

The attachment rate of JAr and JEG-3 spheroids with ECC-1 cells was evaluated in the presence of 1% FBS and without FBS supplementation. In our experiments, the detrimental effect of LPS could be more clearly observed in the absence of FBS supplementation than in the 1% FBS condition due to the fact that FBS contains various factors such as growth factors and adhesion molecules [41]. Bas et al. [42] showed that FBS contains unknown factors and can inhibit TLR activation. For this reason, we designed the attachment and outgrowth experiments of JmJ spheroids without FBS supplementation.

The spheroids prepared by both methods (hanging drop and the Organoid 3D culture kit) were observed histologically using H&E staining. The microscopic observations showed that the Organoid 3D culture kit could provide spheroids with more consistent size and circularity than the hanging drop method. Therefore, spheroids prepared with the Organoid 3D culture kit might be more reliable for growth assays that analyze the ratio of the growth area to the spheroid area. We attempted to identify distinct features of JmJ spheroids compared to JAr and JEG-3 spheroids by histological observations. However, no significant differences were observed between the groups.

Choriocarcinoma JAr and JEG-3 cell lines were used to establish an *in vitro* model to investigate the attachment of trophoblast cells. JAr spheroids were suitable for investigating the effect of LPS on the attachment rate. However, the JAr spheroids did not show any outgrowth areas, indicating that they were not suitable for outgrowth assays. The other trophoblastic spheroids, JEG-3, had a clear outgrowth area for outgrowth assays. However, LPS-treated ECC-1 cells showed no significant difference in the attachment rates of JEG-3 spheroids compared to the control group.

Notably, JmJ spheroids were suitable for both attachment rate and outgrowth assays with ECC-1 cells. The JmJ spheroids showed a significant difference in the attachment rate after treatment with LPS. Moreover, the JmJ spheroids had a clear outgrowth area, which was suitable for outgrowth assays. The JmJ spheroids were used for subsequent experiments involving LPS treatment.

In the attachment rate assay, all spheroids were attached to ECC-1 after 6 hours of co-culture. Usually, the trophoblastic cells of carcinoma cell lines are used for *in vitro* implantation models. Carcinoma cell lines have a stronger invasion ability than normal cell lines [43]. Taken together, using a highly invasive carcinoma cell line could mask the detrimental effects of LPS on the attachment rate after 6 hours of co-culture of trophoblastic spheroids and ECC-1.

The expression of inflammatory cytokines and adhesion molecules was analyzed in the present study. ITGs are adhesion molecules in endometrial, decidual, and extravillous cytotrophoblast cells. They participate in cell-cell adhesion and adhesion between cells and components of the ECM [44]. LPS treatment significantly decreased the mRNA expression of *ITGβ3* and *ITGβ5*. However, the mRNA levels of *ITGβ1* and *ITGαV* were not changed by LPS treatment. A recent study by Guo et al. [45] using a bovine model could help understand these results. The expression of *ITGβ3*, *ITGβ5*, *ITGβ7*, and most transcripts coding for cell adhesion molecules (*PCDH7, PKP1, PKP3, CT, CTNNA3, CTNNAL1*, and *CDH2*) were downregulated after treatment with 2 μg/mL LPS in bovine endometrial epithelial cells. However, transmembrane glycoproteins that mediate cell-cell interactions through calcium binding (i.e., *ITGB6, CDH26, ITGAV*, and *CELSR1*) were overexpressed after LPS treatment [46]. In a preliminary study, we evaluated the relative expression of *ITGβ3* and *ITGβ5* mRNA to determine whether LPS treatment might decrease the attachment between spheroids and ECC-1 (data not shown). However, there were no significant differences among the different types of spheroids (JAr, JEG-3, and JmJ).

In this study, the mRNA expression of the inflammatory cytokines *CXCL1*, *IL-8*, and *IL-33* was higher in LPS-treated ECC-1 cells. CXCL1 and IL-8 are representative inflammatory cytokines in endometrial cells exposed to LPS. IL-8 and CXCL1 are cytokines with neutrophil chemotactic and activating activity and T cell chemotactic activity that play a major role in the recruitment of leukocytes to the endometrium via the CXCR1 and CXCR2 pathways [46-48]. However, the relationship between *IL-33* and LPS in endometrial epithelial cells remains unclear. In a study by Miller et al. [49], IL-33 stimulated the expression of inflammatory cytokines, such as *CXCL1*, IL-6, and IL-15, in the progression of endometriosis. Further studies are needed to understand the relationship between the inflammatory response in endometrial cells and IL-33 expression.

Our study had some limitations. Numerous factors are involved in the implantation process *in vivo*, including immune cells, stromal cells, and hormones [50]. However, in this study, only trophoblastic cells and endometrial epithelial cells were used because of the limitations of the *in vitro* model. Including various other factors in the *in vitro* model would help to understand the details of the implantation process.

This study had several advantages. The implantation process is highly complex and difficult to mimic *in vitro*. Many researchers have used embryo transfer to assess implantation potential. However, embryo transfer into pseudopregnant mice requires expert experimental skills and numerous sacrificial animals [51-53]. An *in vitro* implantation model using the novel trophoblastic JmJ spheroids and ECC-1 endometrial epithelial cells could overcome these problems.

The findings of our study could help to understand the detrimental effects of LPS on the attachment and outgrowth of various types of trophoblastic spheroids and endometrial epithelial cells in an *in vitro* model of implantation (Figure 6). The results of this study suggest that alterations in the expression levels of inflammatory cytokines


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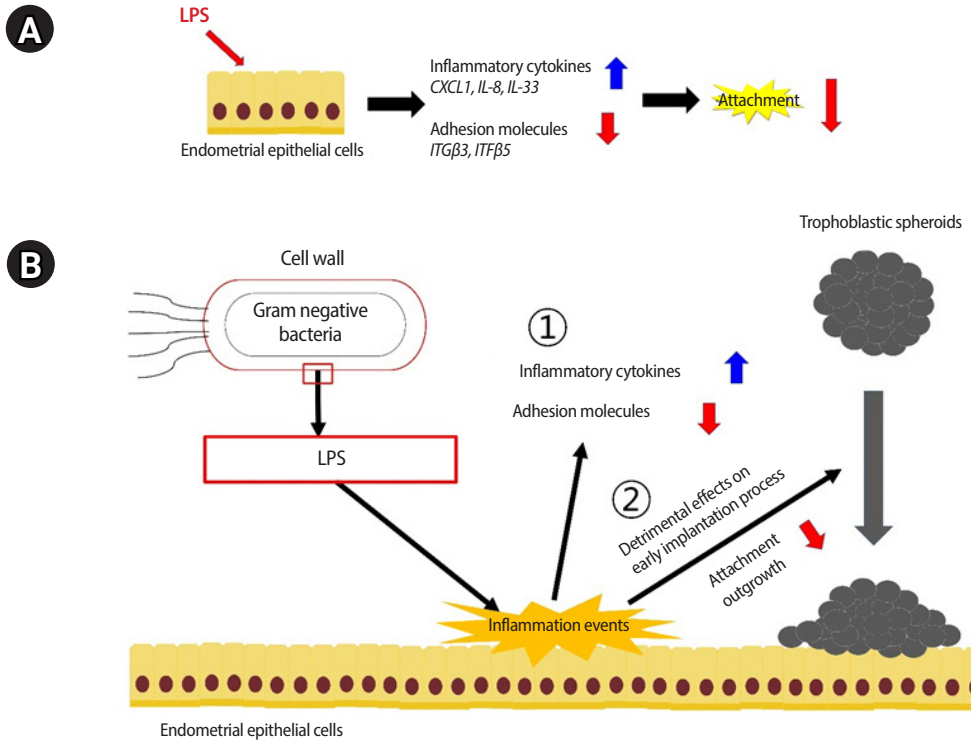


**Figure 6.** Schematic representation of the effects of lipopolysaccharide (LPS) on the attachment and outgrowth of trophoblastic spheroids on ECC-1 endometrial epithelial cells. (A) Gene expression changes of inflammatory cytokines (*CXCL1, IL-8, and IL-33*) and cell adhesion molecules (*ITGβ3, ITGβ5*) were confirmed by quantitative real-time polymerase chain reaction and Western blotting after LPS treatment in ECC-1 endometrial epithelial cells. (B) Treatment with LPS changed the inflammatory cytokines and cell adhesion molecules. The detrimental effect of LPS was shown by a decrease in the attachment rate and outgrowth area of trophoblastic spheroids on endometrial epithelial cells. *CXCL1*, CXC motif ligand 1; *IL*, interleukin; *ITG*, integrin.

(CXCL1, IL-8, and IL-33) and adhesion molecules (ITGβ3 and ITGβ5) by LPS treatment might be related to reduced trophoblastic spheroid attachment and outgrowth on endometrial epithelial cells.

## Conflict of interest

Jin Hyun Jun is an Associate Editor of the journal, but he 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.

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# *Staphylococcus saprophyticus and Escherichia coli*: Tracking from sperm fertility potential to assisted reproductive outcomes

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**Objective:** Bacteriospermia and urogenital infections are common problems in male infertility. This study aimed to evaluate the effects of bacteriospermia on sperm parameters and clinical outcomes in semen samples infected with two common bacteria (*Staphylococcus saprophyticus* and *Escherichia coli*) in northern Iran.
**Methods:** Microbiological tests were performed to isolate and identify organisms from 435 semen samples from infertile couples. Semen samples were assessed according to the World Health Organization criteria. The protamine status, chromatin structure, chromatin condensation, and acrosome reaction of sperm and assisted reproductive outcomes were determined in couples with different male infertility factors.
**Results:** Among the total cases, the two most prevalent pathogens were considered: *S. saprophyticus* (38.2%) and *E. coli* (52.9%). In the semen samples infected with *E. coli*, the spontaneous acrosome reaction and abnormal chromatin condensation were more common ($p<0.05$). Significant increases in abnormal chromatin condensation and deprotamination were seen in the presence of *S. saprophyticus*. In washed semen, tight adhesion between the sperm midpiece and *S. saprophyticus* was observed. There was also a significant decrease in the fertilization rate using semen samples infected with *S. saprophyticus* and *E. coli* during *in vitro* fertilization cycles ($p<0.001$). In addition, the presence of *S. saprophyticus* and *E. coli* in semen samples was associated with a lower likelihood of clinical pregnancy in couples with various factors of male infertility.
**Conclusion:** Poor results of assisted reproductive techniques may be correlated with semen samples infected with two common bacteria in northern Iran.

**Keywords:** Assisted reproductive technique; Bacterial Infections; Fertilization; Pregnancy; Semen analysis

## Introduction

Approximately 15% of cases of male infertility are due to urogenital tract inflammation and infection [1]. Clinical data show that local inflammation or infection can be seen in up to 60% of patients receiving treatment with assisted reproductive technology (ART) [2]. Infections are the main origin of inflammatory disorders in the male genital tract. However, controversy exists regarding the role of different types of bacteria in decreasing sperm parameters. Therefore, male fertility may be inhibited by the negative effects of bacteriospermia and leukocytospermia on sperm parameters [2]. Existing evidence suggests a connection between bacteriospermia and alterations of semen quality. In recent years, the detrimental effects of different types of bacteria strains and/or leukocytes on some sperm parameters were studied [3]. However, the premise that the direct effects of bacteria are the reason for decreasing the chance of success in ART has not been confirmed.

Several studies have investigated the effects of different bacteria

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on semen parameters and male fertility. However, to the best of our knowledge, no study in the literature has yet investigated the impact of *Staphylococcus saprophyticus* on sperm quality and male fertility potential during *in vitro* fertilization (IVF) techniques. However, the effects of *Escherichia coli* have been explored in some research. Therefore, a comparative study of these two bacteria on semen parameters and fertility results of sperm in laboratory conditions would help to better understand the potential effects of bacteria on sperm fertility potential. Since the assisted reproductive outcomes of ejaculated semen with bacteriospermia are an interesting subject, we conducted this study to determine the prevalence of bacteriospermia in infertile men in northern Iran. In addition, the effects of bacteriospermia on sperm quality and assisted reproductive outcomes were studied among couples with different factors of male infertility.

## Methods

### 1. Semen sample collection and preparation

After excluding infertile couples with female-factor infertility, semen samples were collected from 607 infertile couples with male factor infertility who were undergoing IVF-intracytoplasmic sperm injection (IVF-ICSI) cycles at the Alzahra Educational and Remedial Center (IVF center) from May 2017 to June 2019 (Figure 1). The exclusion criteria were men having at least one of the following conditions: (1) taking hormone-containing medications; (2) diabetes; (3); thyroid disease; (4) a history of diseases affecting the reproductive tract (e.g., cryptorchidism, varicocele, testicular torsion, chictic, or other causes of infertility that had been medically proven); (5) a history of surgery of the reproductive system, male factor infertility with previous failed IVF-ICSI cycles, and/or hormonal abnormalities; (6) heavy smoking or alcohol use; and (7) exposure to physical or chemical agents with known negative reproductive effects that may influence the semen quality. In addition, semen samples infected with leukocytes were excluded from this study. Samples infected with other bacterial species than *S. saprophyticus* and *E. coli* were excluded because the number of semen samples infected with other bacterial species was very small. Hence, statistically significant associations with clinical outcomes would not have been found (Figure 1).

The semen samples were analyzed according to the World Health Organization (WHO) criteria [4]. The semen volume, pH, sperm motility, concentration, and viability were evaluated. According to the WHO criteria [4], the semen samples were classified as showing nor-

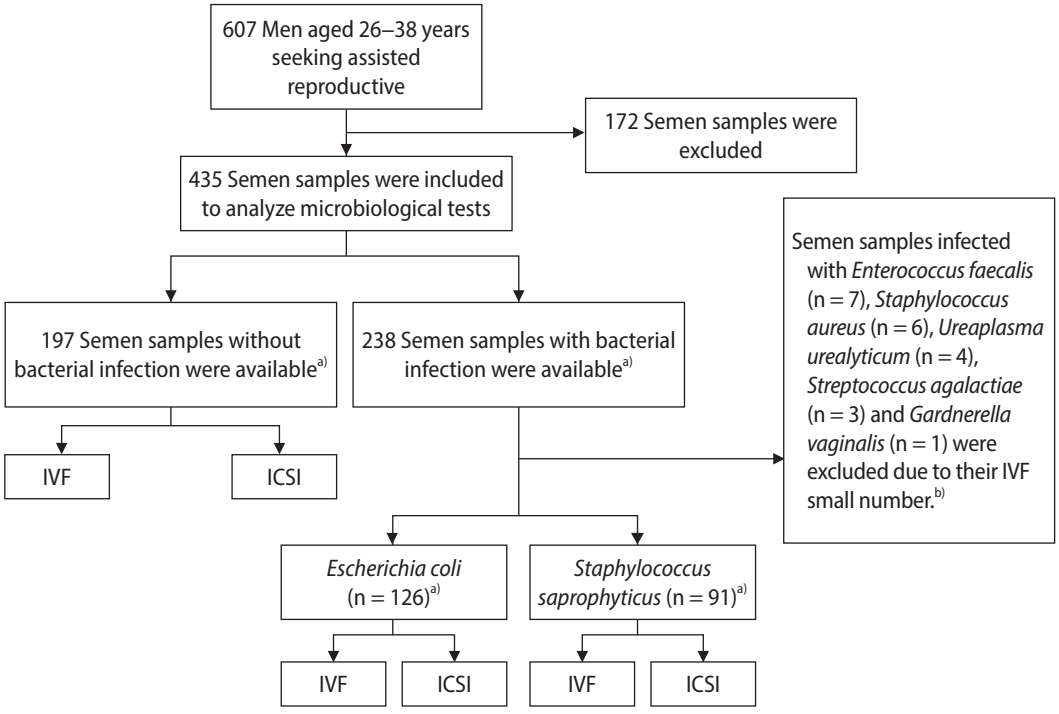


**Figure 1.** Flowchart of the study design. IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection. [a)]All normal and infected semen samples were categorized according to World Health Organization criteria into four groups as following: normozoospermia, asthenozoospermia, teratozoospermia, and oligoasthenoteratozoospermia. ICSI and/or IVF procedures were performed for each group based on sperm parameters, and assisted reproductive outcomes were followed until the live birth stage for each group; [b)]Due to the small number of semen samples infected with these bacteria, statistically significant associations with clinical outcomes would not have been found.

mozoospermia, teratozoospermia, asthenozoospermia, or oligoasthenoteratozoospermia. The severity of abnormalities in the semen samples in each studied group (e.g., asthenozoospermia, teratozoospermia, and oligoasthenoteratozoospermia) was similar. This study was approved by the Guilan University of Medical Sciences committee, and informed consent was obtained from all volunteers to participate in this study.

### 2. Seminal leukocyte quantification

To distinguish white blood cells (WBCs) in semen samples, samples were studied in high-power fields. Only samples with ≥5 round cells were assessed with peroxidase staining based on the Endtz test [5]. Peroxidase-positive leukocytes were indicated as stained brown, counted, and reported as $\times 10^6$ WBC/mL semen.

### 3. Microbiological analysis

Initially, patients received comprehensive information about the method of semen sample collection, the period of sexual abstinence, and the need to avoid taking antibiotics for at least 1 week before semen collection. Semen samples were collected by masturbation after 3–4 days of sexual abstinence. Patients were asked to urinate and wash their hands, penis, and scrotum before ejaculation to avoid possible contamination from the urine or external genitalia. Therefore, semen collection was done in a sterile container, following all given guidance. The semen samples were transported within 1 hour from collection to the Microbiology Laboratory of the University of Guilan to screen for a comprehensive range of microbiological organisms such as anaerobic and aerobic organisms as well as fungi and mycoplasma. First, the semen samples diluted with sterile saline (1:10) were centrifuged at 300 ×*g* for 15 minutes. The sediment was sown using 10-μL calibrated loops on selective specific media. The elimination of seminal plasma and condensation of bacteria increased culture sensitivity [6].

All cultures were incubated at 37°C and the culture media, incubation times, and conditions were as follows: (1) blood agar to culture aerobic bacteria under aerobic conditions for 24 hours; (2) Sabouraud agar to culture fungi under aerobic conditions for 48 hours; and (3) chocolate agar to culture other microorganisms at 5% $CO_2$ for 48 hours. A number of isolated colonies of pathogenic bacteria higher than $1 \times 10^3$ colony-forming units per milliliter was considered as a positive result. Microorganisms were determined using antimicrobial sensitivity testing according to standard conventional techniques such as Gram staining, oxidase, catalase, and other biochemical tests.

### 4. Sperm assays

#### 1) Toluidine blue stain

Toluidine blue (TB) and aniline blue (AB) staining have been introduced as sensitive tests to distinguish abnormalities in sperm chromatin structure and abnormal condensation, respectively. Thin smears were prepared on silane-coated slides. In TB staining, the sperm heads with normal chromatin structure were observed as light blue, while those with diminished integrity and abnormal chromatin structure were shown as deep violet/purple. In addition, in AB staining, at least 200 spermatozoa were counted and recorded as sperm with dark-blue (abnormal) and colorless (normal) heads. The positive group (test group; n=6) was also evaluated following the incubation of semen samples with *S. saprophyticus* and *E. coli in vitro*.

#### 2) Acrosome reaction assessment

Triple staining was used to evaluate the acrosome reaction (acrosome integrity or reacted acrosome) according to the Talbot and Chacon method [7]. In brief, 2% trypan blue was added to spermatozoa (1:1), and incubated at 37°C for 15 minutes. Fixation was done using glutaraldehyde (3% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4) for 30 to 60 minutes. After centrifugation (at 600 ×*g* for 5 minutes), the smear was prepared from fixed sperm and stained with Bismarck Brown Y at 40°C for 5 minutes. The slides were then stained in Rose Bengal at 24°C for 20–45 minutes. Washing and dehydration were performed in water and an alcohol series, respectively. A total of 300 spermatozoa in each slide cleared with xylene and protected with a coverslip were examined using a light microscope. The positive group (test group; n=6) was also evaluated following the incubation of semen samples with *S. saprophyticus* and *E. coli in vitro*.

#### 3) Determination of sperm chromatin packaging by chromomycin A3

Chromomycin A3 (CMA3) stains guanosine-cytosine-rich sequences as a polymerase inhibitor fluorochrome, which distinguishes the degree of sperm protamination [8]. In this way, air-dried smears of spermatozoa were fixed in methanol/glacial acetic acid (3:1) for 20 minutes at 4°C. The slides were then air-dried at room temperature and stained with 100 μL of CMA3 solution (0.25 mg/mL CMA3 in McIlvaine's buffer, containing 10 μM MgCl2) (Figure 2). The positive group (test group; n=5) was also evaluated following the incubation of semen samples with *S. saprophyticus* and *E. coli in vitro*.

#### 4) Scanning electron microscopy

The semen samples prepared by density gradient centrifugation were fixed with Karnovsky solution for 30 minutes at 4°C. Centrifuga-


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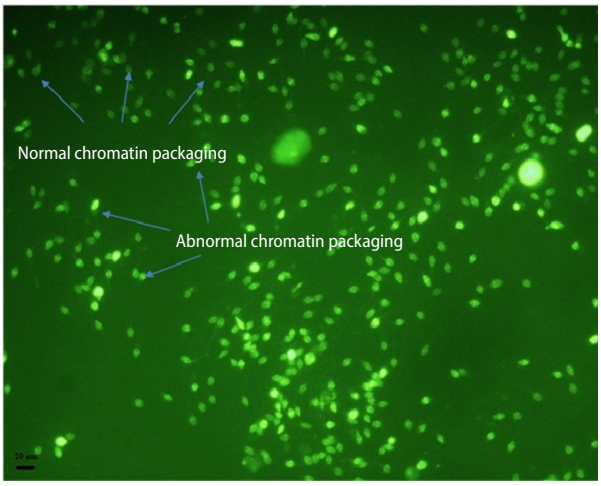


**Figure 2.** Sperm deprotamination. Spermatozoa with negative (dull yellow/normal) and positive (bright yellow/abnormal) chromomycin A3 staining. Scale bar=20 µm.

tion was done at 4,000 × *g* for 15 minutes. After washing the samples, post-fixation was performed with 1% osmium tetroxide for 30 minutes. Dehydration was then done in ascending grades of ethanol, with drying at a critical point using $CO_2$ (Balzers CPD-010). Gold-coated specimens (Balzers MED-010) were examined using a Philips FEM 515 scanning electron microscope (SEM). The positive group (test group; n=4) was also evaluated following the incubation of semen samples with *S. saprophyticus* and *E. coli in vitro*. The adhesion of bacteria to sperm structures was compared to the sham group (human spermatozoa incubated with *S. saprophyticus* and *E. coli* under *in vitro* conditions) [2].

