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**폴리트로프 프리필드시린지** • **성상**: 무색 또는 미약색을 띠는 투명한 액이 들어있는 1회용 주사เข็ม이 달린 투명한 유리제질의 프리필드시린지 • **원료의약품 및 분량**: 주성분 폴리트로핀(제조함-인산포자극호르몬) 75 IU/0.15 mL, 150 IU/0.3 mL, 225 IU/0.45 mL, 300 IU/0.6 mL • **효능 및 효과**: 다음과 같은 임상적 상황에서 여성의 불임증 치료 1, 보조생식프로그램(즉, in vitro fertilization/embryo transfer; IVF/ET, gamete intrafallopian transfer; GIFT, zygote intrafallopian transfer; ZIFT, intracytoplasmic sperm injection; ICSI) 실시 중 다수의 난포를 성숙시키기 위한 조절된 난포자극(controlled ovarian hyperstimulation), 2, Clomiphene citrate로 치료되지 않은 여성의 무배란증 (다낭성난소질환 (PCOD)을 포함하는 WHO 그룹 II에 해당하는 환자들) • **용법 및 용량**: 1, 보조생식술에서 내인성 황체형성호르몬의 급증(LH surge)을 억제하고 황체형성호르몬의 강장정도를 조절하기 위해 여러 가지 자극 프로토콜이 사용되어 질 수 있다. 다수난포의 발달을 위한 난포자극과 배란을 위해 일반적으로 이용되는 방법은 주기의 2일에서 5일째에 시작해서 매일 150 - 300IU를 투여하는 것이다. 치료는 환자의 반응에 따라 용량을 조절하면서 적절한 난포발달이 이루어질 때까지 계속하는데(일정 에스트로겐 농도 모니터링과/혹은 초음파 검사로 평가), 보통 하루에 450IU 이상은 사용되지 않는다. 일반적으로 적절한 난포발달은 평균적으로 치료 10일째에 얻어진다. 최종 난포성숙을 유도하기 위해서는 마지막 약 투여로부터 48시간 이내 태반성 성선자극호르몬(hCG)을 최대 10,000IU 단위 투여한다. 2, 무배란증: 이 약 요법의 목표는 hCG 투여후 난자로부터 방출되는 한개의 성숙된 그라프난포를 발달시키는 것이다. 이 약은 매일 투여도 가능하며, 월경중인 환자의 경우 월경주기의 첫 7일 이내에 치료가 시작되어야 한다. 치료는 각 환자의 반응에 따라 적절히 변경되어야 하며, 각 환자의 반응은 (초음파로 측정된 난포크기와/혹은 (E)에스트로겐 분비로 측정한다. 일반적으로 사용되는 방법은 75 - 150 IU를 매일 투여하다가 필요한 경우 적절한 반응을 얻기 위해 7일 또는 14일 간격으로 75 IU씩 증량하는 것이다. 1일 최대 용량은 225IU를 넘지 않는다. 치료 2주 후에도 충분한 반응이 얻어지지 않을 경우는 이 치료주기를 중단하고 이 주기보다 더 높은 시작용량으로 치료를 재시작하여야 한다. 최적의 반응이 얻어지면, 마지막 약 투여 24 - 48시간 후 태반성선자극호르몬(hCG)을 250µg 또는 5000IU, 최대 10,000IU까지 투여한다. 환자는 태반성선자극호르몬 투여결과 더 이상할 성과를 갖도록 추천된다. 대안으로, 인공수정을 실시할 수도 있다. 단일 또는 다중 난포가 나타나면 치료를 중단하고 태반성선자극호르몬을 보류하며, 다음 주기에 치료를 받는 것보다 낮은 용량으로 실시하도록 한다. • **사용상의 주의사항**: 1, 다음 환자의 경우에는 투여하지 말 것 1) 난소, 유방, 자궁, 시상하부나 뇌하수체에 종양이 있는 환자 2) 임신, 수유부 3) 원발성 또는 2차성 갑상선 기능저하증이 있는 환자 4) 본제의 구성성분에 과민증이 있는 환자 5) 월경성 난소부전 환자 6) 다낭성난소증(PCOD)과 관련된 난소낭종이나 확장된 난소가 있는 경우 7) 임신할 수 있는 생리기의 변화가 있는 경우 8) 임신할 수 없는 자궁의 섬유유종(fibroid tumors)이 있는 경우 9) 치료되지 않은 비생식성 내분비질환이 있는 환자 즉, 갑상선부신이나 뇌하수체 장애 2, 다음 환자에서는 신중히 투여할 것 1) 보조생식술, 특히 체외수정(IVF)중인 불임여성들 종종 나팔관이 기형이기 때문에 자궁 외 임신의 빈도가 증가할 수 있다. 그러므로 초기에 초음파 촬영을 통해 자궁 내 임신을 확인하는 것이 중요하다. 2) 난소과자극 난소과자극의 진단은 초음파검사로 확인될 수 있으므로, 이 약 투여시 약제 투여전과 투여 중 규칙적으로 난포성숙에 대한 초음파검사와 혈중 에스트라디올 농도 측정을 실시해야 한다. 비합법적인 난소과자극이 발생하면 난소과자극증후군이 유발될 수 있으므로 신중히 감사를 실시해야 하며, 이 약의 투여를 중단하고 태반성선자극호르몬(hCG)의 투여도 보류한다. 난소과자극의 초기증상은 하복부통증이며, 오심, 구토, 체중증가가 동반되기도 하며, 드물게 발생하는 심각한 난소과자극의 증상들은 난소의 명백한 증대, 복수증, 흉수증 등이며 파열되기 쉬운 거대 난소낭종의 형성이 특징이다. 드물게 발생하는 심각한 난소과자극은 생명을 위협할 수 있으며, 합병증으로 심각한 혈전 색전증이 발생할 수도 있다. 그러나 태반성선자극호르몬의 투여를 보류하고 적어도 4일간 상고를 금지하면 과도한 에스트로겐 반응이 심각한 난소과자극을 초래하지는 않는다. 다태임신이나 난소과자극증후군(OHSS)의 위험을 최소화하기 위해, 초음파 및 혈중 에스트라디올 농도를 측정한다. 무배란증에서 난소과자극증후군의 위험은 혈중 에스트라디올 농도가 높거나 성숙한 난포가 많을 때 증가한다. 난소의 반응은 개개인(intrapersonal), 개인별(intraindividual)로 변화가 크므로 적절한 난소 반응이 보이도록 추천용량과 투여방법을 잘 준수하고, 주의 깊은 모니터링을 실시하여 난소과자극증후군과 다태임신의 발생을 최소화하도록 한다. • **포장단위**: 1 프리필드시린지 x 자포장단위 • **저장방법 및 사용기간**: 밀봉용기, 자광냉장(2-8°C)보관, 제조일로부터 36개월 • **제조원**: 주식 LG화학 ※ 이 약의 최신정보 확인은 의학전문인사나(https://nedrug.mfds.go.kr)를 참조하시기 바랍니다. [4약제 허가사항 2020.08 기준]

# CERM

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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.

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## REVIEW ARTICLES

- 219 Commentary on the new 2022 European Society of Human Reproduction and Embryology (ESHRE) endometriosis guidelines  
[Eun Hee Yu, Jong Kil Joo](#)
- 225 Non-invasive evaluation of embryo quality for the selection of transferable embryos in human *in vitro* fertilization-embryo transfer  
[Jihyun Kim, Jaewang Lee, Jin Hyun Jun](#)

## ORIGINAL ARTICLES

- 239 Therapeutic effect of *Ferula assa-foetida* oleo-gum resin in rats with letrozole-induced polycystic ovary syndrome  
[Amir Shieh, Seyyed Majid Bagheri, Maryam Yadegari, Davoud Javidmehr, Zeinab Farhadi](#)
- 248 Effects of human chorionic gonadotropin-producing peripheral blood mononuclear cells on the endometrial receptivity and implantation sites of the mouse uterus  
[Delsuz Rezaee, Mojgan Bandehpour, Bahram Kazemi, Sara Hosseini, Zeinab Dehghan, Saiyad Bastaminejad, Mohammad Salehi](#)
- 259 Evaluation of polyglycolic acid as an animal-free biomaterial for three-dimensional culture of human endometrial cells  
[Sadegh Amiri, Zohreh Bagher, Azadeh Akbari Sene, Reza Aflatoonian, Mehdi Mehdizadeh, Peiman Broki Milan, Leila Ghazizadeh, Mahnaz Ashrafi, FatemehSadat Amjadi](#)
- 270 Prolonged semen incubation alters the biological characteristics of human spermatozoa  
[Sayed Abbas Datli Beigi, Mohammad Ali Khalili, Ali Nabi, Mohammad Hosseini, Abolghasem Abbasi Sarcheshmeh, Mojdeh Sabour](#)
- 277 Coenzyme Q10 improves sperm motility and antioxidant status in infertile men with idiopathic oligoasthenospermia  
[Ahmed T Alahmar](#)

## CASE REPORT

- 285 A rare case of primary ovarian mesenchymal chondrosarcoma in pregnancy  
[Sepideh Azizi, Elena Ghotbi, Setare Nassiri](#)





# Commentary on the new 2022 European Society of Human Reproduction and Embryology (ESHRE) endometriosis guidelines

Eun Hee Yu, Jong Kil Joo

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Endometriosis is a prevalent benign illness defined by the presence of endometrial glands and stroma outside of the uterine cavity, primarily on the ovary, pelvic peritoneum, and rectovaginal septum, resulting in a variety of symptoms, including dysmenorrhea and infertility. Traditionally, prolonged medical therapy has been needed in most cases since a conservative approach to surgery has usually been taken, especially in young women. In 2022, new European Society of Human Reproduction and Embryology (ESHRE) guidelines were published that present different directions for diagnosis and treatment from the past. Furthermore, the guidelines for the diagnosis and management of endometriosis are more precise and applicable than in previous editions. Thus, referring to the representative changes in the new guidelines and important updates will be beneficial for the diagnosis and management of endometriosis. This paper provides a brief overview of these developments.

**Keywords:** Endometriosis; ESHRE; Guideline

## Introduction

Endometriosis is a prevalent benign illness defined by the presence of endometrial glands and stroma outside of the uterine cavity, primarily on the ovary, pelvic peritoneum, and rectovaginal septum [1]. It is linked to infertility and a variety of problems, including chronic pelvic pain, dysmenorrhea, deep dyspareunia, dysuria, dyschezia, and fatigue [2,3]. However, symptom severity is not usually proportional to the endometriosis stage, and some women with endometriosis may be asymptomatic.

Traditionally, endometriosis has been diagnosed through diagnostic laparoscopy or the histological identification of lesions. However, with recent developments in imaging modalities, the necessity for diagnostic laparoscopy in cases where endometriosis is relatively obvious has been questioned, and concerns have been raised regarding delays in the endometriosis diagnosis due to the diagnostic laparoscopic and histological confirmation criteria.

In 2022, the new European Society of Human Reproduction and Embryology (ESHRE) guidelines were published [4]. Although the new guidelines are not perfect, they provide clearer guidance on many difficult issues, such as medication selection among combined oral contraceptives (COCs), progestogen, and gonadotropin-releasing hormone (GnRH) agonists, and surgical indications for endometrioma in patients preparing for pregnancy.

The ESHRE guidelines are one of the most frequently cited endometriosis-related guidelines. It is believed that referring to representative changes in the new guidelines and major updates will be beneficial for diagnosing and treating endometriosis (Table 1). This paper provides a brief overview of these developments.

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**Table 1.** Summary of the latest guideline recommendation updates

Category	Updates in the latest version
Diagnosis of endometriosis	· Laparoscopy: no longer the diagnostic gold standard
Treatment of endometriosis-associated pain	· Consideration of a GnRH antagonist as a reasonable second-line treatment · Proceeding to postoperative medical treatment for expected benefits
Treatment of endometriosis-associated infertility	· Ultra-long protocol with GnRH agonist withdrawn from the evidence-based recommendations · Application of the Endometriosis Fertility Index · Importance of fertility preservation
Endometriosis recurrence	· Long-term hormone therapy advised when pregnancy is not desired
Endometriosis and adolescence	· Newly added; diagnosis and management not significantly different from adults
Endometriosis and menopause	· Importance reinforced; related symptoms present even in menopause
Primary prevention of endometriosis	· Revised title from “prevention of endometriosis”; recurrence is dealt with separately in “endometriosis recurrence”
Endometriosis and cancer	· Association of endometriosis with certain cancer risks despite the low absolute risks in people with endometriosis relative to those without; additive reassurance recommended for endometriosis patients

GnRH, gonadotropin-releasing hormone.

**Table 2.** Accuracy of imaging modalities for endometriosis diagnosis

Type of endometriosis	Modality	Number of patients included	Sensitivity (95% CI)	Specificity (95% CI)
Pelvic endometriosis	TVUS	1,222 in 5 studies	0.65 (0.27–1.00)	0.95 (0.89–1.00)
	MRI	396 in 10 data sets	0.79 (0.79–0.88)	0.72 (0.51–0.92)
Ovarian endometrioma	TVUS	765 in 8 studies	0.93 (0.87–0.99)	0.96 (0.92–0.99)
	MRI	179 in 3 studies	0.95 (0.90–1.00)	0.91 (0.86–0.97)
Deep endometriosis	TVUS	1,383 in 12 data sets	0.79 (0.69–0.89)	0.94 (0.88–1.00)
	MRI	289 in 7 data sets	0.94 (0.90–0.97)	0.77 (0.44–1.00)

CI, confidence interval; TVUS, transvaginal ultrasound; MRI, magnetic resonance imaging. Modified from Nisenblat et al. Cochrane Database Syst Rev 2016;2:CD009591 [5].

## Diagnosis of endometriosis

The previous ESHRE guidelines suggested that laparoscopic histologic confirmation of endometriosis was the gold standard for an endometriosis diagnosis. In contrast, according to the new ESHRE guidelines, laparoscopy is no longer the diagnostic gold standard and is now only advised for patients with negative imaging results and/or when empirical treatment is ineffective or unsuitable. This change was based on a meta-analysis regarding endometriosis diagnostic tools. According to a Cochrane review, imaging modalities such as transvaginal ultrasonography and magnetic resonance imaging showed sensitivity and specificity for diagnosing endometrioma and deep endometriosis comparable to a surgical diagnosis (Table 2) [5]. As an added explanation in the new guidelines, the difficulties and limitations of making a noninvasive diagnosis of a superficial disease are described. Despite these limitations, stating that diagnostic laparoscopy is not the gold standard emphasizes the usefulness of imaging modalities for diagnosing endometrioma and deep endometriosis.

## Treatment for endometriosis-associated pain

Discomfort related to endometriosis includes dysmenorrhea, dyspareunia, dysuria, dyschezia, and non-menstrual pelvic pain. In prior Korean and international guidelines, the hormonal medical treatment for endometriosis-associated pain has consisted of COCs, progestogens, anti-progestogens, aromatase inhibitors, and danazol. However, in the new guidelines, GnRH antagonists were introduced as a second treatment option, while anti-progestogens and danazol were withdrawn.

### 1. Introduction of GnRH antagonists as a second-line treatment option

The use of GnRH antagonists to alleviate endometriosis-associated pain can be considered. However, limited data exist on application of GnRH antagonist. Due to their side-effect profile, they are provided as a second-line option (for instance, if hormonal contraceptives or progestogens are ineffective). A study on two identical multi-center double-blind, randomized, placebo-controlled, phase 3 trials of 6-month treatments with oral elagolix at two doses in women with moderate



or severe endometriosis-associated pain provided data on treatment efficacy. The proportion of women who met the clinical response criteria for dysmenorrhea and non-menstrual pelvic pain was significantly higher among women who received each elagolix dose (46% in the lower-dose group, 75.8% in the higher-dose group) than among those who received placebo (19.6%). The reductions in dysmenorrhea and non-menstrual pelvic discomfort were noticeable after 1 month and persisted for 6 months. The most frequently reported adverse reactions were hot flashes, headaches, and nausea [6].

## 2. Changes in other medications

A levonorgestrel-releasing intrauterine system (LNG-IUS) has long been recognized as an effective treatment for endometriosis-associated discomfort. A recent randomized controlled trial (RCT) assigned 103 women with endometriosis-related chronic pelvic pain and/or dysmenorrhea to receive either an etonogestrel (ENG)-releasing subdermal implant or a 52-mg LNG-IUS [7]. Both the ENG implant and the LNG-IUS significantly reduced endometriosis-related pain, dysmenorrhea, and chronic pelvic pain. To alleviate endometriosis-related discomfort, it is recommended that women receive either an ENG implant or an LNG-IUS.

In addition, the Guideline Development Group (GDG) suggested using GnRH agonists as second-line treatments due to their side effect profile. Danazol and anti-progestogens, laparoscopic uterosacral neck ablation, presacral neurectomy, and anti-adhesion medications are no longer included in the recommendations of guidelines because of their harmful effects or lack of extra benefit.

## 3. Effectiveness of postoperative medical treatment

According to previous guidelines, practitioners should not provide adjunctive hormonal treatment for endometriosis-associated pain following surgery because it does not improve the outcomes of surgery to relieve pain. However, a recent Cochrane analysis by Chen et al. suggested that postsurgical medical therapy might reduce pain recurrence and illness recurrence within 12 months [8]. To improve the immediate success of surgery for pain in women with endometriosis who do not desire pregnancy, postoperative hormonal therapy may be administered.

## Treatment of endometriosis-associated infertility

### 1. Introduction of the Endometriosis Fertility Index

The GDG recommends that the decision to perform surgery should be made in consideration of some factors such as the presence or absence of pain symptoms, patient age and preferences, his-

tory of previous surgery, presence of other infertility factors, ovarian reserve, and the estimated Endometriosis Fertility Index (EFI). The EFI was added to the new guidelines for the first time. The EFI staging system predicts non-*in vitro* fertilization pregnancy rates following surgical endometriosis staging and treatment [9]. The EFI is determined by historical variables (age, number of years of infertility, and prior pregnancy) and surgical variables (the American Society for Reproductive Medicine [ASRM] overall score, the ASRM endometriosis score, and the least function score).

### 2. No benefit of ultra-long GnRH agonist prior to assisted reproductive technology

The extended administration of a GnRH agonist prior to assisted reproductive technology treatment to increase the live birth rate in infertile women with endometriosis (ultra-long protocol) is no longer suggested due to the lack of evidence supporting its benefits.

The previous recommendation was based on an older Cochrane review regarding GnRH agonist pre-treatment, which included 228 patients from three studies and showed an increased likelihood of clinical pregnancy by more than 4-fold [10]. However, an updated Cochrane review that included eight parallel-design RCTs with a total of 640 participants, concluded that the effect of GnRH agonist pre-treatment (for at least 3 months) was very uncertain, both on the live birth rate as the primary outcome and on secondary outcomes (clinical pregnancy rate, multiple pregnancy rate, miscarriage rate, the mean number of oocytes, and the mean number of embryos) [11]. Another meta-analysis of studies comparing different GnRH agonist protocols (short, long, and ultra-long) also found that different down-regulation protocols did not significantly improve clinical outcomes (implantation rate, fertilization rate, and clinical pregnancy rate) by analyzing RCTs and observational studies (n = 21) [12].

### 3. Importance of fertility preservation

Prior ESHRE guidelines on fertility preservation considered benign disorders to be an indication for fertility preservation, but did not address whether endometriosis was a reason for fertility preservation in particular [13]. A recent large retrospective study by Cobo et al. [14] evaluated the outcome of fertility preservation utilizing vitrified oocytes in 485 patients with endometriomas of at least 1 cm and an antral follicular count of at least 3, finding oocyte survival rates of 83.2% after warming and a cumulative live birth rate of 46.4%. They concluded that fertility preservation was a valid treatment option in patients with endometriosis. Although several issues such as cost-effectiveness and specific indications remain unsolved, practitioners should explore the advantages and disadvantages of fertility preservation with endometriosis patients who have severe ovarian endometriosis.

#### 4. Impact of endometriosis on pregnancy and pregnancy outcomes

Sparse data with low and moderate quality indicated that the behavior of endometriotic lesions during pregnancy was diverse, ranging from complete elimination to increasing growth [15]. Patients should not be encouraged to become pregnant with the primary aim of treating endometriosis because pregnancy does not necessarily result in symptom improvements or slow disease progression. The decidualization of an endometrioma during pregnancy may resemble malignant ovarian tumors in some circumstances, providing a diagnostic conundrum. However, the incidence of this event is unknown (0%–12% prevalence, 17 studies reporting 60 cases) [16]. Endometrioma can manifest differently throughout pregnancy. If an ultrasound examination during pregnancy reveals atypical endometrioma, it is recommended that the patient be referred to a center with the necessary expertise.

Complications directly connected to pre-existing endometriosis lesions are uncommon and likely underreported. These issues may result from decidualization, adhesion formation/stretching, and endometriosis-related chronic inflammation [16]. Although uncommon, these may pose life-threatening conditions that necessitate surgical treatment. Clinicians should be aware that women with endometriosis may have a higher risk of miscarriages and ectopic pregnancies during the first trimester. They should also be informed of uncommon endometriosis-related problems during pregnancy, which include gestational diabetes, preterm birth, premature rupture of membranes, placenta previa, hypertensive disorders and pre-eclampsia, stillbirth, cesarean section, obstetric hemorrhage (placental abruption, antepartum and postpartum bleeding), small for gestational age, admission to the neonatal intensive care unit, and neonatal death. However, since these results are based on low- or moderate-quality research, they should be regarded with caution, and additional antenatal monitoring is not recommended.

#### Endometriosis recurrence

The recurrence rate of endometriosis has been reported to range from 0% to 89.6% [17]. This variation could be related to differences in the definitions of recurrence, length of follow-up, study design, sample size, type and stage of disease, type of operation, and postoperative medical care [17]. As the high recurrence rate and its significance have been consistently emphasized, they are described in a separate chapter, unlike in the earlier guidelines. For preventing recurrence, ovarian cystectomy instead of drainage/electrocoagulation and postoperative hormonal treatment for at least 18 to 24 months are recommended. The recommended duration of hormonal treatment is based on RCTs. However, in patients who are not immediately

seeking conception, long-term hormonal therapy is recommended.

#### Endometriosis and adolescence

There are scarce data on endometriosis and adolescence. Although there are no major differences between the diagnosis and treatment of endometriosis in adolescents and adults, clinicians treating adolescents should be vigilant regarding several factors. Not only is dysmenorrhea a major symptom of endometriosis, but it is also a highly prevalent occurrence in adolescents, and it takes significantly longer to reach a diagnosis of endometriosis in adolescents than in adults. When previous treatments have failed, clinicians may consider prescribing a GnRH agonist for up to 1 year. If GnRH agonist treatment is considered for young women and adolescents, it should be delivered only after careful evaluation and a discussion of potential adverse effects and long-term health problems with a practitioner in a secondary or tertiary care setting, as recommended by the GDG. As with adults, no studies have addressed the efficacy or utility of fertility preservation—specifically, oocyte cryopreservation—in adolescents with endometriosis. Although the true benefits, safety, and indications for adolescents with endometriosis remain unknown, the GDG recommends informing adolescents about fertility preservation options.

#### Endometriosis and menopause

The amount of data available regarding the prevalence of endometriosis after menopause is extremely limited. A recent retrospective cohort study described a 4% prevalence of postmenopausal endometriosis [18]. It is hypothesized that menopausal hormone therapy can increase the formation of endometriosis [19] and a variety of factors, some of which are unknown, can also affect the growth of endometriosis. Therefore, it is important for clinicians to be aware that endometriosis might continue to be active and cause symptoms after menopause.

For postmenopausal women presenting with signs of endometriosis and/or discomfort, clinicians might consider surgical treatment as a means of enabling histological confirmation of the diagnosis of endometriosis. The GDG suggests that practitioners emphasize the ambiguity regarding the risk of cancer in postmenopausal women. If a mass is found in the pelvis, a diagnostic workup and treatment should be carried out in accordance with the national oncology standards. Clinicians may consider aromatase inhibitors as a potential therapeutic option for endometriosis-related pain in postmenopausal women, particularly if surgery is not a viable option.

Clinicians should be aware that women with endometriosis who undergo early bilateral salpingo-oophorectomy as part of their ther-



apy have a higher risk of decreased bone density, dementia, and cardiovascular disease. It is also essential to emphasize that women with endometriosis have a higher risk of cardiovascular disease, regardless of whether they have undergone early surgical menopause.

For the management of postmenopausal symptoms in women with a history of endometriosis, combined menopausal hormone therapy may be considered; however, tibolone is no longer recommended as a medical treatment for menopausal symptoms in women with a history of endometriosis.

### Extra-pelvic endometriosis

Clinicians should be aware of the symptoms of extra-pelvic endometriosis, which include cyclical shoulder discomfort, cyclical spontaneous pneumothorax, cyclical cough, and enlargement of nodules during menstruation. The diagnosis and treatment of extra-pelvic endometriosis should be discussed by a multidisciplinary team at an institution with sufficient expertise. When possible, surgical excision is the best treatment for alleviating symptoms in women with abdominal extra-pelvic endometriosis. If surgery is inapplicable or unacceptable, hormonal therapy may also be an alternative.

### Asymptomatic endometriosis

Clinicians should not regularly perform surgical excision/ablation for asymptomatic endometriosis discovered incidentally during surgery. Practitioners should not suggest medical therapy to women with an incidental endometriosis diagnosis, but routine ultrasound surveillance must be recommended. Even in the absence of convincing data on the benefits of monitoring asymptomatic endometriosis, the GDG recommends considering ultrasound surveillance due to its low cost and safety.

### Primary prevention of endometriosis

The objective of primary prevention is to prevent healthy and asymptomatic women from developing endometriosis. Although there is no direct proof of the efficacy of a healthy lifestyle and diet in preventing endometriosis, women can be counseled to adopt a

healthy lifestyle and diet, including reduced alcohol use and regular physical activity. The efficacy of hormonal contraceptives for the primary prevention of endometriosis is uncertain. Genetic testing of women with suspected or proven endometriosis should only be undertaken in a research context, citing the newly added text.

### Endometriosis and cancer

According to the 2014 recommendations, ovarian cancer and non-Hodgkin lymphoma were slightly prevalent among endometriosis patients. Nevertheless, according to a recent systematic review and meta-analysis of 49 cohort or case-control studies, endometriosis was related to a very slight and not statistically significant higher risk of cancer overall (summary relative risk [SRR], 1.07; 95% confidence interval [CI], 0.96–1.16) [20]. Endometriosis was related to an increased risk of ovarian cancer (SRR, 1.93), especially clear-cell (SRR, 3.44) and endometrioid (SRR, 2.33) histotypes, breast cancer (SRR, 1.04) and thyroid cancer (SRR, 1.39) [20]. Although endometriosis is associated with an elevated risk of certain cancers, given the low absolute risks of ovarian, breast, and thyroid cancer in people with endometriosis relative to those without (increases of 1.2%p, 0.5%p, and 0.5%p, respectively) and the uncertainty regarding the risk of other cancers, endometriosis patients can be reassured that their cancer risk is low and comparable to that of people without the disease. Clinicians should educate women with endometriosis who request information on their cancer risk that endometriosis is not associated with an increased risk of cancer. Although endometriosis is related to a somewhat higher risk of ovarian, breast, and thyroid malignancies, the absolute increase in risk relative to the general female population is minimal (Table 3).

### Conclusion

The new ESHRE recommendations are clearer and more practical than their predecessors and other guidelines. However, as stated on the opening page of the ESHRE guidelines, clinical practice guidelines do not supersede the clinical decisions made by a healthcare professional for diagnosis and treatment. Ultimately, healthcare professionals must make their own clinical decisions case by case, with consideration of various circumstances.

**Table 3.** Absolute risk for cancer in women with endometriosis [4]

Variable	Absolute risk of developing cancer in a woman's lifetime (%)		Increase in risk in women with endometriosis (%p)
	All women	Women with endometriosis	
Ovarian cancer	1.3	2.5	+1.2
Breast cancer	12.8	13.3	+0.5
Thyroid cancer	1.3	1.8	+0.5

## Conflict of interest

Jong Kil Joo is an editorial board member of the journal but was not involved in the peer reviewer selection, evaluation, or decision process of this article.

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# Non-invasive evaluation of embryo quality for the selection of transferable embryos in human *in vitro* fertilization-embryo transfer

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The ultimate goal of human assisted reproductive technology is to achieve a healthy pregnancy and birth, ideally from the selection and transfer of a single competent embryo. Recently, techniques for efficiently evaluating the state and quality of preimplantation embryos using time-lapse imaging systems have been applied. Artificial intelligence programs based on deep learning technology and big data analysis of time-lapse monitoring system during *in vitro* culture of preimplantation embryos have also been rapidly developed. In addition, several molecular markers of the secretome have been successfully analyzed in spent embryo culture media, which could easily be obtained during *in vitro* embryo culture. It is also possible to analyze small amounts of cell-free nucleic acids, mitochondrial nucleic acids, miRNA, and long non-coding RNA derived from embryos using real-time polymerase chain reaction (PCR) or digital PCR, as well as next-generation sequencing. Various efforts are being made to use non-invasive evaluation of embryo quality (NiEEQ) to select the embryo with the best developmental competence. However, each NiEEQ method has some limitations that should be evaluated case by case. Therefore, an integrated analysis strategy fusing several NiEEQ methods should be urgently developed and confirmed by proper clinical trials.

**Keywords:** Assisted reproductive technology; Cell-free nucleic acids; Culture media; Embryo; MicroRNA; Non-invasive; Secretome; Time-lapse imaging

## Introduction

Over the past half-century, strategies have been developed to evaluate and select competent preimplantation embryos for uterine transfer in human *in vitro* fertilization-embryo transfer (IVF-ET) pro-

grams. In the early days, morphological characteristics including fragmentation and other features of embryos observed by an optical microscope were mainly used to evaluate the quality and developmental potential of embryos [1]. However, simple daily microscopic observations by clinical embryologists had limitations in accurately predicting the developmental capacity of embryos.

Recently, techniques for efficiently evaluating the state and quality of embryos using time-lapse monitoring systems (TLMSs) and various molecular genetic approaches have been introduced. In particular, TLMS could select viable embryos without concerns regarding observer-variability and disturbances of culture conditions [2]. Various studies have searched for optimal morphokinetic parameters during TLMS, which could enhance the probability of blastocyst formation, aneuploidy, and finally implantation. Analyses of implantation-related morphokinetic parameters during TLMS have facilitated the development of several clinical algorithms as promising tools for the evaluation and prediction of embryos destined to become the

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most competent blastocysts [3].

Artificial intelligence (AI) programs based on deep learning technology and big data analysis of TLMS have been developed and applied as a method for the non-invasive evaluation of embryo quality (NiEEQ). The clinical effectiveness of NiEEQ for human IVF-ET programs has just begun to be reported, and there are several algorithms to predict the implantation potential of day-3 or day-5 embryos. Although the application of NiEEQ alone may not be perfect for selecting the best embryos, more advanced information about the physiological and genetic state of embryos could provide insights into all aspects of the embryos' intrinsic characteristics [4].

Furthermore, advanced and sensitive molecular genetic approaches have been successfully applied to spent embryo culture media (SECM), which can be easily obtained during *in vitro* embryo culture. It is possible to analyze small amounts of cell-free DNA (cfDNA), mitochondrial DNA, microRNA, and long non-coding RNA secreted from embryos using real-time polymerase chain reaction (PCR) or digital PCR, as well as next-generation sequencing (NGS) [5-7]. In addition, studies have evaluated the developmental ability of embryos by analyzing substrates and metabolites produced during *in vitro* culture, which will be discussed further below [8-11]. Many recent studies have evaluated the correlation between the results of SECM analysis and the embryos' developmental competence. However, the results obtained from those methods can be affected by various external sources of contamination and have the disadvantages of needing relatively expensive equipment, having high costs, and requiring special expertise.

