#SIMPLE IS GOOD
Aims and Scope

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

Background

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

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REVIEW ARTICLES

273  The impact of COVID-19 on human reproduction and directions for fertility treatment during the pandemic
Dayong Lee

283  Update on genetic screening and treatment for infertile men with genetic disorders in the era of assisted reproductive technology
Seung Ryeol Lee, Tae Ho Lee, Seung-Hun Song, Dong Suk Kim, Kyung Hwa Choi, Jae Ho Lee, Dae Keun Kim

295  Glucocorticoid therapy in assisted reproduction
Yong Jin Kim

303  Decorin: a multifunctional proteoglycan involved in oocyte maturation and trophoblast migration
Beom Seok Park, Jaewang Lee, Jin Hyun Jun

ORIGINAL ARTICLES

311  Progress in human ovarian rejuvenation: Current platelet-rich plasma and condensed cytokine research activity by scope and international origin
E. Scott Sills, Samuel H. Wood

316  The antioxidant roles of L-carnitine and N-acetyl cysteine against oxidative stress on human sperm functional parameters during vitrification
Fatemeh Ghorbani, Zohreh Nasiri, Yeganeh Koohestanidehaghi, Keivan Lorian

322  Adipose tissue-derived mesenchymal stem cells reduce endometriosis cellular proliferation through their anti-inflammatory effects
Fatma Y. Meligy, Dalia A. Elgamal, Lobna A. Abdelzaher, Maha Y. Khassbah, Mohamed A. El-Mokhtar, Ayat A. Sayed, Abeer M. Refaï, Essam R. Othman

337  Administration of red ginseng regulates microRNA expression in a mouse model of endometriosis
Jae Hoon Lee, Ji Hyun Park, Bo Hee Won, Wooseok Im, SiHyun Cho

347  Impact of vitamin D3 supplementation on the in vitro growth of mouse preantral follicles
Yoo Jin Shim, Yeon Hee Hong, Jaewang Lee, Byung Chul Jee

352  Effects of prematuration culture with a phosphodiesterase-3 inhibitor on oocyte morphology and embryo quality in in vitro maturation
Mohammed Ashraf Cherueetil, Prasanna Kumar Shetty, Arya Rajendran, Muhammed Asif, Kamini A Rao

362  Comparison of embryonic competence and clinical outcomes between early and late cumulus cell removal for in vitro fertilization
Pallop Pongsuthirak

368  Anorexigenic peptide (leptin, obestatin, nesfatin-1) levels and their impact on assisted reproductive technology treatment outcomes in patients with polycystic ovary syndrome
Bulut Varlı, Yavuz Emre Sükür, Batuhan Özmen, Berrin İmge Ergüder, Murat Sönmez, Bülent Berker, Cem Atabekoğlu, Ruşen Aytacı

374  Association between the serum estrone-to-estradiol ratio and parameters related to glucose metabolism and insulin resistance in women with polycystic ovary syndrome
Nayoung Kim, Sungwook Chun

CASE REPORT

380  Delayed postpartum regression of theca lutein cysts with maternal virilization: A case report
Sanghwa Kim, Inha Lee, Eunhyang Park, Yeo Jin Rhe, Kyeyongmin Kim, Aminah Ibrahim Aljassim, Joo Hyun Park, Jae Hoon Lee, Bo Hyon Yun, Seok Kyo Seo, SiHyun Cho, Young Sik Choi, Byung Seok Lee
The impact of COVID-19 on human reproduction and directions for fertility treatment during the pandemic

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Since December 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread rapidly, resulting in a pandemic. The virus enters host cells through angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine subtype 2 (TMPRSS2). These enzymes are widely expressed in reproductive organs; hence, coronavirus disease 2019 (COVID-19) could also impact human reproduction. Current evidence suggests that sperm cells may provide an inadequate environment for the virus to penetrate and spread. Oocytes within antral follicles are surrounded by cumulus cells, which rarely express ACE2 and TMPRSS2. Thus, the possibility of transmission of the virus through sexual intercourse and assisted reproductive techniques seems unlikely. Early human embryos express coronavirus entry receptors and proteases, implying that human embryos are potentially vulnerable to SARS-CoV-2 in the early stages of development. Data on the expression of ACE2 and TMPRSS2 in the human endometrium are sparse. Moreover, it remains unclear whether SARS-CoV-2 directly affects the embryo and its implantation. A study of the effect of SARS-CoV-2 on pregnancy showed an increase in preterm delivery. Thus, vertical transmission of the virus from mother to fetus in the third trimester is possible, and further data on human reproduction are required to establish this possibility. Based on analyses of existing data, major organizations in this field have published guidelines on the treatment of infertility. Regarding these guidelines, despite the COVID-19 pandemic, reproductive treatment is crucial for the well-being of society and must be continued under suitable regulations and good standard laboratory practice protocols.