### 5. ICSI and IVF laboratory procedures

On the day of oocyte retrieval, semen samples were collected via masturbation, liquefied, and prepared by density gradient centrifugation (90 and 45% SpermGrade; Vitrolife, Sweden, Göteborg). Semen parameters were assessed according to the WHO [4] criteria. The ICSI or IVF procedure was performed up to 3–4 hours after sperm preparation. The selection of sperm for IVF or ICSI was based on sperm morphology, motility, concentration, patient's history, infertility etiology, and female age. The evaluation of fertilization was performed 16–18 hours after ICSI/IVF with the observation of the 2-pronuclear stage. The embryo development rate was also evaluated at 2–3 days. Intrauterine embryo transfer (ET) was performed at 2–3 days after microinjection. Increasing serum beta-human chorionic gonadotropin concentrations and detection of the fetal heartbeat were defined as biochemical and clinical pregnancy at 2 and 4 weeks after ET, respectively.

### 6. Statistical analysis

The prevalence of bacteriospermia was calculated from the proportion of positive cases relative to the total number of the study population and expressed as a percentage. Using linear regression, the relationship between bacteriospermia and other semen parameters was analyzed. The Fisher exact test was performed to assess the fertilization and cleavage rate, clinical pregnancy rate, and live birth rate. The statistical analysis was done using IBM SPSS ver. 20 (IBM Corp., Armonk, NY, USA). The *P*-values $<0.05$ were considered to indicate statistical significance.

## Results

### 1. Semen samples

In this study, the women and men had a mean age of 26.0±3.2 years (range, 22–32 years) and 32.0±5.9 years (range, 26–38 years), respectively. Statistically significant differences were not found for the age ($p>0.05$) or body mass index (BMI; $p>0.05$) of the female partners in the normozoospermia. In addition, these similar results were seen about groups of asthenozoospermia, teratozoospermia, and oligoasthenoteratozoospermia ($p>0.05$). Men's age and BMI also did not show significant differences in different groups ($p>0.05$). Therefore, positive or negative effects of these parameters on ART outcomes were excluded.

Microbiological analyses were done for semen samples from 435 infertile men, of which 197 semen cultures were negative (45.2%) and 238 (54.7%) were positive. *E. coli* and *S. saprophyticus* were the most commonly isolated pathogens (52.9% and 38.2%, respectively). Semen samples infected with *Enterococcus faecalis* (n=7, 2.9%), *Staphylococcus aureus* (n=6, 2.5%), *Ureaplasma urealyticum* (n=4, 1.6%), *Streptococcus agalactiae* (n=3, 1.2%), and *Gardnerella vaginalis* (n=1, 0.4%) were also detected. Those bacterial species other than *S. saprophyticus* and *E. coli* were excluded to trace reproductive outcomes due to the small number of semen samples, as a result of which statistical significance would not be expected for associations with clinical outcomes. The infection severity of semen samples with *S. saprophyticus* and *E. coli* was almost identical. Mixed infections with both bacteria (*S. saprophyticus* and *E. coli*) were not observed in any of the cultured semen samples. Therefore, the quality of semen samples infected with each type of bacteria (*S. saprophyticus* or *E. coli*) and their clinical outcomes were analyzed.

Among the 414 specimens analyzed based on the WHO criteria [4], 141 (34.05%) had normozoospermia, 100 (24.15%) had teratozoospermia, 103 (24.87%) had asthenozoospermia, and 70 (19.9%) had oligoasthenoteratozoospermia.

### 2. Sperm parameters

As seen in Table 1, the percentage of sperm deprotamination (seen by CMA3 staining) in normozoospermia samples infected with *S. saprophyticus* (258/950, 27.15%) was significantly higher ($p < 0.05$) than that of the non-bacteriospermia samples (116/825, 14.06%). This rate was higher, at 37.8% ($p < 0.01$) and 36.8% ($p < 0.05$), in the asthenozoospermia and teratozoospermia samples infected with *S. saprophyticus*, respectively. The evaluation of acrosome integrity showed that the rate of premature acrosome reaction was higher in the oligoasthenoteratozoospermia samples infected with *E. coli* (279/856, 33.5%; $p < 0.05$). Sperm viability significantly declined in all samples infected with *E. coli* ($p < 0.05$). A decrease of viability was also seen in the asthenozoospermia ($p < 0.001$) and teratozoospermia ($p < 0.05$) samples infected with *S. saprophyticus* (54.9% and 59.02%, respectively). In addition, the evaluation of abnormalities in sperm chromatin condensation using the AB stain showed that the rate of abnormal sperm chromatin condensation (dark sperm) was higher in both bacteriospermia groups in the samples with different infertility factors (Table 1).

### 3. SEM images of bacteriospermia

SEM evaluation was performed for each sample prepared by density gradient centrifugation. The tight adhesion between bacteria and spermatozoa was found. Adhesion of *S. saprophyticus* to the tail of the sperm, especially to the sperm midpiece, was observed. However, the frequency of these adhesions was significantly higher in the asthenozoospermia and teratozoospermia samples from patients with male factor infertility. The adhesion of both bacteria types to spermatozoa was found in the unwashed semen samples; however, after washing, adhesion of *S. saprophyticus* was still observed, but less frequently (Figure 3).

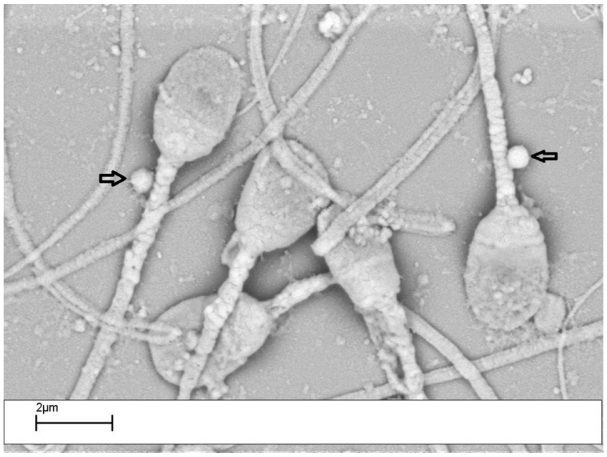


**Figure 3.** Interaction between bacteria and human spermatozoa. Scanning electron micrographs of prepared human spermatozoa after density gradient centrifugation. Adhesion of *Staphylococcus saprophyticus* to the sperm midpiece (arrows) was observed.

**Table 1.** The effect of bacteriospermia on semen quality in men with various infertility factors

| Variable | No. of patients | CMA3 (%) | Spontaneous AR (%) | AB (%) | TB (%) | Viability (%) |
|---|---|---|---|---|---|---|
| Normozoospermia | | | | | | |
| *Staphylococcus saprophyticus* | 41 | 27.2±3.2[a] | 20.1±2.2 | 22.6±2.8 | 25.7±2.6 | 68.6±10.8 |
| *Escherichia coli* | 36 | 20.1±2.2 | 26.2±2.9 | 29.7±2.4[a] | 27.8±2.4 | 64.1±9.4[b] |
| Nonbacteriospermia | 64 | 14.1±2.6 | 18.1±1.9 | 16.8±1.8 | 20.1±2.9 | 82.2±10.9 |
| Asthenozoospermia | | | | | | |
| *Staphylococcus saprophyticus* | 19 | 37.8±2.5[b] | 26.4±2.2 | 31.6±2.7 | 25.8±2.6 | 54.9±9.2[c] |
| *Escherichia coli* | 30 | 23.8±2.2 | 29.9±2.3 | 29.1±2.1 | 26.9±2.4 | 53.4±9.1[c] |
| Nonbacteriospermia | 54 | 19.1±1.9 | 19.2±2.4 | 21.8±2.2 | 22.4±2.6 | 79.5±10.0 |
| Teratozoospermia | | | | | | |
| *Staphylococcus saprophyticus* | 15 | 36.8±3.6[a] | 28.6±2.9 | 36.4±3.3[a] | 30.4±3.8 | 59.0±9.5[a] |
| *Escherichia coli* | 35 | 26.6±2.4 | 31.8±3.3 | 34.7±3.6 | 33.6±3.4 | 53.1±9.1[b] |
| Nonbacteriospermia | 50 | 22.3±3.2 | 24.3±2.4 | 23.6±2.8 | 26.4±2.6 | 70.8±8.3 |
| Oligoasthenoteratozoospermia | | | | | | |
| *Staphylococcus saprophyticus* | 16 | 39.3±3.4 | 27.4±2.2 | 38.7±3.5[a] | 35.7±3.6 | 53.4±9.4 |
| *Escherichia coli* | 25 | 32.4±3.1 | 33.5±2.7[a] | 37.4±2.7[a] | 37.4±2.5 | 51.9±8.3[a] |
| Nonbacteriospermia | 29 | 28.6±2.6 | 20.9±2.2 | 21.6±2.3 | 30.1±2.6 | 61.6±9.1 |

Values are presented as mean±standard deviation. The semen samples infected with *S. saprophyticus* showed significant differences in terms of sperm quality such as sperm deprotamination (normozoospermia, asthenozoospermia, and teratozoospermia), abnormalities in sperm chromatin condensation (teratozoospermia and oligoasthenoteratozoospermia), and viability (asthenozoospermia and teratozoospermia) in comparison to the control group (non-bacteriospermia). In addition, the semen samples infected with *E. coli* showed significant differences in sperm quality such as abnormal chromatin condensation (normozoospermia and oligoasthenoteratozoospermia), spontaneous acrosome reaction (oligoasthenoteratozoospermia), and viability (normozoospermia, asthenozoospermia, teratozoospermia, and oligoasthenoteratozoospermia) in comparison to the control group (non-bacteriospermia).
CMA3, chromomycin A3; AR, acrosome reaction; AB, aniline blue; TB, toluidine blue.
[a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$.



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### 4. ICSI and IVF outcomes

Table 2 shows the clinical outcomes of IVF and ICSI cycles. The clinical results revealed that there was no significant difference in the fertilization rate of ICSI cycles from normozoospermia samples. However, the fertilization rate of normozoospermia samples infected with *S. saprophyticus* ($p < 0.001$) and *E. coli* ($p < 0.05$) was lower in the IVF cycles. No significant difference was found in the embryo development rate of groups (normozoospermia, asthenozoospermia, and oligoasthenoteratozoospermia, $p > 0.05$), except for the teratozoospermia samples infected with *S. saprophyticus* ($p < 0.05$). In contrast, the clinical pregnancy rate was lower for the semen samples infected with *E. coli* from ICSI/IVF cycles in each group. Therefore, in the normozoospermia group, the clinical pregnancy rate decreased in samples infected with *E. coli* during ICSI cycles ($p < 0.001$) and in both bacteriospermia groups during IVF cycles ($p < 0.001$). Bacteriospermia significantly influenced the clinical pregnancy rate in the asthenozoospermia (*S. saprophyticus* and *E. coli*, $p < 0.001$) and teratozoospermia (*E. coli*, $p < 0.05$) groups (Table 2).

## Discussion

The present study investigated the effects of the most common bacteria (*S. saprophyticus* and *E. coli*) on sperm parameters and assisted reproductive outcomes in infertile couples with male factor infertility in northern Iran. Teratozoospermia was the most common abnormality in semen samples infected with *S. saprophyticus*, as manifested by sperm deprotamination, sperm chromatin condensation, and impaired viability. Other semen samples were also influenced by infection with *S. saprophyticus* and *E. coli*. Therefore, semen samples infected with *S. saprophyticus* showed significant differences in terms of sperm quality such as sperm deprotamination (normozoospermia and asthenozoospermia), abnormalities in sperm chromatin condensation (oligoasthenoteratozoospermia), and impaired viability (asthenozoospermia). In addition, semen samples infected with *E. coli* showed significant differences in terms of sperm quality such as abnormal chromatin condensation (normozoospermia and oligoasthenoteratozoospermia), spontaneous acrosome reaction (oligoasthenoteratozoospermia), and impaired viability (normozoospermia, asthenozoospermia, teratozoospermia, and oligoasthenoteratozo-

**Table 2.** The effect of bacteriospermia on assisted reproductive outcomes in men with different infertility factors

| Variable | | Fertilization rate (%) | Cleavage rate (%) | Clinical pregnancy rate P/ET (%) | Live birth LB/IE (%) |
|---|---|---|---|---|---|
| Normozoospermia | | | | | |
| *Staphylococcus saprophyticus* | ICSI | 72.2 | 83.4 | 8/14 (57.1) | 4/8 (50.0) |
| | IVF | 56.2[b] | 78.1 | 8/20 (40.0)[b] | 4/8 (50.0) |
| *Escherichia coli* | ICSI | 67.2 | 85.9 | 8/17 (47.1)[b] | 3/8 (37.5)[a] |
| | IVF | 67.3[a] | 81.6 | 6/13 (46.2)[b] | 3/6 (50.0) |
| Nonbacteriospermia | ICSI | 74.0 | 83.5 | 17/26 (65.4) | 10/17 (58.8) |
| | IVF | 79.6 | 85.5 | 22/30 (73.3) | 13/22 (59.1) |
| Asthenozoospermia | | | | | |
| *Staphylococcus saprophyticus* | ICSI | 68.4 | 82.1 | 7/15 (46.7)[b] | 3/7 (42.8) |
| *Escherichia coli* | ICSI | 69.1 | 83.4 | 12/25 (48.0)[b] | 4/12 (33.3)[a] |
| Nonbacteriospermia | ICSI | 72.7 | 89.3 | 33/49 (67.3) | 18/33 (54.5) |
| Teratozoospermia | | | | | |
| *Staphylococcus saprophyticus* | ICSI | 55.9 | 72.8[a] | 6/13 (46.2) | 2/6 (33.3)[a] |
| *Escherichia coli* | ICSI | 57.1 | 78.2 | 9/28 (32.1)[a] | 3/9 (33.3)[a] |
| Nonbacteriospermia | ICSI | 59.1 | 85.0 | 19/41 (46.3) | 10/19 (52.6) |
| Oligoasthenoteratozoospermia | | | | | |
| *Staphylococcus saprophyticus* | ICSI | 55.7 | 74.1 | 5/14 (35.7) | 1/5 (20.0)[a] |
| *Escherichia coli* | ICSI | 60.3 | 79.8 | 8/21 (38.1) | 2/8 (25.0) |
| Nonbacteriospermia | ICSI | 58.1 | 76.2 | 10/26 (38.5) | 3/10 (30.0) |

Values are presented as number (%) unless otherwise indicated. The semen samples infected with *S. saprophyticus* showed significant differences in terms of assisted reproductive outcomes such as the fertilization rate (normozoospermia), embryo cleavage rate (teratozoospermia), clinical pregnancy rate (normozoospermia and asthenozoospermia), and live birth rate (oligozoospermia and oligoasthenoteratozoospermia) in comparison to the control group (non-bacteriospermia). In addition, the semen samples infected with *E. coli* showed significant differences in the fertilization rate (normozoospermia), pregnancy rate (normozoospermia, asthenozoospermia, and teratozoospermia), and live birth rate (asthenozoospermia and teratozoospermia) in comparison to the control group (non-bacteriospermia).

P, positive cycle; ET, embryo transfer; LB, live birth; IE, implanted embryo; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization.

[a]$p<0.05$; [b]$p<0.001$.

ospermia). The poor sperm quality in the semen samples infected with bacteria may have affected the assisted reproductive outcomes. Furthermore, tight adhesion of *S. saprophyticus* to sperm after density gradient centrifugation was shown, which is reflected in the IVF outcomes (fertilization and clinical pregnancy rates). Although many pathogenic bacteria have been recognized as potential causes of infertility, the resultant clinical outcomes in IVF/ICSI procedures have not yet been elucidated [9]. This study showed that *S. saprophyticus* can interact with spermatozoa, causing reduced acrosomal function, abnormal chromatin condensation (AB staining), sperm deprotamination (CMA3 staining), and tight adhesion to different parts of the tail. Reduced motility was seen in this group. These changes were reflected in IVF outcomes, as the fertilization rate of IVF cycles was lower than that of ICSI cycles for the normozoospermia samples.

The relationship between bacteriospermia and fertilization potential remains unclear [10]. The impact of genital tract infections and bacterial contamination of semen on male fertility potential has been examined in many studies; nevertheless, the impact of bacteriospermia on fertility potential has yet to be elucidated, and there is not complete agreement on the detrimental effect of bacterial infections on sperm quality [10,11]. The most frequently isolated microorganisms in semen have been reported to be *U. urealyticum*, *E. coli, E. faecalis*, and *S. faecalis* [10], but few studies have investigated the role of *S. saprophyticus* in semen quality and male fertility potential. Therefore, the clinical significance of the presence of bacteria in the seminal fluid is still a matter of debate [12]. The present study demonstrates that *E. coli* and *S. saprophyticus* reduce sperm fertility potential and impair assisted reproductive outcomes.

Some researchers have reported that no semen parameters were significantly influenced in specimens with bacteriospermia [13-17]. In addition, Sanocka-Maciejewska et al. [18] observed no effect of bacteria isolated from the genitourinary tract on semen parameters in normozoospermia samples; nevertheless, a reduced antioxidant capacity of sperm was found in infertile patients. In contrast, other studies have reported that bacteriospermia was significantly associated with semen parameters such as sperm count, motility, and morphology [19]. Attachment between *E. coli* and sperm, morphological abnormalities, and sperm agglutination have been reported in some studies [20]. A direct negative effect of certain pathogens on the function of sperm and oocyte quality has been proven [21]. In some works, it has also been reported that sperm parameters such as sperm count, motility (both *in vitro* and *in vivo*), morphology, and viability decreased in the presence of *E. coli* [3,18,19], which is in line with our results. As the results of this study show, the quality and function of sperm decreased in the presence of bacteria, such as a declined protamination rate (positive CMA3 staining), a higher rate of premature acrosome reaction, reduced viability, and abnormal chromatin condensation (AB staining). This negative correlation between sperm quality and bacterial infection may be due to increased reactive oxygen species (ROS) production associated with inflammatory processes in semen infections, as some studies demonstrated that enhanced ROS production led to impairment of sperm DNA and male fertility [9].

In the present study, a diminished quality of sperm infected with *S. saprophyticus* and *E. coli* was followed by a decrease in the clinical pregnancy rate during ICSI/IVF cycles. It can be inferred that bacterial infections of the semen affect the fertility potential of men, even in ART under *in vitro* conditions. Therefore, the existence of bacteria and interactions between bacteria and sperm may explain why the fertilization rate and clinical pregnancy rate were lower in the semen samples infected with some bacteria.

In conclusion, bacterial infections of the male urogenital tract have been correlated with subfertility and infertility. However, these effects on fertility potential are multifunctional and complex. One relevant factor is microbial infection of the semen. This study showed negative correlations between two prevalent bacteria in the north of Iran (*E. coli* and *S. saprophyticus*) and sperm quality and assisted reproductive outcomes such as reduced viability, premature acrosome reaction, a lower protamination rate using CMA3 staining, abnormal chromatin condensation using AB staining, adhesion to sperm, change of sperm function, and lower fertilization and clinical pregnancy rates. To the best of our knowledge, no studies have previously evaluated the presence and effect of these bacteria on sperm quality and fertility potential during ICSI/IVF cycles. Therefore, the results of this study can be useful for promoting assisted reproductive outcomes.

## Conflict of interest

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

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# Coenzyme Q10, oxidative stress markers, and sperm DNA damage in men with idiopathic oligoasthenoteratospermia

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**Objective:** Oxidative stress (OS) plays a key role in the etiology of unexplained male infertility. Coenzyme Q10 (CoQ10) is a potent antioxidant that may improve semen quality and OS in infertile men with idiopathic oligoasthenoteratospermia (OAT), but the underlying mechanism is unknown. Therefore, the present study was undertaken to investigate the effect of CoQ10 on OS markers and sperm DNA damage in infertile patients with idiopathic OAT.
**Methods:** This prospective controlled study included 50 patients with idiopathic OAT and 50 fertile men who served as controls. All patients underwent a comprehensive medical assessment. Patients and controls received 200 mg of oral CoQ10 once daily for 3 months. Semen and blood were collected and analyzed for sperm parameters, seminal CoQ10 levels, reactive oxygen species (ROS) levels, total antioxidant capacity, catalase, sperm DNA fragmentation (SDF), and serum hormonal profile.
**Results:** The administration of CoQ10 to patients with idiopathic OAT significantly improved sperm quality and seminal antioxidant status and significantly reduced total ROS and SDF levels compared to pretreatment values.
**Conclusion:** CoQ10, at a dose of 200 mg/day for 3 months, may be a potential therapy for infertile patients with idiopathic OAT, as it improved sperm parameters and reduced OS and SDF in these patients.

**Keywords:** Coenzyme Q10; Male infertility; Oxidative stress; Sperm DNA fragmentation

## Introduction

Infertility is defined as the failure of conception after at least 1 year of regular unprotected sexual intercourse [1]. The prevalence of infertility among reproductive-aged couples is approximately 8%–15% [2], with male factor infertility accounting for 50% of cases [3]. Infertility in men has been linked to endocrine disorders, developmental anomalies, systemic diseases, and environmental, immunological, and genetic factors [4-6]. However, in approximately 25% of infertile men, no cause can be identified for semen abnormalities; this condition is referred to as idiopathic infertility [7].

Oxidative stress (OS) has been reported as a key factor contributing to idiopathic male infertility [8]. OS may occur as a consequence of higher ratios of oxidants (free radicals and/or reactive oxygen species [ROS]) to antioxidants in the seminal plasma [9]. The etiology of OS can be attributed to several intrinsic or extrinsic factors, including ageing, varicocele, infection, cryptorchidism, testicular torsion, radiotherapy, chemotherapy, and toxins [10,11]. Specific physiological functions, such as sperm capacitation, the acrosome reaction, and fertilization require ROS [6,12]. Nonetheless, overproduction of ROS may impair sperm membrane and DNA integrity, resulting in de-

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creased sperm membrane fluidity and changes in the fertilizing capability of sperm [13]. ROS-induced sperm DNA damage may affect sperm motility and the ability to fertilize an oocyte [14]. Sperm DNA fragmentation (SDF) is irreversible and leads to alteration of sperm function, resulting in infertility [15].