This review provides an overview of the current status of NiEEQ, including TLMS and advanced molecular biological methods in SECM analysis. We also describe the need to develop a method for integrated analysis to overcome the several limitations of each NiEEQ system that has been used in recent years.

## TLMS for the selection of the best embryos for transfer

In human IVF-ET programs, embryo cleavage is observed daily by microscopy during *in vitro* culture, and the quality of embryos is determined by the number of blastomeres, cell symmetry, percentage of fragmentation, and other parameters on the day of transfer. The quality of blastocysts is also assessed according to the blastocysts' expansion state and the appearance of the inner cell mass (ICM) and trophoctoderm cells (TE) [12]. Transferable embryos are traditionally selected through a time-point observation of morphological features by trained clinical embryologists with expertise in embryo evaluation [13]. However, there are some limits in accurately predicting the developmental capacity of embryos by microscopic observations. In-

ter- and intra-observer variations can occur in embryo grading, even when it is performed by expert clinical embryologists [14].

For this reason, the TLMS was developed and applied in human IVF-ET programs [15]. A time-lapse system allows the complete observation of developing embryos in the IVF laboratory within stable culture conditions [16]. Initially, valuable knowledge was obtained through the TLMS during *in vitro* culture of pre-implantation embryos in animal models, such as mice and cows, and the TLMS provided precise information on developmental dynamics by making it possible to recognize important morphological changes of the embryo state [17,18]. The advantages of this system include a reduced need for handling and human risk, uninterrupted culture conditions, the ability to detect abnormal events that would otherwise not be noticed, and reduced inter- and intra-observer variability [16,19].

Through TLMS, various morphokinetic markers of developing embryos to predict blastocyst formation have been proposed, as shown in Table 1 [20-35]. In addition to blastocyst formation, morphokinetic markers associated with embryo implantation have been identified, as shown in Table 2 [22,24,25,34,36-50]. Several algorithms using a combination of morphokinetic variables have been introduced and successfully applied in human IVF-ET to select embryos with higher developmental capacity and implantation potential.

The known implantation data (KID) score is an interesting algorithm to improve embryo selection and predict implantation and live birth. The KID score algorithm attempts to rank embryo quality and optimize embryo selection prior to transfer based on conventional morphology grading [51]. Several reports have demonstrated the efficacy of the KID score algorithm and other similar programs using AI. The KID scores of day-5 blastocysts were found to be inversely proportional to maternal age, but directly proportional to blastocyst morphological grade [52]. This finding indicates that the KID score model works well to select blastocysts with higher implantation potential in patients with advanced maternal age.

More recently, the iDAScore algorithm has been developed; this is a deep learning-based annotation scoring system to predict the viability of embryos and the likelihood of implantation and pregnancy. Automatic embryonic ranking systems with AI have demonstrated higher performance with respect to successful implantation and pregnancy prediction than conventional morphological grading systems for the selection of transferable embryos [53]. The area under the curve (AUC) for the iDAScore was comparable to or higher than those of the KID score and Gardner criteria for young and older groups. In particular, for younger women, the AUC of iDAScore was 0.72, which was greater than those of the other two models.

For the KID score, strongly predictive morphokinetic variables were identified (time to 2 cells, duration of the second cell cycle below or above a threshold) with regard to implantation and live birth



**Table 1.** Various morphokinetic markers to predict blastocyst formation in time-lapse monitoring systems

Study	Study design	No. of evaluated embryos	Origin of embryos	Time-lapse monitoring system	Identified predictive marker
Wong et al. (2010) [20]	Retrospective study	100	Supernumerary frozen 2PN	Modified Olympus IX-70/71; CKX-40/41 BioStation CT	First cytokinesis, P2 and P3
Hashimoto et al. (2012) [21]	Experimental study	80	Donated human embryos for research	BioStation CT	Durations of second and third mitotic divisions
Hlinka et al. (2012) [22]	Retrospective study	180	Clinical IVF routine	Primo vision	c2, c3, and c4; i2, i3, and i4
Cruz et al. (2012) [23]	Retrospective cohort study	834	Oocyte donation cycles	EmbryoScope	t4, s2, DC3 cells, and tM; UN2 cells
Chamayou et al. (2013) [24]	Retrospective study	224	Fresh oocyte ICSI cycles	EmbryoScope	t1, t2, t4, t7, t8, tC–tF, and s3
Kirkegaard et al. (2013) [25]	Prospective cohort study	571	Fresh oocyte ICSI cycles	EmbryoScope	First cytokinesis, t3, and DC3 cells
Conaghan et al. (2013) [26]	Prospective multicenter study	233	Fresh oocyte ICSI cycles	Eeva	P2 and P3
Kirkegaard et al. (2014) [27]	Prospective multicenter study	1,519	Fresh oocyte ICSI cycles	EmbryoScope	P2 and P3
Cetinkaya et al. (2015) [28]	Retrospective observational cohort study	3,354	Clinical IVF routine	EmbryoScope	CS2
Yang et al. (2015) [29]	Prospective observational study	345	Metaphase I donated for research	Primo vision	Cleavage patterns
Milewski et al. (2015) [30]	Prospective observational study	432	Fresh oocyte ICSI cycles	EmbryoScope	t2, t5, cc2, and SC
Storr et al. (2015) [31]	Prospective cohort study	380	Fresh oocyte ICSI cycles	EmbryoScope	s3, t8, and tEB
Motato et al. (2016) [32]	Retrospective study	7,483	Clinical IVF routine	EmbryoScope	tM and t8–t5
Coticchio et al. (2018) [33]	Retrospective observational study	500	Fresh oocyte ICSI cycles	EmbryoScope	PN appearance during fertilization
Zaninovic et al. (2019) [34]	Retrospective multicenter study	27,316	Fresh oocyte ICSI cycles	EmbryoScope	t3, t3–t2, t5, t3–tPNF and t5–tPNF
Desai et al. (2019) [35]	Retrospective observational study	716	Fresh oocyte ICSI cycles	EmbryoScope	Cleavage patterns

PN, pronuclei; CT, computed tomography; P2, time of division from 2 to 3 cells; P3, time of division from 3 to 4 cells; IVF, *in vitro* fertilization; c2, time between 3 and 4 cells; c3, time between 5 and 8 cells; c4, time between 9 and 16 cells; i, interphase; t4, time of cleavage to 4 cells; s2, the second synchronization parameter (t4–t3); DC, direct cleavage; tM, time from insemination to compaction into the morula stage; UN, uneven blastomere size; ICSI, intracytoplasmic sperm injection; t1, time of cleavage to 1 cell; t2, time of cleavage to 2 cells; t7, time of cleavage to 7 cells; t8, time of cleavage to 8 cells; tC–tF, time of pronuclei appearance to disappearance; s3, the third synchronization parameter (t8–t5); t3, time of cleavage to 3 cells; CS2, cleavage synchronicity from 2 cell; t5, time of cleavage to 5 cells; cc2, the second round of cleavage (t3–t2); SC,  $s_{t2} \times \text{odds ratios}_{t2} + s_{t5} \times \text{odds ratios}_{t5} + s_{cc2} \times \text{odds ratios}_{cc2}$ ; tEB, time from insemination to expanded blastocyst; tPNf, time from insemination to pronuclei fading.

following IVF in a retrospective study comprising 2,827 embryos [54]. For the AI-based automated iDAScore, two parameters were identified (blastocyst grading and direct cleavage) using retrospective data from 18 IVF clinics consisting of 115,832 embryos, of which 14,644 embryos were assessed using the KID score [55].

Another AI-based model, termed Life Whisperer (LW), was developed by assessing the images of 8,886 embryos from 11 IVF clinics and provided time-saving and higher accuracy for successful pregnancy [56]. The LW model significantly improves the predictive accuracy of embryologists for viable and non-viable embryos. The weighted overall accuracy was 64.3% for embryo viability, with an improvement of 24.7% over embryologists' accuracy. This model showed a sensitivity of 70.1% and specificity of 60.5% for viable em-

bryos, while still showing a bias toward high sensitivity.

The DynScore, constructed in 2021, is a model calculated with the Gaussian distributions of the “a” coefficients (defined as the estimated number of maximum cells at 72 hours equivalent to the asymptote of the logistic curve). Logistic regression was performed using morphokinetic parameters from the first 3 days of 1,186 embryos, and the model output was highly predictive of blastocyst formation, with an AUC above 0.9 [57]. Although this model used a machine learning system with reinforcement capacity to predict the fate of embryos, it was only useful for specific types of patients, and it was not able to predict pregnancy.

Deep learning models have achieved good prediction results for successful pregnancy and fetal heartbeat following selected blasto-

**Table 2.** Various morphokinetic markers to predict implantation potential in time-lapse monitoring systems

Study	Study design	No. of evaluated embryos	Origin of embryos	Time-lapse monitoring system	Identified predictive marker
Lemmen et al. (2008) [36]	Retrospective study	19	IVF/ICSI cycles	Nikon Diaphot 300 microscope with camera in a closed system	Nuclei appearance in the first blastomere
Meseguer et al. (2011) [37]	Retrospective study	247	ICSI cycles	EmbryoScope	t5, s2, cc2, UN 2 cell, MN 4 cell, and DC 1–3 cells
Azzarello et al. (2012) [38]	Prospective study	159	ICSI cycles	EmbryoScope	PN breakdown
Hlinka et al. (2012) [22]	Retrospective study	114	ICSI cycles	Primo Vision	c2, c3 and c4; i2, i3, and i4
Rubio et al. (2012) [39]	Retrospective multi-center study	5,225 (1,659 transferred)	IVF cycles from donated and autologous oocytes	EmbryoScope	DC 2–3 cells
Freour et al. (2013) [40]	Retrospective analysis and prospectively collected database	191	ICSI cycles	EmbryoScope	t4 and s3
Chamayou et al. (2013) [24]	Retrospective study	178	ICSI cycles	EmbryoScope	cc3
Kirkegaard et al. (2013) [25]	Prospective cohort study	84	ICSI cycles	EmbryoScope	None
Rubio et al. (2014) [41]	Prospective randomized control trial	2,638	ICSI cycles from donated oocytes	EmbryoScope	t5, s2, cc2, UN 2 cell, MN 4 cell, and DC 1–3 cells
Aguilar et al. (2014) [42]	Retrospective cohort study	1,448	ICSI cycles from donated oocytes	EmbryoScope	Time to 2PB, PF, and length of S-phase
Basile et al. (2015) [43]	Retrospective multi-center study	1,122	ICSI cycles from donated and autologous oocytes	EmbryoScope	cc2, t3, t5, UN 2 cell, MN 4 cell, and DC 1–3 cells
Vermilyea et al. (2014) [44]	Retrospective multi-centric study	331	IVF/ICSI cycles	Eeva	P2 and P3
Freour et al. (2015) [45]	Retrospective study	528	ICSI cycles	EmbryoScope	t5, s2, cc2, UN 2 cell, MN 4 cell, and DC 1–3 cells
Dominguez et al. (2015) [46]	Retrospective cohort study	28	ICSI cycles from donated oocytes	EmbryoScope	cc2
Adamson et al. (2016) [47]	Prospective concurrent cohort study		ICSI and IVF cycles from autologous oocytes	Eeva	P2 and P3
Goodman et al. (2016) [48]	Prospective randomized control trial	2,092	ICSI and IVF cycles from autologous oocytes	EmbryoScope	cc2, s2, t5, s3, tSB, MN, and irregular division
Coello et al. (2017) [49]	Retrospective observational cohort study	429	ICSI cycles	EmbryoScope	Vitrified/warmed blastocyst morphology and collapse pattern
Zaninovic et al. (2019) [34]	Retrospective multi-center study	816	Fresh oocyte ICSI cycles	EmbryoScope	t3–t2
Barberet et al. (2019) [50]	Retrospective cohort study	232	ICSI cycles	EmbryoScope	PN appearance and MN 2 cells

IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; t5, time of cleavage to 5 cells; s2, the second synchronization parameter (t4–t3); cc2, the second round of cleavage (t3–t2); UN, uneven blastomere size; MN, multinucleated; DC, direct cleavage; PN, pronuclei; c2, time between 3 and 4 cells; c3, time between 5 and 8 cells; c4, time between 9 and 16 cells; i, interphase; t4, time of cleavage to 4 cells; s3, the third synchronization parameter (t8–t5); cc3, the third round of cleavage (t8–t4); PB, polar body; PF, time of pronuclei fading; t3, time of cleavage to 3 cells; P2, time of division from 2 to 3 cells; P3, time of division from 3 to 4 cells; tSB, time of starting blastulation.

cyst transfer [58,59], and a few studies have reported the prediction of embryo euploidy [60–62]. The correlation between euploidy and embryo morphokinetics has been widely studied, as shown in Table 3 [60–74]. Using a known data set of single static embryo images, the Embryo Ranking Intelligent Classification Algorithm was developed

to rank embryos based on ploidy and implantation potential [75]. The euploid prediction algorithm, with comprehensive consideration of morphokinetic parameters, patient age, and ploidy state determined by preimplantation genetic testing (PGT) improved the predictive efficiency and accuracy (the AUC of 0.80) [76]. To improve the

**Table 3.** Various morphokinetic markers to predict embryo euploidy in time-lapse monitoring systems

Study	Study design	No. of evaluated embryos	Time-lapse monitoring system	Biopsy day after fertilization	Analysis methods of PGD	Identified predictive marker
Chavez et al. (2012) [60]	Prospective observational study	75	Custom-built microscope	Day 3	aCGH	P1, P2, P3, and fragmentation
Campbell et al. (2013) [61]	Retrospective cohort study	98	EmbryoScope	Day 5	aCGH/SNP array	tSB and tB
Basile et al. (2014) [62]	Retrospective cohort study	504	EmbryoScope	Day 3	aCGH	t5–t2 and cc3
Rienzi et al. (2015) [63]	Longitudinal cohort study	455	EmbryoScope	Day 5	CCS	None
Chawla et al. (2015) [64]	Retrospective cohort study	460	EmbryoScope	Day 3	aCGH	tPNf, t2, t5, cc2, cc3, and t5–t2
Vera-Rodriguez et al. (2015) [65]	Prospective observational study	85	Eeva	Day 3	aCGH	Time between PN disappearance and the start of 1st cytokinesis; 3 to 4 cell
Mumusoglu et al. (2017) [66]	Retrospective cohort study	415	EmbryoScope	Day 5	aCGH	t9, tM, tSB, tB, and tEB,
Desai et al. (2018) [67]	Retrospective analysis of prospectively collected data	767	EmbryoScope	Day 5	aCGH/NGS	Dysmorphisms, tSB, tEB, and tEB-tSB
Rocafort et al. (2018) [68]	Retrospective study	1,482	Eeva	Day 5	NGS	P2 and P3
Huang et al. (2019) [69]	Retrospective observational study	188	EmbryoScope	Day 5	aCGH	Starting time to blastocyst expansion
Yap et al. (2019) [70]	Retrospective study	807 Patients	EmbryoScope	Day 5	NGS	KIDscore D5 algorithm (score 6.0–9.9)
Gazzo et al. (2020) [71]	Retrospective study	492	EmbryoScope	Day 5	NGS	KIDscore D5 algorithm
Ozbek et al. (2021) [72]	Retrospective analysis of prospectively collected data		EmbryoScope	Day 5	NGS	Cleavage patterns
Urich et al. (2022) [73]	Prospective observational study	212	EmbryoScope	Day 5	NGS	t2 and t3
De Gheselle et al. (2022) [74]	Retrospective cohort study	539	EmbryoScope	Day 5	NGS	tPB2, tPNa, and t7

PGD, preimplantation genetic diagnosis; aCGH, array-based comparative genomic hybridization; P1, time of division from 1 to 2 cells; P2, time of division from 2 to 3 cells; P3, time of division from 3 to 4 cells; SNP, single-nucleotide polymorphism; tSB, time of stating blastulation; tB, time from insemination to formation of a full blastocyst; t5, time of cleavage to 5 cells; t2, time of cleavage to 2 cells; CCS, comprehensive chromosome screening; tPNf, time from insemination to pronuclei fading; cc2, the second round of cleavage (t3–t2); cc3, the third round of cleavage (t8–t4); PN, pronuclei; t9, time of cleavage to 9 cells; tM, time from insemination to compaction into the morula stage; tEB, time from insemination to expanded blastocyst; NGS, next-generation sequencing; tPB2, time of 2nd polar body extrusion; tPNa, time from insemination to pronuclei appearance; t7, time of cleavage to 7 cells.

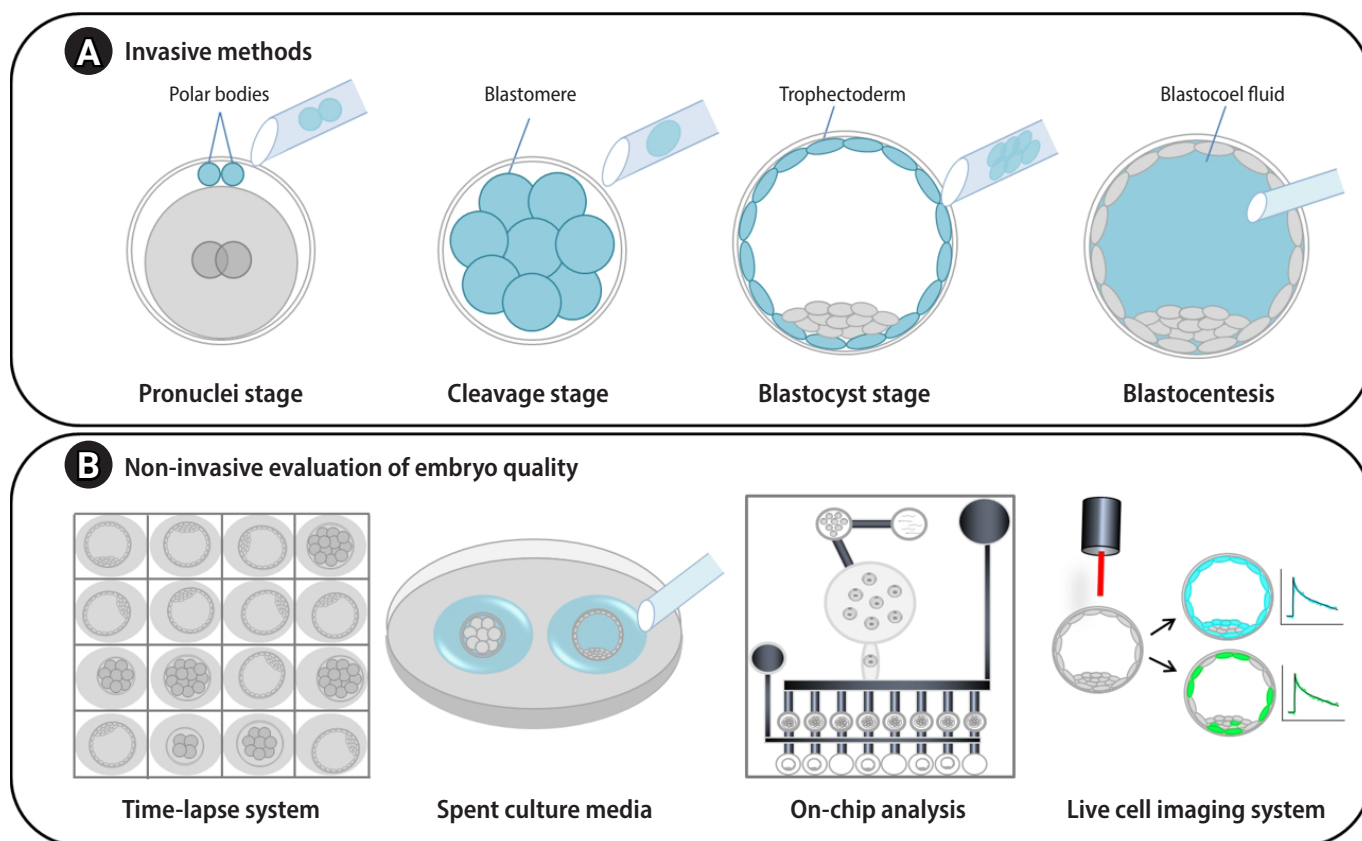
benefits of the AI model in terms of prediction of clinical pregnancy, validation for AI applications with a large-scale dataset is needed. The morphological features of embryos are not absolute, and do not fully represent the potential of embryos for successful implantation.

Despite their widespread application, embryo morphological assessments with TLMS have limited predictive power, especially for genetic variations and metabolic competence. Several studies have clearly demonstrated that embryo morphology and time to blastocyst formation are linked to embryo metabolism. Many approaches rely on intracellular measurements or quantification of metabolites in the spent media to detect the metabolic activity of the whole em-

bryo. Those methods are either invasive or require highly specialized skills. Non-invasive techniques to measure embryonic development and metabolic state may assist in improving embryo selection in clinical laboratories.

### Development and advancement of molecular biological methods for the analysis of SECM

Traditionally, invasive biopsy of pre-implantation embryos is performed for PGT to identify inherited or *de novo* euploidy or aneuploidy (Figure 1A). However, aneuploid cells are preferentially elimi-



**Figure 1.** Invasive and non-invasive methods for evaluation of embryo quality. (A) For invasive methods, genetic materials can be obtained by polar body biopsy at the pronuclei stage, blastomere biopsy for cleavage-stage embryos, and trophoblast biopsy or blastocoel fluid aspiration for blastocyst-stage embryos. (B) For non-invasive methods, image analysis of embryos using time-lapse monitoring systems; spent culture media analysis with quantitative real-time polymerase chain reaction (PCR), digital PCR, and on-chip-analyses; and live cell image analyses using advanced microscopic systems have been applied.

nated from mosaic embryos via processes of apoptosis or expulsion of cells during compaction. This is a cause of misdiagnosis or poor pregnancy outcomes. It was found that autophagy-mediated apoptosis eliminated aneuploid cells in a mouse model of chromosome mosaicism [77].

Many researchers have used non-invasive methods to determine the metabolic and genetic state of embryos concerning their viability and pregnancy outcomes for IVF patients (Figure 1B). During *in vitro* culture of human embryos, a variety of macromolecules, including proteins, nucleic acids, genetic material, and extracellular vesicles are present in SECM. Of many molecules, the level of GDF9 in human SECM was linked to embryo quality and viability [78]. Interestingly, various miRNA populations have been detected in the SECM, and these miRNAs may influence genes impacting early embryo development [79]. Profiling the secretome in SECM provides potential diagnostic biomarkers for embryo quality and ploidy [80-82]. Interestingly, a comparative analysis of the metabolomic profiles of SECM on day 5 found two different clusters of metabolite composition between euploid and aneuploid embryos with good morphology. Fur-

thermore, untargeted metabolomics of SECM by high-performance liquid chromatography–mass spectrometry identified potential biomarkers of embryos with good morphology that would undergo unsuccessful implantation [83]. In a preliminary report, three artificial neural networks that combined morphological variables and proteins using blastocyst image analysis and proteomic profiles of SECM were able to predict live birth, with an AUC of 1.0 in receiver operating characteristic curve analysis [84]. The researchers suggested that their model may provide an efficacious tool to select the embryo most likely to lead to a live birth in a euploid cohort. It may be applied to reduce the number of transferred embryos per patient to prevent complicated multiple pregnancies.

The reported levels of ploidy agreement between non-invasive SECM samples and biopsied embryonic cells vary widely [85]. A study found various cfDNAs in SECM from 57 embryos of seven IVF patients, and their genetic testing by array-based comparative genomic hybridization was consistent with TE biopsy [86]. Furthermore, single-cell bisulfite sequencing of SECM identified cfDNAs derived from human blastocysts, cumulus cells, and polar bodies, and de-



tected cellular origin and chromosome aneuploidy. The DNA methylation-based approach decreases the risk of contamination by maternal components, which interfere with a genetic diagnosis [87].

The greatest advantage of non-invasive genetic testing is cost-effectiveness due to the lack of fees for embryo biopsy, and it is useful as first-line PGT [88,89]. The efficiency of this method has been restricted by technical complications associated with DNA contamination and low sensitivity, resulting in clinical misdiagnoses [90]. In many cases, a small sample size reduces the reliability of the results of non-invasive PGT. Larger-scale and well-designed studies testing embryo-derived and extra-embryonic genetic material are warranted to shed light on the mechanisms and potential dynamics of embryo mosaicism.

Another issue to be considered for non-invasive genetic testing is SECM preparation. Group culture is not suitable, and it is necessary to place only one embryo in each small drop of culture medium. This aspect strongly affects culture conditions by evaporation and leads to excessive use of culture dishes. Modification of the culture platform on which gametes, embryos and media flow are handled may offer benefits including rapid fluid manipulation and feasibility of usage. The microfluidic method utilizes fluid movement along microchannels in a micro- or nano-environment during cell culture, while the embryos remain largely undisturbed [91]. Microfluidics platforms facilitate the easy manipulation or removal of gametes/embryos dealing with small volumes and the examination of metabolomic activity and profiles, offering a feasible non-invasive predictor of embryo quality [91,92]. Some lab-on-a-chip devices have met with a certain degree of success in adherent cell systems [93,94]. The technical development of integrative automation for more complex procedures within the same platform remains a work in progress. Embryo culture and subsequent analysis on the same platform offer the ability to reduce cell handling and the potential introduction of laboratory errors.

Electron carriers, such as nicotinamide adenine dinucleotide and flavin adenine dinucleotide, have recently been used to characterize variations in the metabolic state obtained using fluorescence lifetime imaging microscopy (FLIM) [95]. This measuring system allows the observation of distinct metabolic states between ICM and TE, and makes it possible to detect variations in individual blastocysts from the same patient and between patients. However, the association between FLIM data and embryo ploidy has not yet been fully elucidated.

## Clinical outcomes of TLMS and SECM analysis in human IVF-ET programs

TLMS for pre-implantation embryos in human IVF-ET programs

provides more embryo information as a non-invasive tool. However, it has been debated whether using a TLMS could improve the clinical outcomes compared with conventional evaluation systems. These TLMSs have been applied to clinical practice since the early 2000s, and many clinical trials have been reported. This review discusses the overall trend and future directions through a review of meta-analyses of clinical trials.

The first meta-analysis on the efficiency of TLMS was reported in 2014 [96]. The authors suggested that TLMS does not significantly offer the likelihood of achieving clinical and ongoing pregnancy in blastocyst transfer. They concluded that more research is needed to improve the quality of the available evidence and to investigate the usefulness of TLMS interventions for the selection of transferable embryos.

Thereafter, several meta-analyses were published until 2019, and all suggested that it is difficult to confirm a significant difference between TLMS and conventional methods [97-100]. In a Cochrane review published in 2019, the authors concluded that there was insufficient good-quality evidence of differences in live birth, ongoing pregnancy, miscarriage and stillbirth, and clinical pregnancy rates between TLMS and conventional methods.

However, a recent meta-analysis reported that TLMS interventions were effective [101]. Two randomized controlled clinical trials demonstrated the efficacy of TLMS in various conditions in the last 6 years [102,103]. Many retrospective studies using TLMS have reported statistically significantly higher rates of pregnancy success compared to traditional methods [54,104-107]. In addition, the KID score and iDAScore, using AI algorithms based on deep learning, have been developed and their applications are expanding in human IVF-ET programs [3,51,53,108,109]. The fully automated iDAScore model reduces manual evaluation and eliminates bias due to inter- and within-observer variability [55].

However, a couple of studies have reported that the evidence for significant advantages of TLMS remains unclear [110-112]. Elective single cleavage-stage embryo transfer with TLMS did not have any advantages over conventional observation in young women with good ovarian reserve [111]. That study also suggested that single blastocyst transfer with TLMS does not increase the likelihood of ongoing pregnancy compared to conventional observation; in particular, the use of a TLMS to choose blastocysts for fresh single embryo transfer on day 5 did not improve ongoing pregnancy rate compared to morphology alone [112].

One of the important challenges in the field of PGT of preimplantation embryos is the use of non-invasive procedures [113-115], with the aim of improving PGT cost-efficiency and safety. The collection of SECM is not a difficult procedure, and it can be safely performed on all cultured embryos. It does not require special expertise in embryo

manipulation, unlike invasive biopsies of embryos and blastocysts. Since this method avoids all detrimental effects of suboptimal micro-manipulations and the potential risks caused by invasive procedures, it does not affect the embryo development and reproductive potential. Moreover, SECM can be collected at any pre-implantation developmental stage; even cleaved embryos with fewer than six cells on day 3 and early blastocysts can be tested, unlike invasive PGT, which is based on embryo biopsy or blastocentesis. Hence, PGT by SECM might be particularly suitable for cultured growing embryos with low implantation potential that cannot be tested by invasive PGT. In fact, SECM-PGT is relatively fast, taking less than 12 hours from SECM collection to genetic analysis [116-118], and the results may be available before embryo transfer or cryopreservation. If there is a positive diagnosis, another SECM sample should be collected after 24 hours of incubation for confirmation. Many published reports have suggested that SECM is a potential alternative source of embryonic DNA, indicating that SECM-PGT is a promising procedure for the genetic testing of all developing embryos [119,120]. However, before implementing SECM-PGT in clinical practice, it is necessary to improve its reliability [121]. The standardization of SECM-PGT and establishment of guidelines are also essential to enable reliable comparisons of results and to verify the consistency of results among IVF-ET centers.

Debate continues regarding the reliability of alternative sources of genetic materials for embryo evaluation, although cfDNAs from SECM have been successfully detected and amplified. Discrepancies have been found regarding the concordance of the embryonic genetic state obtained from SECM and other DNA sources, including polar bodies, embryos, and TE biopsies, and whole embryos. There have been many discussions and suggestions on standardization and validation methods in several review papers on this issue [90,113,122-125]. With the latest advanced methods, such as NGS and mass spectrometry, which have recently emerged as superior analysis methods, it has become possible to verify the source of the genetic sample used for analysis and assess the probability of accurately estimating the genetic state of the embryo [126]. However, mosaicism, multinucleation, blastomere fragmentation, and contamination of SECM are still difficult to overcome. In particular, it is not easy to accurately distinguish genetic material of maternal or paternal origin and from the embryo.

## Conclusion

The ultimate goal of human IVF-ET programs is to achieve a healthy pregnancy and birth, ideally from the selection and transfer of the single best, most competent embryo. The effectiveness of NiEEQ in clinical applications of human IVF-ET programs has been pur-

sued intensively. However, each non-invasive method, such as TLMS and SECM analysis, has limitations that must be handled case by case. Since the evaluation of the embryo state using TLMS is based on morphological criteria, it is impossible to confirm variation at the actual genome and gene expression level. It is also difficult to reflect differences according to the culture conditions of each laboratory and the characteristics of each individual. In SECM analysis, alterations of nucleic acids and metabolites may appear depending on the presence or absence of cumulus cells or sperm that can be cultured if they attach to the fertilized oocytes. In order to overcome these limitations of NiEEQ, it would be ideal to develop integrated analysis methods through the fusion of complementary methods.

Rapidly developing, deep learning and AI algorithms with big data analysis can play a crucial role in improving and assisting many methods of both TLMS and SECM analysis. Several studies are being conducted to support the application of various techniques by developing automated annotation programs for the morphological dynamics of TLMS and genetic analysis of SECM. This advanced computational approach is expected to provide fast, robust, and reliable results while reducing bias in the interpretation of data and selection of the best embryo.

In the near future, it is expected that new integrated NiEEQ methods will emerge that can combine the advantages and compensate for the disadvantages of these two methods. We need to develop an integrated NiEEQ for the best embryo selection to achieve a healthy pregnancy and birth.

## Conflict of interest

Jin Hyun Jun is an editorial board member of the journal but was not involved in the peer reviewer selection, evaluation, or decision process of this article.

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Conceptualization: JHJ. Data curation: JK, JL. Formal analysis: JHJ, JK. Funding acquisition: JL, JHJ. Methodology: JK, JL. Visualization: JHJ, JK. Writing—original draft: JK. Writing—review & editing: JHJ, JL.