Keywords: COVID-19; Pregnancy; Reproduction; SARS-CoV-2

Introduction

In December 2019, a novel infectious disease was reported in China, and the World Health Organization defined this viral illness as coronavirus disease 2019 (COVID-19). This disease is mainly a respiratory infection with symptoms including fever, cough, and shortness of breath. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the viral pathogen that causes COVID-19. Coronaviruses are enveloped, single-stranded ribonucleic acid (RNA) viruses that express a characteristic corona-like morphology under an electron microscope. In the last 2 decades, humanity has suffered three major coronavirus outbreaks—severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2—that have evoked considerable worldwide health consternation [1-3]. The differences between these three coronaviruses and the patterns of their outbreaks are summarized in Table 1.

SARS-CoV-2 is mainly transmitted via respiratory droplets, although viral RNA has been detected in various samples, such as nasal secretions, sputum, feces, and rarely in the serum and urine [4]. SARS-CoV-2 has a very effective binding affinity to angiotensin-converting enzyme 2 (ACE2) in human cells. The coronavirus invades the cell by attaching the viral spike (S) glycoprotein to ACE2 and employing the cellular serine protease (transmembrane protease serine...
subtype 2 (TMPRSS2)) to cleave the viral S protein to provoke fusion of the viral and host cell membranes. These enzymes are expressed in a wide range of human organs, including reproductive organs and respiratory tissues [5].

Cardiac, nasal, ocular, and neurological symptoms of COVID-19 have been reported. However, the impact of COVID-19 on the human reproductive system remains debatable and warrants investigation. It is important to establish whether natural and assisted reproduction can be safely accomplished in the era of the COVID-19 pandemic. Thus, the purpose of this review was to investigate how SARS-CoV-2 affects the various stages of human reproduction through available research on the subject.

**Sex differences in the transmission of SARS-CoV-2**

The S glycoprotein of the coronavirus is the key protein present on the outer envelope of the virion and plays a principal role in viral entrance by recognizing host cell receptors and modulating fusion of the viral and cellular membranes [6]. The S protein is synthesized and then cleaves to the S1 subunit of the N terminal and the S2 subunit of the C terminal (Figure 1).

Viral entry into the host requires the SARS-CoV2 S protein to bind to the host ACE2 receptor. A host protease, TMPRSS2, acts to cleave the viral S protein at the host cell membrane. Through this process, priming cleavage of the S protein between S1 and S2 takes place, followed by activating cleavage at the S2 site. Coexpression of ACE2 and TMPRSS2, which is crucial for SARS-CoV-2 infection, has been observed in various tissue types, including the lung, nose, heart, colon, and kidney, where COVID-19 related symptoms have been reported [7].

It is plausible that SARS-CoV2 infection might have a detrimental effect on human reproductive function if cells of the male or female reproductive organs also express these genes. Limited data suggest that the receptor basigin/CD147 (BSG) may induce viral entry, similarly to ACE2 [8], and the cysteine protease cathepsin L (CTSL) can potentially cleave to the viral S protein, similarly to TMPRSS2 [9]. Recently, Shilts et al. [10] reported that recombinant SARS-CoV-2 S proteins do not interact with BSG expressed on the membrane of human cells; therefore, the role of these alternate entry receptors is not definitive.