Coenzyme Q10 (CoQ10) is a potent antioxidant that protects sperm against ROS-induced damage [16]. It is quite ubiquitous, with a high amount in sperm mitochondria [17], and has been reported to enhance sperm motility and concentration [8,11]. Furthermore, insufficient CoQ10 levels have been linked to low sperm count and motility, as well as elevated sperm DNA damage [18]. Our recent study conducted on patients with idiopathic oligoasthenospermia (OA) for 12 weeks showed that CoQ10 therapy substantially enhanced progressive motility, sperm concentration, and seminal fluid CoQ10 concentrations [1]. It also led to an increase in glutathione peroxidase (GPx) levels and total antioxidant capacity (TAC) [1]. Moreover, our recent meta-analysis of three randomized clinical trials on the effect of CoQ10 on semen quality demonstrated beneficial effects of CoQ10 on improving sperm motility, but not on sperm concentration or morphology [18]. The maintenance of testicular scavenging function is normally exerted by intrinsic antioxidants such as GPx, catalase (CAT), and superoxide dismutase (SOD) [19,20].

The present study aimed to investigate the impact of CoQ10 on OS markers and sperm DNA damage in infertile men with idiopathic oligoasthenoteratospermia (OAT) in an attempt to better understand its mechanism of action.

## Methods

### 1. Patients

Fifty patients with idiopathic OAT and 50 fertile men (controls) were recruited at the Fertility Clinic, Hillah, Babyl, Iraq, from July 2018 to January 2019. All patients and controls underwent a comprehensive medical assessment. A prospective controlled study was conducted with a 3-month follow-up. Seven patients dropped out of the study and were therefore excluded. The patients received a daily dose of 200 mg of CoQ10 (in its reduced form as ubiquinol) (America Medic and Science AMS, Woodinville, WA, USA) as a single oral dose for 3 months [21]. Semen analysis, seminal CoQ10 levels, ROS, TAC, CAT, SDF as well as serum hormonal profile (follicle-stimulating hormone [FSH], luteinizing hormone [LH], testosterone, and prolactin levels) in patients after therapy were compared with the baseline values for patients and controls (patients were followed-up from September 2018 to February 2019). Sample size calculation was performed using 80% power and a 5% level of significance and was 42 for each group. Study approval was obtained from the University of Sumer Local Research Ethical Committee (EC/2018/8879). All participants consented to the study prior to enrollment in the study.

### 2. Eligibility criteria

The fertile controls enrolled in the study had fathered a child in the last 24 months and had normal semen analysis results. The patients had a history of infertility of at least 1 year despite regular unprotected intercourse. OAT was defined according to the World Health Organization (WHO) 2010 criteria. Men with varicocele; genital infection; azoospermia; anatomical abnormalities; testicular injury or surgery; endocrine, renal, hepatic, or other systemic illnesses; smoking; alcohol intake; and recent antioxidant intake were excluded, as were those taking relevant medications and male partners in couples affected by female factor infertility.

### 3. Semen analysis

Semen samples were collected by masturbation following abstinence for 2–3 days. A special wide-mouth container was used to collect semen and incubated at 37°C until the semen was liquefied. Semen analysis was then performed within 1 hour following the WHO manual criteria (5th edition, 2010) [22]. Duplicate semen analyses were performed at the beginning and at the end of the study and the average of the two values was used for analysis. The same investigator performed all semen analyses to optimize repeatability.

### 4. Measurement of seminal CoQ10 concentrations

Seminal CoQ10 levels were measured using reverse-phase high-performance liquid chromatography utilizing an ultraviolet light detector at 275 nm, with coenzyme Q9 as an internal standard, and calculated using a published method [23].

### 5. Seminal ROS measurement

Semen samples were centrifuged at 3,000 rpm for 5 minutes to obtain seminal plasma and then were stored at –20°C. A manual method was used for ROS measurement as previously described by Venkatesh et al. [24]. To 400 µL of liquefied neat semen, 10 µL of luminol (5-amino-2,3,-dihydro-1,4-phthalazinedione; Sigma, St. Louis, MO, USA), prepared as 5 mM stock in dimethyl sulfoxide (DMSO), was added. Ten microliters of 5 mM luminol in DMSO served as a blank, while 25 µL of $H_2O_2$ with 10 µL of luminol was used as a positive control. Luminol-dependent chemiluminescence served as indicator of ROS levels.

### 6. Measurement of seminal TAC and CAT

TAC was estimated with a colorimetric method using a total antioxidant capacity assay kit (#E-BC-K136; Elabscience, Houston, TX, USA). Seminal plasma CAT activity was assessed using a CAT assay kit (#E-BC-K031, Elabscience), following the protocol recommended by

the manufacturer.

### 7. Sperm chromatin dispersion test

Sperm chromatin dispersion was tested using the Halosperm kit (Halotech DNA, Madrid, Spain). The principle of the test is that, after acid denaturation and removal of nuclear proteins, sperm with SDF do not exhibit the halo of dispersed DNA loops that is observed in sperm without SDF. The nucleoids from spermatozoa with SDF show no dispersion halo or a minimal halo. Bright-field microscopy with Diff-Quik staining was utilized to examine the halos. SDF, defined as the percentage ratio of sperm with SDF to the total spermatozoa, was calculated using previously published methods [1,25].

### 8. Hormonal assays

Blood samples (5 mL) were collected using venipuncture in clean plain labeled tubes, allowed to clot, and centrifuged at 3,000 rpm for 10 minutes for analysis of hormones. Serum FSH, LH, testosterone, and prolactin levels were measured using enzyme-linked fluorescent assays (mini-VIDAS; Biomerieux, Lyon, France).

### 9. Statistical analysis

IBM SPSS ver. 24 (IBM Corp., Armonk, NY, USA) was used for data analysis. The results were expressed as mean ± standard deviation. The normality of the data distribution was assessed using the Kolmogorov-Smirnov test. One-way analysis of variance was used to compare mean values between subgroups. Pearson correlation coefficients were applied to assess the correlations of seminal fluid parameters with CoQ10 levels and SDF. For all tests, a *p*-value lower than 0.05 was considered to indicate statistical significance.

## Results

The mean age of the control participants and patients was 34.2 ± 13.4 and 31.3 ± 12.6 years, respectively, and the mean duration of infertility in the patients was 7.1 ± 4.8 years. CoQ10 therapy in patients with idiopathic OAT significantly increased the total motility ($p < 0.01$), progressive motility ($p < 0.05$), and sperm concentration ($p < 0.05$) compared with the baseline (Table 1). Treatment with CoQ10 increased progressive motility from 26.5% ± 10.8% to 32.6% ± 15.1%. Sperm concentration also increased from 11.2 ± 6.4 to 13.3 ± 8.6 million/mL following treatment with CoQ10, and total motility also rose from 36.1% ± 10.8% to 44.2% ± 18.5%, with a high level of significance when compared with other sperm parameters.

The results also showed that the seminal antioxidant status was significantly lower in infertile patients than in controls (CoQ10, $p < 0.05$; CAT, $p < 0.001$; and TAC, $p < 0.01$), but ROS levels were significantly higher than in controls ($p < 0.001$) (Table 2). Moreover, treatment with CoQ10 substantially enhanced CAT ($p < 0.001$), TAC ($p < 0.01$), and seminal CoQ10 ($p < 0.001$) levels, while decreasing total ROS levels ($p < 0.05$) in patients with idiopathic OAT. The comparison between fertile controls and idiopathic OAT patients showed that SDF was significantly higher in patients ($p < 0.001$), and was significantly reduced by CoQ10 therapy ($p < 0.001$) (Table 2).

FSH ($p < 0.001$), LH ($p < 0.001$), and prolactin levels ($p < 0.01$) were significantly higher in infertile patients than in controls. Following CoQ10 treatment, LH levels increased significantly in infertile patients compared with their baseline values ($p < 0.05$) (Figure 1). The SDF of infertile patients was negatively correlated with total sperm motility ($r = -0.62$, $p = 0.001$). Furthermore, total sperm motility ($r = 0.56$, $p = 0.005$) was positively associated with CoQ10 levels ($r = 0.23$, $p = 0.12$) in infertile patients (Table 3).

## Discussion

CoQ10 is an essential antioxidant present nearly in all body tissues. It is also present in sperm mitochondria, where it plays a critical role in cellular respiration and energy generation [26]. CoQ10 is also involved in the inhibition of superoxide formation, protecting against

**Table 1.** Comparison of semen parameters in fertile men and infertile patients before and after treatment with CoQ10

| Demographic characteristics and semen parameter | Fertile men (n = 50) | Infertile patients (n = 50) | |
|---|---|---|---|
| | | Before CoQ10 | After CoQ10 |
| Age (yr) | 34.2 ± 13.4 | 31.3 ± 12.6 | - |
| Infertility duration (yr) | - | 7.1 ± 4.8 | - |
| Volume (mL) | 3.5 ± 1.4 | 3.48 ± 1.7 | 3.7 ± 1.8 |
| Concentration (million/mL) | 61.3 ± 34.6 | 11.2 ± 6.4[a)] | 13.3 ± 8.6[c)] |
| Progressive motility (%) | 57.8 ± 12.2 | 26.5 ± 10.8[a)] | 32.6 ± 15.1[a),c)] |
| Total motility (%) | 79.1 ± 15.0 | 36.1 ± 10.8[a)] | 44.2 ± 18.5[b),c)] |
| Normal morphology (%) | 8.2 ± 3.6 | 5.4 ± 3.2[a)] | 5.7 ± 2.9[c)] |

Values are presented as mean±standard deviation.
CoQ10, coenzyme Q10.
[a)] $p<0.05$, vs. baseline; [b)] $p<0.01$, vs. baseline; [c)] $p<0.001$, vs. fertile men.


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**Table 2.** Seminal plasma CoQ10 levels, oxidative stress markers, and sperm DNA fragmentation in fertile controls and infertile patients before and after the administration of CoQ10

| Variable | Fertile men (n = 50) | Infertile patients (n = 50) | |
|---|---|---|---|
| | | Before CoQ10 | After CoQ10 |
| CoQ10 level (ng/mL) | 56.7 ± 37.4 | 41.4 ± 29.3[c)] | 76.2 ± 26.7[b),d)] |
| ROS (× $10^4$ RLU/min/20 million spermatozoa) | 0.08 ± 0.06 | 4.3 ± 1.6[d)] | 3.3 ± 1.5[a),d)] |
| TAC (mmol/L) | 1.4 ± 0.25 | 0.9 ± 0.44[d)] | 1.2 ± 0.51[b),c)] |
| Catalase (U/mL) | 15.54 ± 3.12 | 10.6 ± 2.8[d)] | 12.4 ± 2.61[a),d)] |
| Sperm DNA fragmentation (%) | 16.4 ± 4.7 | 38.6 ± 7.9[d)] | 34.5 ± 9.3[a),d)] |

Values are presented as mean±standard deviation.
CoQ10, coenzyme Q10; ROS, reactive oxygen species; TAC, total antioxidant capacity.
[a)]Significant difference from baseline, $p<0.01$; [b)]Significant difference from baseline, $p<0.001$; [c)]Significant difference from control, $p<0.05$; [d)]Significant difference from control $p<0.001$.

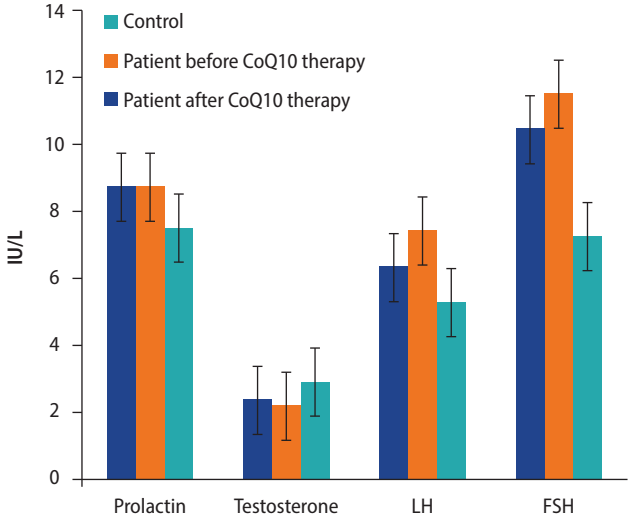


**Figure 1.** Hormonal profiles of controls and infertile patients with oligoasthenoteratospermia before and after coenzyme Q10 (CoQ10) therapy. LH, luteinizing hormone; FSH, follicle-stimulating hormone.

**Table 3.** Correlation of SDF and CoQ10 levels with sperm parameters in infertile patients with idiopathic oligoasthenoteratozoospermia

| Variable | *r* (*p*-value) | | |
|---|---|---|---|
| | Concentration | Total motility | Normal morphology |
| SDF | –0.09 (0.31) | –0.62 (0.001) | –0.18 (0.16) |
| CoQ10 | 0.23 (0.12) | 0.56 (0.005) | 0.14 (0.22) |

SDF, sperm DNA fragmentation; CoQ10, coenzyme Q10.

OS-induced sperm damage [27]. OS-mediated disruptions in fertility parameters entail sperm DNA and cell membrane damage [2].

The role of CoQ10 in cellular respiration and energy generation underscores its usefulness as an antioxidant [28]. In our previous studies, we found that CoQ10 at the dose of 200 mg/day for 3 months improved semen quality and antioxidant status in infertile patients with idiopathic OAT, but the underlying mechanism by which CoQ10 improves fertility parameters are unknown [1,15]. In the present study, we found that CoQ10 therapy in patients with idiopathic OAT significantly improved sperm parameters and antioxidant status, while decreasing OS markers and SDF.

It has been widely reported that SDF is a critical factor that causes male infertility [29]. Thus, the results of the present study may indicate that at the molecular level, CoQ10 acts to ameliorate sperm DNA damage and mitigates OS, thereby leading to improvements in sperm parameters. Similar observations have been published by Suliga and Gluszek [2], who showed that CoQ10 concentrations in seminal plasma were linearly associated with sperm count and motility. Moreover, in a case-control study of 65 idiopathic OA patients, CoQ10 therapy significantly increased progressive motility, GPx levels, sperm concentration, seminal fluid CoQ10 concentration, total motility, and TAC compared with the control group consisting of 45 healthy men [1]. The study concluded that CoQ10 supplementation for 12 weeks led to improvements in OS markers, enhancement of semen parameters, and reduction of SDF in infertile patients with idiopathic OA [1]. Another study conducted in 212 infertile patients with idiopathic OAT, who were on 300 mg of oral CoQ10 for 182 days, showed substantial increases in sperm motility and concentration after CoQ10 treatment [14]. A similar study showed that CoQ10 therapy (200– 300 mg per day) could significantly increase sperm motility and concentration [30]. CoQ10 therapy has also been reported to improve SOD and CAT levels, as well as sperm parameters [14]. A meta-analysis conducted by Lafuente et al. [26], also showed that supplementation with CoQ10 enhanced sperm parameters. Furthermore, our recent meta-analysis of three randomized clinical trials demonstrated that CoQ10 improved sperm motility [18]. In a non-controlled trial [31] in 287 infertile patients with idiopathic OAT, it was found that CoQ10 therapy given orally at the dose of 300 mg twice daily for 1 year significantly improved progressive motility, the proportion of normal morphology, and sperm concentration [2].

A controlled trial conducted among 228 idiopathic infertile OAT patients who received CoQ10 supplementation (200 mg daily for 26 weeks) concluded that CoQ10 increased sperm motility, sperm concentration, and morphology [31]. Another controlled trial including

60 infertile patients with idiopathic OAT treated with CoQ10 (200 mg per day or placebo for 3 months) concluded that there were improvements in semen parameters [32]. The various results from meta-analyses and clinical trials on CoQ10 therapy in men with infertility align with our findings that supplementation with CoQ10 increased seminal CoQ10 levels, sperm motility, and concentration [33]. Generally, controlled clinical trials among men with idiopathic infertility treated with CoQ10 have shown that this treatment leads to a reduction in OS in seminal plasma and lipid peroxidation, as well as an increase in ubiquinol levels and seminal enzymatic antioxidant levels [32].

In a study assessing the effect of 2 doses of CoQ10 on semen quality and OS markers in men with idiopathic OAT, it was found that CoQ10 treatment increased SOD activity, TAC, and CAT activity [34]. CoQ10 inherently inhibits the action of OS by antagonizing any system that increases OS and enhancing systems that could inhibit OS. A prior study showed a strong negative relationship between CoQ10 and hydrogen peroxide levels [6]. Our results, in agreement with the study by Safarinejad et al. [32], showed that CoQ10 therapy decreased FSH and LH, whereas it increased serum prolactin levels. Hyperprolactinemia is among the causes of hypogonadotropic hypogonadism [35].

A study of 20 infertile patients with high SDF levels treated for 3 months with a preparation containing various antioxidants, including CoQ10, showed that SDF levels significantly decreased and sperm concentration significantly increased [36]. In a study of 20 infertile patients with high SDF levels and low-grade varicocele who were treated for 3 months with a preparation containing CoQ10 and other antioxidants, SDF levels significantly decreased and sperm concentration significantly increased [37]. These results support our finding of a significant decrease in SDF levels when infertile patients with idiopathic OAT were placed on CoQ10 therapy (200 mg/day) [1]. It was also shown that treatment with CoQ10 substantially enhanced CAT, TAC, and seminal CoQ10 levels in idiopathic OAT, and that total ROS levels decreased when compared with pretreatment values. These findings suggest that CoQ10 therapy could improve sperm parameters in infertile patients with idiopathic OAT.

The present study showed that CoQ10 increased sperm concentration and sperm progressive motility and decreased SDF levels in infertile patients with idiopathic OAT. These findings suggest that CoQ10 therapy improves sperm parameters by reducing OS and OS-induced sperm damage. Therefore, CoQ10 is a potentially useful antioxidant for the treatment of infertile patients with idiopathic OAT.

## Conflict of interest

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

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Conceptualization, Data curation, Formal analysis, & Methodology: ATA. Project administration: ATA, PS, AEC. Visualization: all authors. Writing–original draft: ATA. Writing & review–editing: PS, SD, AEC.

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# Predictive capability of fasting-state glucose and insulin measurements for abnormal glucose tolerance in women with polycystic ovary syndrome

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**Objective:** The aim of the present study was to evaluate the predictive capability of fasting-state measurements of glucose and insulin levels alone for abnormal glucose tolerance in women with polycystic ovary syndrome (PCOS).
**Methods:** In total, 153 Korean women with PCOS were included in this study. The correlations between the 2-hour postload glucose (2-hr PG) level during the 75-g oral glucose tolerance test (OGTT) and other parameters were evaluated using Pearson correlation coefficients and linear regression analysis. The predictive accuracy of fasting glucose and insulin levels and other fasting-state indices for assessing insulin sensitivity derived from glucose and insulin levels for abnormal glucose tolerance was evaluated using receiver operating characteristic (ROC) curve analysis.
**Results:** Significant correlations were observed between the 2-hr PG level and most fasting-state parameters in women with PCOS. However, the area under the ROC curve values for each fasting-state parameter for predicting abnormal glucose tolerance were all between 0.5 and 0.7 in the study participants, which falls into the "less accurate" category for prediction.
**Conclusion:** Fasting-state measurements of glucose and insulin alone are not enough to predict abnormal glucose tolerance in women with PCOS. A standard OGTT is needed to screen for impaired glucose tolerance and type 2 diabetes mellitus in women with PCOS.

**Keywords:** Fasting; Glucose; Glucose tolerance test; Insulin; Polycystic ovary syndrome

## Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in reproductive-aged women, affecting 5%–10% of women worldwide [1]. PCOS is a reproductive disorder characterized by chronic ovulation and hyperandrogenism, resulting in amenorrhea and infertility; it is also a metabolic disorder based on insulin resistance that leads to future overt diabetes and cardiovascular disease. Insulin resistance and the resulting hyperinsulinemia are known to play a pivotal role in the pathogenesis of PCOS [1-4]. Insulin resistance has been detected in approximately 80% of women with PCOS and in 95% of obese women [5]; therefore, some authors have suggested referring to PCOS as syndrome XX, just as metabolic syndrome is called syndrome X [6]. PCOS is a leading risk factor for prediabetes (comprising impaired fasting glucose [IFG] and impaired glucose tolerance [IGT]) and type 2 diabetes mellitus (T2DM) in reproductive-age women [2,6].

A 2-hour, 75-g oral glucose tolerance test (OGTT) is the standard method used to evaluate glucose tolerance and diagnose diabetes.

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Recent clinical guidelines have recommended a standard OGTT to screen for IGT and T2DM in women with PCOS [7-9]. However, since OGTT involves the inconvenience of evaluating blood glucose every hour for 2 hours, there have been demands to replace it with fasting-state measurement of glucose levels alone in the actual clinical setting of managing PCOS patients; however, some researchers have argued that it is not enough to measure fasting blood glucose levels alone to screen for T2DM [3,10].

Insulin sensitivity is a concept that reflects the opposite of insulin resistance [2]. Currently, the hyperinsulinemic-euglycemic clamp method is regarded as the gold standard for assessing insulin sensitivity/resistance, but this clamp method is used primarily for research purposes only, because it is difficult to apply in real clinical situations due to its cost, invasiveness, time-consuming nature, and dependence on experienced personnel [1,2,11]. Therefore, fasting-state insulin sensitivity assessment indices (ISAIs) derived from fasting glucose and insulin concentrations, such as the homeostatic model assessment of insulin resistance (HOMA-IR), quantitative insulin sensitivity check index (QUICKI), and glucose-to-insulin ratio (GIR), have been widely used to evaluate insulin sensitivity/resistance in epidemiological studies because these indices are uncomplicated and inexpensive quantitative (homeostatic) methods that are strongly correlated with insulin sensitivity as measured with a hyperinsulinemic-euglycemic clamp [2,11,12].

In the present study, we conducted a receiver operating characteristic (ROC) curve analysis to evaluate how well abnormal glucose tolerance can be predicted by measurements of fasting-state parameters alone, such as fasting glucose and insulin concentrations and other fasting-state ISAIs derived from a combination of glucose and insulin levels, without postload glucose measurements, following an oral glucose challenge in women with PCOS.