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# Therapeutic effect of *Ferula assa-foetida* oleo-gum resin in rats with letrozole-induced polycystic ovary syndrome

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**Objective:** Asafoetida is a gum derived from *Ferula assa-foetida*, which is used in traditional Iranian medicine to treat some reproductive system disorders. The effects of asafoetida on ovarian tissue, expression of certain genes associated with polycystic ovary syndrome (PCOS), and levels of liver, kidney, and blood cell factors after treatment in a rat model were investigated.

**Methods:** Thirty rats were divided into five groups: normal, polycystic, and treatment with three doses of asafoetida (12.5, 25, and 50 mg/kg for 3 weeks after PCOS induction). PCOS was induced by letrozole at a dose of 1 mg/kg administered orally for 3 weeks. Blood samples were taken, and the ovaries were removed and prepared for histomorphometric examination. Liver and kidney parameters were measured. The mRNA expression levels of luteinizing hormone receptor, CYP11A1, adenosine monophosphate-activated protein kinase, adiponectin, and adiponectin receptors 1 and 2 were also measured by real-time polymerase chain reaction.

**Results:** The levels of liver, kidney, and blood parameters did not significantly differ between the treatment groups and the control group. At doses of 25 and 50 mg/kg, ovarian histopathology, especially the thicknesses of the theca and granulosa layers, was significantly improved relative to the PCOS group. The expression of target genes also improved in the 25 and 50 mg/kg treatment groups.

**Conclusion:** Asafoetida can be used to treat PCOS as a complementary approach to conventional therapies. Asafoetida appears to act by regulating and activating metabolic and ovarian cycle enzymes.

**Keywords:** Asafoetida; Metabolic syndrome; Polycystic ovary syndrome

## Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder among women that has adverse effects on childbearing. It can be associated with other diseases, including insulin resistance, diabetes mellitus, hyperlipidemia, and cardiovascular disease [1]. Common

symptoms of PCOS include obesity, acne, irregular menstrual cycles, and infertility. Other symptoms such as anovulation, high androgen levels, and the presence of numerous ovarian cysts can also be seen [2]. High androgen levels are observed in approximately three-quarters of patients with PCOS. Hyperandrogenism is thought to be dependent on genetic factors and may also be influenced by environmental factors that impact the hypothalamic-pituitary-ovarian axis [3]. In animal models, letrozole is used to induce PCOS, and follicular atresia and abnormal follicular development have been observed in these models [4]. Several treatments are recommended for PCOS, such as lifestyle modification, pharmacotherapy, and herbal remedies [5]. Nowadays, attention is focused on the efficacy of plants used in traditional medicine because they are inexpensive and have minimal side effects [6]. The genus *Ferula*, belonging to the family of Apiaceae (Umbelliferae), consists of 170 species distributed world-

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wide. Of the 30 species of *Ferula* that have been found in Iran, 16 are native, and many are used in Iranian traditional medicine [7]. The major constituents of *Ferula* plants include sulfur-containing derivatives, coumarins, coumarin esters, sesquiterpenes, sesquiterpene lactones, sesquiterpene coumarins, glucuronic acid, galactose, arabinose, rhamnose, and daucane esters [8,9]. *Ferula assa-foetida*, a well-known species of *Ferula*, has traditionally been used for the treatment of various diseases including gastrointestinal disorders, nervous problems, and some reproductive system disorders, such as decreased libido [10]. In Iranian traditional medicine, *Ferula assa-foetida* oleo-gum resin (asafoetida) has been used as a menstrual enhancer. Also, in some regions of Iran, women with gynecological disorders such as dysmenorrhea [6] and oligomenorrhea [7] use *Ferula assa-foetida* as a remedy. The use of asafoetida during pregnancy has been banned due to its risk of induction of abortion. Modern investigations have shown that asafoetida has antifungal [11], antidiabetic [12], anti-inflammatory [13], antimutagenic [14], anticancer [15], antidementia [16], anticonvulsant [17], and antiviral [18] activities and also has a preventive effect against cuprizone-induced demyelination [19]. In a study on the effect of asafoetida on induced PCOS in rats, asafoetida resin extract was shown to increase the serum concentration of follicle-stimulating hormone and to significantly decrease concentrations of luteinizing hormone and testosterone in the treatment groups [20]. Additionally, Ghavi et al. [21] showed that the number of ovarian follicles and ovarian volume were significantly lower in women treated with asafoetida than in the control group. Based on the evidence and the lack of sufficient experimental studies, we chose to investigate the therapeutic effect of asafoetida on letrozole-induced PCOS in rats.

## Methods

### 1. Animals

Thirty female Wistar rats (200–250 g) were obtained from the animal house of the Faculty of Medicine at Shahid Sadoughi University of Medical Sciences (Yazd, Iran) and kept under a standard cycle of 12 hours of light: 12 hours of darkness. Standard food was fed with water *ad libitum*. Experiments were performed in accordance with the recommendations of the University Ethics Committee of Laboratory Animals of Shahid Sadoughi University of Medical Sciences with approval ID IR.SSU.AEC.1401.005 on 2022-03-09. One week after the animals adapted to the environment, the experiments were initiated. To induce PCOS, letrozole (1 mg/kg) was given orally every day for 3 weeks [22]. To ensure induction of PCOS, the estrous cycle was assessed daily by vaginal smear with a light microscope to examine the ratio of leukocytes, epithelial cells, and cornified cells. The estrous cycle of rats usually lasts approximately 4 days in both control and

PCOS animals, and rats that were in the estrous phase and had more cornified cells in the vaginal smear were selected for the study [23]. Asafoetida at concentrations of 12.5, 25, and 50 mg/kg was given orally every day for 2 weeks.

### 2. Preparation of plant oleo-gum resin

*Ferula assa-foetida* oleo-gum resin was collected from a mountainous area of Tabas (South Khorasan Province, Iran), and the plant species was identified and approved by Dr. Abbas Zarezadeh at the Yazd Agricultural Research Center. After drying in ambient air, the gum was soaked overnight in distilled water at room temperature, and the suspension was used orally. The concentration and dose of the suspension were expressed as the crude amount of dried oleo gum used in the preparation of the stock solution [24].

### 3. Biochemical, hematological, and hormonal analysis

Serum was prepared by collecting blood from the orbital sinuses of rats by centrifugation (3,000 rpm, 20 minutes). The serum was kept frozen until the biochemical assay. Levels of lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea, and creatinine were measured using appropriate kits according to the manufacturers' instructions. To count blood cells, sufficient blood was prepared, mixed with ethylenediaminetetraacetic acid, and counted with a cell counter device. Serum testosterone levels were measured using a testosterone enzyme-linked immunosorbent assay kit (Abcam, Cambridge, UK).

### 4. Histopathological analysis

Ovaries of the rats were removed prior to histological examination. Ovarian tissues were fixed in 10% neutral formalin. Then, all samples were cleaned, dehydrated, and embedded in paraffin. A microtome (Leica, Wetzlar, Germany) was used to prepare 7- $\mu$ m-thick sections, and the slides were stained with hematoxylin and eosin dye. The ovarian morphology and histopathological changes were observed microscopically. Follicles were grouped according to the following definitions: (1) Preantral follicle: a follicle with an intact, enlarged oocyte with a visible nucleus and a single layer of cuboidal granulosa cells. (2) Antral follicle: a follicle with two or more layers of cuboidal granulosa cells, regardless of whether the cavity is apparent. (3) Atretic follicle: a follicle containing a degenerating ovum or pyknotic granulosa cells. (4) Cystic follicle: a follicle appearing as a large fluid-filled structure with an attenuated granulosa cell layer and a thickened theca internal cell layer. For each group, the number of corpus luteum and preantral, antral, atretic, and cystic follicles of the ovaries were evaluated. The thicknesses of the theca interna cells and granulosa layer of the oocyte were evaluated. The layer thickness was mea-

sured using Micrometrics SE Premium Software (Accu-Scope Inc., Commack, NY, USA).

## 5. Reverse transcription and real-time polymerase chain reaction

Total RNA was extracted with RNX-Plus solution (Sinaclon, Tehran, Iran) at the Central Research Laboratory of Shahid Sadoughi University of Medical Sciences in Yazd, Iran in accordance with the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using a Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Parstous, Mashhad, Iran). The cDNA was amplified via reverse-transcription polymerase chain reaction (PCR) using AmpliTaq Gold DNA polymerase and quantitative real-time PCR and by using Premix Ex Taq mix (Yekta Tajhiz, Tehran, Iran) with SYBR Green I dye (Molecular Probes, Eugene, OR, USA) in a Step One Plus system (Applied Biosystems, Waltham, MA, USA). The primers used for reverse-transcription PCR were synthesized by Betagen Inc. (Mashhad, Iran) and are shown in Table 1. All experiments were performed in duplicate, and messenger RNA values were calculated based on the cycle threshold and monitored to obtain an amplification curve.

## 6. Statistical analysis

The results are reported as the mean ± standard error of the mean. Differences between means were obtained using one-way analysis of variance (the Tukey-Kramer method) performed with Graph Pad Prism version 8 software (GraphPad Inc., San Diego, CA, USA).

## Results

### 1. Effect of asafoetida on body weight gain

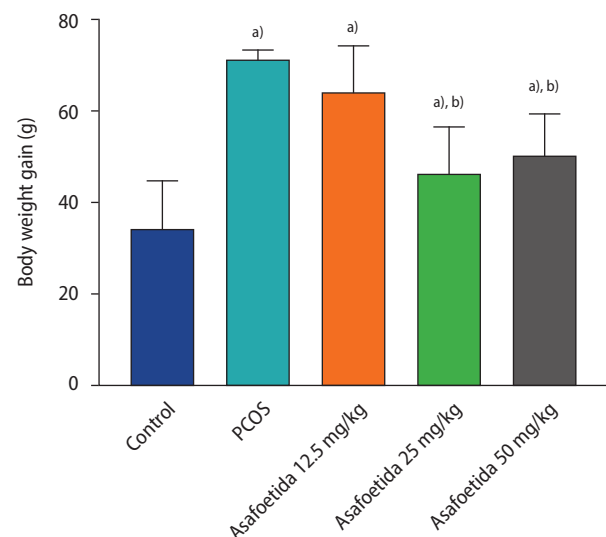
In the present study, a larger amount of body weight gain was observed in the PCOS group than in control rats, but the treatment of PCOS rats with 25 and 50 mg/kg of asafoetida resulted in less weight gain relative to the PCOS group ( $p < 0.05$ ) (Figure 1).

### 2. Biochemical, hematological, and hormonal analysis

Serum biochemical (including urea, creatinine, AST, ALT, ALP, and LDH) and hematological parameters are summarized in Tables 2 and 3. No significant change was observed at any concentration of asafoetida relative to the control group. Serum testosterone levels in the groups treated with 25 and 50 mg/kg asafoetida were significantly lower than in the PCOS group.

### 3. Histopathological results

Histopathological examination of ovaries from the control group revealed numerous ovarian follicles at various grades of maturation surrounded by dense ovarian stroma (Figure 2A). Follicular cysts were observable on the ovaries of the PCOS group as fluid-filled sacs, and histological examination revealed large cystic follicles filled with granulosa cells and surrounded by dense ovarian stroma. PCOS rats



**Figure 1.** Effect of asafoetida on body weight gain by group. Values are presented as mean ± standard error of the mean (n=5). <sup>a)</sup> $p < 0.05$  vs. control group; <sup>b)</sup> $p < 0.05$  vs. polycystic ovary syndrome (PCOS) group.

**Table 1.** Sequence identification and primers used for real-time polymerase chain reaction analysis

No	Gene	Forward primer (5' to 3')	Reverse primer (3' to 5')
1	Adiponectin	AATCTGCCCAGTCATGAAG	CATCTCTGGGTCACCCCTTA
2	Adiponectin receptor 1	TCGACAGGCCTAAGTGCCATC	ACTAGAAGGAGCCCCATTGC
3	Adiponectin receptor 2	GGGGTCAGAGCAGAGTGTT	AGCCAGCCTATCTGCCCTAT
4	CYP11A1	CACTGGAAATGGAGCTGGTATC	GCAGGAGAAGAGTGTCGCT
5	LHR	TCAACCTCGGGAGCTCACAC	GAGATAGGTGAGAGATAGTCTGGC
6	AMPK	TTCGGGAAAGTGAAGGTGGG	GGTTCTGGATCTCTGCGG
7	RPL13A	AAAGGTGGTGGTTGTACGCT	TCCGAGAAAGGCCAGATACT

LHR, luteinizing hormone receptor; AMPK, adenosine monophosphate-activated protein kinase.

**Table 2.** Effects of asafoetida on serum biochemical parameters by group

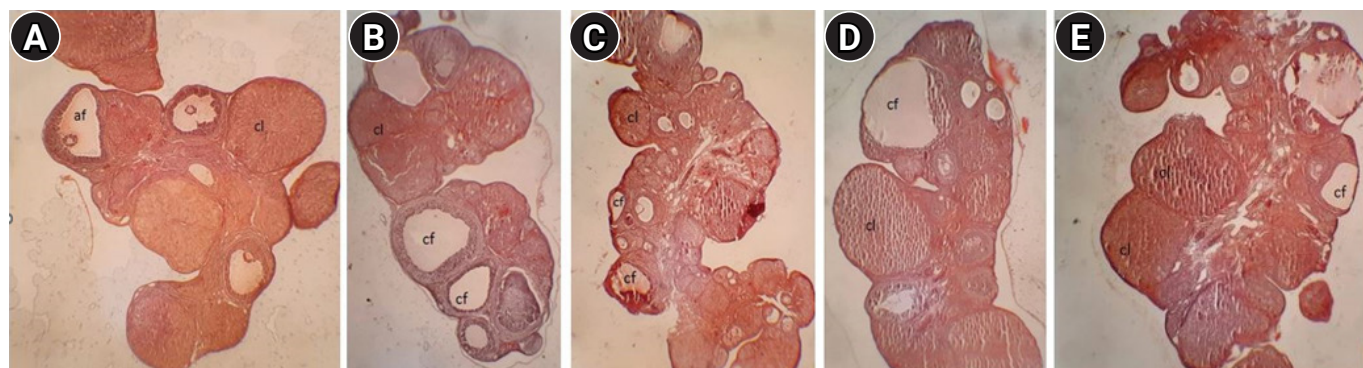
Group	Urea	Creatinine	LDH	AST	ALT	ALP
Normal	52±6.3	1±0.3	44±5.5	87±7.3	86±9.2	469±32
PCOS	49±8.9	1±0.2	46±5.3	96±8.3	69±7.6	432±25
PCOS+asafoetida 12.5 mg/kg	55±5.2	0.5±0.1	39±4.9	118±12	89±9.4	445±45.2
PCOS+asafoetida 25 mg/kg	58±8.6	0.5±0.1	51±4.8	81±6.2	57±6.9	430±28
PCOS+asafoetida 50 mg/kg	60±9.1	1±0.2	41±3.6	93±7.2	79±8.6	481±34

Values are presented as mean±standard error of the mean. Data were analyzed with analysis of variance followed by the Tukey test. LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; PCOS, polycystic ovary syndrome.

**Table 3.** Effect of asafoetida on hematological parameters by group

Group	WBC ( $\times 10^3/\mu\text{L}$ )	RBC ( $\times 10^6/\mu\text{L}$ )	HB	HCT	PLT
Normal	7.1±1.9	7.9±2.1	15±3.2	44±5.9	973±1.6
PCOS	6.2±1.1	7.2±2	14±2.9	41±4.8	1,037±18
PCOS+asafoetida 12.5 mg/kg	4.9±0.9	8±2.3	15±3.6	44±5.2	969±19
PCOS+asafoetida 25 mg/kg	5.2±1.6	7.6±1.9	15±3.8	42±4.8	982±17
PCOS+asafoetida 50 mg/kg	7±2.1	7±1.8	14±2.9	40±4.3	1,021±21

Values are presented as mean±standard error of the mean. Data were analyzed with analysis of variance followed by the Tukey test. WBC, white blood cell; RBC, red blood cell; HB, hemoglobin; HCT, hematocrit; PLT, platelet; PCOS, polycystic ovary syndrome.



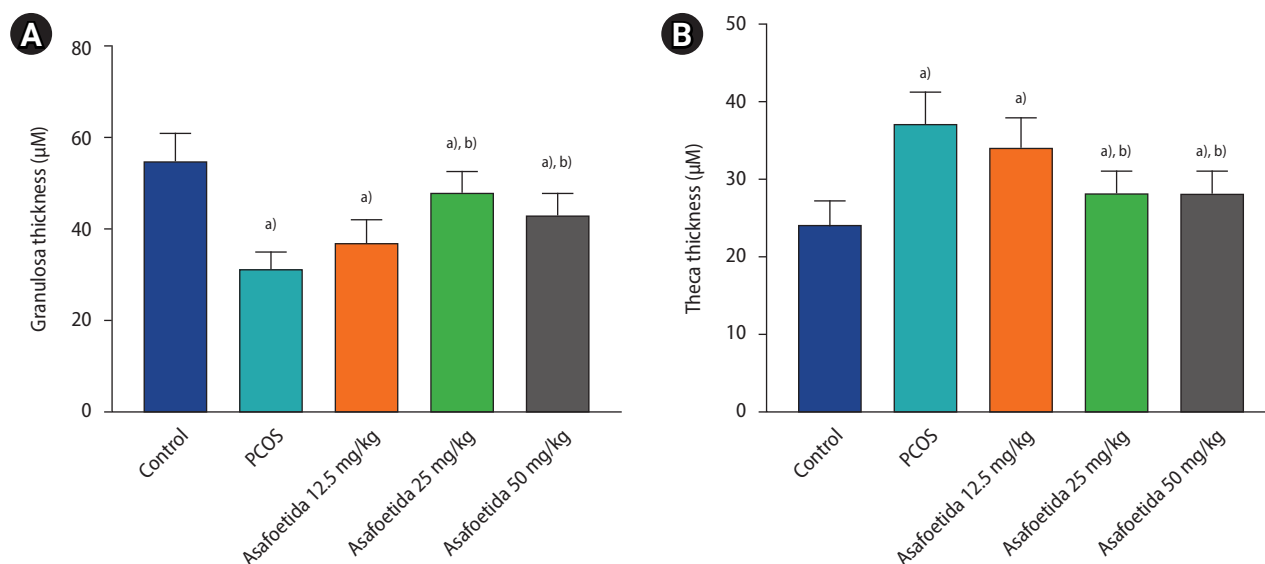
**Figure 2.** Photomicrograph of ovarian tissue from different groups. (A) Control group: section of ovary from a control rat showing follicles at various stages. (B) Polycystic ovary syndrome (PCOS) group: section of ovary from a PCOS rat showing cystic follicles (cf), antral follicles, and a decreased number of corpora lutea (cl). (C-E) Asafoetida group: the ovarian sections indicate a decrease in numbers of atretic and cystic follicles with increases in the number of antral follicles (af) and corpora lutea size (H&E,  $\times 200$ ).

exhibited cystic, atretic, and antral follicles and fewer corpora lutea (Figure 2B). In the groups treated with 25 and 50 mg/kg of asafoetida, increases in the numbers of antral follicles and corpora lutea were observed in the ovarian sections (Figure 2C-E). An increase in the thickness of the theca layer in the cystic follicles was seen in the PCOS group compared with the antral follicles of the control group, and both 25 and 50 mg/kg of asafoetida mitigated this effect significantly ( $p < 0.05$ ) (Figure 3). Plasma testosterone levels were significantly lower in the asafoetida-treated groups than in the PCOS group ( $p < 0.05$ ) (Figure 4).

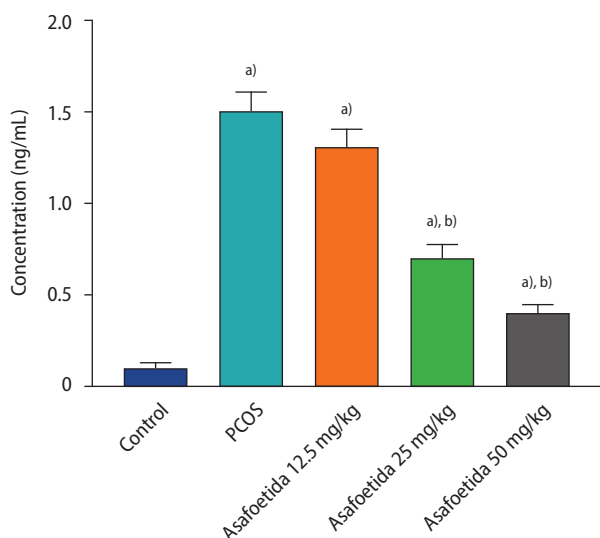
#### 4. Effect of asafoetida on gene expression

The effects of asafoetida on the mRNA expression of luteinizing hormone receptor (LHR), CYP11A1, adenosine monophosphate-activated protein kinase (AMPK), adiponectin, and adiponectin receptor 1 and 2 in ovarian tissue were also determined. Expression levels of LHR, AMPK, adiponectin, and adiponectin receptor 1 and 2 were significantly lower in the untreated PCOS group compared to the control group. However, in the groups treated with 25 and 50 mg/kg of asafoetida, the expression levels of these genes were significantly greater than in the PCOS group. The expression of the CYP11A1 gene was significantly elevated in the PCOS group relative to the





**Figure 3.** Comparison of morphometrical parameters. The thickness of the granulosa layer (A) and the theca interna cells (B) of the oocyte by group. Values are presented as mean±standard error of the mean (n=5). <sup>a)</sup> $p < 0.05$ , vs. control group; <sup>b)</sup> $p < 0.05$ , vs. polycystic ovary syndrome (PCOS) group.



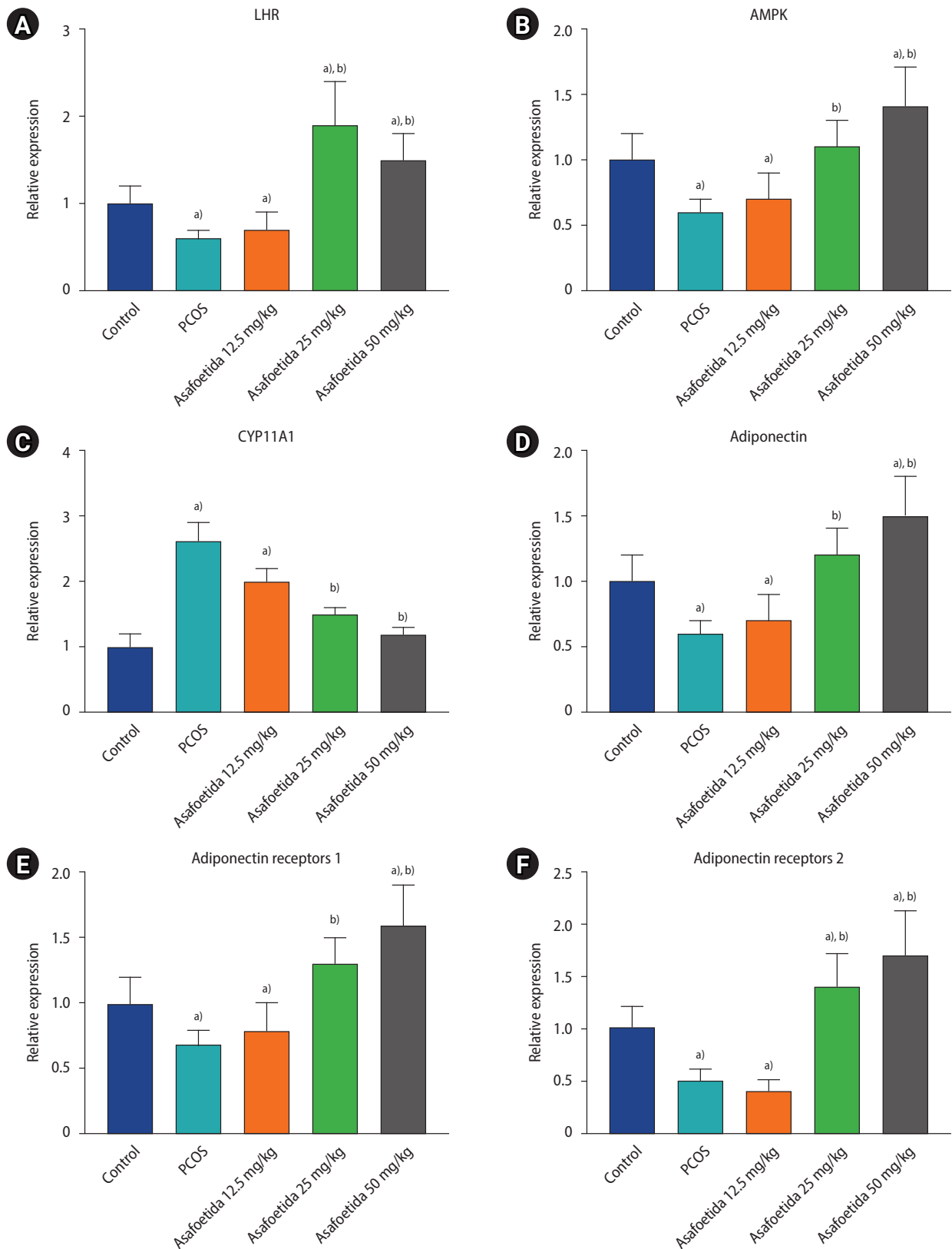
**Figure 4.** Effect of asafoetida on serum testosterone level by group. Values are presented as mean±standard error of the mean (n=5). <sup>a)</sup> $p < 0.05$ , vs. control group; <sup>b)</sup> $p < 0.05$ , vs. polycystic ovary syndrome (PCOS) group.

control and lowered in the groups treated with 25 and 50 mg/kg of asafoetida relative to the PCOS group ( $p < 0.05$ ) (Figure 5).

## Discussion

PCOS is a metabolic disorder related to the disruption of endocrine function regulation. This disorder is characterized by symptoms such

as irregular ovulation, abdominal obesity, and hyperandrogenism [25]. Our study showed that PCOS can cause significant weight gain, and asafoetida in doses of 25 and 50 mg/kg decreases the amount of that gain. Weight gain is one of the metabolic changes associated with PCOS. In a previous study, rats with letrozole-induced PCOS exhibited increased body weight, just like women with PCOS [22]. The results of the present biochemical and hematological analysis indicated that asafoetida did not cause toxic effects in the animals. Although few studies exist regarding the toxic effects of asafoetida, Iranian traditional medicine emphasizes that high doses of asafoetida can lead to swollen lips, gastrointestinal disorders such as bloating and diarrhea, discomfort, and headaches [10]. Bagheri et al. [26] reported that asafoetida had no acute toxic effects, but long-term use, especially at high doses, may damage the liver, kidneys, or bone marrow tissue. Additionally, oral consumption of *Ferula assa-foetida* extract in rats for 28 consecutive days was found to have no significant impact on body weight, blood parameters, and activity levels of AST, ALT, ALP, creatinine, and urea [27]. The histopathological results of the present study showed that asafoetida improved the morphology of the ovarian tissue in rats with PCOS. Follicular dysfunction, such as the presence of large atretic cysts with letrozole-induced small granulosa cells, has been observed in previous studies [28]. In the current study, treatment with asafoetida, especially at doses of 25 and 50 mg/kg, restored the components and morphology of the ovarian follicles to normal. This indicates that the extract is involved in regulating various factors associated with follicular growth in the ovary. A study by Ghavi et al. [21], conducted with 34 women,



**Figure 5.** Effects of asafoetida on the gene expression of luteinizing hormone receptor (LHR; A), adenosine monophosphate-activated protein kinase (AMPK; B), CYP11A1 (C), adiponectin (D), and adiponectin receptors 1 and 2 (E, F) using real-time polymerase chain reaction. Values are presented as mean±standard error of the mean (n=5). <sup>a</sup>*p*<0.05, vs. control group; <sup>b</sup>*p*<0.05, vs. polycystic ovary syndrome (PCOS) group.

showed that receiving 100 mg of asafoetida for 2 months decreased the number of ovarian follicles in women with PCOS. An increase in testosterone level, which causes acne and hair in women with PCOS, is a key challenge in this disease. Elevated testosterone levels have been shown to contribute to the pathogenesis of PCOS, and suppression of these high testosterone concentrations may have beneficial effects in individuals with PCOS disorders [29]. Bagheri et al. [30] found that asafoetida could increase sperm count in male rats after 6 weeks of low-dose treatment, but high doses had detrimental effects on Leydig cells and were associated with decreased testosterone concentration. Another study similarly showed that injection of 75, 150, and 300 mg/kg of asafoetida extract for 14 days thickened the seminiferous layers and decreased the number of Leydig cells and testosterone concentration at a dose of 300 mg/kg [31]. Morovatharifabad et al. [20] also showed that testosterone levels decreased significantly in PCOS rats treated with hydroalcoholic extract of asafoetida gum. LHR gene expression can be an indication of the capacity of drug compounds to treat PCOS. LHR is found on theca and granulosa cell surfaces, and LHR transcript levels are associated with ovulation, corpus luteum formation, and the production of steroids, including estrogen, progesterone, and androgens [32]. In the present study, LHR transcription levels were altered in the ovaries of the PCOS group, and these levels returned to normal with asafoetida treatment. Additionally, the mRNA expression level of *CYP11A1*, which encodes a key enzyme in the synthesis of steroid hormones, was significantly increased in the PCOS group, a trend that was mitigated by treatment with asafoetida. Expression levels of AMPK, adiponectin, and adiponectin receptor 1 and 2 were lower in the PCOS group than the control group, while treatment with asafoetida increased the expression of all of these and may thus restore a regular menstrual cycle. AMPK is a regulator of energy homeostasis that facilitates coordination in metabolic pathways and is a potential therapeutic target in metabolic disorders [33]. In contrast, adiponectin is a hormone that is released from fat tissue, and its levels are inversely related to fat mass [34]. Various animal studies have shown that the expression levels of adiponectin and AMPK are significantly reduced in animals with PCOS, while this effect is reversed in groups with effective treatment [35]. The expression of the adiponectin gene would be expected to increase in the treatment groups. The results of our study are consistent with this evidence, and the expression levels of AMPK, adiponectin, and adiponectin receptors were significantly elevated in the groups treated with asafoetida compared to the PCOS control group. While the mechanism of action of asafoetida in the treatment of PCOS cannot be said with certainty, some evidence may be helpful in clarifying the issue. The potential effect of asafoetida in fat metabolism has been studied, and taking asafoetida has been shown to significantly reduce body weight and abdominal fat

at doses of 25 and 50 mg/kg, an effect that is associated with decreased serum leptin levels [36]. Therefore, the effect of asafoetida on PCOS may be related to energy metabolism [37]. Based on the present findings, asafoetida may exert its protective effects on PCOS rats via the AMPK and adiponectin pathways and, by countering metabolic disorders, aid in PCOS treatment.

The results of the present study suggest that asafoetida may be a potential treatment for hormonal disorders associated with PCOS. These findings may be due to interactions with the hypothalamic-pituitary-ovarian axis or effects on metabolic processes. Despite this, based on the obtained data, the effects of asafoetida on PCOS cannot be clarified for certain, and some aspects are still unclear. Since asafoetida contains different compounds, more studies should be carried out on its different fractions to isolate its active compounds. Also, by conducting controlled studies on humans, the effects of this herbal product in the treatment of PCOS can be better understood.

## Conflict of interest

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

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## Author contributions

Conceptualization: SMB. Data curation: AS, MY. Formal analysis: SMB. Funding acquisition: AS. Methodology: DJ. Project administration: SMB. Visualization: ZF. Writing—original draft: SMB, ZF. Writing—review & editing: MY.

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# Effects of human chorionic gonadotropin-producing peripheral blood mononuclear cells on the endometrial receptivity and implantation sites of the mouse uterus

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**Objective:** This research investigated the effects of human chorionic gonadotropin (HCG)-producing peripheral blood mononuclear cells (PBMCs) on the implantation rate and embryo attachment in mice.