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**Table 1. Biological and clinical characteristics of SARS, MERS, and COVID-19**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pathogen</strong></td>
<td>SARS-CoV</td>
<td>MERS-CoV</td>
<td>SARS-CoV-2</td>
</tr>
<tr>
<td><strong>Reservoir</strong></td>
<td>Bat–musk cat</td>
<td>Bat–camel</td>
<td>Bat–pangolin</td>
</tr>
<tr>
<td><strong>Target enzyme</strong></td>
<td>ACE2</td>
<td>DPP4</td>
<td>ACE2</td>
</tr>
<tr>
<td><strong>Case</strong></td>
<td>8,096 [1]</td>
<td>2,519 [1]</td>
<td>&gt; 161,000,000 [a]</td>
</tr>
<tr>
<td><strong>Death</strong></td>
<td>774 (9.6%) [1]</td>
<td>866 (34.3%) [1]</td>
<td>&gt; 3,343,000 (2.1%) [a]</td>
</tr>
<tr>
<td><strong>Mode of transmission</strong></td>
<td>Droplets</td>
<td>Droplets</td>
<td>Droplets, limited evidence of other routes</td>
</tr>
<tr>
<td><strong>Incubation period (day)</strong></td>
<td>4–7</td>
<td>2–14</td>
<td>1–14</td>
</tr>
<tr>
<td><strong>Key symptom</strong></td>
<td>Cough, fever, diarrhea</td>
<td>Fever, cough, shortness of breath</td>
<td>Fever, cough, shortness of breath</td>
</tr>
<tr>
<td><strong>At-risk group</strong></td>
<td>People with underlying medical conditions</td>
<td>Men &gt; 60 yr</td>
<td>Adults &gt; 60 yr with underlying medical conditions</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td>No specific</td>
<td>No specific</td>
<td>No specific</td>
</tr>
<tr>
<td><strong>Vaccine</strong></td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

SARS, severe acute respiratory syndrome; MERS, Middle East respiratory syndrome; COVID-19, coronavirus disease 2019; CoV, coronavirus; ACE2, angiotensin-converting enzyme 2; DPP4, dipeptidyl peptidase-4.

[a] Figures as of May 2021.
Studies have suggested that females infected with COVID-19 have lower morbidity, mortality, and severe case rates than males, as well as better outcomes. Ding et al. [11] reported that non-menopausal female patients had milder symptoms and better prognoses than age-matched male patients. Among female patients, menopausal women had a longer hospitalization period than non-menopausal women. In that study, both anti-Müllerian hormone (AMH) and estradiol (E2) levels showed significant negative correlations with disease severity. Specifically, E2 levels were negatively correlated with inflammatory markers including interleukin (IL)-2R, IL-6, IL-8, and tumor necrosis factor-alpha in the luteal phase and C3 in the follicular phase. The authors speculated that female hormones in premenopausal women may act as protective factors against COVID-19. Sex differences in the disease severity and prognosis were previously attributed to sex-dependent production of steroid hormones, different copy numbers of immune response X-linked genes, and the presence of disease susceptibility genes, such as TLR7, IFNB1, and IL6 [12]. These results suggest that female hormones in premenopausal women may provide a protective effect against COVID-19. Khan [13] speculated that 17β-E2 would decrease SARS-CoV-2 infection by regulating the renin-angiotensin-aldosterone system, suppressing the inflammatory storm, inducing antiviral immune responses, and promoting the degradation of the virus in endolysosomes. One research group reported that premenopausal women taking combined oral contraceptives had a significantly lower predicted COVID-19 positivity with a reduction in the duration of hospital stay [14]. In another recent study, Ding et al. [15] reported a significant decrease in AMH levels after SARS-CoV-2 infection, suggesting that ovarian injury may occur through COVID-19. Therefore, the causal relationship between the severity of the disease and levels of AMH and estrogen should be carefully considered, and more clinical studies investigating this issue should be conducted.

Cell type-specific expression of genes that produce viral host entry proteins and identification of potential loci of viral infection within the reproductive system are essential to predict whether SARS-CoV-2 has the potential to affect natural and assisted human reproduction. Through studies conducted on gametes, embryos, endometrium, and placenta, we investigated the possible effects of SARS-CoV-2 on human reproduction.

**Effects of SARS-CoV-2 on testes and male gametes**

The presence of ACE2 and TMPRSS2 in reproductive organs is considered a precondition for susceptibility to SARS-CoV-2 infection. Stanley et al. [16] analyzed publicly available single-cell RNA sequencing (scRNA-seq) datasets, their transcriptomic data, and publicly available bulk RNA and proteomics data on human and nonhuman primate reproductive organ tissues. They analyzed the cell-type-specific expression pattern of ACE2 and TMPRSS2. The expression of alternative receptor BSG and protease CTSL were also evaluated. The 11 cell types of the human testes, including somatic niche cells (Leydig cells, endothelial cells, myoid cells, and macrophages) and germ cells (differentiating spermatogonia, early primary spermatocytes, late primary spermatocytes, round spermatids, elongated spermatids, and sperm) were analyzed. A small proportion of spermatogonial stem cells expressed ACE2 and TMPRSS2, although cells coexpressing both genes were extremely rare (0.05% of cells). This low rate of coexpression was similar in other testicular cell types, although the researchers were not able to explore Sertoli cells. These results suggest that sperm cells may not be at increased risk of viral invasion and spread through ACE2 and TMPRSS2. The alternative receptor BSG and protease CTSL were generally expressed in testicular cell types. However, it has not yet been proven whether CTSL can effectively replace TMPRSS2 in viral S protein priming.