## Methods

### 1. Subjects

South Korean women between the ages of 18 and 35 years who first visited Inje University Haeundae Paik Hospital between January 2010 and December 2013 and were diagnosed with PCOS according to the Rotterdam consensus diagnostic criteria [13] were recruited for this retrospective study. Among these patients, only those who met the recently revised diagnostic criteria provided in the international consensus guidelines for PCOS [9] were enrolled. Pelvic ultrasound examinations (through the vagina or rectum) for assessing polycystic ovarian morphology were conducted in the early follicular phase using a Voluson Logiq S7 (GE Ultrasound Korea, Seongnam, Korea) equipped with a transvaginal probe with a frequency range of 3.6–9 MHz, and all of the ultrasound examinations were conducted by the same reproductive endocrinologist in accordance with the international consensus for ultrasound assessment [14]. The exclusion criteria for the present study were as follows [15,16]: patients who were previously diagnosed with thyroid disease or hyperprolactinemia, had a history of ovarian surgery or suspicious ovarian malignancy, or had been taking medications known to affect sex hormone or gonadotropin levels in the 6 months prior to enrollment (oral contraceptives, ovulation induction agents, glucocorticoids, or anti-androgens). Furthermore, patients who were currently taking insulin or oral hypoglycemic drugs were also excluded from the present study. Finally, a total of 153 patients were included.

This study was approved by the Institutional Review Board of Inje University Haeundae Paik Hospital (IRB No. 129792-2014-035), and patient's informed consent in this study was waived by the IRB. Clinical anthropometric parameters were evaluated in all patients when they first visited the outpatient department. Body mass index (BMI) was defined by dividing body weight (kg) by the square of the height ($m^2$), and the waist-to-hip ratio (WHR) was calculated by dividing the waist circumference (cm) by the hip circumference (cm).

### 2. Biochemical measurements and determination of abnormal glucose tolerance

Blood samples were taken from all study participants following overnight fasting in accordance with the guidelines of the Declaration of Helsinki, and sera were obtained by centrifugation to evaluate biochemical parameters. Fasting glucose levels and levels at 2 hours after 75-g glucose ingestion during a 2-hour OGTT were measured using L-Type GluI (Wako Pure Chemical Industries, Osaka, Japan). Fasting insulin levels were evaluated using an Elecsys Insulin assay (Roche Diagnostics Corp., Basel, Switzerland). The intra- and inter-assay coefficients of variation were $< 5\%$ for all measurements.

Fasting-state homeostatic ISAIs derived from a combination of fasting glucose and insulin levels were calculated according to the following formulas:

HOMA-IR = glucose (mg/dL) × insulin (μU/mL)/405,
GIR = glucose (mg/dL)/insulin (μU/mL); and,
QUICKI = 1/{log [insulin (μU/mL)]+log [glucose (mg/dL)]}.

Abnormal glucose tolerance, which comprises IGT and diabetes [17,18] was defined using the criterion established by the American Diabetes Association (ADA) [19]: a 2-hour postload glucose (2-hr PG) concentration ≥ 140 mg/dL after an OGTT.

### 3. Statistical analysis

Values are expressed as the mean ± standard deviation (SD). The unpaired *t*-test was used to compare continuous parameters be-

tween two groups created using a 2-hr PG threshold level of 140 mg/dL. The correlations between the 2-hr PG level and other parameters were evaluated using Pearson correlation coefficients and linear regression analysis, and partial correlation coefficients were used after controlling for confounding variables such as BMI and WHR. For the assessment of the prediction accuracy of fasting-state parameters for abnormal glucose tolerance, areas under the ROC curves (AUCs), sensitivity, and specificity were evaluated. Predictive accuracy using AUCs was categorized in the present study as follows [20,21]: non-informative (AUC = 0.5), less accurate (0.5 < AUC < 0.7), moderately accurate (0.7 < AUC < 0.9), highly accurate (0.9 < AUC < 1), and perfect (AUC = 1). The optimal cutoff value of the fasting-state parameter for identifying abnormal glucose tolerance was defined as the threshold value at which the value of the sensitivity plus the specificity reached a maximum. All statistical analyses were conducted using IBM SPSS ver. 25.0 (IBM Corp., Armonk, NY, USA), and *p*-values of less than 0.05 were considered to indicate statistical significance in all analyses.

## Results

Table 1 shows a comparison of patient baseline anthropometric characteristics and fasting-state laboratory parameters related to glucose and insulin metabolism between the two groups categorized according to a 2-hr PG level of 140 mg/dL. In Table 1, significant differences between the groups were found for all fasting-state laboratory parameters except GIR. Among the anthropometric parameters, WHR and BMI were significantly different between the two groups.

In Table 2, the 2-hr PG level following the 75-g OGTT was significantly related to BMI, WHR, fasting glucose, fasting insulin, HOMA-IR, and QUICKI, which was in agreement with the results shown in Table 1. These results did not change even after we controlled for the effects of variables such as BMI and WHR.

Table 3 and Figure 1 show the results from the ROC curve analyses of various fasting-state parameters to identify their ability to predict abnormal glucose tolerance in the study participants. Despite significant correlations with 2-hr PG on a 75-g OGTT and other fasting-state parameters observed in this study, the calculated AUC values of each fasting-state parameter for predicting abnormal glucose tolerance in women with PCOS were all between 0.5 and 0.7, meaning that the predictive accuracies of all fasting-state parameters fell into the "less accurate" category based on the criteria used in the present study.

## Discussion

It is well established that insulin resistance and compensatory hyperinsulinemia are central components in the pathogenesis of PCOS. Insulin resistance/hyperinsulinemia leads to a high incidence of T2DM and cardiovascular disease in PCOS patients. Up to 35% of

**Table 2.** Correlations of 2-hour postload glucose levels with anthropometric parameters and a variety of fasting-state parameters related to glucose and insulin metabolism

| Variable | *r* | *p*-value | $r^{a)}$ | *p*-value |
|---|---|---|---|---|
| Age | 0.108 | 0.182 | | |
| Body mass index | 0.229 | 0.005 | | |
| Waist-to-hip ratio | 0.343 | < 0.001 | | |
| Fasting glucose | 0.738 | < 0.001 | 0.736 | < 0.001 |
| Fasting insulin | 0.369 | < 0.001 | 0.281 | 0.001 |
| HOMA-IR (fasting) | 0.474 | < 0.001 | 0.442 | < 0.001 |
| GIR (fasting) | –0.204 | 0.203 | –0.157 | 0.07 |
| QUICKI (fasting) | –0.276 | 0.001 | –0.295 | 0.001 |

*r*, Pearson's correlation coefficient; HOMA-IR, homeostasis model assessment of insulin resistance; GIR, glucose-to-insulin ratio; QUICKI, quantitative insulin sensitivity check index.
[a)]Partial correlation coefficient adjusted by body mass index and waist-to-hip ratio.

**Table 1.** Comparison of baseline anthropometric characteristics and laboratory parameters between two groups divided according to 2-hr PG of 140 mg/dL in patients with polycystic ovary syndrome

| Variable | 2-hr PG < 140 mg/dL (n = 129) | 2-hr PG ≥ 140 mg/dL (n = 24) | *p*-value |
|---|---|---|---|
| Age (yr) | 26.35 ± 5.16 | 26.58 ± 5.37 | 0.839 |
| Body mass index (kg/m$^2$) | 21.83 ± 5.06 | 24.38 ± 5.71 | 0.029 |
| Waist-to-hip ratio | 0.79 ± 0.06 | 0.85 ± 0.10 | 0.009 |
| Fasting glucose (mg/dL) | 89.53 ± 6.61 | 103.75 ± 27.74 | 0.020 |
| Fasting insulin (μIU/mL) | 8.16 ± 6.79 | 15.09 ± 14.08 | 0.026 |
| HOMA-IR (fasting) | 1.80 ± 1.51 | 4.46 ± 4.60 | 0.010 |
| GIR (fasting) | 16.83 ± 10.20 | 13.10 ± 9.72 | 0.099 |
| QUICKI (fasting) | 0.37 ± 0.04 | 0.34 ± 0.05 | 0.012 |

Values are presented as mean±standard deviation.
2-hr PG, 2-hour postload glucose level; HOMA-IR, homeostasis model assessment of insulin resistance; GIR, glucose-to-insulin ratio; QUICKI, quantitative insulin sensitivity check index.


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**Table 3.** Areas under the receiver operating characteristic curve for a variety of fasting-state

| Variable | AUC (95% CI) | *p*-value | Cutoff | Sensitivity | Specificity |
|---|---|---|---|---|---|
| Fasting glucose | 0.675 (0.534–0.815) | 0.007 | 103.50 mg/dL | 0.417 | 0.985 |
| Fasting insulin | 0.634 (0.497–0.771) | 0.037 | 9.70 μIU/mL | 0.50 | 0.814 |
| GIR | 0.612 (0.475–0.750) | 0.081 | 5.975 | 0.915 | 0.417 |
| QUICKI | 0.670 (0.535–0.805) | 0.008 | 0.315 | 0.915 | 0.458 |
| HOMA-IR | 0.667 (0.529–0.805) | 0.009 | 4.220 | 0.459 | 0.922 |

Parameters related to glucose and insulin metabolism for predicting abnormal glucose tolerance.
AUC, area under the receiver operating characteristic curve; CI, confidence interval; GIR, glucose-to-insulin ratio; QUICKI, quantitative insulin sensitivity check index; HOMA-IR, homeostasis model assessment of insulin resistance.

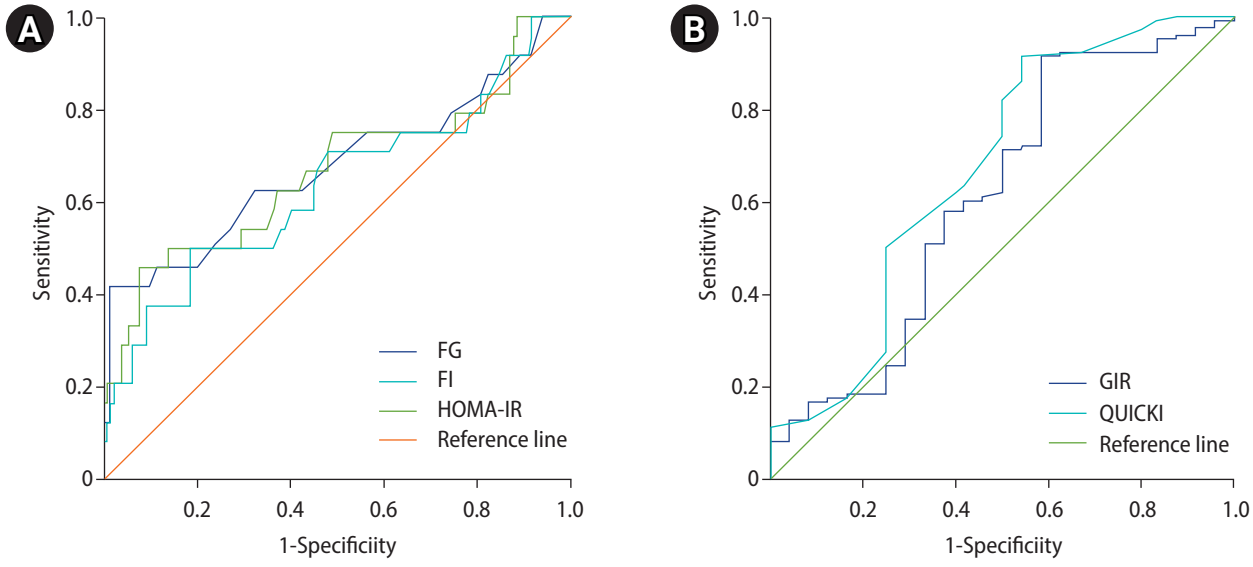


**Figure 1.** Receiver operating characteristic curve analysis to assess the predictive accuracy of fasting-state parameters for abnormal glucose tolerance following an oral glucose tolerance test. (A) Fasting glucose (FG), fasting insulin (FI), and homeostasis model assessment of insulin resistance (HOMA-IR). (B) Glucose-to-insulin ratio (GIR) and quantitative insulin sensitivity check index (QUICKI).

women with PCOS exhibit IGT, while up to 10% meet the criteria for T2DM [2]. Prediabetes (comprising IFG and IGT) and diabetes can be assessed by measuring levels of fasting glucose, postprandial or postload glucose after a glucose challenge, and hemoglobin A1C (HbA1c) [19,22]. The 2-hour OGTT is considered a standard method for screening for IGT and T2DM in women with PCOS [7-9], but the criteria for application of the OGTT are different in each clinical guideline. While an Endocrine Society Clinical Practice Guideline recommends the use of an OGTT to screen for IGT and T2DM in all adolescent and adult women with PCOS [7], it is only recommended in women with PCOS at high risk for future T2DM according to other clinical guidelines [8,9], and fasting plasma glucose or HbA1c can be tested to assess glycemic status in groups that are not at high risk for future T2DM instead of OGTT [9]. Although the OGTT is advantageous over fasting-state measurements of glucose (and other variables) in terms of its ability to define abnormal glucose tolerance and more clearly establish a management plan, it has drawbacks (higher cost, more effort, and time consumption) that may offset its advantages. For this reason, it would be very attractive if those with a high risk of abnormal glucose tolerance could be identified using measurements of fasting-state parameters alone instead of OGTT because this approach can avoid the cost, labor, and inconvenience caused by performing OGTT to screen for T2DM in all PCOS patients. In the present study, we aimed to evaluate the predictive accuracy of the fasting glucose level and other fasting-state indices for assessing insulin sensitivity in women with PCOS using ROC curve analysis for abnormal glucose tolerance following an OGTT, and we found that all of the AUC values for each fasting-state homeostatic parameter fell into the "less accurate" category despite the significant correlations of the 2-hr PG level during the OGTT with fasting glucose, fasting insulin, and other fasting-state ISAIs in the present study. In this study, all AUC values for identifying abnormal glucose tolerance were in the range of 0.5–0.7, which means that these parameters were not much better than a coin toss according to the criteria pre-

sented above [23,24].

Postprandial hyperglycemia and the resulting hyperinsulinemia jointly inhibit hepatic glucose production and stimulate glucose uptake by splanchnic and peripheral (primarily muscle) tissues to dispose of the ingested glucose and restore normoglycemia [12]. In the normal population, postprandial glucose concentrations peak 60 minutes after the start of a meal and return to preprandial levels within 2–3 hours; in contrast, postprandial glucose levels generally peak approximately 2 hours after the start of a meal and do not fall back to the baseline value for 4 to 6 hours in patients with diabetes [25]. The point is that postprandial glucose concentration is influenced both by hepatic and peripheral (muscle) tissue insulin resistance, while fasting blood glucose concentration is mainly affected by hepatic glucose production [12].

IFG and IGT are intermediate states in glucose metabolism that fall between normal glucose homeostasis and overt diabetes [12,26]. Both insulin resistance and impaired β-cell function have been found in subjects with IFG and IGT, but the site of insulin resistance is known to be different between the two disorders based on data from existing clinical studies [10,12,26,27]. Those with IFG predominantly have hepatic insulin resistance with normal muscle insulin sensitivity, while those with IGT have moderate to severe muscle insulin resistance with normal to slightly reduced hepatic insulin sensitivity [12]. In addition, those with isolated IFG show a decreased early-phase (first 30 minutes), but a less severely impaired late-phase (60–120 minutes) plasma insulin response to the oral glucose load during the OGTT; on the contrary, those with isolated IGT have severe impairments in both early- and late-phase insulin responses to glucose load [12,26]. The present study showed low predictive accuracies for all fasting-state indices for abnormal glucose tolerance, which may be attributed to the discrepancy of the pathophysiological mechanisms involved in the abnormal homeostatic control of fasting-state and postprandial blood glucose concentrations mentioned above. HOMA-IR, the most widely used surrogate marker in clinical studies [3,11], mainly reflects hepatic insulin resistance, while the insulin clamp method mainly reflects muscle insulin resistance [12]. Therefore, despite the reported significant association between HOMA-IR and insulin resistance measured by the hyperinsulinemic-euglycemic clamp method, the discrepancy between HOMA-IR and glucose disposal during insulin clamping may occur in the context of a discrepancy between liver and muscle insulin resistance [12,26].

In this study, the cutoff value of fasting glucose for predicting abnormal glucose tolerance was calculated as 103.5 mg/dL; despite its low sensitivity (0.417), it was closer to the ADA criterion of 100 mg/dL [19] than to the World Health Organization criterion of 110 mg/dL [28] for IFG.

An Endocrine Society Clinical Practice Guideline recommends that HbA1c may be considered for screening for IGT and T2DM in adolescents and adult women with PCOS if a patient is unable or unwilling to complete an OGTT [7]. McCartney and Marshall [3] insisted that measurements of fasting blood glucose levels alone should not be recommended for T2DM screening in women with PCOS, and laboratory assessments of insulin resistance (e.g., measurements of fasting insulin levels and other ISAIs) are also not routinely recommended given the imprecision of these assessments and their uncertain clinical usefulness. Instead, they recommended measuring HbA1c for initial screening because it is more convenient for patients than an OGTT in women with PCOS and proposed that a 2-hour OGTT should be considered only for patients who have an HbA1c level that is approaching (but below) the diagnostic threshold for diabetes of 6.5% (e.g., a HbA1c level ≥6%). Of course, their argument may also be controversial in that the high cost of the HbA1c test makes it unavailable or very limited in resource-poor settings [29]; additionally, it runs counter to the recommendations of the Endocrine Society, which prioritizes an OGTT over an HbA1c measurement because of the possibility of a stronger association between IGT and cardiovascular disease in women and the potential to identify women at risk for gestational diabetes before pregnancy [7]. An HgbA1c test may be a feasible alternative to an OGTT, especially for patients who are unwilling or unable to complete an OGTT; however, no assessment of fasting-state HbA1c was made besides fasting glucose and insulin in the present study.

In the present study, we merely measured the fasting insulin level, and we did not assess postload insulin levels following the OGTT. The lack of postload insulin data may be the most important drawback of this retrospective study because the 2-hour postload insulin level has been suggested to be a good indicator of insulin resistance [30].

In conclusion, our results suggest that fasting-state measurements of glucose and insulin measurements alone are not enough to predict abnormal glucose tolerance in women with PCOS; it seems quite difficult to replace the OGTT with only fasting-state measurements of glucose and insulin levels to identify abnormal glucose tolerance in PCOS patients. A standard OGTT is needed to screen for IGT and T2DM in women with PCOS.

## Conflict of interest

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

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# Novel nomogram-based integrated gonadotropin therapy individualization in *in vitro* fertilization/intracytoplasmic sperm injection: A modeling approach

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**Objective:** This study aimed to characterize a validated model for predicting oocyte retrieval in controlled ovarian stimulation (COS) and to construct model-based nomograms for assistance in clinical decision-making regarding the gonadotropin protocol and dose.
**Methods:** This observational, retrospective, cohort study included 636 women with primary unexplained infertility and a normal menstrual cycle who were attempting assisted reproductive therapy for the first time. The enrolled women were split into an index group (n=497) for model building and a validation group (n=139). The primary outcome was absolute oocyte count. The dose-response relationship was tested using modified Poisson, negative binomial, hybrid Poisson-$E_{max}$, and linear models. The validation group was similarly analyzed, and its results were compared to that of the index group.
**Results:** The Poisson model with the log-link function demonstrated superior predictive performance and precision (Akaike information criterion, 2,704; λ=8.27; relative standard error (λ)=2.02%). The covariate analysis included women's age ($p$<0.001), antral follicle count ($p$<0.001), basal follicle-stimulating hormone level ($p$<0.001), gonadotropin dose ($p$=0.042), and protocol type ($p$=0.002 and $p$<0.001 for short and antagonist protocols, respectively). The estimates from 500 bootstrap samples were close to those of the original model. The validation group showed model assessment metrics comparable to the index model. Based on the fitted model, a static nomogram was built to improve visualization. In addition, a dynamic electronic tool was created for convenience of use.
**Conclusion:** Based on our validated model, nomograms were constructed to help clinicians individualize the stimulation protocol and gonadotropin doses in COS cycles.

**Keywords:** Dose-response relationship; Oocytes; Ovarian stimulation; Patient-specific modeling

## Introduction

Improvements in procedural and therapeutic strategies in assisted reproductive technology (ART) have led to a dramatic increase in live birth rates and reduced rates of cycle cancellation [1]. However, poor ovarian response or hyper-response remain limitations. Advances in the design of stimulation protocols with milder properties have reduced the costs of treatment, enhanced oocyte retrieval [2], and led to a significant reduction of the hyper-response rate [3]. Nevertheless, these protocols have been criticized for their possible reduced efficacy in terms of reduced pregnancy and live birth rates [4]. The trade-off between controlled ovarian stimulation (COS) safety and efficacy is further complicated by the complex exposure-response relationship between ovarian response and the gonadotropin dose in each protocol [5]. Therefore, the difficulty in the choosing a stimulation protocol and subsequent gonadotropin dose is a major challenge in designing ART cycles, and it is necessary to personalize COS

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based on the patient's specific factors to optimize both the safety [6] and efficacy [7] of the stimulation. Currently published models for personalized gonadotropin therapy have identified women's age, gonadotropin dosage, and ovarian reserve tests (ORTs), including anti-Müllerian hormone (AMH) levels, follicle-stimulating hormone (FSH) levels, and antral follicular count (AFC), as the most significant predictors of ovarian response [8-11]. Nevertheless, these models have many drawbacks, limiting their validity for application in clinical practice.

First, most of these models define ovarian response as high, good, or poor based on logistic regression models. Different thresholds of ovarian response for each category may potentially bias the predicted response, resulting in misclassification errors [12]. Moreover, modeling the response using categorical variables hides the extensive patterns of variability embedded within each category, resulting in potential information loss [13]. Second, the gonadotropin dose-oocyte relationships have been described using different structural models, with no consensus on which one is optimal. Third, the complex mathematical calculations involved in the final model equations limit the practical application of these models in real-world settings. Fourth, models predicting the probability of COS success in terms of pregnancy rates or live birth rates are potentially confounded by the laboratory phase and luteal phase of embryo transfer, regardless of the specific treatment or clinical factors. Therefore, the current study aimed to test different structural models of the gonadotropin dose-oocyte relationship, to address the limitations of the current models, and to translate the resultant model into an easy-to-use and clinically applicable tool.

## Methods

### 1. Study design

This was an observational, single-center, retrospective cohort study. The study group was split into an index cohort, which was analyzed for model building and optimization, and a validation cohort, which was used for model validation. The candidate women were randomly allocated to these two groups in a 4 to 1 ratio (80% index, 20% validation). Any additional patients included in the analysis phase were assigned to the validation group. The study followed the precepts of the Helsinki Declaration, and was approved by the Ethical Committee of the Faculty of Pharmacy, Helwan University. Written informed consent was obtained from the recruited women.