**Methods:** In this experimental study, a DNA fragment of the *HCG* gene was cloned into an expression vector, which was transfected into PBMCs. The concentration of the produced HCG was measured using enzyme-linked immunosorbent assay. Embryo attachment was investigated on the co-cultured endometrial cells and PBMCs *in vitro*. As an *in vivo* experiment, intrauterine administration of PBMCs was done in plaque-positive female mice. Studied mice were distributed into five groups: control, embryo implantation dysfunction (EID), EID with produced HCG, EID with PBMCs, and EID with HCG-producing PBMCs. Uterine horns were excised to characterize the number of implantation sites and pregnancy rate on day 7.5 post-coitum. During an implantation window, the mRNA expression of genes was evaluated using real-time polymerase chain reaction.

**Results:** DNA fragments were cloned between the BamHI and EcoRI sites in the vector. About 465 pg/mL of HCG was produced in the transfected PBMCs. The attachment rate, pregnancy rate, and the number of implantation sites were substantially higher in the HCG-producing PBMCs group than in the other groups. Significantly elevated expression of the target genes was observed in the EID with HCG-producing PBMCs group.

**Conclusion:** Alterations in gene expression following the intrauterine injection of HCG-producing PBMCs, could be considered a possible cause of increased embryo attachment rate, pregnancy rate, and the number of implantation sites.

**Keywords:** Embryo implantation; Human chorionic gonadotropin; Infertility; Peripheral blood mononuclear cell

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## Introduction

Despite improvements in assisted reproductive technology, the overall fertilization rate in infertile patients referred to infertility centers remains low [1,2]. The failure of embryo implantation in the uterus is a critical factor in patients with infertility. The secretion of steroid hormones prepares the endometrium for embryo receptivity and implantation; these hormones stimulate structural and functional changes in the endometrium and ready the uterus for implantation.

However, steroid hormone supplements administered to women with infertility problems do not have a considerable effect on endometrial function [3,4]. Studies have shown that local immune cells at the implantation site have an important role in the success of implantation. Peripheral blood mononuclear cells (PBMCs) consist of lymphocytes (B and T cells) and monocytes. PBMCs produce various cytokines, such as interleukin (IL)-1, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), and IL-6; these cytokines have a positive impact on the endometrial receptivity of embryos in humans [5,6]. In addition, PBMCs are involved in hemochorial placentation by regulating initiation, controlling invasion, and adjusting immune tolerance in embryo implantation [5]. Hence, immune therapy can be an effective approach for the enhancement of endometrial receptivity [5,6]. Human chorionic gonadotropin (HCG), as a steroid hormone, can initialize arrays of molecular messages between the decidua and blastocyst. HCG promotes progesterone production by corpus luteal cells and also boosts angiogenesis in the uterine vasculature through the activation of endometrial cells [7]. Yoshioka et al. [8] found that intrauterine injection of autologous PBMCs activated with HCG could raise implantation, pregnancy, and live birth rates in infertility patients. Pourmoghadam et al. [9] demonstrated that intrauterine administration of autologous HCG-activated PBMCs in women with recurrent implantation failure (RIF) who had a low Th-17/Treg cell ratio significantly increased as compared to the control group. Furthermore, Okitsu et al. [10] showed that the intrauterine injection of autologous PBMCs beneficially boosted embryo implantation in patients who received frozen-thawed embryo transfer after three or more repeated *in vitro* fertilization (IVF) failures. Another study by Li et al. [11] showed that the intrauterine administration of HCG-activated autologous human PBMCs promoted the live birth rate in frozen/thawed embryo transfer cycles of patients with RIF.

The numerous changes that occur in the endometrium, wherever PBMCs impact endometrial receptivity, may explain these improvements [5,12]. Consequently, we conclude that peripheral immune cells receive signals of the conceptus in the initial stage of pregnancy that activate PBMCs. Activated PBMCs are useful in the expression and production of immune molecules at the implantation site. Immune molecules adjust endometrial receptivity in the maternal-embryonic interface for implantation. All previous studies on this topic have focused on the role of HCG-activated PBMCs by incubating PBMCs with HCG in embryo implantation. We designed this study from a new perspective, exploring the use of HCG-producing PBMCs to increase embryo implantation. Therefore, our objective in the current study was to assess the impact of HCG-producing PBMCs on embryo attachment, the number of implantation sites, and the pregnancy rate in the mice uterus. Furthermore, we investigated the effects of HCG-producing PBMC on the gene expression level of *Il-6*, *Il-1 $\beta$* , leu-

kemia inhibitory factor (*Lif*), vascular endothelial growth factor (*Vegf*), matrix metalloproteinase 9 (*Mmp9*), Janus kinase 2 (*Jak2*), and signal transducer and activator of transcription 3 (*Stat3*) genes in the uterus and the underlying mechanisms.

## Methods

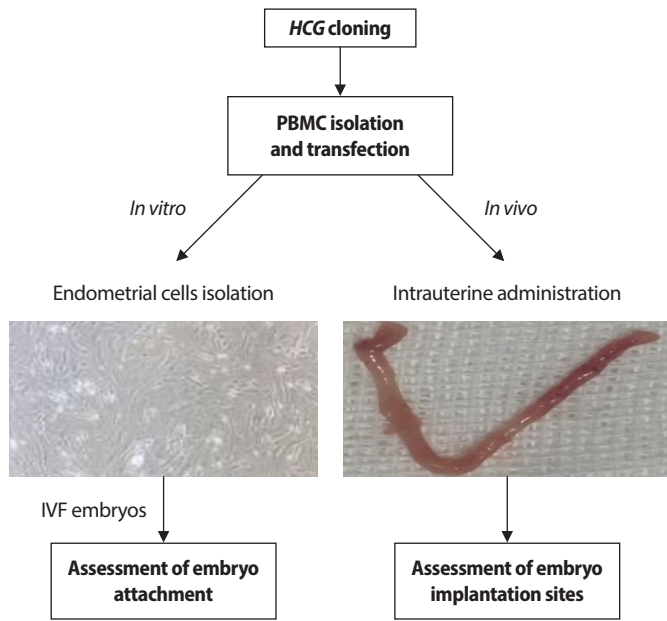
All materials were prepared from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. This study was approved by the Research and Ethics Committee of Shahid Beheshti University of Medical Sciences with the IR.SBMU.RETECH.REC. 1396.1297 design code (Tehran, Iran). B6D2F1 (C57BL/6 $\times$ DBA2) mice (Royan Institute, Tehran, Iran) were used in this experiment. B6D2F1 mice are hybrid and are better for reproductive studies because they have a higher pregnancy rate and release more suitable oocytes. The mice were maintained in standard conditions of a 12-hour light/12-hour dark cycle, at temperatures of 22°C–28°C temperature. Figure 1 provides an outline of the research workflow.

### 1. Cloning of the protein-coding HCG gene into an expression vector

The *HCG* gene sequence was obtained from the Reference Sequence (RefSeq) database of the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). The *HCG* gene was then constructed in a pGH cloning vector (Bioneer, Daejeon, Korea). Plasmid expressing green fluorescent protein-N1 (pEGFP-N1) as an expression vector was selected for insertion of the synthetic *HCG* gene. First, the pGH and pEGFP-N1 vectors were digested with BamHI and EcoRI enzymes (Takara, Dalian, China). An agarose gel extraction kit (Qiagen, Hilden, Germany) was used for DNA extraction and purification of the *HCG* DNA. The purified DNA was inserted into the pEGFP-N1 vector by T4 DNA ligase, and the ligation reaction was transformed into an *Escherichia coli* top 10 competent cell. Clones of bacteria were assayed using colony polymerase chain reaction (PCR). Positive clones were confirmed by digestive enzymes (BamHI and EcoRI) along with DNA sequencing (sanger sequencing; ABI3500 Genetic Analyzer, Foster City, CA, USA).

### 2. PBMC isolation from non-pregnant mice and vector transfection in PBMCs

PBMCs were separated from non-pregnant mice by density gradient centrifugation, using Ficoll-Paque (Sigma Aldrich), collected and washed with phosphate-buffered saline (PBS) and then re-suspended in pre-warmed standard Roswell Park Memorial Institute RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin. pEGFP-N1-*HCG* and pEGFP-N1 (empty vector) vectors were used for transfection of the PBMCs.



**Figure 1.** Outline of research workflow. The *HCG* gene sequence was obtained from National Center for Biotechnology Information (NCBI) database, and synthesized and cloned into an expression vector. Peripheral blood mononuclear cells (PBMCs) were isolated from mouse blood and transfected with a recombinant vector that contained the *HCG* gene by electroporation. In the *in vitro* experiment: transfected PBMCs were co-cultured with endometrial cells and assessed for blastocyst attachment, then the expression gene was investigated by real-time polymerase chain reaction. In the *in vivo* experiment: transfected PBMCs were transmitted into uterine horns in the pregnant mice, on day 1.5 post-coitum, to assess the number of implantation sites. IVF, *in vitro* fertilization.

Transfection of PBMCs with a pEGFP-N1 vector containing the *HCG* gene was done according to our previous study procedure [13]. PBMCs were also transfected with the empty pEGFP-N1 vector (empty vector).

**3. Analysis of HCG concentration by enzyme-linked immunosorbent assay**

The concentration of produced HCG in the transfected PBMCs was estimated by the enzyme-linked immunosorbent assay (ELISA) method. The cell culture supernatants were harvested, and the amount of HCG (at a cell density of  $1 \times 10^6$  cells/mL) was determined by a commercially available ELISA kit (Biocompare, South San Francisco, CA, USA) every 24 hours.

**4. Endometrium isolation from pseudo-pregnant mice**

Epithelial and stromal cells were isolated from the mouse endometrium as follows: the vasectomized male BDF1 mice were mated

with female mice from the same strain. Vaginal plug-positive mice were considered pseudo-pregnant. The pseudo-pregnant mice were killed by cervical dislocation, and the uterus was removed and washed with PBS that contained 300 U/mL penicillin/streptomycin; after that, adipose and connective tissues were separated, and the uterine horns were maintained in pre-warmed medium supplemented with collagenase for 1 hour in a humidified atmosphere of 5% CO<sub>2</sub> and 37°C. Later the uterine horns were scraped and placed in digestion media containing collagenase and dispase enzymes for 2–3 hours in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>. During this time, the tissue pieces were slowly mixed once every 30 minutes; afterwards, the cell suspension was precipitated by centrifugation at  $470 \times g$  for 5 minutes. The supernatant was transferred to 25 cm<sup>2</sup> tissue culture flasks with Dulbecco’s Modified Eagle Medium/Nutrient Mixture F12 plus 15% FBS (DMEM/F12) and maintained in a humidified incubator with a 5% CO<sub>2</sub> atmosphere at 37°C for 72 hours.

**5. Co-culture of isolated endometrial cells with PBMCs**

For co-culture of isolated endometrial cells with PBMCs, passage-2 primary endometrial cells ( $1 \times 10^6$  cells/mL) were added in DMEM/F12 medium supplemented with 15% FBS, 100 nmol/L beta-estradiol, and 10 nmol/L progesterone and placed in a humidified incubator with a 5% CO<sub>2</sub> atmosphere at 37°C for 3 days (72 hours). Endometrial cells and PBMCs were prepared at a 1:1 ratio for the co-culture cells. Isolated endometrial cells without PBMCs were applied as the control group.

**6. *in vitro* model system for the study of mouse embryo attachment**

*1) In vitro fertilization procedure*

On the IVF day, male BDF1 mice were killed by cervical dislocation. Spermatozoa were separated from the cauda epididymis and vasa deferentia. Sperm suspensions were isolated and maintained in a human tubal fluid medium (HTF) containing 4 mg/mL bovine serum albumin (BSA) and incubated for 45 minutes at 37°C. Subsequently, the 6- to 8-week-old female BDF1 mice were superovulated by intraperitoneal injections of 10 IU pregnant mare serum gonadotropin and after 50 hours, to obtain relatively large numbers of oocytes 10 IU hCG were injected intraperitoneally. Next matured MII oocytes were separated from the ampulla of the oviducts of female mice and placed in drops of 100 µL HTF medium supplemented with 4 mg/mL BSA. Immediately, approximately  $1 \times 10^6$  sperm/mL was added to the HTF drops and incubated under the above incubation conditions for 6 hours. After 6 hours, the hypothetical fertilized zygotes were transferred to the potassium simplex-optimized medium containing 4% BSA. One day after IVF, the number of two-cell embryos was counted to assess the fertilization rate by an optical microscope. Four days after IVF, the oocytes were ultimately investigated for blastocyst



formation [14].

## 2) Assessment of embryo attachment

The co-cultured cells (endometrial cells with PBMCs) were seeded in DMEM/F12 medium and incubated for 72 hours. Next, zona-free expanded blastocysts, obtained using Acid Tyrode's solution ( $n = 10$ ), were placed on the cultured cell plates and incubated for 72 hours. The cell culture plates were divided into three groups (endometrial cells, co-culture of endometrial cells with PBMCs, and co-culture of endometrial cells with HCG-producing PBMCs [transfected PBMCs]) and were swirled in a circular motion once every 24 hours, and the numbers of embryos that stayed at the same location were counted for the blastocyst attachment rate. Unattached blastocysts were floated in the medium.

## 7. In vitro animal model for intrauterine transmission of mouse PBMCs into the uterus

### 1) Embryo implantation dysfunctional mouse model establishment

To assess the role of HCG-producing PBMCs on implantation and pregnancy rate, embryo implantation dysfunctional (EID) female B6D2F1 mice models were created using a small dose of mifepristone (0.08 mg/0.1 mL, soluble in propanediol). Mice were randomly divided into three groups: control (without PBMC and mifepristone), EID, EID with PBMCs, EID with HCG-producing PBMCs, and EID with produced HCG. The super-ovulated mice were mated with male mice at a ratio of 2:1. The vaginal plug-positive mice were selected, since the vaginal plug is a symptom of mating, and pregnant mice were identified on the day the vaginal plug was detected, considered as day 0.5 post-coitum. At 9:00 am on day 3.5 post-coitum, 0.1 mL of mifepristone was injected subcutaneously to all groups except the control group [15].

### 2) Intrauterine administration of transfected PBMCs and assessment of implantation site and pregnancy rate

PBMCs and HCG-producing PBMCs were prepared in medium culture as described above. On day 1.5 post-coitum, mice were anesthetized and 5  $\mu$ L ( $10^6$  cells/mL) of HCG-producing PBMCs were injected surgically into the cranial part of each uterine horn using a micro-syringe in the EID with HCG-producing PBMCs group. The EID with PBMCs and EID with HCG groups also received 5  $\mu$ L of PBMCs ( $1 \times 10^6$  cells/mL) and 450 pg/mL of produced HCG, respectively. An empty culture medium was used in the control and EID groups. On day 3.5 post-coitum at 22:00, some of the studied mice in each group were sacrificed. Next, the uterus was isolated and cryopreserved at  $-70^\circ\text{C}$  for a real-time PCR test, while the other studied mice were sacrificed on day 7.5 post-coitum and the bifurcated uterus was removed. The numbers of pregnant mice and implantation sites

were assessed in each group.

### 3) RNA extraction, reverse transcription, and real-time PCR

Total RNA was extracted from endometrial tissues using a commercial mini kit (GeneAll Hybrid-R RNA Purification Kit, Seoul, Korea) according to the manufacturer's instructions. The quantity and quality of the RNA were confirmed by spectrophotometry (Eppendorf, Hamburg, Germany) and gel electrophoresis. Samples were reverse-transcribed by the protocol described in previous studies [13]. Briefly, 3  $\mu$ L of random hexamers, 5  $\mu$ L of nuclease-free water, and 4  $\mu$ L of RNA were added to each microtube. The microtubes were incubated in a GeneMate series thermal cycler (Fgeng02FT, Saint Paul, MN, USA) for 5 minutes at  $75^\circ\text{C}$ . The tubes were held on ice and cDNAs were synthesized using  $5 \times$  reverse-transcriptase buffer, 200 U reverse transcriptase (RT) enzyme, 10 mmol/L dNTP, and 10 U RNase inhibitor. The reverse transcription reaction was carried out as follows:  $25^\circ\text{C}$  for 10 minutes,  $37^\circ\text{C}$  for 15 minutes,  $42^\circ\text{C}$  for 45 minutes, and  $72^\circ\text{C}$  for 10 minutes. DNA Master SYBR Green I Mix (Yekta Tajhiz, Tehran, Iran) was used for the quantitative real-time PCR (qRT-PCR) reactions for *Il-6*, *Il-1 $\beta$* , *Lif*, *Vegf*, *Mmp9*, *Jak2*, and *Stat3* according to the manufacturer's recommendations. Amplification was performed in the following conditions:  $95^\circ\text{C}$  for 2 minutes,  $95^\circ\text{C}$  for 5 seconds,  $60^\circ\text{C}$  for 30 seconds,  $72^\circ\text{C}$  for 10 seconds, and 40 cycles of extension. Reverse transcription polymerase chain reaction (RT-PCR) was carried out in a Step One instrument (Applied Biosystems, Waltham, MA, USA) with beta-2-microglobulin (beta-2m) as the reference gene. Relative expression was estimated by the  $2^{-\Delta\Delta\text{CT}}$  method [16]. The primer information is listed in Table 1.

## 8. Statistical analysis

Data analysis was carried out with IBM SPSS ver. 20 (IBM Corp., Armonk, NY, USA). One-way analysis of variance and the Tukey test were used to calculate the rate of blastocyst attachment and implantation sites. REST 2009 Software (Qiagen, Hilden, Germany) was used to analyze RT-PCR data. A  $p$ -value  $< 0.05$  indicated statistical significance.

## Results

### 1. Confirmation of cloned DNA

The accuracy of cloned *HCG* was checked by colony PCR, enzymatic digestion, and sequencing. Colony PCR was done with universal PE primers F: 5' CGCAAATGGCGGTAGGCGTG 3' and R: 5' GGCCCCGTTACGTCGCCGTC 3'. A PCR product of approximately 670 bp proved the correctness of cloning in the expression vector (Figure 2A). The target insert band (560 bp) was determined in the enzyme digestion in Figure 2B, after double digestion with the BamHI and EcoRI enzymes.

**Table 1.** List of primers employed in quantitative real-time polymerase chain reaction

Primer name	Sequence
<i>Lif</i>	F: CCCATCACCCCTGTAAATGCC R: CGCACATAGCTTTCCACGTTG
<i>Vegf</i>	F: TCATGCGGATCAAACCTCAC R: CGGGATTCTTGCGCTTTCG
<i>Mmp-9</i>	F: CTCTGCATTCTTCAAGGACGG R: TACACCACATTTGACGTCCAG
<i>Jak2</i>	F: CTGTCATCCGTGATCTTAACAGC R: CACTCCCGAAGTTACCTTTGCC
<i>Stat3</i>	F: TTCAACATCAGCAGCAACCACC R: TTCTTCAGCACCTCCATCACCC
<i>Il-6</i>	F: CTTCATCCAGTTGCCTTCTTG R: AATTAAGCCTCCGACTTGTTC
<i>Il-1β</i>	F: GGCTCCGAGATGAACAACAAAA R: CACTTTGCTCTTGACTTCTATCT
<i>Beta 2m</i>	F: GCTATCCAGAAAACCCCTC R: CCCGTTCTTCAGCATTG

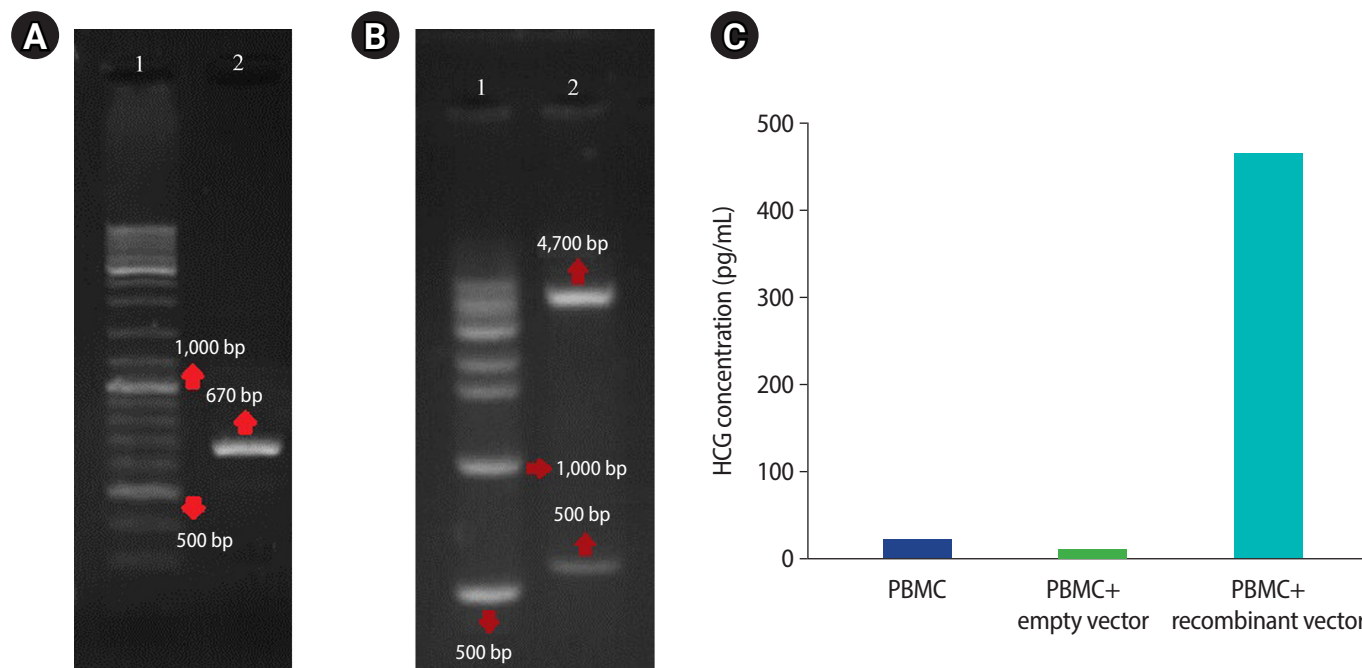
*Lif*, leukemia inhibitory factor; *Vegf*, vascular endothelial growth factor; *Mmp-9*, matrix metalloproteinase9; *Jak2*, janus kinase 2; *Stat3*, signal transducer and activator of transcription 3; *Il-6*, interleukin 6; *Il-1β*, interleukin 1 beta; *Beta 2m*, beta-2-microglobulin; F, forward; R, reverse.

## 2. Transfection of PBMCs with recombinant vector and production of HCG

PBMCs were transfected with a recombinant vector using an electroporation procedure. Protein production observed in transfected PBMCs was high at 48 hours after transfection, and the highest concentration of produced HCG was 465 pg/10<sup>6</sup> cells in transfected PBMCs (Figure 2C).

## 3. Isolation of endometrium cells and investigation of *in vitro* blastocyst attachment

The microscopic examination of isolated endometrial cells showed that the epithelial cells were cuboidal to columnar and the stromal cells were spindle-shaped. Table 2 presents the developmental competence rate in IVF; approximately 84% of embryos developed into blastocysts. The blastocysts (no zona pellucida) were attached and migrated to the co-cultured cells within 24–72 hours, and the attachment and migration of the blastocysts are shown in Figure 3A–C. The blastocyst attachment rate was calculated as 82.5% ± 2.4% in the transfected PBMCs (HCG-producing PBMC) group, 59.5% ± 0.74% in the PBMCs group, 35% ± 1.92% in the endometrial cells with produced HCG group, and 26.11% ± 1.4% in the control group (endome-



**Figure 2.** (A) Colony polymerase chain reaction for the confirmation of *HCG* gene cloning in the plasmid expressing green fluorescent protein-N1 (PEGFP-N1) vector. Lane 1: DNA ladder (1 kb). Lane 2: positive clone (670 bp). (B) Enzymatic digestion of the PEGFP-N1-HCG vector (recombinant vector) by BamHI and EcoRI. Lane 1: DNA ladder (500 bp) and Lane 2: the lower band (560 bp) is the *HCG* gene, and the upper band (4,700 bp) is the PEGFP-N1 vector. (C) The enzyme-linked immunosorbent assay method was applied to determine the concentration of produced HCG in the different groups. HCG, human chorionic gonadotropin; PBMC, peripheral blood mononuclear cell.

trial cells without PBMCs and HCG). The results showed that the percentage of attached blastocysts was significantly higher in the transfected PBMCs group compared with the other groups (Figure 3D).

#### 4. Analysis of the number of implantation sites in the presence and absence of produced HCG by PBMCs

To determine the uterine receptivity, the pregnancy rate and the number of implantation sites were investigated on day 7.5 post-coitum. The pregnancy rate was lower in the EID group (33.33%) than in the control group (88.8%) ( $p=0.012$ ). The pregnancy rates in the EID with produced HCG (77.7%) ( $p=0.038$ ), EID with PBMCs (77.7%)

( $p=0.031$ ), and EID with HCG-producing PBMCs (88.8%) ( $p=0.021$ ) groups were higher than in the EID group (Figure 4A). The number of implantation sites in the EID group ( $1.66 \pm 1.52$ ) was also lower compared to the control group ( $12.33 \pm 5.1$ ) ( $p=0.014$ ). After the intra-uterine administration of produced HCG and PBMCs, the number of implantation sites was higher than in the EID group. Specifically, the numbers of implantation sites in EID with produced HCG group and EID with PBMCs were  $6.0 \pm 1.0$  ( $p=0.04$ ) and  $8.3 \pm 1.52$  ( $p=0.032$ ), respectively. The EID with HCG-producing PBMCs group showed a remarkably higher implantation site number ( $14.33 \pm 2.51$ ) ( $p=0.001$ ) than the EID group. Examples of the implantation sites of pregnant

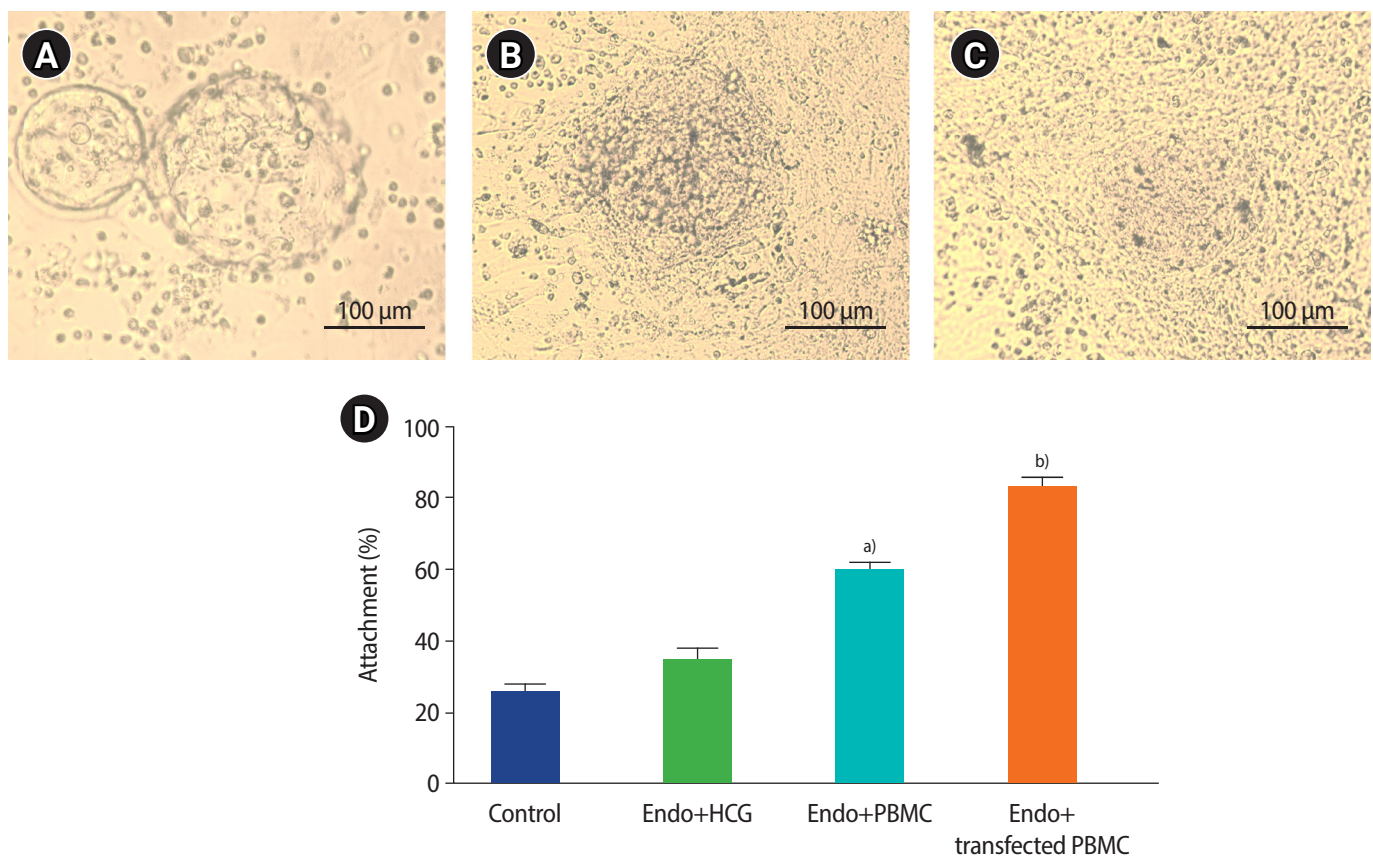
**Table 2.** Developmental competence rate for mouse embryos after IVF

Group	Number of oocytes	Two-cell stage (%)	Four-cell stage (%)	Eight-cell stage (%)	Morula (%)	Blastocyst (%)	$p$ -value <sup>a)</sup>
IVF	120	$95.8 \pm 0.61$	$91.6 \pm 1.8$	$90.0 \pm 2.5$	$87.5 \pm 2.65$	$84.1 \pm 1.7$	0.044

Values are presented as mean $\pm$ standard error.

IVF, *in vitro* fertilization.

<sup>a)</sup>One-way repeated-measures analysis of variance.



**Figure 3.** Assessment of blastocyst attachment in the co-cultured endometrial cells and peripheral blood mononuclear cells (PBMCs). (A-C) Attachment and migration of the blastocysts occurred within 24–72 hours. (D) The percentage of blastocyst attachment in the study groups. Values are presented as mean $\pm$ standard deviation. <sup>a)</sup> $p=0.014$  compared with control group; <sup>b)</sup> $p=0.002$  compared with control group; analysis of variance, Tukey test. Endo, endometrial cells; HCG, human chorionic gonadotropin.



mice in each group are shown in Figure 4B-F on day 7.5 post-coitum.