Testicular pain and discomfort have been observed in some male patients infected with COVID-19 [17]. Compensated hypogonadism with no statistically significant difference in serum testosterone and follicle-stimulating hormone levels, but significantly higher serum luteinizing hormone levels, has also been reported [18]. These symptoms and signs suggest that SARS-CoV-2 infection may affect the testes. Although SARS-CoV-2 was detected in the testis by reverse transcription-polymerase chain reaction in one case [19], it is still unclear whether SARS-CoV-2 can directly penetrate the testis because there is no additional direct evidence of this possibility. However, in that study, a postmortem examination of the testes of 12 patients revealed significant seminiferous tubular injury, reduced Leydig cells, and mild lymphocytic inflammation.

SARS-CoV-2 may disrupt spermatogenesis through multiple pnenomcious mechanisms that include febrile conditions and oxidative stress. Febrile conditions can decrease the concentration of sperm and increase the proportion of abnormal sperm [20]. SARS-CoV-2 infection activates oxidant-sensitive pathways via inflammatory responses, and reactive oxygen species are produced during this process [21]. This stress stimulates the release of cytokines, causing an exaggeration of the inflammatory responses. Holtmann et al. [22] reported that men who recovered from moderate COVID-19 infection showed a statistically significant impairment in semen parameters compared with men who recovered from mild symptoms and the control group. The detection of SARS-CoV-2 RNA in semen was reported by Li et al. [23], though the methodology of sample collection appears flawed and there was a possibility of contamination. In other studies, no virus was detected in semen samples [22,24,25].

These results suggest that long-term effects of SARS-CoV-2 on male reproductive function are unlikely, and the possibility of transmission of the virus from men through sexual intercourse and assist-
Effects of SARS-CoV-2 on ovaries and female gametes

To date, there are insufficient data on scRNA in human ovaries, specifically the outer ovarian cortex. Published scRNA-seq datasets on nonhuman primate ovarian tissue including oocytes and six somatic cell types (stromal cells, granulosa cells, smooth muscle cells, natural killer cells, macrophages, and endothelial cells) were analyzed [16]. According to these data, a subpopulation of oocytes in nonhuman primate ovarian tissue coexpressed ACE2 and TMPRSS2, while coexpression was not observed in ovarian somatic cells. The coexpression of ACE2 and TMPRSS2 in oocytes increased according to follicular maturation: primordial follicles expressed minimal coexpression, while 62% of antral follicles had detectable coexpression. Considering that antral follicles either undergo ovulation or regression within several days of the follicular phase in each menstrual cycle, a sustained impact of the virus on oocytes seems unlikely. A transcriptomic and proteomic analysis by Virant-klun and Strle [26] also revealed that human oocytes expressed both genes and proteins for ACE2 and BSG. Essahib et al. [27] visualized the viral entry protein by immunohistochemistry using a donated human oocyte. In their study, BSG was mainly present on the oolemma, while ACE2 was not expressed in primary oocytes.

The transcriptome analysis of human cumulus cells using novel RNA-seq datasets revealed that ACE2 is widely expressed in these cell types. By contrast, the expression of TMPRSS2 was reported to be absent or extremely low in human cumulus cells, which is concordant with the primate scRNA-seq results. Therefore, cumulus cells may serve as a physical barrier, and oocytes enclosed with cumulus cells are unlikely to be susceptible to SARS-CoV-2 infection. However, the virus may affect oocytes in other ways, such as through oxidative stress, inflammatory cytokines, distorted metabolism, and changes within the ovarian follicle or ovarian cortical tissues.

Effects of SARS-CoV-2 on embryos

Available research results on human gametes indicate that SARS-CoV-2 infection may not have a long-term effect on male and female reproductive function. Although this statement cannot be interpreted as a definitive conclusion, it suggests that there is a low risk of viral transmission from gametes to embryos during ART, including oocyte retrieval and in vitro fertilization. Although there is currently no evidence that SARS-CoV-2 is transmitted through coitus, ART minimizes the risk of exposing reproductive cells to viral infection compared to natural conception.