### 2. Patients

Women undergoing intracytoplasmic sperm injection (ICSI) between January 2015 and May 2019 at Midwest Reproductive Center (Giza, Egypt) were assessed for eligibility to be included in the study. The inclusion criteria were an adequate trial of spontaneous conception for at least 1 year, the first trial of *in vitro* fertilization (IVF)/ICSI cycles, and normal menstrual cycle. Patients were excluded if they were more than 40 years old or presented with a secondary cause of female infertility, in order to avoid the potential confounding of their underlying pathologies or special treatment plans with our model. Therefore, women who presented with any explained cause of infertility, including tubal, uterine, or ovarian pathologies, as well as those with neurological or endocrine causes of infertility, were excluded. Moreover, women were excluded if they reported recent administration of hormonal contraceptives within the last 3 months. Since conventional IVF was not available at our center, all candidate women underwent standard IVF/ICSI. Data retrieved from the local center registry included basal FSH measured on day 2–3 of the menstrual cycle, AMH levels, total AFC (including follicles measuring 2–10 mm), the starting and total gonadotropin dose, duration of the stimulation, type of the stimulation protocol, number of metaphase II oocytes, and the total count of oocytes retrieved following COS.

### 3. Treatment protocols

According to the treating physician's clinical experience, women underwent pituitary desensitization using a long, short, or antagonist protocol. For the long protocol, women were initiated on a gonadotropin-releasing hormone (GnRH) agonist (Triptorelin; Decapeptyl; Ferring Pharmaceuticals, Saint-Prex, Switzerland) on day 20 of the menstrual cycle preceding the stimulation cycle. When pituitary downregulation was attained, as evidenced by a serum estradiol level $< 50$ pg/mL, gonadotropin stimulation was administered as human menopausal gonadotropin (Merional; IBSA, Lugano, Switzerland) or recombinant FSH (Gonal-F; Merck Serono, Eysins, Switzerland) started at empirical doses determined by the physician based on age, serum AMH levels, and body mass index (BMI) [14]. For the short protocol, a GnRH agonist was started on the first day of the ART cycle; then, gonadotropin doses were started on day 3 until the day of human chorionic gonadotropin (hCG) administration [15]. Finally, women on the antagonist protocol started gonadotropin stimulation doses daily on day 1 of the stimulation cycle (day 2–3 of the menstrual cycle). A GnRH antagonist (Cetrorelix; Cetrotide, Merck Serono) with a daily dose of 0.25 mg/day (subcutaneous) was then initiated when at least 1 follicle measured 14 mm and continued until the target follicular response [4]. For each protocol, the starting gonadotropin dose ranged from 150 to 450 IU/day and was modified subsequently if needed on a weekly basis in 75 IU increments according to the results of ultrasonographic follicular tracking. Final oocyte maturation was triggered with hCG (Choriomon, IBSA) administered at a dose of 10,000 U when at least three follicles measured $\geq 18$ mm, and ovum pick-up was then facilitated by transvaginal ul-


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trasonography 36 hours later. Embryo transfer was performed on days 2–4 after pick-up.

### 4. Ultrasonographic and hormonal measurements

Basal antral follicles were measured in both ovaries by qualified radiographers on day 3 of the menstrual cycle using transvaginal ultrasonography (ClearVue 350; Philips, Louisiana, USA). Longitudinal AFC evaluation 1 week after the start of ovarian stimulation was carried out to guide the incremental gonadotropin dose modification. For hormonal assays, cubital vein blood samples were taken in the early follicular phase (day 3), prior to the start of ovarian stimulation regimens. Serum AMH was analyzed by enzyme-linked immunosorbent assay (ELISA) using the Beckman Coulter AMH ELISA kit (Immunotech, Marseilles, France). Levels of basal FSH were determined in a similar fashion using ELISA (Immulite 2000 analyzer; Siemens, Munich, Germany) on day 3 of the cycle. All samples were analyzed on the day of collection using the same kits and operators to minimize intra-assay variability.

### 5. Outcomes

The primary outcome investigated was the absolute count of oocytes retrieved following COS in ICSI cycles. The secondary outcomes were the rates of hypo- or hyper-response and the ovarian response prediction index (ORPI). Poor response was defined as the attainment of fewer than eight oocytes at the end of the stimulation cycle, whereas hyper-response was defined as the retrieval of 15 oocytes or more [6]. The ORPI was calculated as (AMH level × AFC)/age [9].

### 6. Statistical analysis

Continuous data are described as mean ± standard deviation or median and interquartile range (IQR), while categorical variables are presented as the count with corresponding percentages (n, %). For comparisons, the Mann-Whitney *U*-test was used for continuous non-normally distributed data, the *t*-test for normally distributed data, and the chi-square test for ratios. An a priori alpha of less than 0.05 was set. The process of model development involved a three-step approach: structural model development, building a covariate model, and validation of the final model.

#### 1) Structural model development

The hypothesized models for investigation of the dose-response relationship included modified Poisson, negative binomial, linear, and hybrid $E_{max}$ models with the log-link function for counts. The hybrid $E_{max}$ model is a proposed structural model that combines the $E_{max}$ model [16] and the Poisson model [17] in the same structure to account for non-linearity in the dose-response relationship between FSH doses and oocyte retrieval. The precision of the tested models was evaluated by comparing the estimated percentages of relative standard error (%RSE) calculated for each model. To characterize the interindividual variability, mixed-effect modeling was applied at this stage.

#### 2) Covariate selection

After the determination of the best structural model, potential covariates, including AFC, AMH and FSH levels, age, gonadotropin dose, and protocol type, were tested for their impact on the Akaike information criterion (AIC) of the structural model. Stepwise forward-inclusion covariate modeling was applied. The difference in AIC values between the candidate models was approximately chi-square–distributed and was considered significant when the AIC changed by more than 3.84 when testing a single covariate ($\chi^2$, df = 1; $p = 0.05$).

#### 3) Model validation

Non-parametric bootstrapping of the original dataset was performed with 500 replicates of the same sample size, and the results were compared to those of the base model. Furthermore, model metrics including the root mean squared error considering the log error model, C-index, chi-square model score, and pseudo-$R^2$ were calculated from the validation group analysis and compared to their corresponding estimates in the index group.

### 7. Software

Descriptive statistics and group comparisons were performed using IBM SPSS ver. 26.0 (IBM Corp., Armonk, NY, USA). The structural model assessment was performed using Monolix software ver. 2019R2 (Lixoft, Paris, France). Covariate modeling, validation, and nomogram development were performed using R ver. 3.5.0 (R Foundation, Vienna, Austria).

## Results

### 1. Baseline and clinical characteristics

In total, 636 women were enrolled in the present study, with a mean age of 29.87 ± 5.26 years. The median AFC was nine follicles (range, 1–33 follicles), while the mean AMH level was 2.54 ± 1.31 ng/mL (range, 0.6–14.9 ng/mL), and the mean FSH level was 6.88 ± 3.79 mIU/mL (range, 1.2–27.6 mIU/mL). COS was performed using long (32.9%), short (56.6%), or antagonist (10.5%) protocols, resulting in poor (41.5%), normal (50.6%), and hyper-response (7.9%). No patients presented with severe ovarian hyperstimulation syndrome. The starting gonadotropin dose applied in different COS cycles ranged between 150 and 450 IU. The majority of women received a dose of 225 IU (54.6%). The median number of retrieved oo-

cytes at the end of stimulation was 8 (IQR, 6–11), and the mean units required to obtain one oocyte was 35.85 ± 26.96 IU/oocyte. Of the 636 patients, 497 (78%) were assigned to the index group and 139 (22%) to the validation group. Pairwise comparisons between the index group and the validation group demonstrated statistically non-significant differences. Table 1 summarizes the baseline characteristics of the study groups.

### 2. Structural model building

Among the screened dose-response models, the modified Poisson model (with log-dose) demonstrated the highest parameter precision (λ = 8.27, RSE(λ) = 2.02%), lowest unexplained interindividual parameter variability (28%; RSE, 7.8%), and the best predictive performance as evidenced by the lowest AIC compared to all other models (AIC, 2,704). Our novel hybrid model demonstrated convincing precision to estimate the $E_{max}$ and $ED_{50}$ parameters (%RSE, 2.17% and 0.44%, respectively); however, it was excluded due to an apparently higher AIC (AIC, 2,752). Finally, the linear models presented the worst performance, as shown by the highest AIC and %RSE. A summary of the structural model assessment is provided in Table 2.

### 3. Covariate selection

Based on the modified Poisson structural model, univariate analysis was performed. Statistical significance was demonstrated for age, AFC, AMH and basal FSH levels, gonadotropin dose, and stimulation protocol type, while BMI was found to be a non-significant predictor ($p = 0.57$). Despite significance in the univariate analysis ($p < 0.001$), AMH was found to be nonsignificant in the multivariate model ($p = 0.21$), possibly due to multicollinearity with other covariates. Centering AMH around the median or correcting the independent variables for AMH resulted in imprecise estimates of model coefficients and poor model stability. Therefore, AMH was dropped from

**Table 1.** Baseline clinical and demographic characteristics of the study groups

| Baseline characteristics | Total (n = 636) | Index group (n = 497) | Validation group (n = 139) | *p*-value |
|---|---|---|---|---|
| Age (yr) | 29.87 ± 5.26 | 29.97 ± 5.27 | 29.52 ± 5.22 | 0.38 |
| Body mass index (kg/m$^2$) | 29.58 ± 3.41 | 29.6 ± 3.41 | 29.47 ± 3.44 | 0.69 |
| AFC | 9 (6–13) | 9 (6–13) | 9 (6–12) | 0.70 |
| AMH (ng/mL) | 2.54 ± 1.31 | 2.55 ± 1.37 | 2.49 ± 1.08 | 0.78 |
| Basal FSH (mIU/mL) | 6.88 ± 3.79 | 6.89 ± 3.85 | 6.82 ± 3.6 | 0.82 |
| ORPI | 0.65 (0.35–1.24) | 0.64 (0.34–1.25) | 0.68 (0.37–1.2) | 0.71 |
| Protocol of stimulation | | | | 0.69 |
| Long | 209 (32.9) | 166 (33.4) | 43 (30.9) | |
| Short | 360 (56.6) | 277 (55.7) | 83 (59.7) | |
| Antagonist | 67 (10.5) | 54 (10.9) | 13 (9.4) | |
| Starting gonadotropin dose (IU) | | | | 0.22 |
| 150 | 123 (19.3) | 98 (19.7) | 25 (18) | |
| 225 | 347 (54.6) | 265 (53.3) | 82 (59) | |
| 300 | 97 (15.3) | 78 (15.7) | 19 (13.7) | |
| 375 | 53 (8.3) | 40 (8) | 13 (9.4) | |
| 450 | 16 (2.5) | 16 (3.2) | 0 | |
| Total gonadotropin dose (IU) | 2,844 ± 1,710 | 2,881 ± 1,795 | 2,710 ± 1,361 | 0.88 |
| Duration of stimulation (day) | 11 ± 3.34 | 11 ± 3.44 | 10.8 ± 2.93 | 0.77 |
| Gonadotropin dose adjustment | 121 (19.2) | 96 (19.3) | 25 (18) | |
| Dose step-up | 85 (13.4) | 65 (13.1) | 20 (14.4) | 0.72 |
| Dose step-down | 36 (5.6) | 31 (6.2) | 5 (3.6) | 0.47 |
| Starting gonadotropin units per oocyte | 35.85 ± 26.96 | 36.62 ± 28.46 | 33.08 ± 20.59 | 0.46 |
| No. of retrieved oocytes | 8 (6–11) | 8 (6–11) | 9 (6–11) | 0.21 |
| No. of retrieved MII oocytes | 2 (1–4) | 2 (1–4) | 2 (1–4) | 0.53 |
| Response to ovarian stimulation | | | | 0.57 |
| Poor responders | 264 (41.5) | 211 (42.5) | 53 (38.1) | |
| Normal responders | 322 (50.6) | 249 (50.1) | 73 (52.5) | |
| High responders | 50 (7.9) | 37 (7.4) | 13 (9.4) | |

Values are presented as mean±standard deviation, median (interquartile range), or number (%).
AFC, antral follicular count; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; ORPI, ovarian response prediction index; MII, metaphase II.



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the final multivariate model. The final multivariate regression model identified age, AFC, FSH levels, protocol type, and the starting gonadotropin dose as significant independent predictors of the oocyte retrieval count (Table 3). The gonadotropin dose initially demonstrated a negative relationship with oocyte count in the univariate analysis (regression coefficient, –0.221; $p < 0.001$); however, after adjusting for confounding variables, a positive relationship was shown in multivariate regression (adjusted odds ratio, 1.11; 95% confidence interval [CI], 1.004–1.24).

**Table 2.** Comparison of different structural models of the oocyte count (dependent variable) and gonadotropin dose (independent variable)

| Model | Parameter | Estimate | SE (%RSE) | AIC |
|---|---|---|---|---|
| Modified Poisson | λ (oocyte) | 8.27 | 0.167 (2.02) | 2,704 |
| | UIIV (λ) | 0.28 | 0.022 (7.8) | |
| Negative binomial | λ (oocyte) | 8.35 | 0.164 (1.96) | 2,709 |
| | ο (oocyte) | 0.021 | 0.008 (36.2) | |
| | UIIV (λ) | 0.019 | 0.043 (22.3) | |
| | UIIV (ο) | 1.09 | 0.34 (31.2) | |
| Hybrid $E_{max}$ and Poisson | $E_{max}$ | 11.8 | 0.26 (2.17) | 2,752 |
| | $ED_{50}$ | 101 | 0.447 (0.44) | |
| | UIIV ($E_{max}$) | 0.322 | 0.02 (6.41) | |
| | UIIV ($ED_{50}$) | 1.61 | 0.435 (27) | |
| Linear | Slope | 0.007 | 0.002 (24.5) | 3,522 |
| | Intercept | 7.16 | 0.376 (5.25) | |
| | UIIV (slope) | 0.008 | 0.001 (12) | |
| | UIIV (Intercept) | 1.94 | 0.216 (11.2) | |

SE, standard error; RSE, relative standard error; AIC, Akaike information criterion; UIIV, unexplained interindividual variability; $E_{max}$, the maximum therapeutic effect; $ED_{50}$, the dose associated with half-maximal therapeutic effect.

## 4. Model validation

The results obtained from 500 rounds of bootstrapping indicated a close match between the mean coefficients of the bootstrapped samples and those of the original model with little bias. All of the final model coefficients lay within the 95% CI of the mean coefficients estimated from bootstrapping, indicating stable and robust predictive performance of the final model. Table 4 summarizes the results of bootstrapping. Comparing the mean actual versus predicted oocyte count across the different studied gonadotropin doses resulted in nonsignificant differences, confirming the reliability of the model predictions (Figure 1).

The validation group (n = 139) demonstrated similar discrimination criteria compared to the index group (Table 5). For both groups, the developed model explained approximately 50% of the variability in the observed oocyte count (pseudo-$R^2$: 0.48 vs. 0.49 for the index and validation groups, respectively). The concluded model achieved statistical significance when analyzing both groups ($p < 0.001$ for both groups). The C-statistic was reasonable for both models, con-

**Table 4.** Estimates from 500 bootstrapped samples of the original dataset

| Covariate | Mean coefficient | SE | 95% CI |
|---|---|---|---|
| Age | –0.017 | 0.003 | –0.023 to –0.011 |
| AFC | 0.034 | 0.003 | 0.028 to 0.04 |
| FSH | –0.028 | 0.005 | –0.033 to 0.023 |
| Protocol type | | | |
| Long | | Reference | |
| Short | 0.07 | 0.04 | –0.01 to 0.15 |
| Antagonist | 0.18 | 0.06 | 0.06 to –0.3 |
| Dose | 0.11 | 0.06 | –0.01 to 0.23 |

SE, standard error; CI, confidence interval; AFC, antral follicular count; FSH, follicle-stimulating hormone.

**Table 3.** Regression analysis of oocyte retrieval versus candidate covariates

| Covariate | Univariate regression | | | Multivariate regression | | | Included (yes/no) |
|---|---|---|---|---|---|---|---|
| | *r* | Change in AIC | *p*-value | *r* | aOR | *p*-value | |
| Age | –0.028 | 85 | < 0.001 | –0.019 | 0.98 (0.976–0.987) | < 0.001 | Yes |
| BMI | –0.003 | 2 | 0.57 | - | - | - | No |
| AFC | 0.042 | 186 | < 0.001 | 0.033 | 1.033 (1.028–1.038) | < 0.001 | Yes |
| AMH | 0.053 | 23 | < 0.001 | - | 0.15 (0.99–1.04) | 0.21 | No |
| FSH | –0.032 | 54 | < 0.001 | –0.028 | 0.972 (0.965–0.980) | < 0.001 | Yes |
| Protocol | | | | | | | Yes |
| Long | | | Reference | | | | |
| Short | 0.118 | 15 | 0.001 | 0.093 | 1.098 (1.034–1.156) | 0.002 | |
| Antagonist | 0.2 | | < 0.001 | 0.208 | 1.231 (1.124–1.349) | < 0.001 | |
| Log (dose) | –0.221 | NA[a)] | < 0.001 | 0.107 | 1.11 (1.004–1.235) | 0.042 | |

*r*, regression coefficient; AIC, Akaike information criterion; aOR, adjusted odds ratio; BMI, body mass index; AFC, antral follicular count; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; NA, not applicable.

[a)]The logarithm of the starting gonadotrophin dose is an essential component of the base structural model.

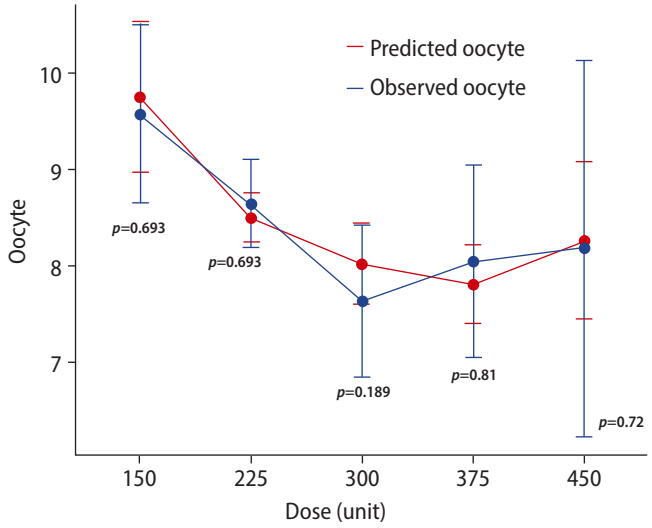


**Figure 1.** Comparison of the predicted versus observed oocyte yield for each gonadotropin dose level. Data are shown as mean±standard error of the mean. Error bars represent 95% confidence intervals. The Wilcoxon signed-rank test was applied to calculate the *p*-value for each pairwise comparison.

**Table 5.** Assessment of model performance in the validation group compared to the index group

| Parameter | Index group (n = 497) | Validation group (n = 139) |
|---|---|---|
| RMSE(%)[a] | 3.04 (34.88) | 3.88 (43.11) |
| C-index (SE) | 0.71 (0.01) | 0.72 (0.02) |
| Chi-square (*p*-value) | 319.28 (< 0.001) | 92 (< 0.001) |
| Pseudo-$R^2$[b] | 0.48 | 0.49 |

RMSE, relative mean squared error; SE, standard error.
[a]Estimated as exponents of log-linear mean squared error; [b]Calculated based on the Cragg-Uhler method.

firming the agreement between predicted and actual oocyte counts (C-statistic, 0.71 vs. 0.72, respectively). The RMSE estimates for both models were also comparable. The estimated odds of oocyte yield from both models demonstrated a distinct similarity (Figure 2).

### 5. Nomogram development

Based on our validated final model, a point-based static nomogram was built to help individualize treatment decisions based on patient-specific characteristics (Figure 3). The prognostic weight of each independent variable is depicted by the length of the corresponding point scale. Gonadotropin dose and protocol type are the shortest scales; hence, they have a lower weight than the other patient-related factors. The nonlinear exposure-response relationship is well-captured by the point system scaling. The points attained from incremental increases of the gonadotropin dose non-linearly decrease as the dose increases; moreover, the total number of points required to increase the oocyte count is much higher in cases with a predicted retrieval of fewer than 8 oocytes than in those with a predicted retrieval of more than 15 oocytes (Figure 3). A dynamic individualization tool was constructed to aid electronic manipulation for more convenient institutional applications (Figure 4).

## Discussion

The present study is the first to introduce a nomogram-based clinical tool that can be utilized to individualize both the protocol and the starting gonadotropin dose selection for IVF/ICSI candidates. The nomograms were built based on a robust validated model that predicted the number of retrieved oocytes from the stimulation cycles. In line with many previous studies [8,18,19], the choice of retrieved oocytes as a target outcome to model was suggested in the current work to overcome confounding with different non-therapeutic factors encountered in models predicting IVF/ICSI success in terms of pregnancy or live birth rates. Moreover, the definition of response to COS in terms of absolute oocyte count enables a realistic representation of ovarian response as a continuous spectrum instead of "black and white" categories in binary logistic models for COS individualization [11,12,20,21].

Our developed model identified women's age, basal FSH levels, AFC, stimulation protocol type, and gonadotropin dose as significant predictors of oocyte retrieval. The prognostic value of each of these identified predictors has been well established in previous reports [8,11,12,22].

One of the main findings of the current model is that treatment-related factors, including the stimulation protocol and gonadotropin dose, may have little influence compared to the patient's intrinsic factors, including age and ORTs, when predicting oocyte retrieval. This suggests that the matter of optimal response to gonadotropin therapy is more related to the patient herself rather than the specific characteristics of the therapeutic approach. Similarly, Rustamov et al. [23] concluded that only 10% of the total observed variance of retrieved oocytes could be explained by treatment-related factors, while 53% could be attributed to patient-specific factors in their mixed-effects Poisson model.