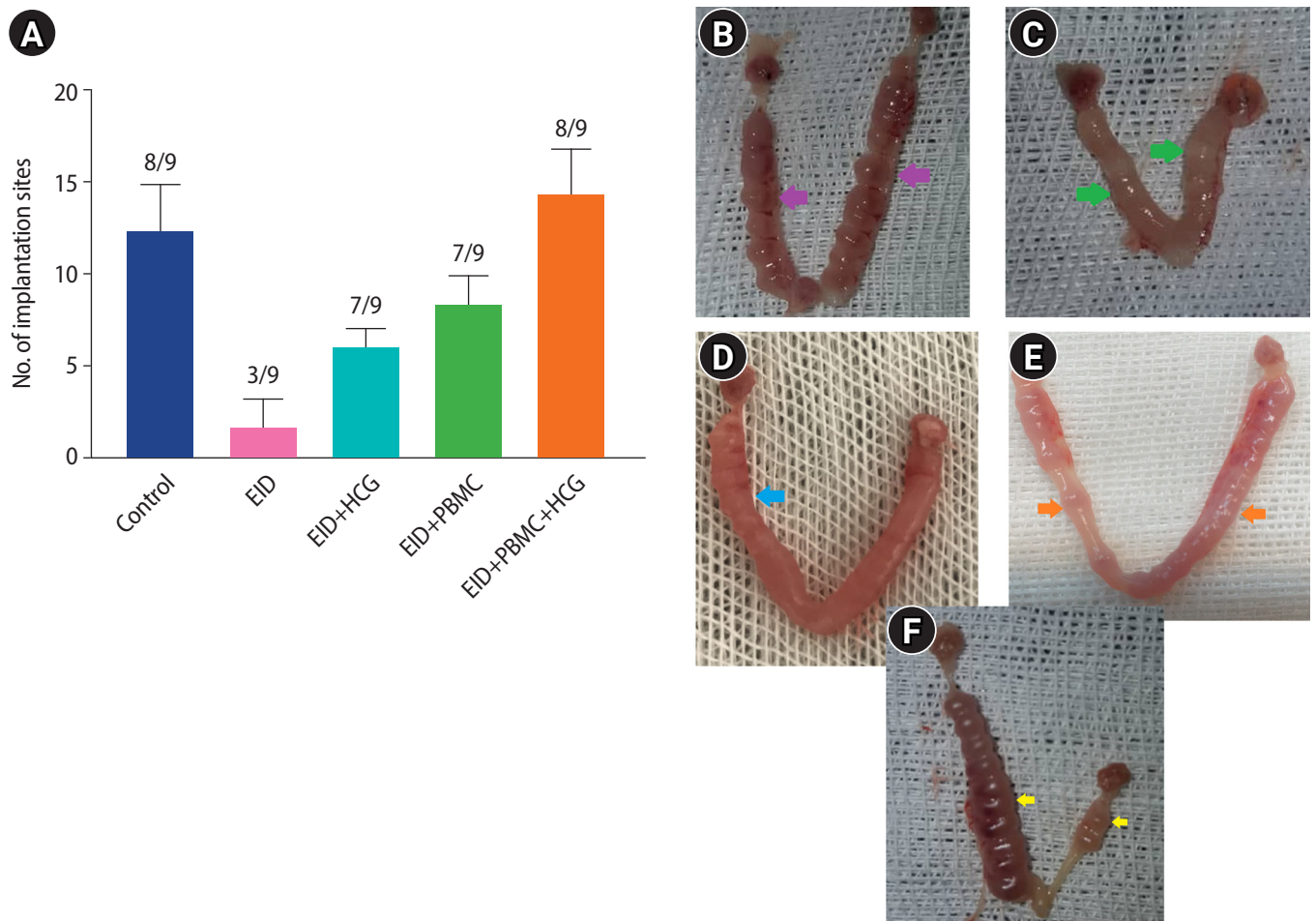
### 5. Monitoring gene expression by qRT-PCR

The expression levels of *Mmp-9*, *Lif*, *Jak2*, *Stat3*, *Vegf*, *Il-β*, and *Il-6*, normalized by the level of beta-2m expression, were assessed in the endometrial tissues. The qRT-PCR analysis showed that *Mmp-9*, *Lif*, *Jak2*, *Stat3*, *Vegf*, *Il-β*, and *Il-6* mRNA was upregulated in the endometrium of mice during the implantation window in the control group, EID, EID with HCG, EID with PBMCs, and EID with HCG-producing PBMCs (EID+recombinant vector) groups. Compared with the EID group, *Lif*, *Jak2*, *Stat3*, *Vegf*, *Il-β*, and *Il-6* mRNA expression levels in the control group were significantly higher. The mRNA expression levels of genes were higher in the EID with produced HCG group, EID with PBMCs, and EID with HCG-producing PBMCs (EID +recombinant

vector) groups than in the control group (Figure 5). The assessment of gene expression with real-time PCR was repeated three times.

### Discussion

The importance of implantation as a crucial process in reproduction has been proven, and one of the main reasons for the failure of embryo implantation in RIF patients is that the embryo does not implant well in the uterus [1,2]. In the maternal-fetal interface at the site of implantation, the presence of immune-related cells plays an important role in the creation of immune tolerance and moderate inflammatory response during implantation [17]. Our result showed that uterine administration of HCG-producing PBMCs had positive



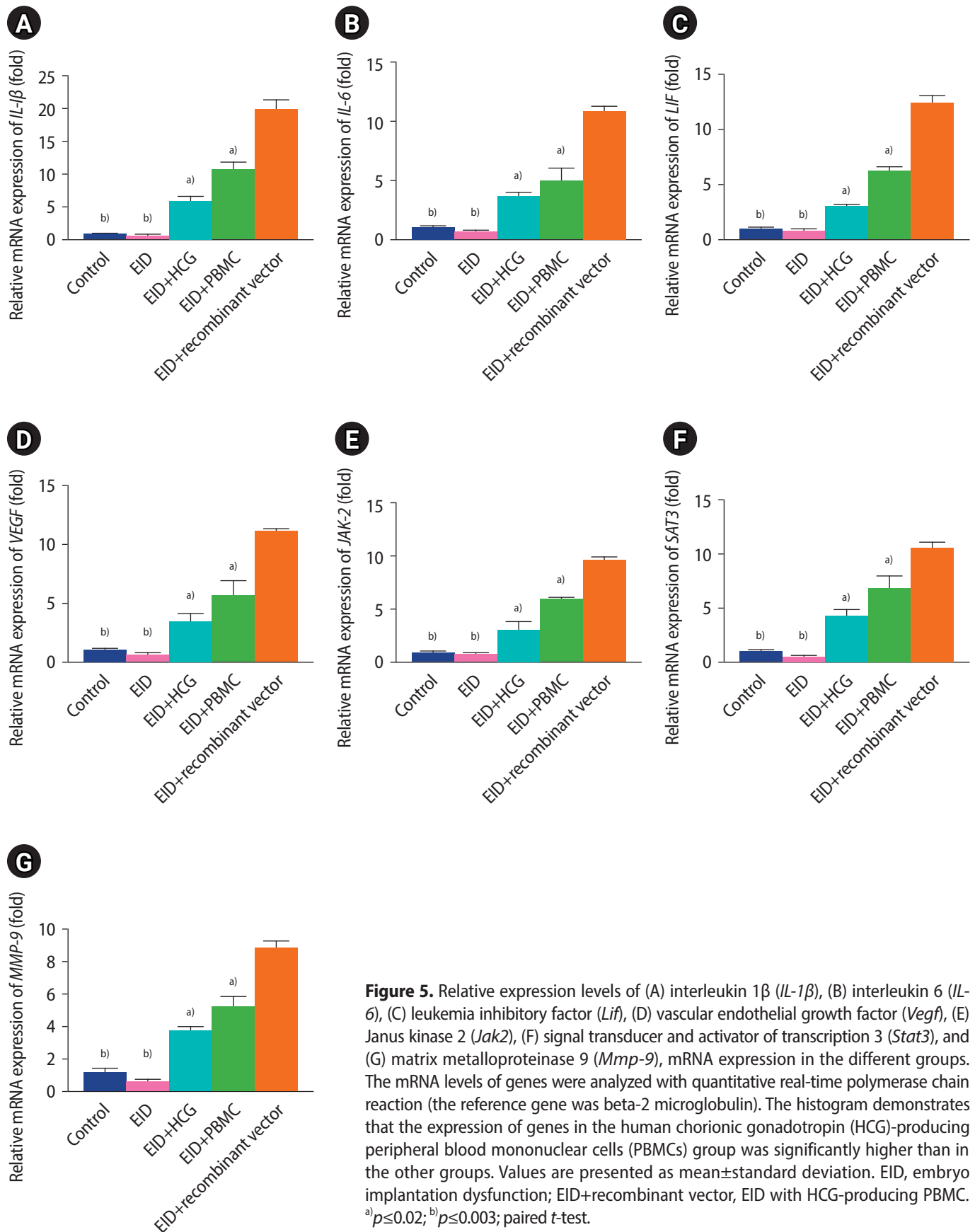
**Figure 4.** *In vivo* animal experiments. The histogram shows pregnancy rates. (A) Implantation sites on day 7.5 post-coitum in the different groups. In the transfected peripheral blood mononuclear cells (PBMCs) with recombinant vector (human chorionic gonadotropin [HCG]-producing PBMCs), the pregnancy rate and the number of implantation sites were significantly higher than in the other groups (analysis of variance, Tukey test). The number of pregnant mice out of the tested mice is shown, for example (8/9) means that out of 9 mice tested, 8 mice became pregnant. Values are presented as mean±standard deviation. (B-F) Examples of implantation sites in the uteri of studied pregnant mice in each group on day 7.5 post-coitum: (B) control, (C) embryo implantation dysfunction (EID), (D) EID+PBMCs, (E) EID+HCG, (F) EID+PBMC+HCG (HCG-producing PBMCs) groups. Control group, 16 implanted embryos; EID group, 3 implanted embryos; EID+HCG group, 6 implanted embryos; EID+PBMC group, 9 implanted embryos; and EID with HCG-producing PBMC group, 12 implanted embryos.



impacts on embryo attachment, blastocyst implantation in the uterus, and pregnancy rates. Also, mRNA levels of *Mmp-9*, *Lif*, *Jak2*, *Stat3*, *Vegf*, *Il-1 $\beta$* , and *Il-6* were higher in the HCG-producing PBMCs group than in the PBMCs group. The abnormal immune environment of the endometrium is involved in improper embryo attachment [6]. Researchers have shown that 80% of patients with prior implantation failures had an imbalance of the immune system in the uterus. This issue could be a reason for the poor receptivity and attachment of the embryo into the endometrium [18]. It has been revealed that immune therapy such as lymphocyte therapy [19] or intravenous immunoglobulin [20] influences the development of pregnancy in RIF patients. Intrauterine injection of HCG-exposed PBMCs before transferring the embryo into the uterus has produced better pregnancy outcomes in RIF patients [11]. We found that intrauterine infusion of HCG-producing PBMCs could effectively enhance the number of implanted embryos in EID mice. It was also discovered that the attachment of blastocysts onto endometrial cells significantly increased in the presence of HCG-producing PBMCs. To investigate and determine the specific mechanism of HCG-producing PBMCs in the EID mice, we used qRT-PCR to examine the expression of the *IL-1 $\beta$* , *IL-6*, *Lif*, *Vegf*, *Mmp-9*, *Jak2*, and *Stat3* genes, which are involved in regulating embryo implantation. Our results showed that the presence of PBMCs and produced HCG upregulated the mRNA expression of inflammatory cytokines such as *IL-1 $\beta$*  and *IL-6*, and positively improved endometrial receptivity in the EID mice. PBMCs are engaged in the production of inflammatory cytokines, such as *IL-1 $\beta$*  and *IL-6* [15]. A study in baboons showed that intrauterine injection of *IL-1 $\beta$*  and chorionic gonadotropin induced changes in the endometrium that mimicked the early events in pregnancy [15,21]. The current study demonstrated that HCG-producing PBMCs stimulated the production of *IL-1 $\beta$*  and *IL-6*, and subsequently showed improvement in embryo implantation and endometrial receptivity. Inflammatory cytokines such as *IL-1 $\beta$*  and *TNF- $\alpha$*  noticeably influence the production and secretion of *Mmp-9* and *Mmp-2*, which increase the capability of human embryo invasion into the uterus [22,23]. Our data displayed that *Mmp-9* was downregulated in the EID group compared with the control group, but it was upregulated with PBMCs and HCG therapy. One member of the *IL-6* family is *LIF*, a cytokine associated with the Th2 response, which leads to the production of *IL-4* [24]. *LIF* regulates numerous activities, including endometrium preparation for implantation, decidualization, growth, and development of the embryo, interaction between uterus and blastocyst, attachment of trophoblast into the endometrium, and the regulation of immunity involved in pregnancy [25]. The vital role of *LIF* has led to suggestions that *LIF* could be used as a biomarker for the assessment of endometrial receptivity [26]. The binding of *LIF* to its receptor activates the *JAK/STAT* signaling cascade. Activation of the *JAK/*

*STAT* pathway is involved in regulating several biological responses such as cell growth, differentiation, longevity, and migration. Stimulating *Stat-3* through *LIF* affects the expression of various genes. The expression of these genes controls different actions, for example cytokines and signaling (*IL-6*, *OSMR*, *SOCS3*, and *JUNB*), adhesion (*CE-CAM1*, *PDPN*, and *ITGB3*), invasion (*PAPPA*, *caspase 1*, *SER PINB3*, *TIMP1*, *TIMP2*, and *TIMP3*), and angiogenesis (*ID1*, *ICAM1*, *EDIL3*, and *CCL2*) [27]. We observed that HCG-producing PBMCs upregulated the expression of *LIF* and the *Jak2/Stat3* genes. This suggests that intrauterine injection of these cells has a positive effect on uterine receptivity. *VEGF*, as a vascular permeability factor, regulates the proliferation of endothelial cells at the implantation site [28]. *VEGF* in rodents leads to permeability and uterine edema, and inhibition of *VEGF* is implicated in embryo development and implantation outcome [29]. Real-time gene expression analysis showed that during the implantation window, the expression level of *Vegf* was significantly upregulated in mice under conditions of EID with HCG-producing PBMCs. Our results showed that infusion of HCG-producing PBMCs into the uterus enhanced endometrial receptivity in mice; this increase in uterine receptivity may be due to improved physiological endometrial secretory changes or surface modification of expression molecules in endometrial cells [3].

Progesterone is one of the main factors in embryo implantation, and studies have indicated that the stimulation of progesterone production by luteal cells is mediated by PBMCs [4]. According to the evidence provided by previous studies, PBMCs receive information about the presence of the embryo at the sites of embryo implantation at the beginning of pregnancy. Next, PBMCs transfer information through the blood circulation into the ovaries and then regulate the function of the corpus luteum during pregnancy [30,31]. Therefore, PBMCs indirectly enable the embryo to be implanted in the uterus in keeping with HCG [32]. In pregnant women, PBMCs induce embryo invasion and also are directly involved in embryo outgrowth [32]. These results imply that PBMCs are very effective at indirectly regulating ovarian function and using directly inducing embryo invasion in early pregnancy [12,32]. A study showed that the spreading and invasion of mouse embryos were not affected by PBMCs and HCG; however, it was also observed that the presence of activated PBMCs with HCG in the uterus promoted the spreading and invasion of the embryos. This difference in outcomes may be related to a lack of chorionic gonadotropin production at the implantation site of the mouse embryos [32]. One of the limitations of our study was the different immunological environments of individual mice. Therefore, the use of this method in some people may not show a positive effect due to immunological dysfunction. Furthermore, our study did not keep track of the embryos for assessment of the live birth rate. Our results provide theoretical support for the potential clinical ap-



plication of PBMCs and HCG simultaneously in patients with RIF. The effect of using expression vectors on human cells must be investigated, and precautions for clinical use related to the insertion and expression of the vector must be carefully studied. In conclusion, this study has shown that the HCG-producing PBMCs enhanced the number of implantation sites and the rate of embryo attachment in the endometrial cells. Alteration in cytokine expression could be a possible cause of the high embryo attachment rate, pregnancy rate, and the number of implantation sites. The results of our study show that it is possible that HCG-producing PBMCs could be used for patients suffering from RIF.

## Conflict of interest

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

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# Evaluation of polyglycolic acid as an animal-free biomaterial for three-dimensional culture of human endometrial cells

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**Objective:** Animal-free scaffolds have emerged as a potential foundation for consistent, chemically defined, and low-cost materials. Because of its good potential for high biocompatibility with reproductive tissues and well-characterized scaffold design, we investigated whether polyglycolic acid (PGA) could be used as an animal-free scaffold instead of natural fibrin-agarose, which has been used successfully for three-dimensional human endometrial cell culture.

**Methods:** Isolated primary endometrial cells was cultured on fibrin-agarose and PGA polymers and evaluated various design parameters, such as scaffold porosity and mean fiber diameter. Cytotoxicity, scanning electron microscopy (SEM), and immunostaining experiments were conducted to examine cell activity on fabricated scaffolds.

**Results:** The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay and SEM results showed that endometrial cells grew and proliferated on both scaffolds. Immunostaining showed cytokeratin and vimentin expression in seeded cells after 7 days of culture. On both scaffolds, an epithelial arrangement of cultured cells was found on the top layer and stromal arrangement matrix on the bottom layer of the scaffolds. Therefore, fibrin-agarose and PGA scaffolds successfully mimicked the human endometrium in a way suitable for *in vitro* analysis.

**Conclusion:** Both fibrin-agarose and PGA scaffolds could be used to simulate endometrial structures. However, because of environmental and ethical concerns and the low cost of synthetic polymers, we recommend using PGA as a synthetic polymer for scaffolding in research instead of natural biomaterials.

**Keywords:** Fibrin-agarose; Human endometrial cells; Polyglycolic acid; Scaffold; Three-dimensional culture

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## Introduction

The endometrium is a unique tissue lining the uterus that plays a critical role in the reproductive system by preparing a site for embryo implantation [1]. Advances in designing experimental models have resulted in a better understanding of the human endometrial environment and function. For decades, standard two-dimensional monocultures were typically used to study endometrial cell function; however, these traditional cultures fail to represent the complex three-dimensional (3D) architecture of the tissue [2]. Recent advancements in tissue engineering have led to the development of



3D tissue constructs utilizing matrices and a scaffold-based approach. A scaffold provides a mechanical framework that mimics the extracellular matrix (ECM) composition for cell growth [3-7]. Developing 3D cell culture models improves the ability to investigate the cellular and molecular features of tissue function and allows more accurate research on therapeutics [8]. The optimal 3D culture system conditions vary widely for each cell type or cell line [9]. Natural biomaterials are highly biocompatible and readily available, showing great potential for cell viability. Hydrogel-forming natural polymers have several advantages, including biomimicking of the ECM and self-assembling capability. They have excellent biocompatibility due to their high hydration rate and ability to engage in diffusion and exchange, facilitating cell function and viability [10-15].

One of the most commonly used hydrogel substrates in tissue engineering is fibrin, which has advantages such as a low price, good interactions between cells and the biomaterial, and a fibrillary and porous pattern. Fibrin is easy to handle [16,17], and fibrin hydrogels can be created from the patient's plasma; thus, they can be considered as an autologous therapeutic product. Despite these advantages, the biomechanical properties of natural polymeric materials, such as fibrin hydrogels, are typically relatively poor compared to native tissues in terms of stiffness, flexibility, resistance, and strength. Hence, researchers have attempted to improve the biomechanical properties of fibrin hydrogels by combining them with another biomaterial. From this perspective, some studies have shown that the addition of agarose enhanced the biomechanical properties of fibrin hydrogels, mainly when chemical crosslinkers were used [18-22].

In tissue engineering, polymers derived from animal materials, such as fibrin and collagen, are used to create scaffolds structurally identical to those of the native ECM [23,24]. However, the dependence on animals has made these methods undesirable due to variability [25], environmental issues [26], and ethical concerns [27]. Non-animal or synthetic materials have emerged as a promising potential source for consistent, chemically defined, low-cost scaffolds. Synthetic or natural animal component-free polymers such as cellulose [28,29], chitin/chitosan [30], alginate [31], recombinant silk [32], polyglycolic acid (PGA) [1], polylactide [33], and polycaprolactone [34] provide low-cost, stable, and tunable scaffolds.

PGA is a common synthetic polymer that has been used to support diverse cell types, including fibroblasts and epithelial cells, to regenerate abdominal wall, urethral, and gut tissues [35-37]. The PGA polymer has good potential for high biocompatibility with reproductive tissues, and it is therefore a recommended suture material for perineal repair. It is also associated with a minimal tissue inflammatory response when used as a suture material for oral tissues compared to other sutures [38,39]. An electrospun PGA scaffold was selected to grow a 3D model of primary bovine endometrial epithelial

and stromal cells that reflected the endometrium's architecture [1]. However, PGA scaffolds have not yet been studied for the 3D culture of human endometrial cells. Considering the advantages and disadvantages of natural and synthetic scaffolds, this study was designed to compare the functional reconstitution of the human endometrium using epithelial and stromal cells between synthetic polymer scaffolds and natural scaffolds.

## Methods

### 1. Endometrial cell isolation

The Research Committee of Iran University of Medical Sciences reviewed and approved all aspects of this project with regard to ethical issues (IR.IUMS.REC 1396.32888). Written informed consent was obtained from all subjects. Endometrial biopsies (n = 10) were obtained using a pipeline aspirator (Prodimed, Neuilly-en-Thelle, France) from fertile women during the proliferative phase of the uterus. Healthy fertile women (with regular menstrual cycles and at least one spontaneous conception) aged 20–35 years volunteered to participate in this study. Women who used intrauterine devices or received hormonal therapy during the previous 3 months were excluded. None of the participants had any gynecological pathologies.

All tissue samples were collected and labeled in Hank's balanced salt solution (HBSS; Sigma, St. Louis, MO, USA) supplemented with 20% fetal bovine serum (FBS; Gibco, Waltham, MA, USA) and 1% penicillin/streptomycin (Sigma). The samples were processed immediately following primary isolation and washed by Dulbecco's phosphate-buffered saline (DPBS; Sigma). Next, the samples were minced into 1 × 1 mm pieces using a scalpel in a Dulbecco's modified Eagle medium/F-12 nutrient mixture (DMEM-F12; Sigma). The dissected tissue was incubated for 1 hour at 37°C in a 10 mL digest solution, containing trypsin/EDTA (2.5 BAEE units/mL, Sigma), collagenase I (2 mg/mL, Sigma), and DNase I (0.1 mg/mL, Sigma) in HBSS. After incubation, the digested tissue was strained in DMEM-F12 supplemented with 20% FBS (Gibco) and 1% penicillin/streptomycin (Sigma) through a 40 µm nylon mesh cell strainer. The stromal cells were passed through the filter and collected. The epithelial glands that were kept in the strainer were back-washed and collected. The stromal and epithelial glands were cultured into 75 cm<sup>2</sup> and 25 cm<sup>2</sup> culture flasks, respectively. All cell cultures were incubated at 37°C in a humid atmosphere with 5% CO<sub>2</sub>. Once the cell populations were ~70% confluent, they were frozen and stored in liquid nitrogen until further usage.

### 2. Three-dimensional endometrial cell co-culture

#### 1) Three-dimensional cultures using fibrin-agarose scaffolds

Approximately  $1.0 \times 10^6$  stromal cells were suspended in 32.7 µL

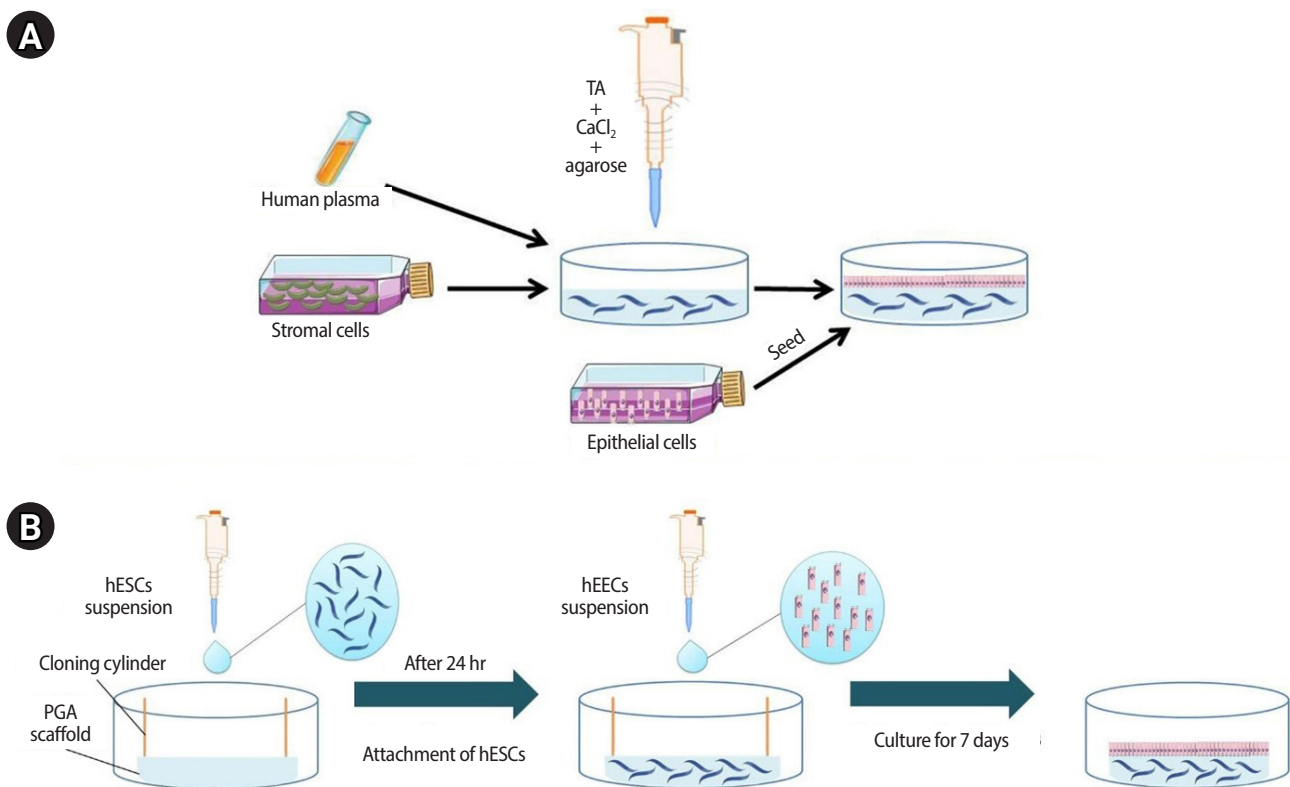
of culture media, and 400  $\mu\text{L}$  of human plasma was added to the mixture. In all steps of the 3D endometrial cell co-culture, 1 nM estrogen and 0.902 nM progesterone were added to the DMEM-F12 medium without phenol red, which was supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Sigma) and used as the culture medium. To prevent fibrinolysis, 3.8  $\mu\text{L}$  of tranexamic acid (Amchafibrin; Rottapharm, Monza, Italy) was added. After that, 25  $\mu\text{L}$  of melted type VII agarose (2% in DPBS) was added to achieve a final concentration of 0.1%. Finally, 38.5  $\mu\text{L}$  of 100 mM  $\text{CaCl}_2$  was added to start fibrin polymerization. The mixture (total volume of 500  $\mu\text{L}$ ) was instantly transferred to a 24-well cell culture plate and incubated at 37°C to solidify. After 20 minutes, 500  $\mu\text{L}$  of culture medium was added. Twenty-four hours after solidification,  $3.0 \times 10^5$  epithelial cells were seeded on top of the scaffold. This was a modified version of Alaminos' 3D rabbit cornea culture protocol [40]. Figure 1A presents a schematic diagram of the 3D cultures performed using the fibrin-agarose scaffold.

## 2) Three-dimensional cultures using PGA scaffolds

The first step was to fabricate PGA scaffolds by the electrospinning

process. A 11.5% w/v solution of PGA (Lakeshore Biomaterials; Birmingham, AL, USA) in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Fluka, Milwaukee, WI, USA) was prepared, and complete overnight dissolution was performed using an orbital shaker. The solution of PGA in HFIP was dispensed through four blunt 21-gauge steel needles connected to a 10 mL plastic syringe with two syringe pumps at a flow rate of 0.04 mL/min. A 20-cm-long, 5-cm-thick steel mandrel covered in a sheet of non-stick release paper was placed 15 cm from the needle tip and rotated at 50 rpm. An 11.0 kV electrical field was applied between the paper-covered mandrel and the needle tip to perform electrospinning. Fibers were collected on the paper-covered mandrel and formed a nonwoven scaffold sheet. The electrospinning process was performed at 19°C and around 38% humidity. When deposition ended, the scaffold was removed and dehydrated under vacuum at 25°C for at least 72 hours. Next, 13-mm discs were cut from these scaffolds and kept in moisture-barrier pouches including a desiccant. The pouches were sterilized using gamma irradiation.

For 3D endometrial cell co-culturing, the PGA scaffold (13-mm diameter) was fixed to the well of a culture plate by an 8-mm cloning cylinder. To pre-wet the scaffolds, we filled the cloning cylinder with



**Figure 1.** Diagrammatic scheme of the development of the three-dimensional (3D) endometrial culture systems. (A) A 3D matrix consists of stromal cells, human plasma, tranexamic acid (TA),  $\text{CaCl}_2$ , and agarose. After 24 hours, epithelial cells are seeded on the top of the 3D matrix to form a monolayer. (B) Human endometrial stromal cells (hESCs) cultured on the polyglycolic acid (PGA) scaffold. After 24 hours, human endometrial epithelial cells (hEECs) are seeded on the top of the scaffold.

300  $\mu\text{L}$  of DMEM-F12. Approximately  $1.0 \times 10^6$  stromal cells per scaffold in 200  $\mu\text{L}$  of culture medium were seeded onto the wetted scaffold. After 4 hours, another 3,000  $\mu\text{L}$  and 100  $\mu\text{L}$  of DMEM-F12 was added to the outer and inner parts of the cloning cylinder, respectively. The stromal cell-seeded PGA scaffolds were incubated at 37°C in a humid atmosphere with 5%  $\text{CO}_2$ . After 24 hours,  $3.0 \times 10^5$  epithelial cells were seeded upon the scaffold. The culture medium was changed every 48 hours. Scaffolds were cultured for up to 7 days before being removed from the culture medium for histological examinations on days 1, 2, 5, and 7. [Figure 1B](#) presents a schematic diagram of the 3D cultures using the PGA scaffold.

### 3. Cytotoxicity assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was applied to evaluate changes in the proliferation of viable cells seeded on the scaffolds. In the 24-well culture plates, the scaffolds consisted of epithelial and stromal cells that were free of culture medium and washed by DPBS. Next, 200  $\mu\text{L}$  of MTT solution (5 mg/mL) was mixed with 800  $\mu\text{L}$  of culture medium. The mixture was added to the scaffold and incubated at 37°C in a humid atmosphere with 5%  $\text{CO}_2$  for 3 hours. The MTT solution was removed. For cell lysis and the dissolution of formazan crystals, 200  $\mu\text{L}$  of dimethyl sulfoxide (DMSO) was added. Then, 100  $\mu\text{L}$  of DMSO-formazan solution was transferred to each well of a 96-well plate (SPL, Pocheon, Korea) and its optical density (OD) was measured at 570 nm absorbance with a plate reader (Polarstar Omega; BMG Labware, Aylesbury, UK). The MTT results for the scaffolds are reported as OD values.

### 4. Cell attachment assay

For the cell attachment assay, the culture medium was removed, and the scaffold structures were washed twice in DPBS for 5 minutes for fixation. The scaffolds were immersed in 2% paraformaldehyde for 5 minutes before being washed three times in DPBS. The scaffold structures were stored at 4°C in glutaraldehyde (2%) or paraformaldehyde (2%), respectively, for further processing (scanning electron microscopy [SEM] imaging and wax embedding).

#### 1) Immunohistochemistry

The embedded scaffolds were evaluated with dual immunocytochemistry. Epithelial cells were identified with rabbit anti-cytokeratin (Abcam, Cambridge, UK) and stromal cells with mouse anti-vimentin (Sigma). Goat anti-rabbit immunoglobulin G conjugated with FITC (Abcam) and rabbit anti-mouse polyclonal antibody conjugated with Texas red (Jackson ImmunoResearch, West Grove, PA, USA) used as secondary antibodies were diluted to 1:800 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). The scaffold

sections were de-waxed in xylene two times, for 2 minutes each. Then, a graded series of ethanol (100%, 90%, 70%, and 50%) and de-ionized water were used for rehydration (2 minutes each). The slides were incubated in boiling sodium citrate buffer (pH 6.0) for 3 minutes, and then cooled and washed in PBS with 0.025% Triton X-100 (Sigma). Next, they were blocked in blocking solution (5% donkey serum+PBS+1% BSA) for 2 hours. Diluted primary antibody (1:100 in PBS containing 1% BSA) was added to the slides and incubated overnight at 4°C. Later, the slides were washed three times in PBS containing 1% BSA for 5 minutes each. Diluted secondary antibody was applied to the slides and incubated at room temperature for 1.5 hours in darkness. The slides were subjected to three washes in PBS containing 1% BSA and mounted using 4',6-diamidino-2-phenylindole as the last step.