Some viruses can infect developing embryos. For example, the Zika virus can infect and propagate into the trophoderm cells of a preimplantation human embryo, causing apoptosis of neural progenitor cells, microcephaly, and miscarriage [28]. Because SARS-CoV-2 is highly contagious, and a significant number of infected individuals are asymptomatic, certain women are likely to become pregnant during the course of subclinical infection. This acts as a risk not only for women who are pregnant naturally, but also for women who are pregnant through ART.

Analysis of publicly available scRNA-seq datasets of zygotes, four-cell and eight-cell embryos, morulae, inner cell masses, epiblasts, primitive endodermers, and trophectodermers for the coronavirus receptors (ACE2, BSG), and the S protein cleavage enzymes (TMPRSS2, CTSL) showed that ACE2 and BSG are expressed in the cells from the zygote to blastocyst stages, including the trophodermal lineage [29]. ACE2, TMPRSS2, BSG, and CTSL are coexpressed in some proportion of epiblast cells and most trophectoderm cells. Moreover, the embryonic and trophodermal cells also express genes for viral replication, interaction with SARS-CoV-2, and endosomal sorting complexes required for transport. Similarly, Weatherbee et al. [30] showed the expression and coexpression of ACE and TMPRSS2 genes in the trophoblast of the blastocyst and syncytiotrophoblast, as well as in the hypoblast, which develop into placental tissues that interact with the maternal blood supply for oxygen and nutrient exchange. Visualizing viral receptors through immunohistochemistry revealed that in preimplantation and peri-implantation blastocysts, ACE2 and BSG were present on the membrane of trophoderm and hypoblast cells, which will both develop into the embryonic part of the placenta (chorion). Both receptors were also present on the membrane of epiblast cells, which will differentiate into the embryo [27].

These results imply that SARS-CoV-2 is theoretically able to bind and invade human pre- and peri-implantation embryos. Both embryonic and extra-embryonic cell lineages display these receptors. This indicates that the virus could invade the embryo at distinct time points before, during, and after implantation through vertical transmission. Further research is needed to determine whether viral infection occurs in vivo or whether it causes failure of implantation, miscarriage, pregnancy complications, fetal compromise, and/or congenital malformations.

Effects of SARS-CoV-2 on the endometrium and implantation

The effects of SARS-CoV-2 on endometrial tissue and implantation remain inconclusive. In this situation, to determine the potential susceptibility of the endometrium to infection by SARS-CoV-2 and possible damage thereafter, Henarejos-Castillo et al. [31] assessed SARS-CoV-2 infection-related gene expression of ACE2, TMPRSS2, TMPRSS4, BSG, cathepsin B (CTSB), CTSL, FURIN, and MX1 from endometrial transcriptomic data sets. The results revealed that TMPRSS4 increased viral susceptibility on its own, and this was observed in the
epithelial cells of gut tissue [32].

Transcriptomic data sets available across the phases of an endometrial cycle were analyzed to evaluate the molecular risk of SARS-CoV-2 infection. Gene expression data on normal endometrium from five studies were assembled throughout the menstrual cycle and included samples from the proliferative phase, early secretory phase, mid-secretory phase, and late secretory phase. ACE2, the principal receptor for SARS-CoV-2 cell entry, showed low expression in the endometrium. The expression of ACE2 increased (fold change, 2.47) from the early secretory to mid-secretory phases, implying an increase of ACE2 expression during the window of implantation and a higher risk of viral infectivity at this stage. TMPRSS2, the major protease involved in SARS-CoV-2 cleavage together with ACE2, had medium endometrial expression throughout the menstrual cycle. There was no correlation between TMPRSS2 and ACE2. These results imply that the endometrium is safe against SARS-CoV-2 infection. Other minor proteases such as TMPRSS4, CTSL, CTSB, and furin showed high expression during certain periods of the menstrual cycle, although none of the highly expressed proteases in this study were reported to initiate SARS-CoV-2 infection. The positive correlation between age and ACE2 from the proliferative to mid-secretory phases, especially in the early secretory phase, suggests that the endometrium in older women could be more susceptible to SARS-CoV-2 infection. However, no prospective studies have assessed the real-world clinical implications of this possibility, and verification is needed.

Overall, the endometrium appears to have low susceptibility to SARS-CoV-2 infection based on low ACE2 and TMPRSS2 expression. Further research on endometrial tissue of women of reproductive age infected with SARS-CoV-2 will help determine whether the causal implications deduced from molecular characterization of the endometrium translates to actual clinical manifestations.