Interestingly, the evaluation of the predictive performance of different ORTs demonstrated superior predictive performance of basal FSH levels compared to AMH levels. Similarly, Magnusson et al. [24] concluded that AMH levels did not improve the rates of targeted ovarian response when added to AFC. Nevertheless, the results of similar analyses should be interpreted with caution, especially when comparing our results, due to differences in sample sizes, the inclusion of confounding conditions (e.g., polycystic ovaries), and application of different stimulation protocols. For instance, Moon et al. [19] reported that age, basal FSH levels, AFC, and AMH levels were signifi-


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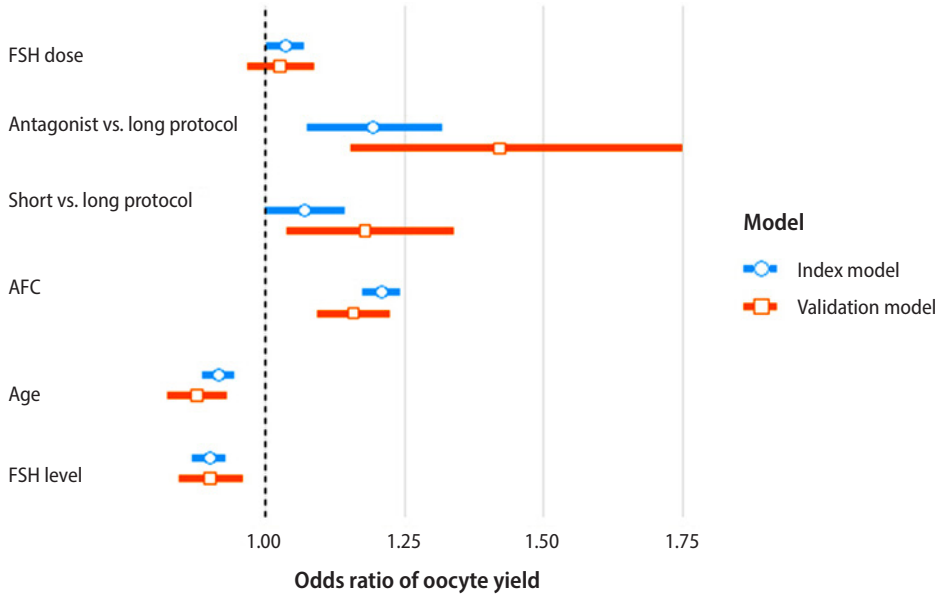


**Figure 2.** Forest plot comparing the odds ratios estimated from the index and validation models. Corresponding point estimates are depicted by blue circles (index group) or orange squares (validation model). Horizontal lines represent the corresponding 95% confidence intervals of the estimated odds ratios for each model. FSH, follicle-stimulating hormone; AFC, antral follicle count.

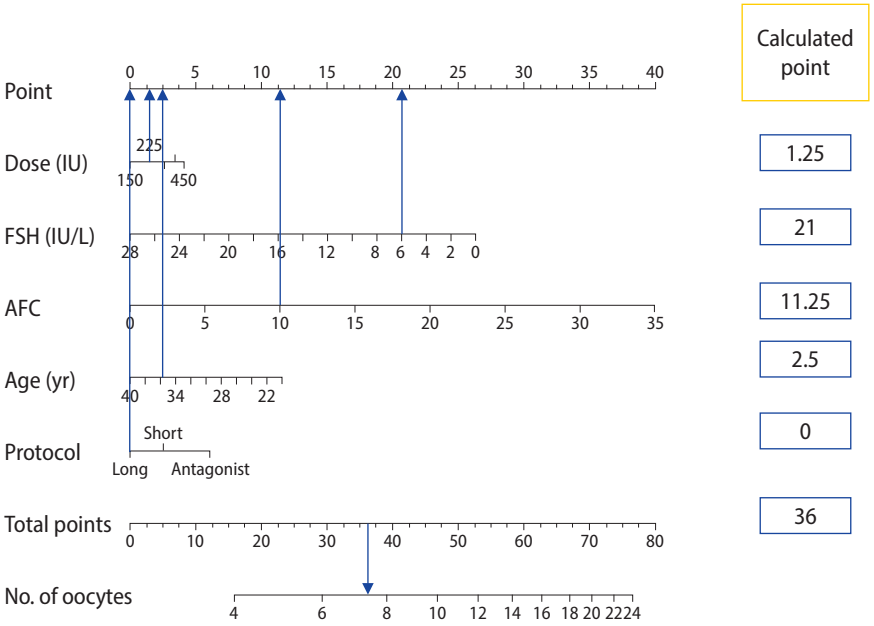


**Figure 3.** Static nomogram developed for individualizing controlled ovarian stimulation. In this example, a 36-year-old woman with an antral follicle count (AFC) of 10 and a basal follicle-stimulating hormone (FSH) level of 6 IU/L was evaluated for a starting empirical gonadotropin dose of 225 IU as a part of long protocol. Based on our developed score, the woman was assigned a total of 36 points, corresponding to a predicted oocyte yield of fewer than 8 oocytes. To overcome the problem of poor response, a change in the stimulation protocol to an antagonist protocol only could be recommended (adding 6 points to the total score, thereby increasing the predicted oocyte count to 8, corresponding to a good response). The instructions for the nomogram are as follows. (1) Based on the patient's specific data, extrapolate a vertical line from the specific values for each variable to the points on the axis in the upper panel. (2) For each variable, record the corresponding points in the corresponding boxes in the right panel. (3) Calculate the sum of assigned points and record the result in the box corresponding to the total points in the right panel. (4) Extrapolate a vertical line from the total points axis to the "no. of oocytes" axis, which gives an average prediction of oocyte count based on the selected treatment scenario.

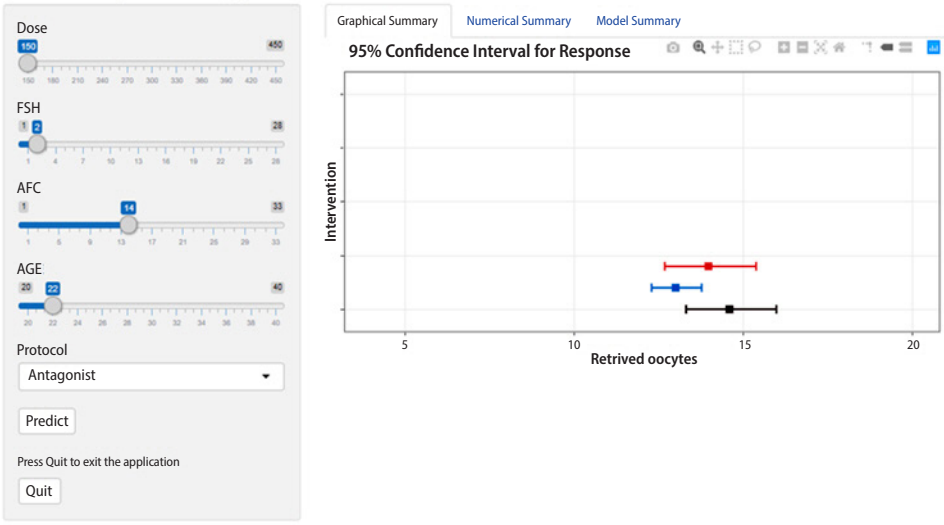


**Figure 4.** Application of the dynamic individualization tool in optimization for gonadotropin therapy. The black bar describes a predicted 95% confidence interval (CI) of the retrieved oocyte count based on a hypothetical selection of an antagonist protocol and a gonadotropin dose of 225 IU daily for a 22-year-old woman with a basal follicle-stimulating hormone (FSH) level of 2 IU/mL and an antral follicle count (AFC) of 14 follicles. Due to the possibility of hyper-response, we note two possible modifications to the therapeutic regimen. First, the antagonist protocol can be changed to the short protocol (blue bar). Second, the dose can be reduced to 150 IU (red bar). Both modifications seem to be safe (the mean predicted oocyte count is between 8 and 14 oocytes); however, changing the protocol seems to be safer, as there would be no risk of hyper-response (the 95% CI of oocyte yield is less than 15). By clicking on the numerical summary tab on the upper right panel, a detailed summary of the three scenarios is provided. The developed tool is freely available at https://individualization.shinyapps.io/dynnomapp/ for institutional use [25].

cant predictors of the oocyte count; however, in their final model, they excluded FSH due to its relatively weak predictive performance. This low weight of basal FSH may be related to their relatively small sample size (n = 141), and the high inter-subject variation that was encountered with FSH levels in their work (range, 2.9–26.6 mIU/mL; coefficient of variation, 45%) [19].

In particular, the impact of gonadotropin dose on oocytes remains debatable. It was suggested that a positive relationship may exist between gonadotropin doses from 50 IU to 225 IU and oocyte yield, whereas doses more than 225 IU failed to demonstrate an extra benefit, indicating a possible nonlinear relationship [26]. Possible explanations for this phenomenon include limited capacity of the follicular pool, large variability in the AFC, and/or sensitivity to exogenous gonadotropin stimulation [27]. Unfortunately, the characterization of the nonlinear dose-response relationship has largely been overlooked in previous similar reports. It is therefore not surprising that our results identified the linear models as the worst ones, highlighting a major criticism of the currently published linear regression models for gonadotropin dose individualization [28-30]. In the current study, two possible models were proposed to capture these nonlinear patterns: hybrid $E_{max}$ and modified Poisson models. In pharmacodynamic terms, the $E_{max}$ model assumes that a drug has a maximum effect at a certain dose, while beyond this dose, no additional response will be obtained. The $E_{max}$ model is parameterized with $E_{max}$, a metric for the average maximum response with escalating FSH doses, and $ED_{50}$, a metric that corresponds to the dose that yields half of the maximum response [31]. The idea behind our proposed hybrid $E_{max}$ model is that the traditional version of $E_{max}$ predicts continuous responses, which is not suitable for modeling oocytes as discrete outcomes [16], while the Poisson model correctly predicts oocyte count as a discrete variable, but assumes a linear relationship between FSH dose and oocyte count, which is not true [19]. In the proposed hybrid model, we replaced the traditional exponential linear function with the exponential nonlinear $E_{max}$ function to account for the relatively flat dose-response relationship attained at higher FSH doses, while enabling oocytes to be predicted as discrete counts. In contrast, the proposed modified Poisson model introduced the FSH dose covariate in log terms instead of the linear scale implemented in conventional Poisson models to account for the nonlinear FSH dose-oocyte count relationship. Comparing both models, the modified Poisson model demonstrated the best fit with the highest precision and showed a significant effect of gonadotropin dose on oocytes ($p = 0.042$).

In contrast to our results, a recent meta-analysis concluded that escalating FSH doses had similar efficacy and safety profiles in both poor and hyper-responders. However, the authors reported that the


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included studies might have suffered from limited sample sizes and heterogeneous comparisons of different doses of FSH [32]. Borges et al. [29] demonstrated that escalating doses of FSH were not associated with an increased oocyte count. However, they reported lower FSH requirements per oocyte retrieved at a younger age ( ≤ 35 years), suggesting that the impact of gonadotropin dose on oocytes may be masked by intrinsic poor ovarian function, primarily due to aging.

Regarding the protocol type, our findings indicate that milder protocols were associated with higher oocyte yield than the conventional long stimulation protocol. This finding is supported by Pinto et al. [20], who found that antagonist protocols were associated with a significantly higher mean number of retrieved oocytes than long protocols. In contrast, three different meta-analyses reported statistically significant lower oocyte yields with antagonist protocols than with agonist (long) protocols [33-35]. Of note, Lambalk et al. [35] demonstrated a non-significant difference among protocol types in poor responders, supporting our theory that the intrinsic response to gonadotropins is basically related to the characteristics of the patient herself. To our knowledge, no study has adjusted for the effect of the higher gonadotropin doses involved in the long protocol. It should be noted that the longer duration of stimulation, with the subsequent higher total gonadotropin dose per cycle in the long protocol, may potentially confound this relationship [14]. Whether a higher oocyte count is related to the specific type of stimulation or the higher gonadotropin dose in the long protocol remains questionable.

To translate models into practice, nomograms provide rigorous tools for model visualization, helping clinicians to optimize treatment decisions on an individual basis [36]. The nomogram-based model for oocyte prediction previously introduced by La Marca et al. [8] was limited by the analysis of a homogeneous group of predicted normal responders only, with a single stimulation protocol (long protocol only). In contrast to our model findings, they demonstrated enhanced predictive performance of their multivariate linear model, which reached up to 50% correct FSH dose prediction in predicted normal responders aged less than 35 years. Moreover, the external validation of the nomograms developed by La Marca et al. [8] resulted in acceptable performance in individualizing FSH doses [7,37]. This suggests that the application of linear models in predicting a suitable gonadotropin dose may be appropriate when analyzing a more homogeneous population of predicted normal responders. Despite comparable predictive performance, our results should be interpreted with great caution when compared to the findings of La Marca et al. [8] due to the implementation of a different dose-response model and the analysis of a more heterogeneous group that included different patterns of response other than predicted normal response. More recently, Moon et al. [19] presented an appropriate oocyte model in terms of Poisson regression for counts; however, their nomogram was criticized for failure to interpret the contributions of both protocol type and gonadotropin doses to oocyte retrieval due to their relatively small sample size.

Taken together, the current study exhibits several strengths. First, the fitted model adequately describes the complex nonlinear relationship between gonadotropin doses and oocyte retrieval. Second, the developed model dealt with the large heterogeneity in the definitions of ovarian response by modeling the response to COS as the absolute oocyte count. Third, the developed nomogram is user-friendly and does not require the user to apply any sophisticated mathematical or statistical techniques, as were used in the process of model building. Fourth, our nomogram is the first to introduce a method for integrated gonadotropin therapy personalization, enabling both the protocol and subsequent gonadotropin dose to be tailored. Nevertheless, the current work has several limitations. Since external validation was not possible during the development phase, the generalizability of the proposed nomogram remains unknown. The exclusion of women with explained causes of female infertility further limits the generalizability of the developed nomogram. The model used for oocyte retrieval did not account for intra-cycle variability due to its dependence on baseline clinical and demographic characteristics only. Additionally, the literature has presented many factors that may have an influence on oocyte prediction, including estradiol levels, *FSHR* gene polymorphisms, and smoking status, which were unavailable for the included women during the data collection phase. Since the study population included only naïve IVF/ICSI candidates, the role of previous stimulation cycles in predicting oocyte count could not be investigated in the current work. The retrospective nature of the study may pose a threat of information bias. The retrospective design allowed data collection from a relatively large population for optimal model building. The exact predictive performance of the total gonadotropin doses administered during the entire cycle could not be investigated due to a lack of monitoring of patient adherence to the prescribed gonadotropin therapy, which might have resulted in mismatching between the recorded doses and the actual administered doses. Similarly, this point could be raised to criticize previous studies [19,38], which assessed the effect of total gonadotropin doses on ovarian response without reporting how patient adherence was monitored, introducing a risk of potential information bias [39]. Future studies should apply intensive monitoring schedules for different ORTs during IVF/ICSI cycles for further validation of our nomograms with more detailed data collection to address all possible sources of oocyte yield variability.

## Conflict of interest

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

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Conceptualization: AHIME, Formal analysis: MIM. Methodology: SMAM, Writing–original draft: MIM. Writing–review & editing: MMAS.

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# Comparison of pregnancy outcomes using a time-lapse monitoring system for embryo incubation versus a conventional incubator in *in vitro* fertilization: An age-stratification analysis

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**Objective:** The aim of this study was to compare the pregnancy outcomes of *in vitro* fertilization with embryo transfer between embryos cultured in a time-lapse monitoring system (TLS) and those cultured in a conventional incubator (CI).
**Methods:** The medical records of 250 fertilized embryos from 141 patients undergoing infertility treatment with assisted reproductive technology at a tertiary hospital from June 2018 to May 2020 were reviewed. The study population was divided into TLS and CI groups at a 1 to 1 ratio (125 embryos per group). The primary outcome was the live birth rate.
**Results:** The TLS group had a significantly higher clinical pregnancy rate (46.4% vs. 27.2%, $p$=0.002), implantation rate (27.1% vs. 12.0%, $p$=0.004), and live birth rate (32.0% vs. 18.4%, $p$=0.013) than the CI group. Furthermore, subgroup analyses of the clinical pregnancy rate and live birth rate in the different age groups favored the TLS group. However, this difference only reached statistical significance in the live birth rate in women aged over 40 years and the clinical pregnancy rate in women aged 35–40 years ($p$=0.048 and $p$=0.031, respectively). The miscarriage rate, cleavage rate, and blastocyst rate were comparable.
**Conclusion:** TLS application improved the live birth rate, implantation rate, and clinical pregnancy rate, particularly in the advanced age group in this study, while the other reproductive outcomes were comparable. Large randomized controlled trials are needed to further explore the ramifications of these findings, especially in different age groups.

**Keywords:** Embryo culture techniques; Incubator; Live birth rate; Pregnancy rate; Time lapse

## Introduction

With the increasing prevalence of infertility and subfertility worldwide [1,2], more people are seeking fertility treatment to achieve pregnancy. In Thailand, the prevalence of infertility is about 12% [3]. The major causes of infertility can be divided into female factors, male factors, and unexplained infertility. Advanced age, tubal occlusion, or severe endometriosis in women, or severe male-factor infertility, may require the use of assisted reproductive technology (ART) to achieve pregnancy [4-6]. Although major advances in ART have been made since the first successful *in vitro* fertilization (IVF)-conceived birth in 1978, the live birth rate has remained low, at only 10%–30%, in patients with good embryo transfer [7]. Several factors impact the success rate of ART, although these predominantly involve embryo quality and endometrial receptivity.

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Embryo quality assessment is a crucial step in ART that influences pregnancy outcomes. Many methods, including both invasive and non-invasive techniques, have been developed to evaluate embryo quality for better implantation prediction [8]. Morphological assessment in a conventional incubator (CI) is one of the standard noninvasive methods in this step [9]. This is a familiar method for both clinicians and embryologists to grade embryos to select the best one for transfer. However, there are several limitations of this technique [10]. First, the embryologist must remove the embryo from the incubator at least once daily for assessment. This can impact the environment surrounding the embryo and can cause deleterious effects [11]. Moreover, segmental monitoring of the embryo in CI can miss some phenomena that occur during the developmental process, such as direct or reverse cleavage. These kinetic changes are associated with poor embryo quality and low implantation potential [12,13]. The time-lapse monitoring system (TLS), comprising an automatic device with a built-in camera, is a novel incubator that provides an undisturbed culture system and permits regular, uninterrupted monitoring of the developing embryo [14]. Therefore, TLS provides another dimension of monitoring the dynamic morphokinetic changes of embryos in addition to CI [15-17]. It is able to detect kinetic changes and abnormal cleavage patterns that could be missed in the CI system and enhances the consistency of embryo scoring by different embryologists [18]. In 2011, Meseguer et al. [19] conducted a retrospective study to create a model for assessing the kinetic parameters from TLS to classify embryos according to their probability of successful implantation. Several studies have shown associations of kinetic markers from TLS with pregnancy outcomes. In particular, the selection of a good-quality embryo that has been monitored in TLS for transfer enhances the implantation rate, increases the cumulative pregnancy rate, decreases early pregnancy loss, and reduces the time to pregnancy [14,19-24].

However, several confounding factors need to be clarified before concluding that this hypothesis has been confirmed, especially regarding patients' reproductive baseline and the culture environment conditions. Although several studies have provided support for the role of TLS in promoting good embryo development, others have not [14]. One randomized controlled trial that compared embryos cultured in TLS with embryos for which conventional once-daily morphologic screening was performed with additional kinetic data did not show an improvement in predicting clinical pregnancy or implantation success [17]. That study did not find an advantage of having additional morphokinetic data from an undisturbed incubation environment. Moreover, several studies have found that TLS did not contribute to improving the developmental potential of embryos, especially in patients with a favorable prognosis [25,26]. Consequently, to date, there is no consensus regarding the benefits of TLS over CI [17,27], and the advantage of utilizing TLS over CI to improve pregnancy outcomes has not yet been established.

In addition, the generalized application of TLS might not be cost-effective in all populations. To guide clinical practice, more high-quality evidence needs to be obtained from a larger trial or verified data from subgroup analyses specifically related to an age-adjusted population [28,29]. Consequently, the present study aimed to compare pregnancy outcomes between TLS and CI in different age groups to determine the most appropriate indicators for identifying patients who would benefit from utilizing TLS for embryo culture and for monitoring embryo development.

## Methods

This retrospective study was conducted from June 2018 to May 2020. Electronic medical records of infertile women who underwent intracytoplasmic sperm injection (ICSI) with embryo transfer at the Infertility Unit of the Department of Obstetrics and Gynecology, Faculty of Medicine Siriraj Hospital, Mahidol University were reviewed, with the data collected after Institutional Review Board approval (COA no.SI338/2018). There was no informed consent due to we conducted the study as a retrospective study. Patients aged 18–45 years undergoing autologous ICSI cycles with blastocyst transfers were included in this study. Embryos that had been rewarmed more than 4 times were excluded.

The controlled ovarian hyperstimulation (COH) protocols of both groups were chosen through a multifactorial approach, including age, antral follicle count, basal hormone levels, cause of infertility, and history of a previous treatment cycle. Exogenous follicle-stimulating hormone with or without luteinizing hormone (LH) was used for follicle stimulation under antagonist, agonist, or another protocol for suppressing premature LH surges. When two or more follicles reached 17 mm in diameter, ovum pick-up (OPU) was performed 34–36 hours later, after the induction of maturation by recombinant human chorionic gonadotropin (rhCG) or a gonadotropin-releasing hormone (GnRH) agonist. Fertilization by ICSI was performed in all patients. The study participants were classified into two groups (TLS and CI) based on the type of incubator used. The designation of the monitoring modality was made at the discretion of the physician and embryologist.

### 1. Fertilization and embryo culture

Following follicle aspiration, oocytes were placed in 0.5 mL Universal IVF Medium (Origio; Cooper-Surgical, Malov, Denmark) in a four-well dish in an MCO-230AICUVH $CO_2$ incubator (Panasonic, Osaka, Japan) under 37°C and 6% $CO_2$. The cumulus– oocyte complexes were soaked in a medium containing hyaluronidase (Sage, Coo-

per-Surgical) first and then the cumulus cells were removed by mechanical pipetting 2–3 hours after OPU.

All metaphase II oocytes were fertilized using standard ICSI procedures at ×400 magnification with an inverted microscope (Olympus IX71, Tokyo, Japan) 39–41 hours after rhCG or GnRH agonist administration. The injection was performed in Sydney IVF Cleavage Medium (Cook Medical, Bloomington, IN, USA) under an oil overlay. Fertilization was assessed 16–18 hours after insemination. The normally fertilized zygotes were transferred to a pre-equilibrated culture plate that had been prepared overnight within the incubator (Panasonic Healthcare, Osaka, Japan) at 37°C with 6% $CO_2$ and 5.5% $O_2$.