#### 2) Scanning electron microscopy

For SEM, the scaffold structures were rinsed three times in DPBS and fixed in 2% glutaraldehyde for 3 days at 4°C. Then after washing with deionized water, a graded series of ethanol (30%, 50%, 70%, 95%, 100%, and 100% dry ethanol) was used to dehydrate the scaffolds. After dehydration, the specimens were frozen in an ultra-low temperature freezer (-80°C) and moved to a freezer dryer (Edwards Super Modulyo) for drying. Then, specimens were mounted and sputter-coated with gold. Samples were observed under SEM (AIS2300; Seron Technology, Uiwang, Korea) that was run at 20.0 kV.

### 5. Statistics

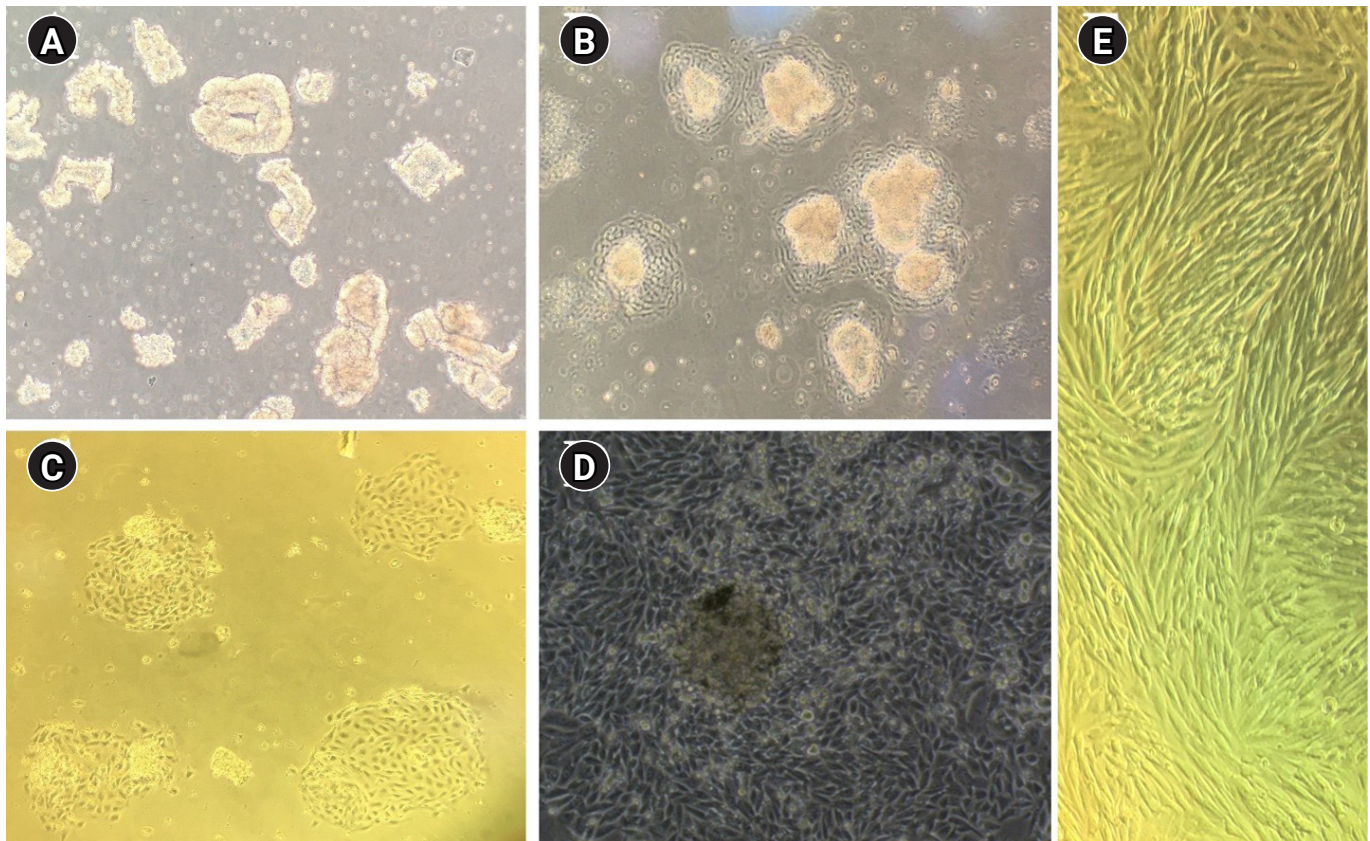
Data were presented as the mean  $\pm$  SEM and were analyzed using GraphPad Prism v9 (GraphPad, La Jolla, CA, USA). Data for the physical characteristics of the scaffolds and proliferation on scaffolds were compared using analysis of variance. A  $p$ -value  $< 0.05$  was considered to indicate statistical significance.

## Results

### 1. Isolation and culture of endometrial epithelial and stromal cells

Human endometrial tissues obtained by a pipelle aspirator were enzymatically digested and expanded in two-dimensional cultures to achieve a sufficient number of cells. As described, the retaining epithelial glands in the strainer were back-washed and cultured in 25  $\text{cm}^2$  culture flasks ([Figure 2A](#)). Epithelial glands attached to the flask within 24 hours ([Figure 2B](#)). Epithelial glands attached to each other extended and formed clusters that resembled islands ([Figure 2C](#)). These clusters eventually formed a monolayer of epithelial cells. In 5–7 days, a monolayer of confluent epithelial cells was achieved. At this stage, the epithelial cell monolayer folded over and formed





**Figure 2.** Light microscopy of endometrial cells. (A) Epithelial glands. (B) Initial attachment of epithelial cells into the flask. (C) Island-shaped clusters of epithelial cells. (D) Confluent monolayer of epithelial cells with a dome-like structure. (E) Confluent stromal cells.  $\times 50$  magnification.

dome-shaped structures (Figure 2D). The collected stromal cells from the filtrate were also cultured in a 75 cm<sup>2</sup> culture flask. After 12 hours, the culture medium was changed to remove tissue debris, blood cells, and unattached epithelial cells. In 3–4 days, the stromal cells were confluent (Figure 2E). The medium in the cell culture was changed every 48 hours.

## 2. Initial assessment of the scaffolds

Electrospun PGA fibers were assembled into a dense mesh-like layer, as visualized by SEM (Figure 3). The physical features of both PGA and fibrin-agarose scaffolds were compared (Figure 4). The mean diameter of the fibers and porosity properties were not significantly different (the mean diameter of the fibers was 10.43  $\mu\text{m}$  and 10.24  $\mu\text{m}$  in the PGA and fibrin agarose scaffolds, the mean pore diameter of the PGA and fibrin-agarose scaffolds was 52.06  $\mu\text{m}$  and 49.2  $\mu\text{m}$ , and the largest pore size diameter in the PGA and fibrin-agarose scaffolds was 101.35  $\mu\text{m}$  and 98.05  $\mu\text{m}$ , respectively). Cell proliferation on scaffolds seeded with stromal and epithelial cells was assessed using the MTT assay. The number of growing cells in the co-culture could not be calculated from the OD values because the co-culture did not

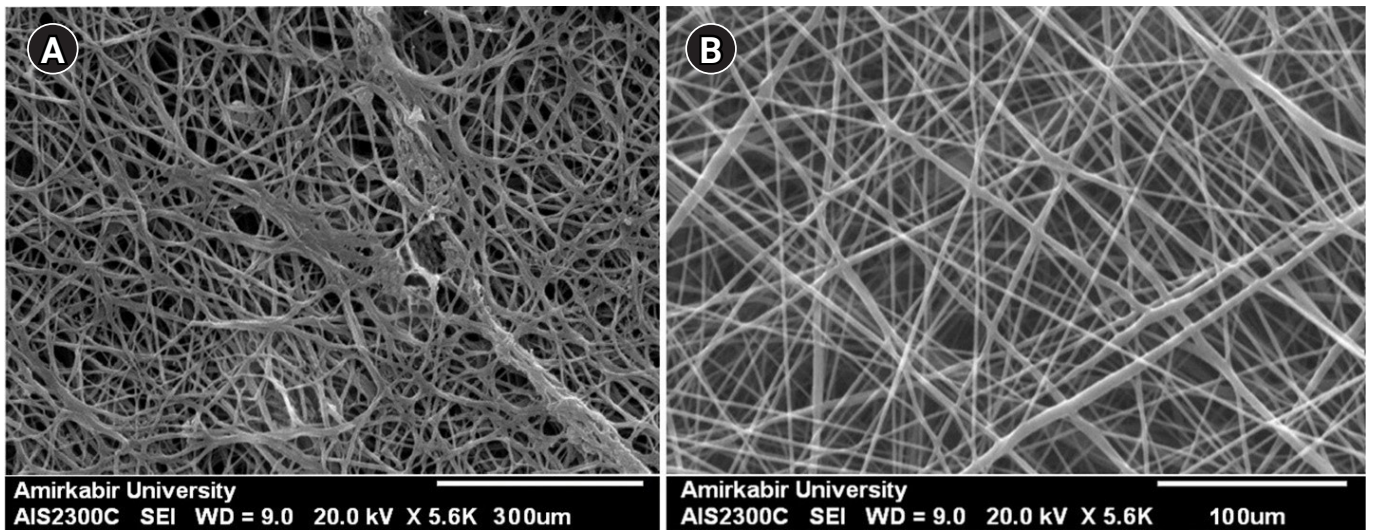
contain a unique cell type, and the MTT OD standard curve against cell number is specific for the cell type (Figure 5) [41].

The MTT OD of the fibrin-agarose scaffold seeded with stromal and epithelial cells increased during 7 days of cell culture ( $p < 0.05$ ). The MTT OD of the electrospun PGA scaffold seeded with stromal and epithelial cells also increased during 7 days of cell culture ( $p < 0.05$ ). The MTT OD of the fibrin-agarose scaffold was higher than that of the electrospun PGA scaffold at the same times of culture, but the difference was not statistically significant ( $p \geq 0.05$ ) (Figure 5).

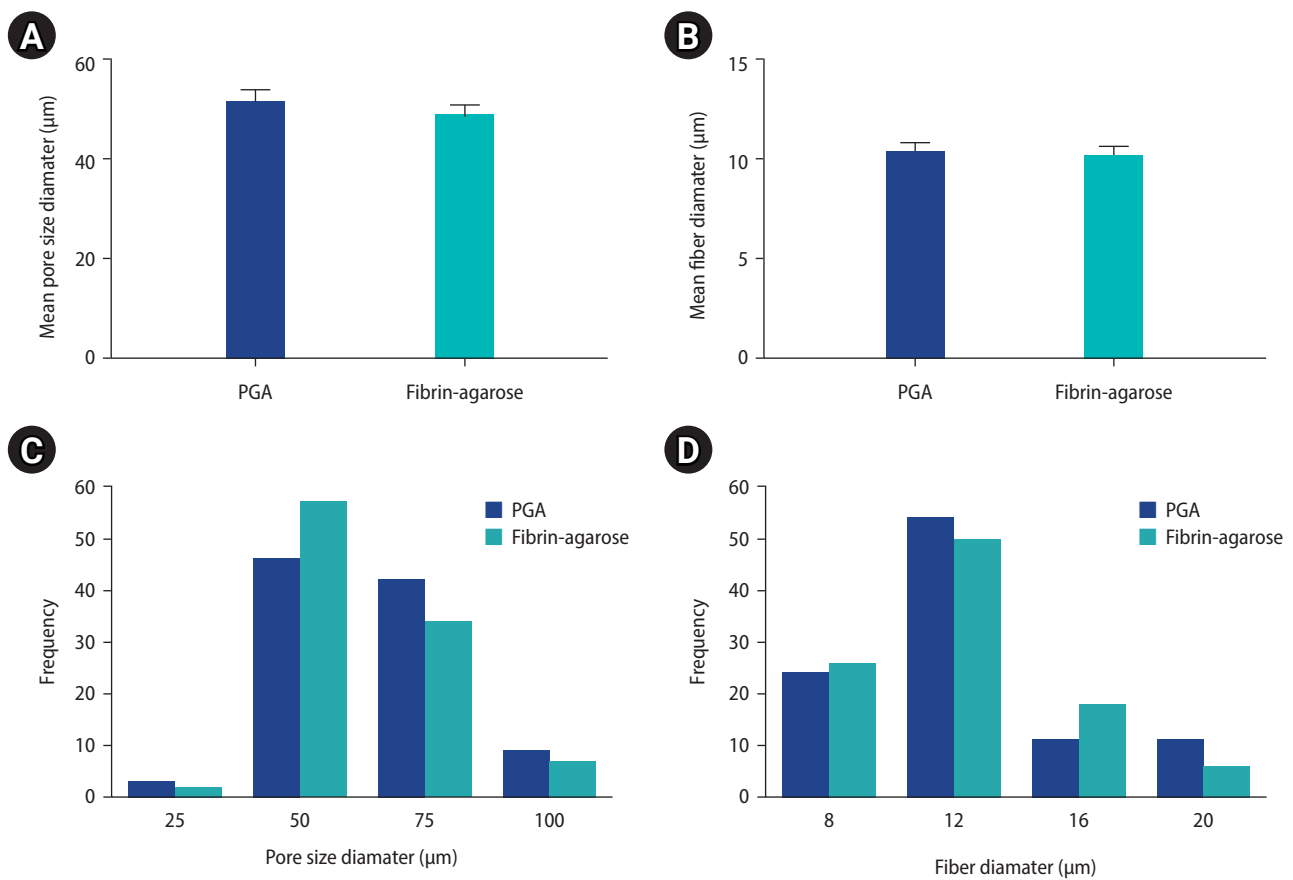
## 3. Description of the 3D co-culture of primary human endometrial epithelial and stromal cells

In the 3D culture, the origins and location of the epithelial and stromal cells were defined by cytokeratin and vimentin immunostaining, respectively. Immunohistochemistry (IHC) for cytokeratin was positive only for epithelial cells in the surface epithelium (Figure 6). IHC for vimentin was positive for the stromal cells located in the 3D matrix (Figure 6). On the top part of both 3D culture systems, epithelial cells formed a constricted cell monolayer. The stromal cells combined with the fibrin-agarose gel or PGA scaffolds became

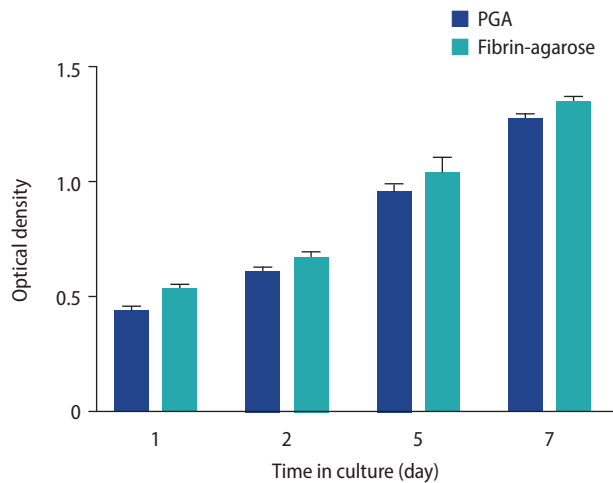




**Figure 3.** Scanning electron microscopy images of scaffolds prior to cell seeding. (A) Fibrin-agarose scaffold. (B) Electrospun polyglycolic acid scaffold.



**Figure 4.** Comparison of physical characteristics between the fibrin-agarose scaffold and the electrospun polyglycolic acid (PGA) scaffold. Comparison of the mean pore size diameter (A) and the mean fiber diameter (B) in two scaffolds; these parameters were not significantly different ( $p \geq 0.05$ ). Histogram of pore size diameter (C) and fiber diameter (D).



**Figure 5.** The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was used to measure the cell viability of stromal and epithelial cells co-cultured with the electrospun polyglycolic acid (PGA) scaffold or the fibrin-agarose scaffold. The MTT optical density of both the PGA and fibrin-agarose scaffolds seeded with stromal and epithelial cells increased during 7 days of cell culture. The cell viability did not show any significant between-group differences over time ( $p \geq 0.05$ ).

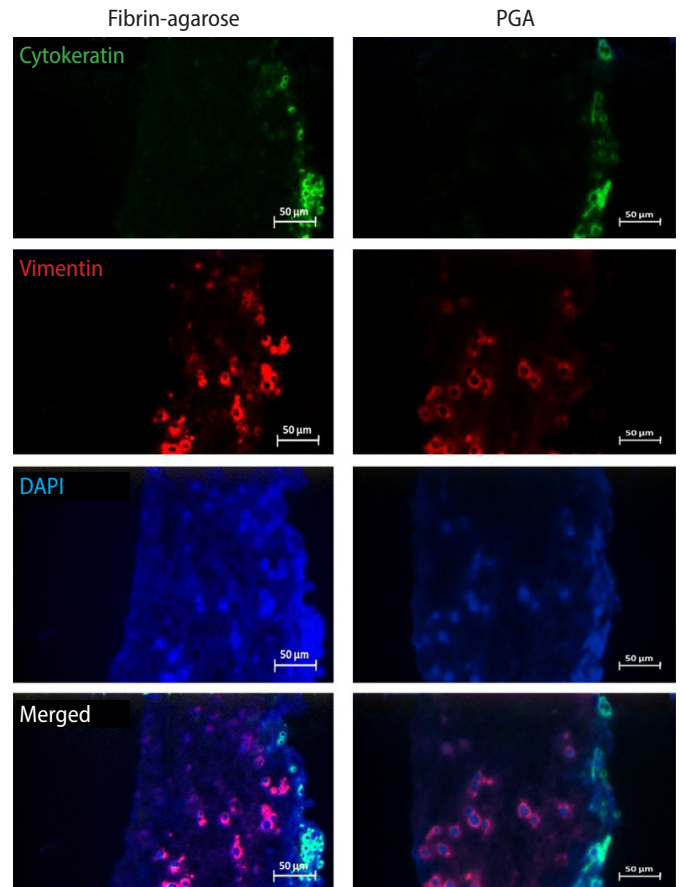
lengthened and expanded, displaying that the 3D culture systems supplied a suitable environment for the growth of endometrial cells.

These results showed that both scaffolds—fibrin-agarose and PGA—could simulate the structure of the human endometrium. SEM was used to assess epithelial cell growth and proliferation on the scaffolds' surfaces. The epithelial cell clusters were more distinct on the fibrin-agarose scaffold than on the PGA scaffold, which may indicate epithelial gland formation in this structure (Figure 7).

## Discussion

In this study, fibrin-agarose (a natural biomaterial) and electrospun PGA (a synthetic, animal-free polymer) were selected as scaffolds to support the proliferation and growth of human endometrial constructs. The results of the MTT assay showed that neither of these two scaffolds had a toxic effect on the survival of endometrial cells. IHC showed that stromal cells grew inside the scaffold and epithelial cells formed a monolayer on the matrix. These cells were able to grow on the scaffolds, and these scaffolds were able to create an endometrial-like structure, as IHC proved.

MacKintosh et al. [1] designed a new 3D culture system for bovine endometrial cells using electrospun PGA as a scaffold, and Wang et al. [42] used a fibrin-agarose scaffold to simulate the human endometrial structure. As shown by the results for cell adhesion and proliferation on the scaffolds, both scaffolds were suitable for primary human endometrial epithelial and stromal cells. The scaffolds main-

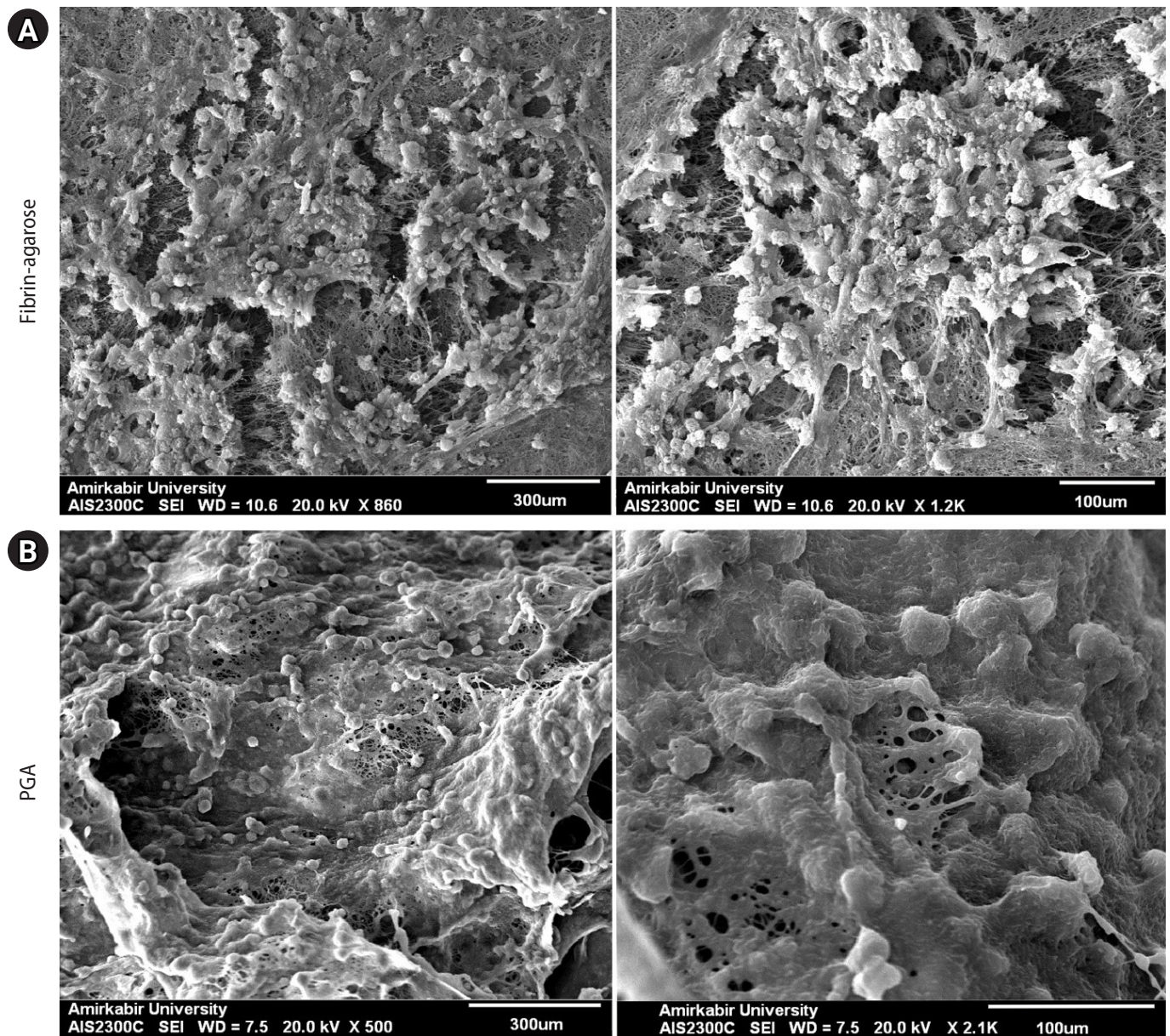


**Figure 6.** Immunohistochemistry images of co-cultured epithelial and stromal cells seeded on fibrin-agarose and electrospun polyglycolic acid (PGA) scaffolds on day 7 of culture. Cross-sectional expression of vimentin (red) or cytokeratin (green) by endometrial stromal and epithelial cells on both scaffolds, respectively. DAPI, 4',6-diamidino-2-phenylindole.

tained the growth of a monolayer of epithelial cells upon multiple layers of stromal cells, similar to natural endometrial tissue architecture. The PGA electrospun scaffold has a significant advantage, in that it synthetically mimics the ECM protein, collagen, and provides a perfect context to support the growth of tissue [43,44]. However, PGA nonwoven scaffolds have several limitations, such as a high degradation rate and poor mechanical properties. PGA electrospun scaffolds have been broadly used in the cell culture of different tissues, including human skin and vascular tissue and bovine endometrial and aortic endothelial cells [45,46]. However, to the best of our knowledge, this report is the first model of human endometrial cells growing on PGA electrospun scaffolds.

A scaffold is expected to support the attachment and growth of cells, provoke ECM deposition, and have proper porosity to facilitate gas diffusion, molecular signaling, and waste and nutrient products to enable the differentiation and survival of cells [47]. As demonstrat-





**Figure 7.** Evaluation of epithelial cell growth and proliferation on the surface of fibrin-agarose (A) and electrospun polyglycolic acid (PGA; B) scaffolds by scanning electron microscopy on day 7 of culture. Epithelial cell clusters were more distinct on the fibrin-agarose scaffold than on the PGA scaffold.

ed using IHC, both scaffolds simulated the human endometrial structure. In both culture systems, epithelial cells formed a single layer of tight cells on the top of the scaffolds. Stromal cells were embedded into the fibrin-agarose gel, or PGA scaffolds became extended and spread out, proving that these systems of 3D culture provided a proper environment for cell growth.

In an endometrial 3D model, Wang et al. [42] detected spontaneous gland formation by epithelial cells in the 3D constructs of stromal cells, probably from epithelial cell contamination within the population of stromal cells or from differentiation of uterine stem

cells into epithelial cells during culture. We did not recognize epithelial glands by IHC on either scaffold. However, SEM imaging showed that clustered forms of epithelial cells grew upon the fibrin-agarose scaffolds, representing endometrial glands. The proliferation of stromal cells co-cultured with epithelial cells was observed over time on both PGA electrospun and fibrin-agarose scaffolds. The capability of these scaffolds to facilitate the growth of both stromal and epithelial cells was proven using histology and the MTT assay. We focused on embedding stromal cells inside the scaffold, on top of which a suspension of epithelial cells would be seeded. The growth of stromal

cells within the scaffolds and growth of the epithelial cells on the superficial layer of the scaffold was confirmed using immunocytochemistry. On the electrospun scaffold, most stromal cells were concentrated on the upper part of the scaffold and developed into and on top of the scaffold, which led to an increase in the thickness of the entire structure. The growth and expansion of stromal cells within the scaffolds showed that the scaffold's porosity was adequate for cell transfer, and stromal cells were also detected in the deeper parts of the scaffolds.

Our results showed that both scaffolds facilitated the growth and proliferation of endometrial cells and could create endometrial-like structures. Therefore, due to the environmental and ethical concerns about animal resources in relation to natural polymers such as fibrin and the reasonable price of synthetic scaffolds, we recommend using synthetic scaffolds such as PGA to create endometrial-like structures in research studies. While the endometrial arrangement reported in this study only included epithelial and stromal cells, normal endometrium also contains glands, a vascular system, and immune cell populations. Although establishing an endometrial culture system using the two major cell types (epithelial and stromal cells) is beneficial, the involvement of other related cell types in the co-culture model could be investigated in further studies using methods such as organoids.

This study presents a straightforward model in which, similar to a fibrin-agarose (a natural biomaterial) scaffold, multiple layers of human endometrial stromal cells were cultured in 3D on a PGA electrospun scaffold (a synthetic, animal-free polymer) overlaid by a monolayer of human endometrial epithelial cells, and the overall arrangement was similar to the native endometrium. Replacing animal-derived hydrogels using a synthetic scaffold, such as electrospun PGA, has many potential benefits in clinical applications in terms of physiological, and structural properties, as well as environmental and ethical considerations. The accessibility of an advanced 3D model of endometrial tissue will also be useful for studying diseases and developing treatment methods. However, further studies are needed to assess and improve these models.

## Conflict of interest

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

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## Author contributions

Conceptualization: FSA, MA. Data curation: SA, FSA. Formal analysis: ZB, MM, PBM, RA. Methodology: SA, AAS, LG. Project administration: MA. Visualization: SA, FSA. Writing—original draft: SA. Writing—review & editing: FSA, AAS, ZB.

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# Prolonged semen incubation alters the biological characteristics of human spermatozoa

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**Objective:** The present study assessed the biological characteristics of human spermatozoa at different time intervals (0, 1, 1.5, and 2 hours) after incubation at 37°C.

**Methods:** Twenty-five normozoospermic semen samples were incubated at 37°C. Incubation was performed at four time intervals of 0 (after liquefaction), 1, 1.5, and 2 hours. The samples were evaluated for sperm parameters at each time interval.

**Results:** The rate of sperm progressive motility decreased at 1.5 hours compared to 0 hours as well as 2 hours compared to 1 hour and 0 hours. The rate of non-motile spermatozoa also decreased after 2 hours compared to after 0 hours. No significant changes were observed in sperm viability ( $p=0.98$ ) and non-progressive motility ( $p=0.48$ ) at any time intervals. Abnormal sperm morphology increased at 1.5 hours of incubation time ( $p<0.001$ ). No significant changes were observed in DNA fragmentation at 1 hour compared to 0 hours (median [interquartile range]: 19.5 [4] vs. 19 [4]), as well as at 1.5 hours compared to 1 hour (20 [5]). However, a significant increase in DNA fragmentation was observed at 1.5 hours compared to 0 hours. The mitochondrial membrane potential decreased remarkably after 1 hour of incubation time. No significant differences were observed in the acrosome reaction or malonaldehyde levels at any time point ( $p=0.34$  and  $p=0.98$ , respectively).

**Conclusion:** The incubation of normozoospermic samples before use in assisted reproductive technology should be less than 1.5 hours to minimize the destructive effects of prolonged incubation time on general and specific sperm parameters.

**Keywords:** Acrosome reaction; DNA fragmentation; Incubation time; Mitochondrial membrane potential; Spermatozoa

## Introduction

Infertility or low fertility refers to the inability of couples to achieve pregnancy for 1 year, despite regular unprotected sexual intercourse, according to the World Health Organization [1]. About 15% of couples have infertility-related problems and seek medical treatment for infertility [2]. In general, 20% to 50% of infertility cases are related to spermatozoa parameters [3]. At many clinics, common semen pa-

rameters, such as count, morphology, and motility, are used to assess sperm quality. The seminal fluid that is delivered to the laboratory for analysis and use in assisted reproductive technology (ART) must be sampled and stored under special conditions based on the World Health Organization (WHO) protocol [4].

One of the most important factors affecting sperm quality is time: both the time taken by an individual to deliver his sample to the laboratory, and the time that the samples remain in the laboratory for analysis. According to a previous study, the time from sample collection to transfer to the laboratory should not exceed 1 hour [5]. Sample incubation in the laboratory should also not take more than 2 hours [6]. In an andrology laboratory, semen samples are incubated before analysis or use in ART. This incubation can be done at room temperature (RT) or at 37°C, the latter of which is the usual incubation temperature [7]. The purpose of this incubation is the liquefaction process, which may take about 15 to 30 minutes. However, ejac-

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ulates may be incubated longer in the laboratory before analysis or use in ART.

Other specific parameters can be also assessed in order to assess sperm health. One of the main factors is the sperm DNA status, which can be checked for fragmentation, denaturation, or chromatin compression [8]. Sperm DNA integrity plays an important role in ART treatment cycles, and DNA damage has a considerable effect on clinical outcomes [9]. Studies have shown that there is a significant reverse relationship between sperm DNA damage and embryo formation, fetal growth, and pregnancy [10]. The most common cause of sperm DNA breakdown is oxidative stress [11]. In the male reproductive system, reactive oxygen species (ROS) are involved in many physiological processes, including capacity building, hyperactivity, acrosomal reaction, and fertilization process [12]. Spermatozoa are vulnerable to oxidative stress due to the presence of abundant unsaturated fatty acids on the surface of the plasma membrane and the lack of protective enzymes in the cytoplasm [13]. The presence of ROS produced by sperm and multinucleated leukocytes in semen can damage sperm DNA [14]. In addition, the mitochondria are another factor affecting sperm health. The main function of sperm mitochondria is to synthesize adenosine triphosphate (ATP) through oxidative phosphorylation. Although, the true contribution of mitochondrial-produced ATP in sperm is not fully understood, mitochondrial function is related to sperm quality. Mitochondrial status plays an important role due to its relationship with cell energy status and motility related to male fertility [15].