Effects of SARS-CoV-2 on the placenta and pregnancy

SARS-CoV-2 has the potential to affect the embryo. During the early developmental stage, the cells of the embryo are enclosed by a layer of trophodermal cells that differentiate to form the placenta. ACE2 and TMPRSS2 are highly expressed in the developing trophoderm and human placental cells. This suggests that the early stage of a placenta may be permissive to viral infection through vertical transmission of SARS-CoV-2 during early pregnancy via maternal blood. Indeed, virions were detected in the trophoblast cells of the placenta in pregnant women infected with COVID-19 [33-35]. Furthermore, placental damage was observed in pregnancies complicated by SARS-CoV-2 infection [36,37]. However, there are little experimental and clinical data concerning the actual risks of vertical transmission at this earlier stage of pregnancy. Likewise, there is a dearth of information on maternal and neonatal outcomes after SARS-CoV-2 infection in the first to second trimester of pregnancy.

An existing systematic review and meta-analysis on pregnancy and neonatal outcomes reported an increased risk of preterm birth, preeclampsia, cesarean section, and perinatal mortality if COVID-19 infection occurs in the early third trimester [38,39]. Consistently, a Centers for Disease Control and Prevention report on the U.S. population showed that among 3,912 infants born to mothers with SARS-CoV-2 infection, 12.9% were preterm (< 37 weeks), which is higher than the US national estimate of 10.2% [40]. Among neonates with test results, 2.6% tested positive for SARS-CoV-2, and they were born primarily to mothers who were infected at delivery. The occurrence of preterm birth did not differ by the status of maternal symptoms. With regard to pregnancy outcomes, 99.3% had a live birth, 0.3% miscarried before 20 weeks, and 0.4% miscarried after 20 weeks. These study results are compared with those of previous coronavirus infections (SARS-CoV and MERS-CoV) in Table 2 [3,38-41].

Conflicting clinical reports have been published regarding SARS-CoV-2 infection in neonates born to mothers infected with SARS-CoV-2 before childbirth. Some studies reported no evidence of vertical transmission in this situation, while others found that some neonates were born with the SARS-CoV-2 infection [42,43]. However, the methods of sample detection or processing in many studies were flawed. Some studies failed to detect SARS-CoV-2 in neonates or only reported the presence of specific antibodies associated with the viral infection. Others detected the virus, but the route of transmission was not clear, as maternal or newborn blood, amniotic fluid, and placenta were not thoroughly investigated in order to ascertain the maternal and neonatal relationship. Besides, in many of these cases, the possibility that secondary infection occurred immediately after birth among the caregivers cannot be excluded.

A case report describing transplacental transmission of SARS-CoV-2 in a neonate born to a mother infected at the end of the third trimester (35+5 weeks of gestational age) suggests that vertical transmission may be possible at this stage [44]. In this case, (1) maternal viremia; (2) placental infection demonstrated by immunohistochemistry and a high viral load, and placental inflammation confirmed by histological examination and immunohistochemistry; and (3) neonatal viremia following placental infection were confirmed. These comprehensive virological and pathological investigations strongly suggest vertical infection. The neonate presented with neurological manifestations indicated by white matter injury, gradually recovered, and was subsequently discharged from the hospital. Based on this case, transplacental transmission may be possible in the last weeks of pregnancy. Nonetheless, possible transmission and fetal consequences in the first or second trimester are not definitive, and further studies focusing on long-term outcomes in these cases are required.
Conclusions

Through a review of the existing published studies, the possible effects of COVID-19 on human reproduction are summarized in Table 3. With regard to human gametes, sperm cells may not be at an increased risk of viral invasion and spread. Oocytes within antral follicles are surrounded by cumulus cells, which rarely express ACE2 and TMPRSS2. Thus, a sustained impact of the SARS-CoV-2 on oocytes also seems unlikely. These results suggest that infertility treatment can be performed safely if human gametes are obtained and manipulated in line with the established principles for preventing contamination in ART. Additional studies and data are needed to determine the possibility of SARS-CoV-2 affecting the endometrium during the implantation period or the embryo in the early stages of development, and thereby interfering with implantation or increasing the rate of miscarriage. Since the probability of preterm delivery or pregnancy-related complications increases when a pregnant woman is infected with SARS-CoV-2, more emphasis should be placed on preventing infection in these women. However, additional studies on how asymptomatic or self-limiting infection during pregnancy may affect long-term pregnancy outcomes or fetal and neonatal health should be conducted. Nevertheless, it is necessary to be cautious when considering the causal relationships in existing research findings, and the results of molecular biological and clinical studies that are actively being conducted in this area should be regularly reviewed.