The embryos were sequentially cultured in Sydney IVF Cleavage Medium and Sydney IVF Blastocyst Medium (Cook Medical), using either TLS (Embryoscope time-lapse system; Vitrolife, Gothenburg, Sweden) or CI under an oil (Sage) overlay. For standard cultivation or CI, a 35-mm dish was prepared with 10 μL of Sydney IVF Cleavage Medium. The media was changed to Sydney IVF Blastocyst Medium at day 3 under 37°C with a 5% $CO_2$, 5% $O_2$, and 90% $N_2$ atmosphere in a mini-incubator (Planer Benchtop Incubator BT37, Cooper-Surgical). The embryos were scored according to the blastomere numbers, size, and amount of fragmentation daily in the morning following the consensus checkpoints for standard embryo assessment [10]. For the time-lapse imaging system (EmbryoScope time-lapse system, Vitrolife), a 12-well Embryoslide culture dish was prepared with 25 μL of Sydney IVF Cleavage Medium and the medium was refreshed after 48 hours with Sydney IVF Blastocyst Medium under the same temperature and gas concentration settings as CI. Embryo images were captured from seven focal planes every 10 minutes. The embryos in the TLS group were selected for transfer based on morphology, the presence or absence of abnormal cleavage, and morphokinetic parameters, including time to 2 cells (t2), 4 cells (t4), 5 cells (t5), and the interval between 3 and 4 cells (s2).

### 2. Embryo transfer method

Fresh or frozen embryo transfer (FET) was selected by the physician based on the number of oocytes retrieved, endometrial morphology, and pre-implantation genetic test planning. The blastocysts were transferred 5 days after oocyte retrieval in fresh embryo transfer. Vaginal progesterone for luteal support was administered on the day of OPU or 1 day after. For FET, vaginal progesterone was added 5–6 days before the blastocysts were transferred after artificial endometrial preparation by estradiol. Embryo transfer was performed under transabdominal ultrasound guidance in all cases.

### 3. Outcome measurements

The patients' baseline characteristics, including age, body mass index (BMI), and type and cause of infertility, were collected. Cycle characteristics, including the stimulation protocol, duration of stimulation, number of oocytes retrieved, maturation rate, and fertilization rates, were also recorded. The live birth rate, which was the primary outcome, was defined as delivery of the fetus beyond 28 weeks of gestational age. The secondary outcomes were the implantation rate, clinical pregnancy rate, cleavage rate, blastocyst rate, miscarriage rate, and age-stratification of the live birth rate and clinical pregnancy rate. The implantation rate was defined as the number of gestational sacs identified by ultrasonography divided by the number of embryos transferred, while the clinical pregnancy rate was defined as the presence of gestational sacs on ultrasonography.

The number of oocytes retrieved was defined as the total number of oocytes that were collected, while the maturation rate was calculated as the number of metaphase II oocytes divided by the total number of oocytes retrieved. The fertilization rate was defined as the number of two pronuclear (2PN) zygotes divided by the total number of inseminated mature oocytes. The total number of day 3 embryos divided by the number of 2PN zygotes gave the cleavage rate, and the total number of blastocysts divided by the number of 2PN zygotes was defined as the blastocyst rate.

### 4. Statistical analysis

Based on a previous study, the live birth rates of embryo transfers from the use of TLS and CI were 45% and 28%, respectively [4]. Considering a power of 80% for the study and a type 1 error of 5%, our study required 125 embryo transfers per group. All statistical analyses were performed using PASW ver. 18.0 (SPSS Inc., Chicago, IL, USA). The baseline characteristics and cycle outcomes in the TLS and CI groups were defined as the number (%) and mean ± standard deviation when normally distributed. Categorical outcomes between the TLS and CI groups were compared using analysis of variance, the Fisher exact test, or the chi-square test as appropriate. The independent Student *t*-test or Mann-Whitney *U*-test was used for a comparative analysis of continuous variables. Subgroup analysis was used to evaluate the primary outcome in the three different age groups. The baseline parameters that were significantly different between the TSL and CI groups and those that may impact the primary endpoint were considered to be possible confounding factors and were analyzed by logistic regression. All the significance tests were two-tailed, with an a level of 0.05. A *p*-value of less than 0.05 was considered to indicate statistical significance.

## Results

In total, 141 patients underwent ICSI cycles with 250 embryo transfers (125 in the TLS group and 125 in the CI group) and were enrolled in the study. The baseline characteristics of the patients are


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**Table 1.** Baseline characteristics of participants with embryos cultured in the TLS and CI groups

| Variable | TLS (n = 125) | CI (n = 125) | *p*-value |
|---|---|---|---|
| Age (yr) | 36.86 ± 3.85 | 36.85 ± 3.39 | 0.614 |
| BMI (kg/m$^2$) | | | 0.025[a] |
| Underweight ( < 18.5) | 16 (12.8) | 7 (5.6) | |
| Normal (18.5–23.5) | 85 (68) | 77(61.6) | |
| Overweight (23.6–27) | 14 (11.2) | 29(23.2) | |
| Obese ( > 27) | 10 (8) | 12 (9.6) | |
| Cause of infertility | | | < 0.001[a] |
| Endometriosis | 5 (4) | 14 (11.2) | |
| Ovulation factor | 5 (4) | 6 (4.8) | |
| Other female factor only | 46 (36.8) | 51 (40.8) | |
| Male factor only | 5 (4) | 27 (21.6) | |
| Male+female factor, tubal excluded | 3 (2.4) | 5 (4) | |
| Tubal+male factor | 0 | 1 (0.8) | |
| PGD | 33 (26.4) | 6 (4.8) | |
| Unexplained infertility | 25 (20) | 15 (12) | |
| Others | 3 (2.4) | | |
| Type of infertility | | | 0.003[a] |
| Primary infertility | 86 (68.8) | 63 (50.4) | |
| Secondary infertility | 39 (31.2) | 62 (49.6) | |

Values are presented as mean±standard deviation or number (%).
TLS, time-lapse monitoring system; CI, conventional incubator; BMI, body mass index; PGD, preimplantation genetic diagnosis.
[a] $p<0.05$.

demonstrated in Table 1. The mean age in both groups was 36.8 years. The participants in both groups predominantly had a BMI in the normal range. Female factors were the major cause of infertility in both groups. The second most common cause of infertility in the CI group was male factors (21.6%), while ICSI for preimplantation genetic diagnosis (PGD) was the second most common reason for ART in the TLS group (26.4%). Most patients had primary infertility in both groups. The COH cycle characteristics are presented in Table 2. The mean duration of stimulation, mean number of retrieved oocytes, and the stimulation protocol used were comparable in both groups. The maturation rate and fertilization rate were also similar. However, there was a higher number of participants in the TLS group than in the CI group who underwent fresh embryo transfer ($p = 0.002$). More preimplantation genetic screening (PGS) or PGD was performed in the TLS group than in the CI group ($p < 0.001$). The reproductive outcomes are shown in Table 3. TLS demonstrated a higher live birth rate (32.0% vs. 18.4%, $p = 0.013$), implantation rate (27.1% vs. 12.0%, $p = 0.004$) and clinical pregnancy rate (46.4% vs. 27.2%, $p = 0.002$) than the CI group. The miscarriage rates in the TLS and CI groups were 6.4% and 5.6%, respectively ($p = 0.79$). There were 10 patients (8%) in the TLS group and four patients (3.2%) in the CI group who developed chemical pregnancy followed by a spontaneous decrease in beta-human chorionic gonadotropin. The cleavage rate was over 90% and the blastocyst rate was over 70% in both groups. The results from the subgroup analysis of the live birth rate, clinical pregnancy rate, and miscarriage rate stratified by age groups are listed in Table 4. There were 65 embryo transfer cycles for patients aged < 35 years (group A), 141 embryo transfer cycles for patients aged 35–40 years (group B), and 44 embryo transfer cycles for patients aged > 40 years old (group C). Statistical significance was retained regarding the live birth rate in group C (34.8% vs. 9.5%, $p = 0.048$) and the clinical pregnancy rate for group B (45.7% vs. 28.2%, $p = 0.031$). The live birth rates in the TLS and CI groups were 40.6% and 21.2% ($p = 0.090$), 27.1% and 19.7% ($p = 0.298$), and 34.8% and 9.5% ($p = 0.048$) in groups A, B, and C, respectively, as shown in Figure 1. The miscarriage rates in the three age groups were not significantly different. The live birth rate adjusted by confounding factors (PGS and type of embryo transfer) is presented in Table 5 and logistic regression analysis of these parameters is displayed in Table 6. There was no significant difference in the live birth rate between PGS and non-PGS, between fresh and FET embryo transfer cycles, or according to BMI range. These parameters did not affect the favorable outcomes of TLS over CI, although this did not reach statistical significance.

**Table 2.** Controlled ovarian hyperstimulation cycle characteristics in the TLS and CI groups

| Characteristics | TLS (n = 125) | CI (n = 125) | *p*-value |
|---|---|---|---|
| Duration of stimulation (day) | 9.4 ± 1.3 | 9.4 ± 1.2 | 0.950[a] |
| No. of oocytes retrieved | 15.64 ± 13.50 | 13.8 ± 6.95 | 0.966[a] |
| Maturation rate (%) | 79.22 ± 16.74 | 78.42 ± 16.33 | 0.510[a] |
| Fertilization rate (%) | 80.78 ± 17.51 | 81.69 ± 16.32 | 0.880[a] |
| Stimulation protocol | | | |
| Antagonist | 108 (86.4) | 114 (91.2) | 0.504[b] |
| Short agonist | 6 (4.8) | 2 (1.6) | |
| Long agonist | 4 (3.2) | 3 (2.4) | |
| Other | 7 (5.6) | 6 (4.8) | |
| Type of embryo transfer | | | |
| Fresh | 32 (25.6) | 13 (10.4) | 0.002[b] |
| FET | 93 (74.4) | 112 (89.6) | |
| Number of embryos transferred | 1.51 ± 0.63 | 1.75 ± 0.56 | < 0.001[b] |
| PGS/PGD cycle | | | |
| No PGS/PGD | 65 (52) | 110 (88) | < 0.001[b] |
| PGS | 31 (24.8) | 9 (7.2) | |
| PGD | 29 (23.2) | 6 (4.8) | |

Values are presented as mean±standard deviation or number (%).
TLS, time-lapse monitoring system; CI, conventional incubator; FET, frozen embryo transfer; PGS, preimplantation genetic screening; PGD, preimplantation genetic diagnosis.
[a]Independent sample *t*-test; [b]Chi-square test.

**Table 3.** Reproductive outcomes of transfer cycles with embryos cultured in the TLS and CI groups

| Variable | TLS (n = 125) | CI (n = 125) | *p*-value |
|---|---|---|---|
| Live birth rate | 40 (32.0) | 23 (18.4) | 0.013[a] |
| Clinical pregnancy rate | 58 (46.4) | 34 (27.2) | 0.002[a] |
| Implantation rate (%) | 27.1 | 12.0 | 0.004[b] |
| Chemical pregnancy rate | 10 (8) | 4 (3.2) | 0.099[a] |
| Miscarriage rate | 8 (6.4) | 7 (5.6) | 0.790[a] |
| Cleavage rate (%) | 93.26 ± 14.88 | 93.54 ± 12.66 | 0.370 |
| Blastocyst rate (%) | 71.84 ± 23.29 | 79.28 ± 19.00 | 0.399 |

Values are presented as number (%) or mean±standard deviation.
TLS, time-lapse monitoring system; CI, conventional incubator.
[a]Chi-square test; [b]Independent Student *t*-test.

## Discussion

One of the key factors for a successful pregnancy from ART is good embryo quality. An embryo monitoring incubator is essential equipment that also impacts the embryo quality. Good embryo grading for embryo transfer depends on the optimal culture environment and having precise morphological information. A standard incubator (or CI) provides a stable culture environment, including the temperature, pH, humidity, and gas concentration [23]. Usually, when a CI is used, embryo morphology is assessed under an inverted microscope by an embryologist once daily. This process has the advantage that information can be obtained on the embryo development and some dysmorphic events can be observed, but at the same time, the use of a CI can lead to a disturbance of the culture environment [23]. Moreover, some significant kinetic changes in the embryo cannot be tracked without continuous observation [10,30,31]. The development of TLS, a novel automated embryo incubator, can overcome the limitations of a CI by allowing continuous monitoring of embryo development in favorable constant culture conditions.

Our study clearly showed better reproductive outcomes, including a higher live birth rate, implantation rate, and clinical pregnancy rate with a lower miscarriage rate, in the TLS group than in the CI group. The purification of gas by constant recirculation through an active filter in TLS may facilitate embryo development. Moreover, the tem-


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**Table 4.** Reproductive outcomes in subgroup analyses of TLS and CI based on patients' age groups

| Variable | TLS (%) | CI (%) | Difference (%) | *p*-value |
|---|---|---|---|---|
| Live birth rate | | | | |
| < 35 yr | 40.6 | 21.2 | 19.5 | 0.090 |
| 35–40 yr | 27.1 | 19.7 | 7.4 | 0.298 |
| > 40 yr | 34.8 | 9.5 | 25.3 | 0.048 |
| All | 32.0 | 18.4 | 13.6 | 0.013 |
| Clinical pregnancy rate | | | | |
| < 35 yr | 50.0 | 30.3 | 19.7 | 0.105 |
| 35–40 yr | 45.7 | 28.2 | 17.5 | 0.031 |
| > 40 yr | 45.3 | 19.0 | 24.5 | 0.082 |
| All | 46.4 | 27.2 | 19.2 | 0.002 |
| Miscarriage rate | | | | |
| < 35 yr | 0 | 9.1 | 9.1 | 0.083 |
| 35–40 yr | 10.0 | 4.2 | 5.8 | 0.182 |
| > 40 yr | 4.3 | 4.8 | 0.5 | 0.947 |
| All | 6.4 | 5.6 | 0.8 | 0.790 |

Age: <35 (n=65), 35–40 (n=141), >40 (n=44), all (n=250).
TLS, time-lapse monitoring system; CI, conventional incubator.

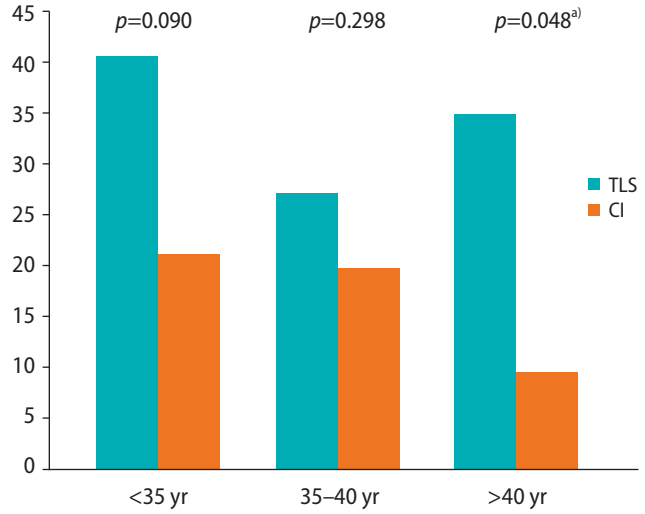


**Figure 1.** Live birth rate in time-lapse monitoring system (TLS) and conventional incubator (CI) stratified by age groups. a)$p<0.05$.

**Table 5.** Live birth rate adjusted by non-PGS versus PGS and fresh versus FET in the TLS and CI groups

| Variable | TLS (%, n = 125) | CI (%, n = 125) | *p*-value |
|---|---|---|---|
| Non-PGS | | | |
| Fresh (n = 44) | 38.7 | 23.1 | 0.318 |
| FET (n = 166) | 23.8 | 16.5 | 0.247 |
| PGS | | | |
| Fresh (n = 1) | 100 | 0 | NA |
| FET (n = 39) | 40 | 33.3 | 0.718 |

PGS, preimplantation genetic screening; FET, frozen embryo transfer; TLS, time-lapse monitoring system; CI, conventional incubator; NA, not available.

perature and gas concentrations in TLS recover more quickly to optimal conditions after doors are opened than in CI [23]. In the age subgroup analysis, the live birth rate was higher in participants whose embryos were monitored in TLS in all subgroups, although, the result only reached statistical significance in participants aged > 40 years. This may be a result of age-related changes in embryo quality. Given the high prevalence of good embryo quality in younger patients, this suggests that TLS might not be necessary to discriminate embryo quality for better pregnancy outcomes in this age group. On the contrary, in advanced-age patients with a high prevalence of poor-quality embryos, specifically with aneuploidy [32], TLS assists in identifying embryos at high risk of complex aneuploidy by allowing the monitoring of some critical morphokinetic parameters, specifically the time point of the second cell division (t3) and the time interval between 2 and 5 cells (t5–t2) [33]. Therefore, it can play a crucial role in improving pregnancy outcomes by enabling the clinician to select and discard low-implantation-potential embryos. Moreover, the cleavage pattern has been suggested to be associated with embryo quality. Direct and reverse cleavage, for example, are phenomena associated with poor embryo quality [13]. In the CI group, embryos with direct or reverse cleavage may be lost to tracking and may be mislabeled as good embryos instead [12,34,35].

Not only the cleavage sequence, but also atypical embryo phenotypes, including abnormal syngamy, abnormal first cytokinesis, abnormal cleavage, and chaotic cleavage associated with poor implantation potential, can be detected by TLS [13,34]. Both the morphology and development series were considered during embryo selection in the TLS group. As a result, only the embryos that followed the general timeline were considered the best for transfer. In other words, TLS can facilitate the prioritization of embryo transfer.

Many previous studies have mentioned the benefits of TLS for pregnancy outcomes; however, there is still no consensus yet [36]. The study of Siristatidis et al. [4], similar to ours, showed a higher live birth rate in the TLS monitoring group, especially in participants aged > 40 years old. They proposed that having more details for the embryological assessment and the stable culture conditions of TLS contributed to these outcomes. In the present study, as has been reported in other studies, TLS allowed the continuous monitoring of embryo development in an uninterrupted environment and enabled the detection of morphokinetic events that can help predict implantation potential and pregnancy outcomes [12,19,23,30,37-39]. In contrast to the present study, however, other reports did not find any significant improvement in reproductive outcomes even when TLS was applied [14,17,25]. In 2014, Polanski et al. [40] reported two randomized controlled trials comparing efficacy and safety between TLS and CI. Their study did not find a substantial benefit of TLS over CI, although they also stated that the use of this method did not pose a

**Table 6.** Evaluation of possible confounding factors on the live birth rate by logistic regression analysis (including the type of incubator, PGS vs. non- PGS, fresh vs. FET, and BMI)

| Variable | B | OR (95% confidence interval) | *p*-value |
|---|---|---|---|
| Method | | | |
| CI | - | 1 | |
| TLS | 0.478 | 1.61 (0.85–3.06) | 0.142 |
| PGS and type of embryo transfer | | | |
| Non-PGS, fresh | - | 1 | 0.090 |
| Non-PGS, FET | –0.641 | 0.527 (0.25–1.31) | 0.100 |
| PGS, FET | 0.119 | 1.127 (0.45–2.86) | 0.802 |
| BMI (kg/m$^2$) | | | |
| Underweight | - | 1 | 0.978 |
| Normal | –0.226 | 0.798 (0.29–2.18) | 0.660 |
| Overweight | –0.179 | 0.836 (0.25–2.77) | 0.770 |
| Obese | –0.231 | 0.794 (0.19–3.27) | 0.749 |

PGS, preimplantation genetic screening; FET, frozen embryo transfer; BMI, body mass index; OR, odds ratio; CI, conventional incubator; TLS, time-lapse monitoring system.

risk or cause any harm. In addition to the study of Polanski et al. [40], other systematic reviews have revealed insufficient data to support the routine use of TLS for embryo selection, and TLS has typically been considered an experimental intervention since then [27,41,42]. It should be noted that these studies were performed on participants with different individual and cycle-level characteristics from our study. In particular, studies that showed a significant improvement in the pregnancy rate were mainly undertaken with cleavage transfer [4,37]. In light of the scarcity of high-quality literature, several trials have been conducted to determine the exact roles for which TLS is most suitable for embryo monitoring. A significant advantage of TLS was shown in a recent meta-analysis [24], which demonstrated that the application of TLS was associated with an increased live birth rate, higher ongoing pregnancy rate, and lower early pregnancy loss, although the quality of the study was still not good enough to draw a firm conclusion.

At our center, we usually prefer to place embryos that need PGD or PGS in TLS for blastocyst culture. Thus, significantly more PGD or PGS tests were performed in the TLS group. For this reason, we utilized a logistic regression model to analyze all the significant confounding factors that may impact pregnancy outcomes from the different types of incubators. Although several studies have demonstrated a significant improvement in the ongoing pregnancy rate and implantation rate in embryos with PGS testing [43-45], the present study did not find any statistically significant differences in the live birth rate according to whether PGS was performed in both groups. As with PGS, fresh or FET and BMI were not identified as significant factors contributing to improvements in the live birth rate in the logistic regression analysis. After accounting for all the confounding factors, the effect of TLS over CI had an odds ratio of 1.61 (95% confidence interval, 0.85–3.06), although it did not reach statistical significance. Our study presented the clinical significance of TLS for improving the live birth rate compared to CI. It should be noted that the live birth rate achieved with TLS was higher than that for CI in all subgroups, although the population was too small to reach statistical significance.

Our study was conducted in a population with a variety of baseline characteristics. Only patients who underwent blastocyst transfer were recruited and embryos that were cultured for at least 300 runs every 10 minutes in the TLS group were selected. In this context, we assumed that the time period in the TLS group was adequate to reflect the environmental effect of this incubator system. Moreover, we analyzed the live birth rate to determine the final outcome of pregnancy, which might give information on the cost-effectiveness of applying TLS to improve pregnancy outcomes. Nevertheless, the retrospective nature of the study could be a limitation of our study. The causes of infertility were different between the groups. Although the most common causes of infertility were the same, more participants who underwent PGD were present in the TLS group. Some patients need PGD to prevent pregnancy with a diseased child, not to treat infertility, such as high-risk couples with alpha- and beta-thalassemia. Thus, the higher number of other causes in the CI group may have led to poorer reproductive outcomes in this group. Other confounding factors and selection bias should also be considered when interpreting the results. Moreover, the different culture conditions in the two types of incubators might have affected the embryo quality in different ways.

In conclusion, TLS application improved the live birth rate, implantation rate, and clinical pregnancy rate in patients with an older maternal age. The differences in outcomes between the two types of incubators studied (TLS and CI) tended to become more evident as



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age increased. A study with a larger sample size is needed to find strong evidence to specifically support the advantage of TLS in different age groups.

## Conflict of interest

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

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Conceptualization: PC, IT. Data curation: PC, BT. Formal analysis, Methodology, & Investigation: PC, IT. Writing–original draft: PC. Writing–review & editing: PC, IT, PL.