It should be noted that sperm cells are not able to fertilize an egg immediately after ejaculation. The acrosome reaction refers to the structural and metabolic changes during which sperm acquire the ability to fertilize an egg [16]. Studies have shown that damage to the acrosome reaction may lead to infertility [17]. The aim of this study was to assess the sperm parameters, ROS production, sperm mitochondria, sperm chromatin, and acrosomal reaction in samples of normozoospermic men at different time intervals (0, 1, 1.5, and 2 hours) after incubation at 37°C. To our knowledge, no previous studies have assessed the biological characteristics of spermatozoa at different time intervals using neat seminal samples.

## Methods

### 1. Participants and study design

Samples were collected from 25 normozoospermic men (aged 20–40 years) referred to the Yazd Reproductive Science Institute from August to October 2021. The samples were prepared in sterile containers by masturbation after 2–5 days of sexual abstinence. A consent form was signed by all participants. This study was approved by the Ethics Committee of Shahid Sadoughi University (IR.SSU.MEDI-

CINE.REC.1400.132).

### 2. Semen preparation and analysis

The semen samples were incubated at 37°C without washing or adding solution. Incubation was performed for four different time intervals: 0 (after liquefaction for 10–15 minutes), 1, 1.5, and 2 hours. Samples were evaluated for sperm parameters at each time interval.

Sperm analysis was performed according to the WHO guidelines [18]. To evaluate sperm motility, 10 µL of the sample was placed on the slide. A contrast phase microscope (Nikon, Tokyo, Japan) was used to assess 200 spermatozoa in five fields at ×200 magnification. Sperm motility was reported as the non-motile, non-progressive, and progressive motility percentages [18]. Sperm viability was assessed by eosin-Nigrosin staining. In this method, 10 µL of the sample was mixed with 10 µL of Eosin-Nigrosin. After 30 seconds, 10 µL of the mixture was placed on a slide and a smear was prepared. Then, 200 sperm cells were examined with a light microscope (×1,000 magnification). Dead sperm cells were stained pink or red, while viable sperm remained colorless [18]. Finally, the percentage of live spermatozoa was reported.

Diff-Quick staining kit (Dian Bio assay, Tehran, Iran) was used to evaluate sperm morphology. In this method, 10 µL of the sample was placed on a slide and a smear was prepared. After drying, the smear was fixed with 95% ethanol. The slide was then stained with eosin for 10 seconds and methylene blue for 5 seconds. The slides were finally rinsed with tap water. After slide drying, 200 sperm cells were evaluated by light microscopy (×1,000 magnification). The percentage of normal spermatozoa was reported [18].

### 3. Sperm chromatin dispersion test

A halo sperm kit (SDFA kit; Ideh Varzan Farda, Tehran, Iran) was used to assess the sperm chromatin condition. This method is not complicated and time-consuming, although it is economical [19]. In brief, 20 µL of low-melting agarose was combined with 50 µL of semen samples. Then, 20 µL of the mixture was placed on the precoat-ed glass slide. A coverslip was placed on the drop to spread evenly over the slide. The slide was placed in the refrigerator at 4°C for 5 minutes. Next, the coverslip was slowly removed from the slide and the denaturing solution (A) was added. The slides were kept in a dark room at RT for 7 minutes. The lysing solution (B) was added to the slide and placed at RT for 15 minutes. The slide was washed with distilled water for 5 minutes. For dehydration, the slides were immersed in 70%, 90%, and 100% ethanol solutions for 2 minutes, respectively, and dried for 2 minutes at RT. First, the slides were stained with solution C for 75 seconds, then solution D for 3 minutes and finally solution E for 2 minutes. After that, the slides were washed with distilled water and 200 sperm cells were examined with bright-field micros-

copy ( $\times 1,000$ ). The DNA fragmentation rate was evaluated according to halo formation around the sperm head. A medium or large halo around the sperm head indicated DNA without fragmentation, and the absence of a halo represented fragmented DNA [20]. The percentage of sperm with fragmented DNA (no halo) was reported.

#### 4. Acrosome reaction assessment

For acrosome reaction evaluation, 10  $\mu\text{L}$  of the sample was placed on a slide and a smear was prepared. After smear drying in air, the sample was fixed with 95% ethanol. Then, the slides were stained with 25  $\mu\text{L}$  of fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin solution and incubated at 4°C for 1 hour. The slides were washed with distilled water and air-dried. Later, the slides were evaluated with a fluorescent microscope at 450–495 nm under  $\times 400$  magnification. The sperm with the acrosome reaction had a fluorescent band in the middle, while the rest of the parts did not have a fluorescent color, and more than half of the head of the sperm without the acrosome reaction was bright [21]. The percentage of spermatozoa with the acrosome reaction was reported.

#### 5. Evaluation of sperm mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was evaluated using a JC1 kit (Cayman, Ann Arbor, MI, USA). First, 25  $\mu\text{L}$  of the sample was mixed with 25  $\mu\text{L}$  of JC-1 dye in a microtube and incubated in a CO<sub>2</sub> incubator at 37°C for 15–30 minutes. After incubation, 100  $\mu\text{L}$  of phosphate-buffered saline was added to the samples. Each sample was centrifuged at RT for 5 minutes at 400  $\times g$  and then the supernatant was gently removed. Then, 10  $\mu\text{L}$  of the sample was placed on a slide and a smear was prepared. After the smear was dried, 200 spermatozoa were assessed with a fluorescent microscope under  $\times 1,000$  magnification at 520–570 nm. Sperm cells with a red or

shiny middle piece were considered to have high mitochondrial potential, or JC-1(+), and sperm cells with a green middle piece were considered to have low mitochondrial potential, or JC-1(-) [22]. The percentage of cells with high mitochondrial potential, denoted as JC-1(+), was reported.

#### 6. Assessment of semen malondialdehyde levels

The amount of malondialdehyde (MDA) in the samples was measured by a TPR kit (Teb Pazhoohan Razi, Tehran, Iran). First, 100  $\mu\text{L}$  of the sample was poured into a microtube and 100  $\mu\text{L}$  of the standard solution was added. Then, 100  $\mu\text{L}$  of reagent (R4) and 200  $\mu\text{L}$  of chromogen were added to the microtube. The microtube was first placed in a hot water bath for 1 hour, and then on ice for 10 minutes. The sample was finally centrifuged at 4°C for 15 minutes, and 200  $\mu\text{L}$  of the samples were placed in the plate wells and inserted into the plate reader. The absorption rate was recorded by the device at 530–540 nm.

#### 7. Statistical analysis

Data were analyzed in IBM SPSS ver. 26 (IBM Corp., Armonk, NY, USA). The Kolmogorov-Smirnov test was used to evaluate the normality of the data. Certain data was shown to be non-parametric distribution, the Friedman test was used to evaluate the trends. In addition, multiple comparisons among different times were performed, and the Bonferroni correction was used to adjust the statistical significance. A  $p$ -value  $< 0.05$  was considered to demonstrate a statistically significant difference in the Friedman test.

## Results

### 1. Effect of different incubation times on sperm parameters

The results of different incubation times on sperm parameters are

**Table 1.** Effect of different incubation times on sperm parameters

Sperm parameter	Incubation time (hr)				$p$ -value
	0	1	1.5	2	
Progressive motility (%)	55.14 $\pm$ 12.03 <sup>a)</sup>	52.82 $\pm$ 9.59 <sup>b)</sup>	50.45 $\pm$ 10.25	48.45 $\pm$ 10.22	$< 0.001$ <sup>e)</sup>
Median (IQR)	20 (17)	50 (15)	48 (14)	49 (13)	
Non-progressive motility (%)	2.86 $\pm$ 2.35	2.86 $\pm$ 2.25	2.64 $\pm$ 2.77	2.82 $\pm$ 2.73	0.997
Median (IQR)	2 (4)	2 (3)	2 (3)	2 (5)	
Non-motility (%)	42.50 $\pm$ 11.65 <sup>c)</sup>	44.32 $\pm$ 9.48	46.41 $\pm$ 10.34	48.68 $\pm$ 10.22	$< 0.001$ <sup>e)</sup>
Median (IQR)	46 (17)	48.5 (12)	48.5 (16)	50.5 (12)	
Viability (%)	80.91 $\pm$ 11.50	80.05 $\pm$ 13.81	78.95 $\pm$ 15.52	79.41 $\pm$ 13.72	0.986
Median (IQR)	85.5 (15)	85.5 (17)	85 (18)	84.5 (17)	
Normal morphology (%)	7.55 $\pm$ 5.51 <sup>d)</sup>	7.32 $\pm$ 6.46	6.00 $\pm$ 5.72	5.68 $\pm$ 6.21	$< 0.001$ <sup>e)</sup>
Median (IQR)	5 (4)	4.5 (5)	4.5 (4)	3.5 (5)	

Values are presented as mean  $\pm$  standard deviation unless otherwise indicated.

IQR, interquartile range.

<sup>a)</sup> $p < 0.001$ : vs. 1.5 hr,  $p < 0.001$ : vs. 2 hr; <sup>b)</sup> $p < 0.001$ : vs. 2 hr; <sup>c)</sup> $p < 0.001$ : vs. 2 hr; <sup>d)</sup> $p = 0.004$ : vs. 1.5 hr,  $p < 0.001$ : vs. 2 hr; <sup>e)</sup>Considered significant.



shown in Table 1. Briefly, after 1.5 hours and 2 hours, both progressive motility and normal morphology decreased compared to 0 hours and 1 hour. Furthermore, the percentage of non-motile sperm increased after 2 hours compared to 0 hours. No significant changes were observed in non-progressive motility ( $p=0.48$ ) and viability ( $p=0.98$ ) at any time intervals.

## 2. Effect of different incubation times on sperm chromatin/DNA and the acrosome reaction

After incubation, sperm DNA fragmentation increased significantly (Figure 1). No significant changes were observed at 1 hour compared to 0 hours (median [interquartile range]: 19.5 [4] vs. 19 [4]), as well as at 1.5 hours compared to 1 hour (20 [5]). However, a significant increase in DNA fragmentation was observed at 2 hours compared to 0 hours and at 1 hour and at 1.5 hours versus 0 hours. The effects of incubation on the acrosome reaction are shown in Figure 2. No significant differences were observed at 0, 1, 1.5, or 2 hours.

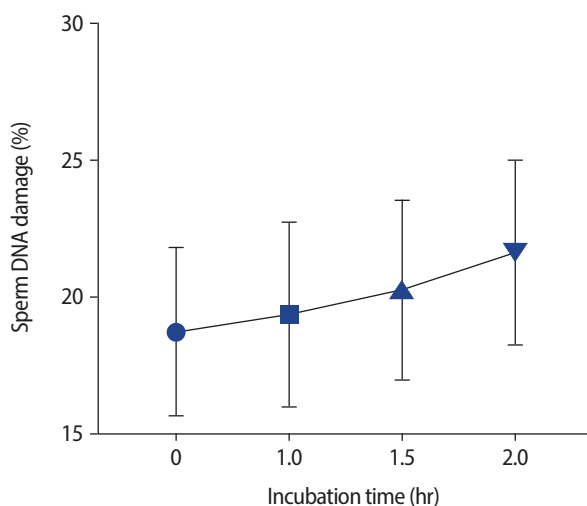
## 3. Effect of different incubation times on MDA levels and MMP

As presented in Figure 3, no significant changes were noted after incubation in semen MDA levels. The effects of different incubation times on the MMP are presented in Figure 4. No significant difference in the MMP was observed at 1 hour compared with 0 hours ( $p=0.11$ ) and at 1.5 hours versus 1 hour (median [interquartile range]: 79.5 [11] vs. 80.5 [10]). However, the sperm MMP decreased significantly at 2 hours compared to 0 and 1 hours and at 1.5 hours versus 0 hours ( $p<0.001$ ).

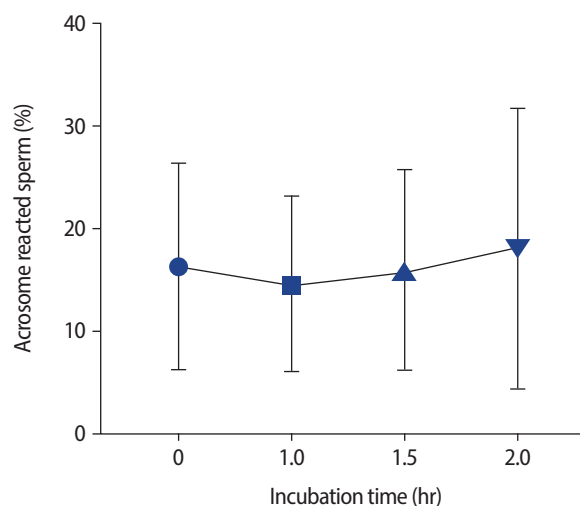
## Discussion

The aim of this research was to investigate the most suitable time intervals for semen incubation in the laboratory for common and specific sperm parameters, such as DNA fragmentation, the acrosome reaction, MMP, and MDA production levels. The significant contribution of this research was its documentation of the effects of different time intervals on the semen preparation procedure, which is expected to help professionals choose the optimal conditions. The data showed that sperm motility changed at different time intervals. A decreasing trend was observed in the percentage of progressive motility after longer incubation periods. The percentage of progressive motile sperm decreased after 1.5 hours compared to the previous times. Moreover, when the incubation time exceeded 1.5 hours, the percentage of immotile spermatozoa in semen samples increased significantly. Sperm abnormal morphology, as well as DNA fragmentation, increased beyond 1.5 hours of incubation time. The MMP decreased remarkably after 1 hour of incubation time.

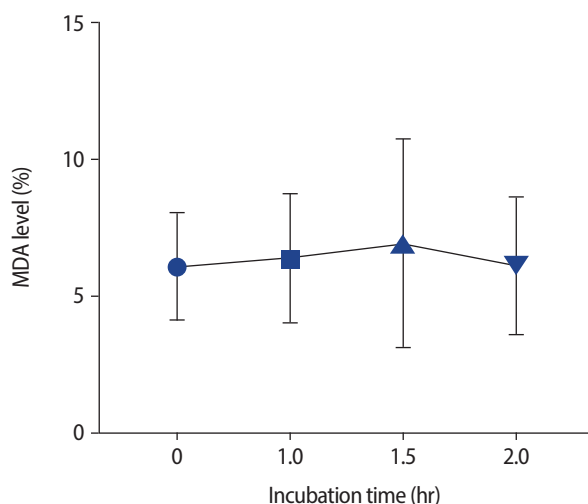
In the process of sperm preparation for different uses, especially in ART procedures, the incubation of semen at 37°C plays a crucial role [6]. Prolonged incubation of spermatozoa is performed in order to accommodate delays in oocyte preparation and pick-up, oocyte maturation, and rescue intracytoplasmic sperm injection (ICSI) [23]. Since the last decade, the assessment of spermatozoa incubation in laboratories has been a matter of debate. Although the influence of incubation time on conventional parameters of semen was investigated previously [24], specific factors, such as the acrosome reaction,



**Figure 1.** Effects of different incubation times on sperm DNA integrity. There were significant changes at 1.5 hours versus 0 hours ( $p<0.001$ ) and 2 hours ( $p<0.001$ ). Furthermore, at 1 hour versus 2 hours ( $p<0.001$ ), a significant increase in DNA fragmentation was observed.

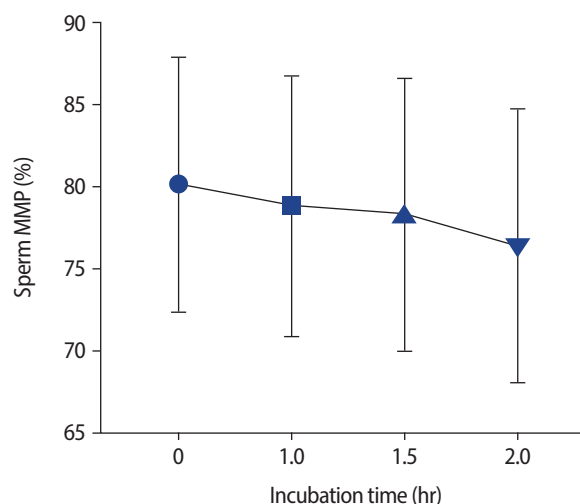


**Figure 2.** Effect of different incubation times on the acrosome reaction (AR) in sperm. No significant differences were found in the AR according to time ( $p=0.34$ ).



**Figure 3.** Effect of different incubation times on semen malonaldehyde (MDA) levels. No significant correlation was found between incubation time and semen MDA levels ( $p=0.98$ ).

DNA fragmentation, MMP, and MDA production levels, required more attention. Ouitrakul et al. [25] demonstrated that sperm motility decreased after 1 hour of incubation at RT, while the sperm viability was affected after 2 hours. The viability assessment in their study did not significantly change before 2 hours of incubation, which is in agreement with our findings. Furthermore, progressive motility decreased significantly after 2 hours, which is in agreement with the above study. Nematollahi et al. [26] showed that the percentage of progressive motile spermatozoa decreased in both the RT and 37°C groups compared to the fresh group (control). Furthermore, similar to our findings, more immotile spermatozoa were observed at 24 hours at 37°C than in the fresh group. In the current study, we observed that semen incubation induced morphological changes in sperm heads. Peer et al. [27] showed that the percentage of spermatozoa with vacuolated nuclei increased after 2 hours of incubation. Ahmed et al. [28] also reported that abnormal sperm morphology increased after 5 hours of incubation at 37°C. Our findings showed an increase in sperm abnormal morphology at 1.5 hours of incubation, which is similar to the previous studies. Another adverse effect of incubation is DNA fragmentation. Our findings were similar to those of Nabi et al. [6], who reported a significant increase in DNA fragmentation after 2 hours of incubation. Alvarez Sedo et al. [29] also showed that DNA fragmentation of semen prepared by the swim-up method significantly increased after 4 hours of incubation, which is in agreement with this study. The production of ATP is one of the main functions of mitochondria, which are required primarily for sperm motility and cellular events involved in capacity, hyperactivity, and the acrosome reaction. The MMP decreased notably after 1 hour of incubation. Zhu et al. [30] showed that incubation of sperm after 6 hours in



**Figure 4.** Effect of different incubation times on the mitochondrial membrane potential (MMP), as measured using the JC-1 assay. The mitochondrial membrane temperature was reduced with long incubation of spermatozoa. Significant changes were observed at 1.5 hours versus 0 hours ( $p<0.001$ ), as well as at 2 hours versus 0 hours ( $p<0.001$ ) and at 2 hours versus 1 hour ( $p<0.001$ ).

a low-glucose solution led to reductions in both ATP levels and the MMP. An acrosomal cap covers about 40% to 70% of the anterior region of the sperm nucleus. Acrosomes contain hydrolyzing enzymes that are released to digest oocytes during the acrosomal reaction. The hyperactive conditions related to the acrosome reaction result in the penetration of sperm into the zona pellucida and interactions with the oocyte. In the current study, the acrosome reaction did not change significantly at different time intervals. By increasing time intervals, adverse results may be encountered. It has been suggested that sperm DNA fragmentation may be related to apoptosis events and oxidative stress. Sperm cells are very vulnerable to damage induced by ROS, because the sperm membrane contains large amounts of polyunsaturated fatty acids. MDA, as an indicator of oxidative damage, is an end product of lipid peroxidation of the sperm membrane, which can affect sperm structure, and function.

At infertility centers, to perform ART cycles, sperm cells should be prepared and kept for approximately 2 hours in a 37°C incubator or at RT before microinjections, which may have destructive effects on clinical outcomes. In this regard, Pujol et al. reported that the rate of clinical pregnancy decreased due to a long time period between oocyte pick-up and ICSI [31]. Altogether, this study suggests that the ideal incubation time to reduce the adverse effects on vital semen parameters should be less than 1.5 hours, which may contribute to better clinical outcomes for infertile individuals. This information may also encourage patients to deliver their samples to the laboratory within the optimal period of time. Therefore, in andrology labora-

tories, paying attention to the incubation time in semen preparation protocols and ART performance is a noteworthy issue.

The incubation of normozoospermic samples before use in ART should be less than 1.5 hours to minimize the possible destructive effects of a prolonged incubation time on specific sperm parameters. Although sperm with prolonged incubation can fertilize oocytes, the clinical outcomes may be altered.

## Conflict of interest

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

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Conceptualization: SADB, MAK. Methodology: MH, AN, MS. Project administration: SADB, MAK. Writing—original draft: all authors. Writing—review & editing: AAS.

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# Coenzyme Q10 improves sperm motility and antioxidant status in infertile men with idiopathic oligoasthenospermia

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**Objective:** Oxidative stress is a key player in the development of idiopathic male infertility (IMI), and various antioxidants have been used for the treatment of IMI with inconsistent results. Coenzyme Q10 (CoQ10) is a cofactor and an antioxidant that may improve semen parameters and reduce oxidative stress in patients with idiopathic oligoasthenospermia (OA). Therefore, this study aimed to explore the effect of CoQ10 on semen parameters and antioxidant markers in patients with idiopathic OA.

**Methods:** Fifty patients with idiopathic OA and 35 fertile controls were enrolled in this prospective controlled study. All participants underwent a comprehensive fertility assessment. All patients received CoQ10 (300 mg/day) orally once daily for 3 months. Semen parameters, seminal CoQ10 levels, reactive oxygen species (ROS) levels, total antioxidant capacity (TAC), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were measured in patients and controls at the start of the study and after 3 months.

**Results:** Treatment with CoQ10 resulted in increased sperm progressive motility ( $p < 0.05$ ), total motility ( $p < 0.01$ ), seminal TAC ( $p < 0.01$ ), SOD ( $p < 0.05$ ), GPx ( $p < 0.001$ ), and seminal CoQ10 ( $p < 0.001$ ) levels and reduced ROS ( $p < 0.01$ ) in patients as compared to baseline. Sperm concentration and motility were also significantly correlated with antioxidant measures and seminal CoQ10 levels ( $r = 0.38-0.57$ ).

**Conclusion:** CoQ10 therapy (300 mg/day for 3 months) improved sperm motility and seminal antioxidant markers in patients with idiopathic OA. Therefore, CoQ10 could be a promising treatment for patients with idiopathic infertility and may improve their fertility potential.

**Keywords:** Antioxidants; Coenzyme Q10; Male infertility; Oligoasthenospermia; Oxidative stress

## Introduction

Infertility refers to the inability of a couple to conceive after 12 months of regular unprotected intercourse [1]. Infertility affects approximately 10% to 15% of couples worldwide [2,3]. Male factors contribute to approximately half of these cases [4]. Oligoasthenospermia (OA) is diagnosed when a low sperm concentration ( $< 15$  million/mL) and motility (progressive motility  $< 32\%$ , total motility

$< 40\%$ ) are identified according to World Health Organization (WHO) guidelines [5]. Several factors have been linked to OA, including autoimmunity, cryptorchidism, varicocele, systemic diseases, endocrine diseases, genital trauma, genital infection, medications, and toxins [6]. However, in approximately 25% of cases, the underlying causes of OA cannot be identified; these cases are designated as idiopathic male infertility (IMI) [7]. Several mechanisms have been suggested for IMI, including genetic and epigenetic factors, oxidative stress (OS), and sperm DNA fragmentation (SDF).

OS is the result of an imbalance between pro-oxidants and antioxidant defense mechanisms, which results in a state of redox paradox [8]. Reactive oxygen species (ROS) are essential for certain physiological functions required for male reproduction. However, high levels of ROS are detrimental to sperm, as sperm have limited intrinsic antioxidant capacity. OS could be caused by endogenous factors such as metabolic processes, immature sperm, and leukocytes, as well as ex-

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ogenous factors such as infection, varicocele, smoking, alcohol, obesity, malignancy, radiotherapy, chemotherapy, environmental toxins, and systemic disease. The seminal fluid contains a set of antioxidants that protect spermatozoa from oxidative damage. These antioxidants include enzymatic antioxidants, such as catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) [9], as well as non-enzymatic components including vitamins A, C, and E, coenzyme Q10 (CoQ10), pyruvate, L-carnitine, glutathione, taurine, urate, zinc, and selenium [10]. Excessive production of ROS and OS could lead to sperm membrane lipid peroxidation, increased sperm membrane permeability, reduced sperm motility, reduced fertilization, SDF, and poor pregnancy and assisted reproductive techniques (ART) outcomes [11]. These adverse effects may reduce male fertility potential and increase the risk of poor pregnancy outcomes. Increased SDF may also result in abnormal sperm function, reduced male fertility potential, and reduced pregnancy and ART success rates [12]. Recent studies have also linked OS and SDF to the development of autosomal dominant genetic diseases, childhood malignancies, birth defects, and neurological diseases in newborns [13].

Previous studies have shown that oral antioxidants may enhance semen parameters and seminal antioxidant status [14,15]. However, there is a lack of consensus on the type of antioxidant, dosing, length of therapy, and target group [16]. Several studies have tried different doses of the same antioxidant or compared two or more antioxidants and different treatment durations, which illustrates this inconsistency. In addition, the excessive use of antioxidants and high doses may reduce ROS and may shift the redox balance into a reductive status, leading to a state of reductive stress [17]. CoQ10 is a cofactor, and its reduced form (ubiquinol) has antioxidant and mitochondrial bioenergetic properties [18]. It transfers electrons in the mitochondria from complex I/II to complex III. CoQ10 is widely distributed in human tissues, and its main dietary sources include oily fish, meat, and whole grains. Reduced CoQ10 levels are associated with cardiovascular diseases, diabetes mellitus, and cancer, and seminal CoQ10 levels are also reduced in infertile men, which correlates with sperm concentration and motility [19]. We and others have also shown that CoQ10 treatment improves semen measures and seminal antioxidant status in patients with infertility [20,21]. Furthermore, our recent meta-analysis of three randomized controlled trials (RCTs) also confirmed beneficial effects for CoQ10 on sperm motility with a longer duration of treatment of 6 months [22]. A single study, however, demonstrated a lack of improvement in semen measures following CoQ10 treatment [23]. While the aforementioned studies explored the impact of various antioxidants on semen measures in patients with infertility and reported beneficial effects, studies on the effect of CoQ10 on seminal OS levels in patients with idiopathic OA are

scarce. Furthermore, inconsistencies regarding the optimal dose, duration of treatment, and the target group for CoQ10 therapy exist. In addition, managing the challenging group of men with IMI requires the development of an optimal treatment protocol for oral antioxidant therapy including CoQ10. Therefore, this study aimed to explore the effect of CoQ10 therapy for 3 months on improving semen measures and seminal antioxidant status in men with idiopathic OA and fertile controls. To our knowledge, the study is the first to explore the impact of CoQ10 (300 mg/day) on seminal antioxidant markers in infertile patients with idiopathic OA.

## Methods

Ethical approval was received from the University of Sumer, Iraq (EC/2018/8876) and informed consent was obtained before enrollment.

### 1. Patients

In this prospective controlled study, 50 infertile men with idiopathic OA (mean age,  $27.28 \pm 8.47$  years; mean duration of infertility,  $5.62 \pm 3.49$  years) and 35 fertile healthy control subjects (mean age,  $29.61 \pm 9.33$  years) were included. Five patients did not complete the study and therefore were excluded. The patients were selected from the Fertility Clinic, Hillah, Iraq from May to September 2018. A special questionnaire was designed to collect data. All patients underwent a detailed fertility assessment by a fertility specialist to exclude known causes of OA. For all participants, semen analysis (WHO guidelines), seminal antioxidant measures (total antioxidant capacity [TAC], SOD, GPx, and ROS), and seminal fluid CoQ10 levels were assessed at the start of the study and after 3 months. Patients received CoQ10 (300 mg/day) orally in a single dose for 3 months and their measures were compared between baseline and after CoQ10 therapy. The dose of CoQ10 was similar to the dose used in previous studies [24]. The sample size, which was calculated using 80% power and a 5% significance level, was 34 for each group. The test is based on superiority and we used a previously published method for sample size calculation [25].

### 2. Eligibility criteria

Patients had a history of infertility of at least 1 year despite regular unprotected intercourse. OA was diagnosed when abnormal sperm concentration and motility according to the fifth-edition WHO criteria were detected in semen analysis [26]. Patients who had varicocele, genital tract infection, scrotal trauma, scrotal surgery, undescended testis, azoospermia, systemic illness, smoking, alcohol use, female factor infertility, and recent antioxidant intake in the last 6

months were excluded from the study. Fertile controls were required to have fathered a child in the last 2 years and have a normal semen analysis.

### 3. Semen analysis

Semen samples were obtained by masturbation after sexual abstinence for 2–3 days, collected into a special container, held at 37°C for liquefaction, and then semen analysis was performed within 1 hour according to the fifth edition WHO guidelines [26] for all semen parameters and the fourth edition WHO guidelines for sperm morphology depending on the available laboratory facilities [27]. Semen analyses were performed by the same researcher. Two semen samples were assessed at the start of the study and after 3 months, and their average value was used for this study.

### 4. Seminal TAC

Semen samples were centrifugated at 3,000 rpm for 5 minutes, and seminal plasma was stored at –20°C. The TAC level was assessed with the total antioxidant capacity assay kit (#E-BC-K136; Elabscience, Houston, TX, USA). Colorimetric optical absorbance was measured at 520 nm using the standard procedure.

### 5. Seminal SOD activity

SOD activity was assessed according to the method reported by Magnani et al. [28]. The test principle is based on the competition between pyrogallol autoxidation by  $O_2^{\cdot-}$  and scavenging by SOD. Colorimetric optical absorbance was measured at 420 nm using the standard procedure.

### 6. GPX activity

GPX was assessed with the glutathione peroxidase (GSH-PX) assay kit (#E-BC-K096, Elabscience). Colorimetric optical absorbance was measured at 412 nm using the standard procedure.

### 7. Measurement of seminal ROS and COQ10 levels

ROS was assessed using a manual method reported by Venkatesh et al. [29]. The level of ROS was measured using luminol-dependent chemiluminescence. Reverse-phase high-performance liquid chromatography was utilized to assess seminal CoQ10 levels using an ultraviolet detector at 275 nm according to the method described by Li et al. [30]. Coenzyme Q9 was used as the internal standard.

### 8. Statistical analysis

The statistical analysis was conducted using IBM SPSS ver. 24 (IBM Corp., Armonk, NY, USA). The results were described as mean  $\pm$  standard deviation. The normality of data was explored using the Kolmogorov-Smirnov test, and a normal distribution was shown ( $p > 0.05$ ). The dependent  $t$ -test was used for comparisons of parameters before and after treatment, and the independent  $t$ -test was used to compare seminal measures between patients and fertile controls. Correlations between seminal parameters and TAC, SOD, GPx, ROS, and seminal CoQ10 levels were estimated using Pearson correlation coefficients. A  $p$ -value below 0.05 was adopted as the level for statistical significance.