Over the last year, accumulated data on molecular virology, immunobiology, and clinical manifestations of SARS-CoV-2 infection have improved the understanding of the effect of this novel disease on human reproduction. As knowledge of the infection increased, the guidelines of major academic societies on the implementation of ART also changed. The guidelines published by major organizations on human reproduction are listed in chronological order and sum-
In the early stages of the COVID-19 pandemic, the effects of SARS-CoV-2 on ART or pregnancy outcomes were unclear, and these concerns suggested that ART should be postponed except for emergent cases. However, one of the most important factors in determining fertility is the woman’s age, and the more deferred the infertility treatment, the worse the outcomes of ART. Moreover, the detrimental effects of SARS-CoV-2 infection increase in severity with age, and this fact implies that older women of reproductive age undergoing ART could be at higher risk of severe symptoms and worsened ART and pregnancy outcomes. Accordingly, the time variable is crucial in specific subgroups of infertile couples, particularly women with a diminished ovarian reserve who tend to lose their fertility potential rapidly with an expected poor prognosis for ART.

In light of the rapidly accumulated knowledge and consensus that infertility treatment should not remain suspended for a long time for the well-being of infertile couples and the health of society, ART should be maintained despite the pandemic. Regardless of the clinical indications, ART should resume under established regulations for local circumstances and characteristics. Given the fact that SARS-CoV-2 has high surface stability on various materials, there are many potential routes by which cross-contamination of reproductive tissue within the laboratory can take place. Patients should be screened before any procedures, and active COVID-19 infections and suspected

Table 4. Representative international guidelines for infertility treatment during the COVID-19 pandemic

<table>
<thead>
<tr>
<th>Organization</th>
<th>Publication date</th>
<th>Major points in the guidelines</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIGO [46]</td>
<td>March 20, 2020</td>
<td>Avoid pregnancy and discontinue fertility treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Suspend the initiation of all new treatment and postpone all non-urgent fertility interventions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oocyte/embryo cryopreservation to postpone pregnancy</td>
</tr>
<tr>
<td>ESHRE [47]</td>
<td>April 23, 2020</td>
<td>ART treatments can be resumed for any clinical indication, in line with local regulations.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vigilance and measured steps must be taken for safe practice</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Discussion, agreement, and consent before starting treatment</td>
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<tr>
<td></td>
<td></td>
<td>Staff and patient triage: 2 weeks before treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Access to advice and treatment; patient education</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adaptation of ART services: sanitation/access restriction/mini teams</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment cycle planning: minimal exposure/re-triage</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Code of conduct for staff and patients</td>
</tr>
<tr>
<td>ASRM [48]</td>
<td>May 11, 2020</td>
<td>Clinics should weigh the benefits and risks of proceeding for the involved individuals.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Only the person undergoing the procedure should be present.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Preoperative viral testing (for SARS-CoV-2) should also be strongly considered.</td>
</tr>
<tr>
<td>ARCS and BFS [49]</td>
<td>June 12, 2020</td>
<td>Resumption of fertility services must take place in a manner that minimizes the chances of spread.</td>
</tr>
<tr>
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<td>Patients considering treatment should be fully informed about the effect of the ongoing pandemic on their treatment and give informed consent.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The fertility sector should adopt sustainable changes in working practices that help to build resilience against any future increases in the spread of COVID-19.</td>
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<tr>
<td></td>
<td></td>
<td>Minimizing clinical risk including OHSS</td>
</tr>
<tr>
<td>ASRM, ESHRE and IFFS [50]</td>
<td>July 13, 2020</td>
<td>Reproductive care is essential for the well-being of society and for sustaining birth rates at a time that many nations are experiencing declines.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>During the pandemic, reproductive medicine professionals should continue working.</td>
</tr>
<tr>
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<td>Develop clear and codified plans to ensure the ability to provide care while maximizing the safety of patients and staff.</td>
</tr>
<tr>
<td>ASPIRE [51]</td>
<td>November 26, 2020</td>
<td>Active SARS-CoV-2 infections and suspected cases should be excluded.</td>
</tr>
<tr>
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<td>Tele-health and social distancing</td>
</tr>
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<td>Air quality control, including the use of air filtration and air pressurization, particularly in surgical and laboratory areas</td>
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<tr>
<td></td>
<td></td>
<td>Shared decisions and informed consent, detailing the risks of attending the facility during the SARS-CoV-2 pandemic</td>
</tr>
</tbody>
</table>