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# Successful pregnancy following transmyometrial embryo transfer after robot-assisted radical trachelectomy

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Radical trachelectomy is a fertility-preserving alternative to radical hysterectomy in carefully selected young women with early-stage cervical cancer. However, in cases with subsequent severe cervical stenosis, assisted reproductive techniques can be difficult. This is a case report of a 34-year-old patient who underwent robot-assisted radical trachelectomy and cerclage for early-stage (IB2) adenosquamous carcinoma. Three months after surgery, the patient underwent ovarian stimulation using a gonadotropin-releasing hormone antagonist protocol. As it was impossible to perform transcervical embryo transfer due to the almost complete absence of the cervical opening, transmyometrial embryo transfer under ultrasound guidance was performed. This resulted in a successful singleton pregnancy. This is the first case of successful pregnancy conceived by *in vitro* fertilization with transmyometrial embryo transfer in a patient who had previously undergone robot-assisted radical trachelectomy.

**Keywords:** Embryo transfer; Fertility preservation; Oocyte retrieval; Trachelectomy; Uterine cervical neoplasms

## Introduction

Early detection of cervical cancer increases the chance that fertility-preserving surgery can be performed. However, embryo transfer can be complicated by cervical abnormalities, such as cervical stenosis, atresia, or previous trachelectomy. According to two previous studies, the live birth rate after radical trachelectomy appears to be around 64%–67%, and most pregnancies are supported by intrauterine insemination or *in vitro* fertilization (IVF). Reasons for infertility after trachelectomy include cervical stenosis, decreased cervical mucus, and decreased vasculature supplying the uterus, according to previous studies [1,2]. The rate of cervical stenosis due to cervical cancer only or cervical trachelectomy in cases of difficult embryo transfer is challenging to obtain. In about 1% of cases, experienced experts have found cervical embryo transfer to be difficult due to anatomical and pathological cervical disorders [3]. Doctors may try traumatic transcervical embryo transfer, cervical dilation (with or without hysteroscopy), and transmyometrial embryo transfer (TMET) in such patients.

Kato et al. [4] introduced the Towako method for TMET and achieved several successful cases of pregnancy using this method. The Towako set consists of a needle/stylet and transfer catheter. Under transvaginal ultrasonography (TV-USG) guidance, a needle is inserted transmyometrialy between the upper cervix and lower bladder. When the needle reaches the uterine cavity, it is pulled back until the tip becomes clearly visible at the center of the endometrium. The inner catheter— loaded with one embryo in the culture medium— is then moved through the outer catheter, and the embryo is deposited in the uterine cavity [4]. A retrospective study showed an im-

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plantation rate of 9.5%, with pregnancy and miscarriage rates of 25% and 30%, respectively, after TMET [3].

There have been no reports of pregnancy with TMET after robotic trachelectomy. Herein, we introduce the first successful case of pregnancy with TMET after robotic trachelectomy.

## Case report

A 34-year-old woman was diagnosed with cervical intraepithelial neoplasia grade 3 with human papillomavirus 16 after a cervical punch biopsy at a local gynecological hospital in December 2019. Soon after, she was transferred to Seoul National University Bundang Hospital for further evaluation. As a result of a loop electrosurgical excision procedure, a 6-mm-deep and 11-mm-wide adenocarcinoma was found. According to magnetic resonance imaging and positron emission tomography, there was no lymph node or distant metastasis. The patient was nulligravida and wanted to become pregnant.

The oncologist classified her disease as stage IB1 (FIGO Committee on Gynecologic Oncology, 2019). Robotic radical trachelectomy, sentinel lymph node mapping, and cervical cerclage were successfully performed in March 2020. The final pathology report confirmed that the adenocarcinoma component was a mixture of the usual type and mucinous carcinoma, and that cancer might remain in the uterus.

The disease classified as stage IB2p without parametrial or lymphovascular invasion. Therefore, an additional margin was resected and an endometrial biopsy was performed; no tumor was observed. In February and May 2020, it was shown that the squamous cell carcinoma antigen level was 0.6 ng/mL, and the patient decided to postpone additional surgery and get pregnant quickly.

She had been married for 8 years, had a regular menstrual cycle from the age of 15 years, and had no history of other chronic medical or surgical disease; she was a non-smoker and had no history of hirsutism or galactorrhea. Her physical examination was uneventful, except for severe cervical stenosis without a cervical opening. A hormonal work-up showed a normal profile with a day-3 follicle-stimulating hormone (FSH) concentration of 2.05 mIU/mL and an anti-Müllerian hormone level of 1.52 ng/mL. Hysterosalpingography was normal. Her male partner was 39 years old and had no significant medical history. A semen analysis showed decreased motility (33%) 5 years ago.

Ovarian stimulation using a gonadotropin-releasing hormone (GnRH) antagonist protocol and IVF/intracytoplasmic sperm injection (ICSI) were selected, considering the patient's age, cervical status, and borderline ovarian reserve. The patient's last menstrual period was June 12 and human FSH (300 IU/day; Gonal-F, Merck Serono, Middlesex, UK) was injected subcutaneously from June 14 to 21. Day 5 TV-USG showed a normal uterus and ovaries. Each ovary had dominant follicles measuring 11–12 mm and a total antral follicle count of eight follicles. Subcutaneous injection of 0.25 mg of GnRH antagonist (Cetrotide; Serono, Geneva, Switzerland) was performed on day 6 for 4 days. On day 9, TV-USG showed two dominant follicles with mean diameters of 18 mm or more and an endometrial thickness of 9.7 mm. On the same day, 250 μg of recombinant human chorionic gonadotropin (hCG; Ovidrel, Sereno) was administered subcutaneously on the same day to induce ovulation.

The estradiol level on the hCG day was 2,604 pg/mL, and the progesterone level was 0.84 ng/mL. Oocyte retrieval was performed 36 hours after hCG injection under TV-USG guidance. A total of 11 oocytes were retrieved, including three oocytes in metaphase II. ICSI was performed for all available oocytes due to the poor sperm parameters. On day 1, four oocytes showed two pronuclei, which were cleaved into two good-quality embryos (10A and 6A-com). Two embryos were discarded, and one embryo was cryopreserved in a blastocyst (ErB-AB).

As it was impossible to perform transcervical embryo transfer due to the almost complete absence of the cervical opening, TMET under ultrasound guidance was performed using a Towako transfer set (Towako needle; Cook, Eight Mile Plains, Australia) (Figure 1). The pa-

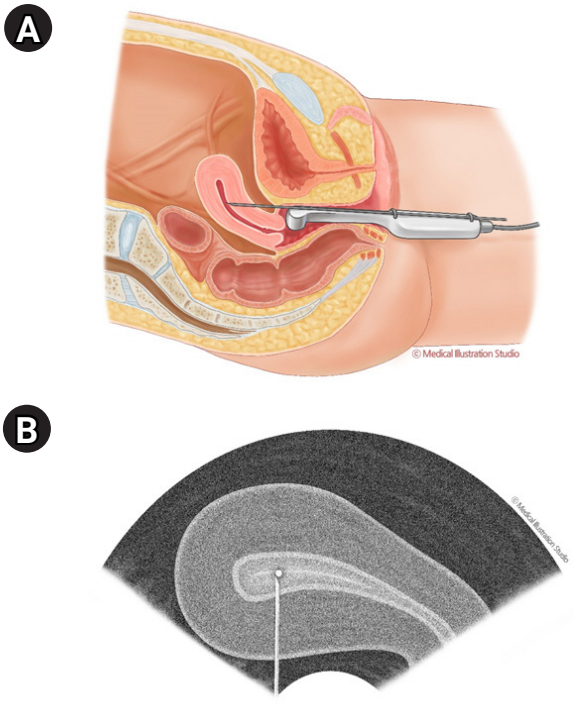


**Figure 1.** Transmyometrial embryo transfer under ultrasound guidance. (A) Schematic diagram of the procedure: the needle is inserted transmyometrialy and reaches the uterine endometrium. (B) Transvaginal ultrasonography of catheter placement and embryo transfer.

tient was premedicated with 3 mg of midazolam administered intravenously (IV) and fentanyl (100 μg; IV); both were administered 10 minutes before the procedure. One embryo (EdB-AB) was transferred successfully on the fifth day after oocyte retrieval. There were no major complications, including pain, bleeding, infection, or injuries. Biochemical pregnancy was confirmed 15 days after post-oocyte retrieval by a measurement of serum β-hCG (140.83 mIU/ mL). An intrauterine fetal heart was confirmed by TV-USG at 7 weeks' gestation.

## Discussion

As early detection of gynecological cancers has become possible, there have been increasingly many patients desiring fertility preservation, particularly in patients with cervical cancer. In cases of stage IB1 cancer and when the tumor 2 cm or smaller, radical trachelectomy and pelvic lymph node dissection with (or without) para-aortic lymph node dissection may be presented as an option, and abdominal trachelectomy can be performed even if the tumor is 2 cm or larger [5]. After trachelectomy, cervical dilation is difficult, and it is difficult to reach the fundus.

Our patient underwent robot-assisted radical trachelectomy with clinical-stage IB1 disease, but the final pathology report confirmed that the adenocarcinoma component was a mixture of the usual type and mucinous carcinoma, and it was found that cancer might remain in the uterus. Thus, additional resection margins and an endometrial biopsy were conducted, with no residual cancer as a result. Because the patient prioritized pregnancy, the consideration of any additional surgery was postponed.

Unlike other cases reported previously, the patient presented herein prioritized pregnancy and attempted to become pregnant immediately after surgery without proper follow-up to determine whether there was recurrence. In our case, for a fast pregnancy, TMET was performed immediately after trachelectomy, and a successful pregnancy was achieved.

With the development of assisted reproductive technology technology, it is important to increase the implantation and pregnancy rates through appropriate embryo transfer. According to a retrospective observational study, it was estimated that a poor embryo transfer technique accounted for 30% of all IVF failures. Embryo transfer was difficult in about 7.7% of all procedures, and the clinical pregnancy rate (CPR) was significantly lower than in non-difficult procedures (27.1% vs. 38.2%, $p < 0.001$). The CPR decreased progressively with the use of additional maneuvers during embryo transfer, including the use of an outer catheter sheath, Wallace stylet, and tenacula [6].

If there are cervical abnormalities, such as cervical stenosis, atresia, or previous trachelectomy, the likelihood of needing an additional maneuver increases. In such cases, TMET can be considered. According to Khairy et al. [7], when performing TMET, endometrial trauma and myometrial contraction can be reduced by minimizing the procedure time, and the risk of contamination of the embryo catheter and infection can be reduced by not passing through cervical secretions.

In a previous report, a patient had cervical cancer (1A2) and attempted a natural pregnancy for 6 months after radical vaginal trachelectomy and laparoscopic pelvic lymph node dissection 3 years previously; however, she was unsuccessful. As a result, she tried IVF using a GnRH agonist long protocol with TMET and was presented as a case of successful pregnancy [8]. Another patient had a distorted internal cervical canal and cervical dilation failed repeatedly. She also achieved pregnancy by TMET [9].

Khairy et al. [7] compared the outcomes of "very difficult transcervical embryo transfer" (vdTCET) versus TMET in 174 patients. They defined vdTCET as requiring more than two tenacula or stylets, changing the embryo transfer catheter, reloading of the embryos or canceling the procedure, and freezing the embryo to transfer after cervical dilatation. The clinical pregnancy and live birth rates for TMET (32.6% and 25%, respectively) were higher than those for vdTECT (26.1% and 16.4%, respectively). These studies have shown that TMET is a good alternative for difficult embryo transfer cases where cervical embryo transfer cannot be achieved.

During the follow-up period, it is necessary to check for miscarriage, live birth, or recurrence of cervical cancer. This is the first case of successful pregnancy conceived by IVF with TMET for a patient who had previously undergone robot-assisted radical trachelectomy. In the future, it may be meaningful to consider providing the options of ART and TMET in women of childbearing age diagnosed with cervical cancer when additional surgery is needed after a quick pregnancy.

## 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.

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Conceptualization: SKK. Data curation: HJH. Formal analysis: HJH, SKK. Project administration: SKK, JYP, DHS, JRL, BCJ, HJC. Writing–original draft: HJH. Writing–review & editing: HJH, SKK.

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# INSTRUCTIONS TO AUTHORS

CERM

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## I. ABOUT THE JOURNAL

*Clinical and Experimental Reproductive Medicine (CERM)* is an international peer-reviewed journal and is an official journal of the Korean Society for Reproductive Medicine, the Korean Society for Assisted Reproduction, the Pacific Society for Reproductive Medicine and Korean Society for Fertility Preservation. Official abbreviated title is *Clin Exp Reprod Med*.

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## II. ETHICS IN PUBLISHING

The journal adheres to the guidelines and best practices published by professional organizations, including ICMJE Recommendations and the Principles of Transparency and Best Practice in Scholarly Publishing (joint statement by the Committee on Publication Ethics, COPE; the Directory of Open Access Journals, DOAJ; the World Association of Medical Editors, WAME; and Open Access Scholarly Publishers Association, OASPA; https://doaj.org/bestpractice). Furthermore, all processes of handling research and publication misconduct shall follow the applicable COPE flowchart (https://publicationethics.org/resources/flowcharts).

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Clinical research should be done in accordance of the WMA Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/). Clinical studies that do not meet the Helsinki Declaration will not be considered for use in the publication. Human subjects should not be identifiable, such that the confidentiality of the patient's names, initials, hospital numbers, dates of birth, or other protected healthcare information should not be disclosed. For animal subjects, research should be performed based on the National or Institutional Guide for the Care and Use of Laboratory Animals, and the ethical treatment of all experimental animals should be maintained.

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A written statement must be described in the original articles indicating whether or not Institutional Review Board (IRB) approval was obtained or equivalent guidelines followed in accordance with the Helsinki Declaration; if not, an explanation must be provided. In addition, a statement of IRB status (approved, waived, or other) must be included in the Methods section of the manuscript. Similarly, a written statement confirming approval by appropriate Institutional Animal Care and Use Committee (IACUC) must be included for research involving animals. Any manuscript submitted without appropriate IRB or IACUC approval will not be reviewed and be returned to the authors.

### 3. Registration of clinical trial research

Any researches that deal with clinical trial should be registered to the primary national clinical trial registration site such as Korea Clinical Research Information Service (CRiS, http://cris.nih.go.kr), other primary national registry sites accredited by World Health Organization (https://www.who.int/ictrp/network/primary/en/) or ClinicalTrial.gov (https://clinicaltrials.gov/), a service of the Unite States National Institutes of Health.

### 4. Authorship

The *CERM* follows the recommendations for authorship by the International Committee of Medical Journal Editors (ICMJE, http://www.icmje.org). Authors should have made significant conceptual, intellectual, experimental, and analytical contributions to the research, as well as having participated in writing and revising the manuscript. Each author should have participated sufficiently in the work to take public responsibility for its content. Authorship credit should be based on: (1) substantial contributions to conception and design, acquisition of data, and/or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; (3) final Enacted: December 31, 1974 Revised: November 31, 2018 ii approval of the version to be published; and (4) agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of it are appropriately investigated and resolved. Every author should meet all 4 of these conditions. Copyright assignment must also be completed by every author.

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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.

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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 spublication 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.

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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.

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- 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 preiv pared 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.
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- 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.
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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.

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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

experimental methodology is the main issue of the paper, describe the process in detail so as to recreate the experiment as closely as possible. The sources of the apparatus or reagents used should be given along with the source location (name of company, city, and country). Ensure correct use of the terms sex (when reporting biological factors) and gender (identity, psychosocial or cultural factors), and, unless inappropriate, report the sex and/or gender of study participants, the sex of animals or cells, and describe the methods used to determine sex and gender. If the study was done involving an exclusive population, for example in only one sex, authors should justify why, except in obvious cases (e.g., prostate cancer). Authors should define how they determined race or ethnicity and justify their relevance. If needed, include information on the IRB/IACUC approval and informed consent. Methods of statistical analysis and criteria for statistical significance should be described.

5) **Results:** The results should be presented in logical sequence in the text, tables, and illustrations. Do not repeat in the text all data in the tables or figures, but describe important points and trends.

6) **Discussion:** Observations pertaining to the results of research and other related materials should be interpreted for your readers. Emphasize new and important observations; do not merely repeat the contents in the Introduction or Results. Explain the meaning of the observed opinion along with its limits, and within the limits of the research results connect the conclusion to the purpose of the research.

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Examples of authors' contributions are as follows:
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**Examples of references**

(1) **Journal article**
Kim SG, Kim YY, Park JY, Kwak SJ, Yoo CS, Park IH, et al. Early fragment removal on in vitro fertilization day 2 significantly improves the subsequent development and clinical outcomes of fragmented human embryos. Clin Exp Reprod Med 2018;45:122-8.

(2) **Website**
American Society for Reproductive Medicine. Headlines in reproductive medicine [Internet]. Birmingham: American Society for Reproductive Medicine; 2010 [cited 2018 Jan 10]. Available from: http://www.asrm.org/headlines/.

(3) **Book**

Suikkari AM. Use of in vitro maturation in a clinical setting. In: Gardner DK, Weissmaan A, Howles CM, Shoham Z, editors. Textbook of assisted reproductive technologies. 3rd ed. London: Informa Healthcare; 2009. p. 155-62.

(4) **In press**

Yang XL, Chen F, Yang XY, Du GH, Xu Y. Low molecular weight heparin does not reduce miscarriages in non-thrombophilic IVF/ICSItreated women. Acta Obstet Gynecol Scand 2018 Oct 14 [Epub]. https://doi.org/10.1111/aogs.13483.

11) **Tables:** Tables should be typed double-spaced on separate pages within manuscript, and they should be titled and numbered in Arabic numerals (not Roman numerals) in the order of their first citation in the text. Give each column a short heading. Place explanatory matter in footnotes, not in the heading. For footnotes vi use the following symbols, in this sequence: a), b), c), d), e), f) in superscript. Do not use internal vertical lines.

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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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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

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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.
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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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- ✔ 투여시간 **단축**


**Product Information**

【성상】 무색 또는 담황색이고 투명 또는 약간 혼탁한 액이 무색 투명한 바이알에 든 주사제입니다. 【원료약품의 분량】 1mL 중 주성분 : 사람면역글로불린-지 (별규) 100mg, 안정제 : 글리신 (KP) 18.8mg, 용제 : 주사용수 (EP) 적 량 【효능·효과】 1. 저 및 무감마글로불린혈증 2. 중증감염증에 항생물질 병용 3. 특발혈소판감소자색반병(타제가 무효로서 현저한 출혈경향이 있고, 외과적 처치 또는 출산 등 일시적 지혈관리를 필요로 하는 경우) 4. 길랑바레 증후군(급성특발다발신경염) 5. 가와사키병(관상동맥합병증 예방목적) 【용법·용량】 1. 저 및 무감마글로불린혈증 : 1회 사람면역글로불린-지로서 체중 kg당 200~600mg을 3~4주 간격으로 점적 정맥주사 또는 직접 정맥주사합니다. 2. 중증감염증에 항생물질 병용 시 : 1회 사람면역글로불린-지로서 성인 2,500~5,000mg, 소아 50~150mg/kg을 점적 정맥주사 또는 직접 정맥주사합니다. 3. 특발혈소판감소자색반병 : 이 약으로서 1일 1000mg/kg, 2일간 사용하여도 증상의 개선이 보이지 않으면 중지합니다. 4. 길랑바레 증후군 : 400mg/kg 1일 1회 5일간 투여합니다. 5. 가와사키병 : 1일 400mg/kg, 5일간(증감), 혹은 2,000mg/kg을 1회 점적 정맥주사합니다 (적의감량). 가와사키병에 사용하는 경우에는 발병 후 7일 이내에 투여를 개시하는 것이 바람직합니다. 2,000mg/kg를 1회 투여할 경우 기본적으로 아래 투여속도를 준수하는 것으로 하지만 기준으로서는 12시간 이상에 걸쳐 점적 정맥주사합니다. 투여속도 초기 30분 동안은 0.01~0.02mL/kg/min으로 투여하고 (예, 체중 60kg 인 경우 0.6~1.2mL/min 투여) 환자의 상태에 이상이 없으면 0.06mL/kg/min까지 점차적으로 투여속도를 증가할 수 있습니다. 투여 후 환자의 상태를 충분히 관찰하고, 만약 투여속도 증가로 인해 환자의 상태에 이상이 발생할 경우 즉시 투여속도를 줄이거나 증상이 호전될 때까지 투여를 중단합니다. 【사용상의 주의사항】 1. 경고 1) 이 약은 사람 혈장으로부터 제조되어 현재의 과학기술 수준에서 혈액 매개 바이러스 또는 다른 종류의 감염원(이론적으로 CJD)의 감염 위험을 완전히 배제할 수 없습니다. 따라서 혈우병 환자 또는 면역기능이 현저히 저하된 환자는 A형 간염 백신 등 적절한 백신 접종이 권장되며, 동 제제 투여 시 의사는 정기적으로 감염 여부를 모니터 해야 합니다. 또한 사람 혈액을 원재료로 하고 있는 것에 의한 감염증 전파의 위험을 완전히 배제할 수 없으므로 투여시 환자에게 충분히 설명을 하고 질병 치료상의 필요성을 충분히 검토한 후에 필요한 최소한의 사용에 그치도록 합니다. 2) 이 약 투여를 통한 혈전증 발생 위험은 완전히 배제할 수 없으며, 위험요인 및 투여 경로에 무관하게 발생할 수 있습니다. 고령자 등 혈전증 발생 위험요인(고령, 장기간 부동상태, 과응고 상태, 정맥 또는 동맥 혈전증 병력, 에스트로겐 사용, 중심정맥카테터 삽입, 고점도 및 심혈관장애 위험요인)이 있는 환자의 경우, 가능한 최소농도를 최저 주입속도로 신중 투여하여야 합니다. 또한 투여 전 환자가 적절한 수분을 섭취할 수 있도록 하여야 하며, 투여 후 혈전증 증상 및 징후를 관찰하고 고점성 위험이 있는 환자의 혈액점성을 평가하는 등 환자의 상태를 관찰하여야 합니다. – 첨부문서 참조. 【포장단위】 10, 25, 50, 100, 200 mL/Vial × 1 【저장방법 및 유효기간】 밀봉용기에 넣어 1~25℃ 에서 차광하여 보관 제조일로부터 18개월까지입니다. ※ 유효기한 또는 사용기한이 경과되었거나 변질, 변패 또는 오손된 제품은 약국, 병·의원, 도매상에 한하여 각 영업소에서 교환하여 드립니다. ※ 이 첨부문서의 작성일자(2017년 07월 03일) 이후 변경된 내용은 홈페이지(www. greencross.com)나 소비자 상담실 (☎ 080-260-8232)로 문의하시기 바랍니다.