## Results

Patients' and controls' demographics and semen parameters are shown in Table 1. Sperm concentration and motility in the patient group were significantly lower than in the fertile controls ( $p < 0.001$ ). Following 3 months of CoQ10 therapy, sperm progressive motility ( $p < 0.05$ ) and total motility ( $p < 0.01$ ) in patients improved significantly in comparison to pretreatment levels. Normal sperm morphology was reduced after CoQ10 therapy, but not to a statistically significant extent ( $p > 0.05$ ). Infertile patients demonstrated lower seminal TAC, SOD, GPX, and CoQ10 levels, and higher seminal ROS than fertile controls ( $p < 0.01$ ) (Figure 1). CoQ10 administration in patients

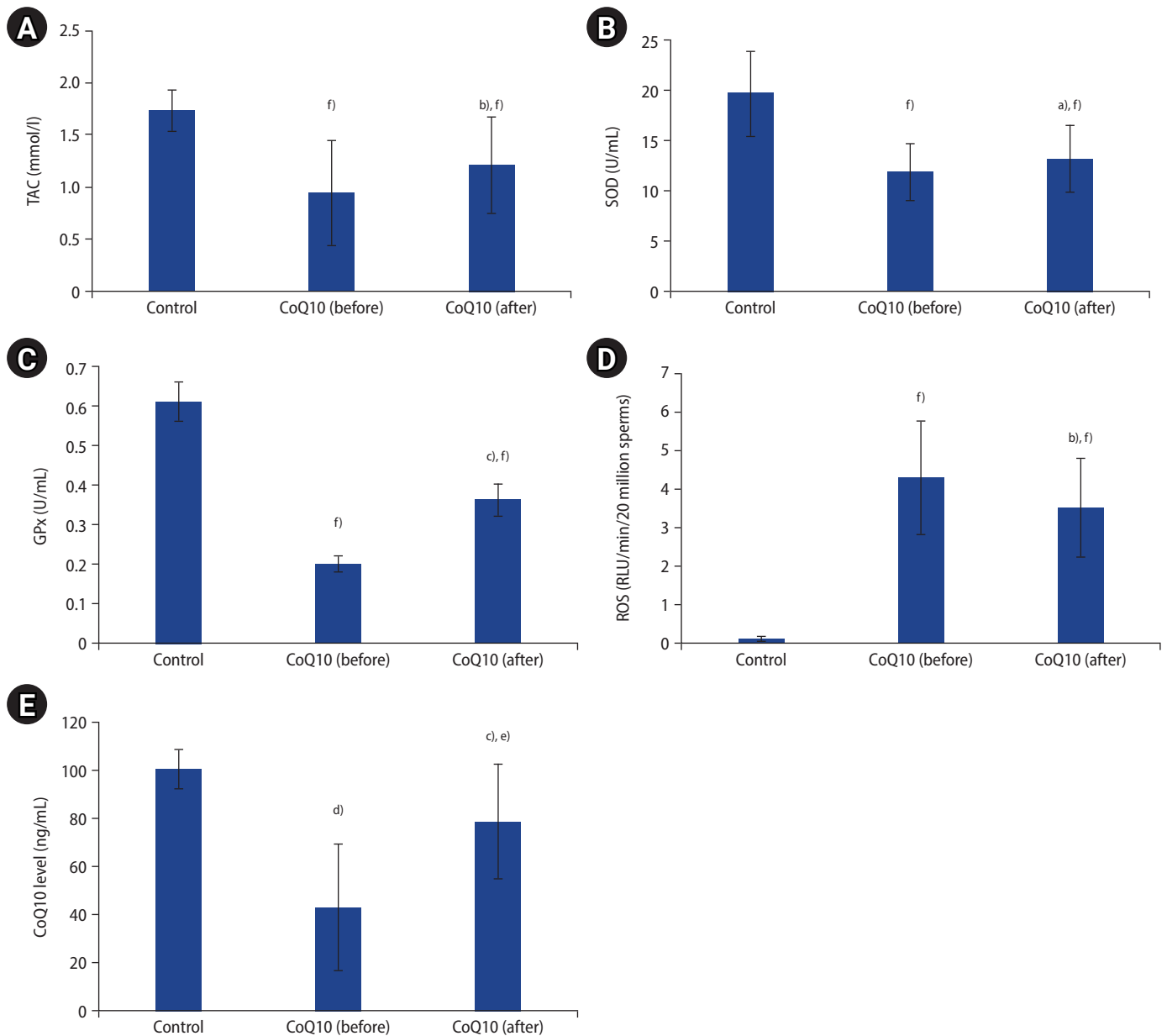
**Table 1.** Patients' and controls' characteristics and semen parameters

Semen parameter	Fertile control (n = 35)	Patient before CoQ10 (n = 50)	Patient after CoQ10 (n = 50)
Age (yr)	29.61 $\pm$ 9.33	27.28 $\pm$ 8.47	-
Infertility duration (yr)	-	5.62 $\pm$ 3.49	-
Volume (mL)	2.75 $\pm$ 0.67	2.72 $\pm$ 0.85	2.92 $\pm$ 0.78
Concentration (million/mL)	46.58 $\pm$ 24.5	8.62 $\pm$ 4.51 <sup>d</sup>	10.3 $\pm$ 5.22 <sup>d</sup>
Progressive motility (%)	43.5 $\pm$ 9.05	20.37 $\pm$ 7.86 <sup>d</sup>	24.6 $\pm$ 11.42 <sup>a),c)</sup>
Total motility (%)	60.4 $\pm$ 11.26	27.4 $\pm$ 9.44 <sup>d</sup>	33.8 $\pm$ 12.61 <sup>b),c)</sup>
Normal morphology (%)	39.58 $\pm$ 7.64	38.5 $\pm$ 7.18	36.9 $\pm$ 8.37

Values are presented as mean  $\pm$  standard deviation.

CoQ10, coenzyme Q10.

Significant difference from patients' baseline: <sup>a)</sup> $p < 0.05$ , <sup>b)</sup> $p < 0.01$ ; <sup>c)</sup>Significant difference from control:  $p < 0.001$ .



**Figure 1.** (A) Seminal plasma total antioxidant capacity (TAC), (B) superoxide dismutase (SOD), (C) glutathione peroxidase (GPx), (D) reactive oxygen species (ROS), and (E) coenzyme Q10 (CoQ10) levels in controls and infertile patients pre-and post-coenzyme Q10 treatment. Significant difference from patients’ baseline: <sup>a)</sup> $p < 0.05$ , <sup>b)</sup> $p < 0.01$ , <sup>c)</sup> $p < 0.001$ ; Significant difference from control: <sup>d)</sup> $p < 0.05$ , <sup>e)</sup> $p < 0.01$ , <sup>f)</sup> $p < 0.001$ .

also resulted in significantly higher seminal TAC ( $p < 0.01$ ), SOD ( $p < 0.05$ ), GPx ( $p < 0.001$ ), and CoQ10 levels ( $p < 0.001$ ), and lower seminal ROS levels ( $p < 0.01$ ) post-therapy.

We detected significant and direct correlations of sperm concentration and motility with seminal TAC, SOD, GPx, and CoQ10 levels. Additionally, we observed significant and inverse correlations of sperm concentration and motility with ROS. Seminal CoQ10 levels also showed significant correlations with seminal TAC and SOD (Table 2).

## Discussion

The recent decades have witnessed a decline in semen quality among men worldwide. Infertility represents a global health issue, and IMI is challenging both for science and clinical practice. Infertility imposes medical and psychological consequences on infertile couples as well as a financial burden on health system resources. The exact mechanisms underlying semen abnormalities in men with IMI



**Table 2.** Correlations between semen parameters and seminal plasma CoQ10 and antioxidant levels post-CoQ10 treatment

Variable	<i>r</i> ( <i>p</i> -value)			
	Concentration	Motility	Morphology	CoQ10
TAC	0.45 (0.03)	0.6 (0.01)	0.21 (0.06)	0.58 (0.01)
SOD	0.48 (0.03)	0.52 (0.02)	0.18 (0.07)	0.43 (0.03)
GPx	0.38 (0.04)	0.35 (0.04)	0.25 (0.04)	0.22 (0.06)
ROS	-0.51 (0.02)	-0.47 (0.03)	-0.11 (0.09)	-0.16 (0.07)
CoQ10	0.48 (0.03)	0.57 (0.02)	0.05 (0.12)	

CoQ10, coenzyme Q10; *r*, Pearson's correlation coefficient; TAC, total antioxidant capacity; SOD, superoxide dismutase; GPx, glutathione peroxidase; ROS, reactive oxygen species.

remain unknown. Furthermore, many treatment modalities for this condition have yielded inconsistent results. Patients with IMI represent a significant proportion of infertile couples and the condition has an impact on infertile couples, as well as on the community and health systems due to the need for long, extensive, and costly investigations and treatment. This study showed that treatment with CoQ10 for 3 months improved sperm progressive and total motility in infertile men with idiopathic OA. Our results are in parallel with other studies that demonstrated increments in sperm motility following the administration of oral antioxidants, including vitamin C, vitamin E, CoQ10, L-carnitine, zinc, selenium, and N-acetylcysteine in infertile men [10,31]. However, these studies demonstrated a lack of agreement on standardized antioxidant regimens. Further, some studies used one or two oral antioxidants, while others explored the effects of combined antioxidants with variable doses for each antioxidant. Balercia et al. [20] conducted a RCT on 60 patients with idiopathic asthenospermia who received CoQ10 (200 mg/day) for 6 months. The study detected an increment in progressive motility in the treatment group. In another RCT, infertile patients with idiopathic oligoasthenoteratospermia (OAT) were treated with CoQ10 (300 mg/day) for 26 weeks and demonstrated improvements in semen parameters post-therapy [32]. Furthermore, a study on men with idiopathic OAT reported an increment in all semen measures following CoQ10 (300 mg/day) for 12 months [33]. Another study investigated the effect of CoQ10 (100 mg/day for 6 months) in patients with idiopathic asthenospermia and reported improvements in both sperm motility and normal morphology [34]. The use of different doses of CoQ10 in the aforementioned studies highlights the need for optimization of the dose and treatment protocol of CoQ10 in infertile patients with IMI.

Our results are also in parallel with our other studies that reported improvements in semen parameters in patients with idiopathic OA and OAT who received CoQ10 (200 mg/day) for 3 months [9,21,35]. Further, our recent meta-analysis of three RCTs and two other recent meta-analyses have also confirmed beneficial effects for CoQ10 on semen parameters (mainly sperm motility) and with a long duration

(6 months) of treatment [22,36,37]. Another study, however, demonstrated no significant changes to seminal fluid parameters after CoQ10 treatment [23]. The observed improvement in sperm motility in men with idiopathic OA could be the result of reduced ROS and increased antioxidant capacity (TAC, GPx, and SOD), which could be due to the antioxidant characteristics of CoQ10, its key role as a cofactor for the mitochondrial respiratory chain, and its mitochondrial bioenergetics effects. These effects counteract the detrimental effects of OS on sperm membranes, motility, and fertilization. Additionally, recent reports have suggested anti-apoptotic, anti-inflammatory, and gene modulation effects for CoQ10, which could explain the use of CoQ10 for the treatment of other diseases such as ischemic heart diseases, Parkinson's disease, diabetes mellitus, and malignant tumors in addition to infertility [36,38,39]. We [12,35,40] and others [41-43] have also detected higher SDF in men with IMI, and these levels were ameliorated by CoQ10 therapy. These findings augment the role of CoQ10 as a gene modulator. One possible explanation for the different results of the previous studies is the heterogeneity of the patient groups (OA, OAT, or asthenospermia), different durations of treatment (3-26 weeks), and the lack of a consensus on the optimal dose of CoQ10. Establishing the optimal dose of CoQ10 and duration of treatment could increase the efficiency of the treatment protocol and may help patients avoid exposure to an unnecessarily high dose of CoQ10 or long treatment.

In our study, CoQ10 therapy in patients also resulted in significantly higher seminal TAC, SOD, GPx, and CoQ10 levels, as well as lower seminal ROS levels, post-treatment. Significant and direct correlations between sperm concentration and motility with antioxidant measures and seminal CoQ10 levels were also detected. Studies have reported that it is essential to identify new biomarkers and predictors of pregnancy and ART outcomes for the evaluation of male infertility, such as sperm function tests, OS markers, and SDF. Further, the correlations detected in this study between semen parameters and antioxidant markers could provide a rationale for the use of different antioxidants as a potential treatment for patients with IMI through reducing seminal OS and increasing antioxidant defenses.

Our results are congruent with those of other studies that have shown reduced seminal antioxidant defenses and CoQ10 levels in infertile patients [41,44]. Administration of CoQ10 could increase seminal CoQ10 and may ameliorate seminal OS [20,45]. Safarinejad et al. [24] reported an increment in semen measures and TAC in patients with idiopathic OAT following CoQ10 (200 mg/day) treatment. Correlations between CAT, SOD, and semen parameters were also observed in this study.

Our findings are also in parallel with our previous studies, which demonstrated that CoQ10 therapy (200 mg/day) in men with IMI increased TAC, SOD, CAT, GPx, and seminal CoQ10 levels and reduced seminal ROS levels [9,12,21,35,40]. The detection of higher seminal CoQ10 levels in these patients after CoQ10 treatment also suggests that oral administration of CoQ10 could be effective in increasing seminal CoQ10 levels and seminal antioxidant defenses against OS. It is worth mentioning that in our previous studies, we used a lower dose of CoQ10 (200 mg/day), meaning that a direct comparison of the results may not be possible. In these studies, correlations between sperm concentration and motility with antioxidant markers and seminal CoQ10 levels were also observed. In addition, our recent systematic review and another recent systematic review confirmed the beneficial effects of CoQ10 supplementation on improving semen parameters in men with infertility [36,46]. The improvement in seminal antioxidant capacity in patients with idiopathic OA in our study could have been due to higher seminal CoQ10 levels, antioxidant features of CoQ10, its role as a cofactor in the mitochondrial respiratory chain, and its bioenergetic effects that counteract the detrimental effects of OS on sperm and the fertilization process. Furthermore, recent reports have highlighted the impact of CoQ10 on gene modulation as well as inflammatory and apoptotic inhibitory effects [38,39]. Previous studies have used a wide range of CoQ10 doses (100–400 mg/day), but with inconsistent results. Therefore, our study explored the effect of CoQ10 (300 mg/day) on semen parameters and antioxidant markers in men with idiopathic OA. Data available on the effect of this dose in men with idiopathic OA are limited. The improvement in progressive and total sperm motility, TAC, SOD, GPx, CoQ10 levels, as well as the reduction in ROS detected in our study, were greater than those observed in other studies that have used CoQ10 doses of 100 or 200 mg/day [20,21,34,47,48]. This finding could be attributed to the stronger antioxidant effects of CoQ10, its bioenergetic properties, and enhanced antioxidant defenses, as well as the reduction of ROS with a higher dose of CoQ10. In contrast, recent studies have reported reductive stress with high doses of antioxidants [17]. Therefore, optimizing the dose of antioxidants, including CoQ10, is essential for the effective management of infertile men with IMI. It is worth mentioning that comparing our results with previous studies is challenging due to variability in study design, patient

groups, and therapeutic protocols. Some of the studies mentioned above have shown beneficial effects for CoQ10 with a duration ranging from 3 months to 26 weeks, and the fact that more effective results were obtained with a longer duration could be attributed to longer antioxidant effects and the duration of the spermatogenesis cycle of 72 days.

Our study was limited by the small number of control group participants due to consent, and we did not assess dietary CoQ10 intake, although a dietary assessment could be limited by the complexity of dietary intake and recall bias. We did not assess the pregnancy rate because the follow-up was limited by time and cost constraints. Another limitation is the selection of participants from a single area, so our findings may not be generalizable to all regions, and further large and multicenter studies are warranted.

CoQ10 therapy (300 mg/day for 3 months) in patients with idiopathic OA could improve sperm progressive motility, total motility, and seminal antioxidant markers. Therefore, CoQ10 could be a promising treatment for patients with IMI and may enhance their fertility and pregnancy outcomes.

## Conflict of interest

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

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# A rare case of primary ovarian mesenchymal chondrosarcoma in pregnancy

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Mesenchymal chondrosarcoma is a rare tumor that is more common in young people; it is an uncommon type of chondrosarcoma with a poor prognosis. In two-thirds of cases, it affects the bone, especially the spine. However, parts of the body other than the skeletal system are occasionally involved. These rarer types have a worse prognosis, with a high likelihood of metastasis and death. Due to the possible misdiagnosis of mesenchymal chondrosarcoma, the integrated use of imaging, immunohistochemistry, and pathology can be helpful.

**Keywords:** Mesenchymal chondrosarcoma; Ovarian; Pregnancy

## Introduction

The purpose of this report is to describe the clinical and pathologic features of primary mesenchymal chondrosarcoma of the ovary as an exceedingly rare entity, specifically during pregnancy [1]. Chondrosarcoma is a malignant cartilaginous tumor characterized by the formation of cartilage, but not of bone, by tumor cells and it is the third most common primary malignant bone tumor in adults [2,3].

## Case report

The present report describes a case of adnexal chondrosarcoma affecting a 31-year-old woman (gravida 1) at 22 weeks of pregnancy. The patient presented with chronic lower abdominal pain, was diagnosed with a 25-cm solid-cystic pelvic mass detected on ultrasonography, and was referred to our gynecologic oncology clinic. The pa-

tient was from an average Iranian middle-class family with no significant family history of gynecologic malignancy. Her pregnancy was otherwise uncomplicated, and all pregnancy screening tests were normal. The only medication at that time was pregnancy supplements. She had noticed pain in her lower abdomen over the previous month and experienced urinary retention related to the mass effect of the tumor. On a vaginal bimanual examination, a non-tender, non-mobile mass was palpable in the left adnexa that was also palpable over the abdomen and beneath the costal margin. The rest of the detailed systemic examination did not reveal any abnormalities. The patient's white blood cell count was 12,000/mm<sup>3</sup> with 30% lymphocytes and 60% leukocytes. Her hemoglobin level was 6 g/dL, and she received 8 units of packed cells and fresh frozen plasma before and during surgery to maintain intraoperative hemostasis and prevent dilutional coagulopathy. The platelet count was within normal limits. Her C-reactive protein concentration was 5 mg/L. Renal function, liver function, serum electrolytes, and other biochemical investigation results were normal. A magnetic resonance imaging scan of her abdomen revealed a left adnexal mass with an internal echo (Figure 1). Since a malignancy was suspected, the patient underwent midline laparotomy and resection of the entire left adnexa and the mass. Further exploration of the resected mass revealed acute massive internal hemorrhage, estimated to be about 2,400 mL, which was the underlying cause of the patient's severe acute anemia. No additional abnormalities were found in the uterus and right adnexa

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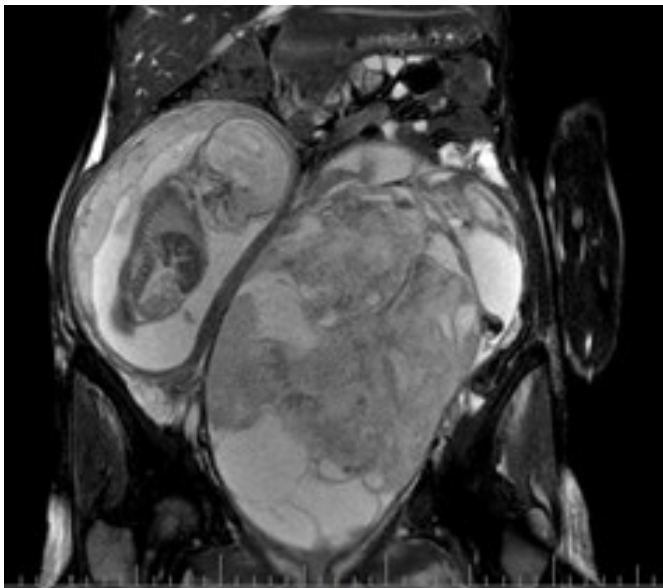
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and upon exploration of the abdominal and pelvic cavities (Figure 2). The pathological report of the mass revealed grade 3 mesenchymal chondrosarcoma with rhabdomyoblastic differentiation and without lymphovascular invasion. The mitotic rate was more than 18 per 10 high-power fields (Figure 3). Immunohistochemistry of cellular areas was positive for CD99 and CD34 with patchy reactivity for desmin and myogenin. CD10 was positive in areas with low cellularity. S100 and CD34 highlight chondroid islands. Smooth muscle actin, epithelial membrane antigen, Wilms' tumor protein, estrogen receptor, CD117, and cyclin D1 were negative, and the percentage of Ki-67

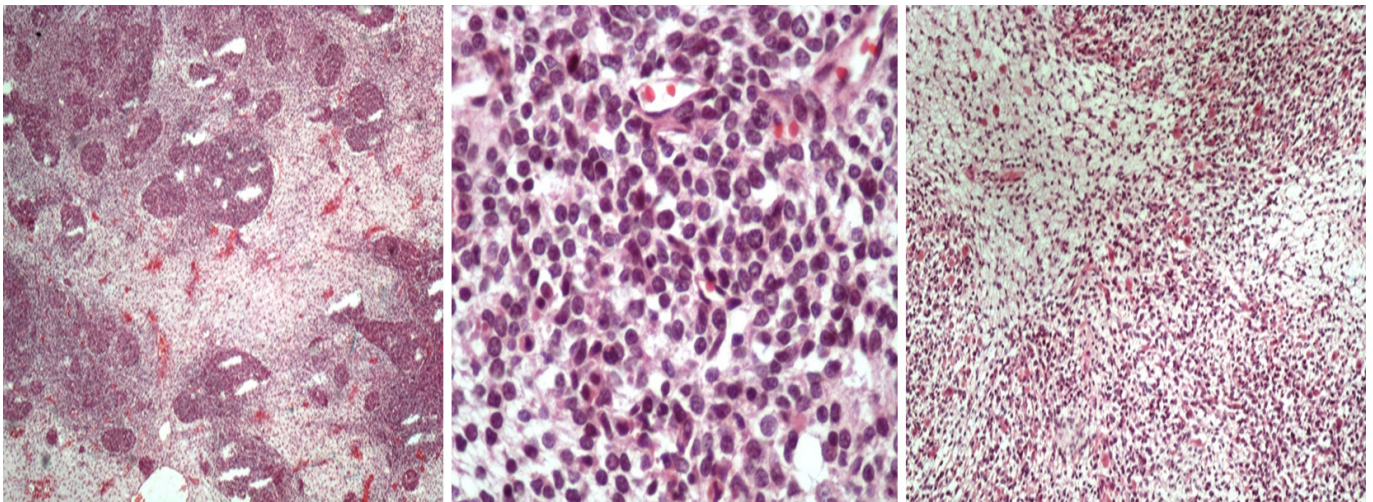
positivity was estimated to be 80%. Given the fact that the patient was 22 weeks pregnant by the time of surgery and the fetus was aborted spontaneously within a week after surgery, we initiated chemotherapy in agreement with the entire treatment team, including a gynecologic oncologist, fertility specialist, and radiotherapist. Post-operatively, the patient underwent 6 sessions of chemotherapy (vincristine, doxorubicin, and cyclophosphamide). Three months after surgery, follow-up magnetic resonance imaging after the fifth round of chemotherapy showed a soft tissue mass measuring 112 × 44 mm in the left adnexa with central cystic changes and an irregular and



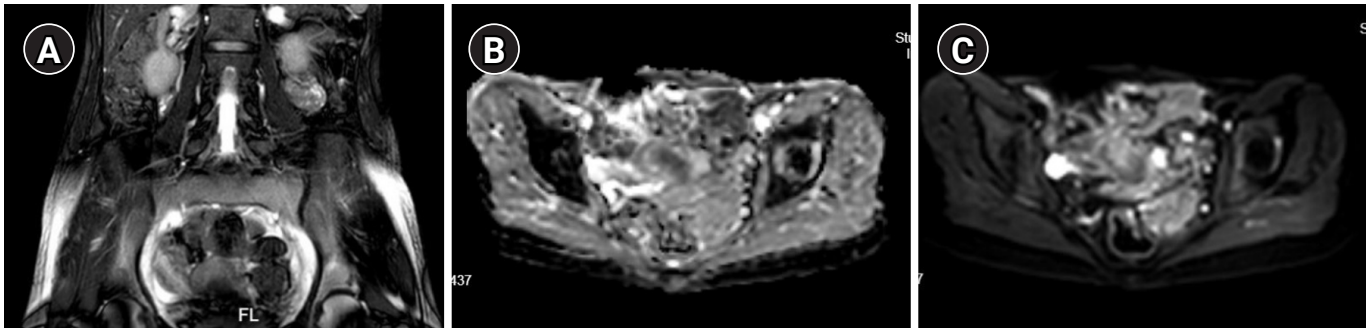
**Figure 1.** Magnetic resonance imaging, showing the fetus in the uterus on the right and the tumor in the left adnexa.



**Figure 2.** Intraoperative image of the mass.



**Figure 3.** Mesenchymal chondrosarcoma pathology with rhabdomyoblastic differentiation and without lymphovascular invasion. The mitotic rate was more than 18 per 10 high-power fields. There was a biphasic mesenchymal and chondrocyte pattern. The assay was performed by immunohistochemistry technique (x400).



**Figure 4.** Post-chemotherapy (A) T2 axial, (B) apparent diffusion coefficient coronal, and (C) diffusion-weighted imaging coronal views on magnetic resonance imaging. An 86×36-mm mass in the left adnexa with central cystic changes suggested a hemorrhagic component, with no evidence of tumor recurrence.

thick wall with high signal in the T1 sequence, suggesting a hemorrhagic component, and no enhancement after contrast injection. The mass shrunk in further follow-up imaging (Figure 4). The patient had no recurrence and signs of metastases as of 2 years after the last chemotherapy session.

## Discussion

Mesenchymal chondrosarcoma was first recognized in 1959. Two years after its original description, nine cases were reported from the files of the Mayo Clinic. The first case of extraskeletal mesenchymal chondrosarcoma (EMC) was described in 1964. EMC accounts for about 1% of all chondrosarcomas. Mesenchymal chondrosarcoma was initially considered restricted to the bone, but that is no longer the case [4]. A more recent analysis of reported cases concluded that 20% to 33% of these tumors occur in extraskeletal tissues. EMC is a rare subtype of chondrosarcoma, of chondroprogenitor cell origin [4]. The most common sites of origin of chondrosarcoma are the lower extremities, which are known as the primary sites, followed by the meninges, lung, neck, mandible, heart, and orbit. It is more common in women and mainly presents in the second to third decades of life [4,5]. Although most cases of uterine chondrosarcoma have been reported in postmenopausal women, our case was in reproductive age. In the head and neck, chondrosarcoma usually begins between the fourth and seventh decades of life. It has also been suggested that primary pure heterologous sarcomas may arise from primitive embryonal cell remnants or from a complete stromal overgrowth of a Mullerian mixed tumor. Another etiology appears to be related to the uncontrolled proliferation of Merkel cells (embryonic remains) [1,2]. Chondrosarcoma is also uncommon during pregnancy, and to the best of our knowledge no cases of proven primary ovarian chondrosarcoma in pregnancy have been reported in the literature; with regard to chondrosarcoma arising primarily in the ovary, only two cases have been reported in the English-language literature [6]. There are

also some reports of primary carcinosarcomas of the uterus in the literature, although this is also a very rare condition, with 17 cases reported to date [7]. In fact, only 10 cases of gestational chondrosarcomas have been described in the literature and none of these cases affected the ovaries. The effect of pregnancy on the growth features of chondrosarcomas remains unclear. Some experts have suggested a growth-enhancing effect of altered hormone levels on various bone tumors during pregnancy [2]. In addition, it still remains unclear whether chondrosarcoma during pregnancy may show progression or dedifferentiation according to the changes in hormonal status [1]. There are reports of G1 skeletal chondrosarcoma with fast progression during the course of pregnancy in the literature. The survival rates of pregnant women with chondrosarcoma of the extremities are not likely to differ from those of non-pregnant women. However, no epidemiological data exist on the prognosis and survival rate of ovarian chondrosarcoma during pregnancy. Complications during pregnancy and delivery have only been reported for pelvic tumors; these complications include distant metastases, recurrent pain, and fetal growth restriction. As our patient's pregnancy was terminated spontaneously after surgery, we cannot add any data on this issue [1]. EMC has a biphasic histologic pattern comprising small cells and islands of hyaline cartilage [4]. Chondrosarcoma is divided into several subtypes, and 90% are considered as mainly slow-growing tumors with a low risk of metastases. Histologically, chondrosarcoma is graded as G1–3, although G1 has recently been named atypical cartilaginous tumor. G3 tumors are known to have a likelihood of metastasis [1]. These tumors are diagnosed primarily based on morphology, as they have a nonspecific immune profile, and no molecular diagnostic characteristics of these tumors have been specified. On immunohistochemistry, the cartilaginous component of these tumors is strongly positive for S100 protein and the undifferentiated component is positive for CD99, as in our case. Some recent studies have discovered Sox9 as a marker that shows nuclear positivity in both undifferentiated and cartilaginous components. Other markers, including cytoke-



atin, epithelial membrane antigen, and muscle markers, are usually negative. However, rare cases showing scattered tumor cells with desmin, myogenin, and myoD1 positivity have also been described in tumors with areas of rhabdomyosarcomatous differentiation. As described above, in our case with rhabdomyoblastic differentiation, the specimen was positive for myogenin. Overall, no single best stain for this tumor has been identified, and a group of stains should be used to reach a certain diagnosis. Molecular genetic characteristics of these tumors can now be detected, including translocation (9;22) (q22; q11), resulting in the EWSR1/NR4A3 sequence in the majority of patients [5]. Wide and adequate resection of the involved area to treat localized disease is the most promising treatment option for EMC, followed by adjuvant systemic chemotherapy to treat metastatic cases. Adjuvant chemotherapy has been reported to increase the survival rate in some studies, but there is not enough evidence to reach a consensus on this matter [4,5,7]. In general, EMC is known as a highly potent tumor for distant metastasis. Most tumors are low- to intermediate-grade, although our case involved a high-grade tumor, and the clinical course tends to be prolonged in most patients. The prognosis and survival rate depend on the location of the tumor [3,4]. There is no strong evidence favoring a specific optimal option for systemic therapy. Given the aggressive nature of this tumor and poor response to treatment, some experts prefer watchful waiting in metastatic cases following wide local excision [5]. Due to the rarity of this entity, there is no established treatment protocol, and we speculated that this chemotherapy might be of benefit in this case, as the tumor was grade 3 and had a high mitotic rate. The patient had no signs of recurrence 2 years after initial wide local surgery [6]. Data on systemic treatment during pregnancy have not been reported. Despite its ethical issues, termination of pregnancy in non-obstetric chondrosarcoma may also be an option, especially during the first trimester, but it has not been recommended routinely. The absence of other valid therapy options provides the strongest arguments for a surgical approach with the maintenance of pregnancy in cases of chondrosarcomas of the extremities during pregnancy, specifically in the second and third trimesters [1]. A recent retrospective review reported a 68.2% 5-year disease-free survival rate of this malignancy [4]. In another study, the authors concluded that EMC has a relatively favorable prognosis. Some studies have detected relatively long survival times in EMC, even with metastatic disease (2–8 years). A study reported greater cellularity and a higher mitotic rate with a higher likelihood of distant metastases. Nonetheless, in another two studies, the investigators found no relationship between cellularity and prognosis in EMC [3].

Our case involved a tumor with a mitotic rate greater than 18 per 10 high-power fields and grade 3 with no metastasis at the time of diagnosis. However, there are existing data on metastatic lesions ob-

served in the liver in abdominal tomography of grade 1 chondrosarcoma [7]. The present case occurred in a pregnant woman with primary ovarian mesenchymal chondrosarcoma that was treated with surgical resection and systemic chemotherapy after spontaneous termination of pregnancy with no signs of metastasis.

## Conflict of interest

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

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## Author contributions

Conceptualization: SA. Data curation: SA. Formal analysis: SA. Methodology: EG. Project administration: EG. Visualization: SN. Writing—original draft: SN. Writing—review & editing: SN.

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Examples of authors' contributions are as follows:

Conceptualization: BCJ. Data curation: DL. Formal analysis: YIA. Funding acquisition: JHA. Methodology: BCJ. Project administration: MYP. Visualization: MHC. Writing – original draft: DL. Writing – review & editing: BCJ.

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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/ICSI-treated women. *Acta Obstet Gynecol Scand* 2018 Oct 14 [Epub]. <https://doi.org/10.1111/aogs.13483>.

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