COVID-19, coronavirus disease 2019; FIGO, International Federation of Gynecology and Obstetrics; ESHRE, European Society of Human Reproduction and Embryology; ART, assisted reproductive techniques; ASRM, American Society for Reproductive Medicine; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ARCS, Association of Reproductive and Clinical Scientists; BFS, British Fertility Society; OHSS, ovarian hyperstimulation syndrome; IFFS, International Federation of Fertility Societies; ASPIRE, Asia Pacific Initiative on Reproduction.
patients should be excluded. Both patients and healthcare providers should adhere to the use of appropriate personal protective equipment designated for each phase of the ART procedure. Infertility treatment laboratories manipulating gametes and embryos should establish and strictly follow good standard laboratory practices.

**Conflict of interest**

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

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**References**


Update on genetic screening and treatment for infertile men with genetic disorders in the era of assisted reproductive technology

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A genetic etiology of male infertility is identified in fewer than 25% of infertile men, while 30% of infertile men lack a clear etiology, resulting in a diagnosis of idiopathic male infertility. Advances in reproductive genetics have provided insights into the mechanisms of male infertility, and a characterization of the genetic basis of male infertility may have broad implications for understanding the causes of infertility and determining the prognosis, optimal treatment, and management of couples. In a substantial proportion of patients with azoospermia, known genetic factors contribute to male infertility. Additionally, the number of identified genetic anomalies in other etiologies of male infertility is growing through advances in whole-genome amplification and next-generation sequencing. In this review, we present an up-to-date overview of the indications for appropriate genetic tests, summarize the characteristics of chromosomal and genetic diseases, and discuss the treatment of couples with genetic infertility by microdissection-testicular sperm extraction, personalized hormone therapy, and in vitro fertilization with pre-implantation genetic testing.

Keywords: Azoospermia; Genetics; Infertility; Spermatozoa

Introduction

More than 30% of cases of infertility are related to male factors alone, and male factors alone or in combination with female factors contribute to up to 50% of all cases of infertility among couples [1,2]. Male infertility is related to men's overall health, and genetic abnormalities are thought to cause 13%–30% of cases of male infertility, with azoospermia being the most common phenotype associated with genetic abnormalities [3-5]. However, genetic analyses show no clear etiology of infertility in up to 72% of affected men, leading to a diagnosis of idiopathic male infertility [6].

Advancements in reproductive genetics have provided insights into the mechanisms of male infertility, and an important task in male reproductive medicine is to classify idiopathic male infertility based on its causes and elucidate the details of the pathogenesis of this condition. Based on recent progress in genome-analysis technologies, many genetic variations have been shown to be associated with male infertility [7]. Next-generation sequencing technologies have made particularly significant contributions to the search for candidate genes. Therefore, elucidation of the pathophysiology of male infertility through genetic approaches may contribute to increased pregnancy rates in the era of artificial reproductive technology.

Recently, the indications for genetic screening have been updated to achieve the best sensitivity and specificity from genetic tests. For the treatment of severe oligozoospermia and nonobstructive azoospermia (NOA) with genetic disease, hormone therapy or microdissection-testicular sperm extraction (m-TESE) is required. Following rapid technological advances in pre-implantation genetic testing.
(PGT), more technologies have been developed to enable couples known to be at risk for genetic diseases to avoid passing these diseases on to the next generation [8]. Indeed, these approaches can be used to differentiate affected embryos during in vitro fertilization (IVF) by biopsying embryos to test for structural chromosomal abnormalities and single-gene mutations before implantation [9].

In this review, we present updated genetic screening indications for idiopathic infertility in men, discuss the characteristics of structural chromosomal abnormalities and single-gene mutation diseases, and suggest appropriate treatments for infertility-related diseases caused by genetic abnormalities.

Screening for genetic analysis

1. Updated indications for karyotype analysis

In 2021, the American Urological Association (AUA) and American Society for Reproductive Medicine (ASRM) joined to review the diagnosis and treatment of infertility in men and indicated karyotype analysis for azoospermia or severe oligozoospermia (sperm concentration < 5 million/mL) with elevated follicle-stimulating hormone (FSH) levels, testicular atrophy, or a presumed diagnosis of impaired sperm production as the cause of azoospermia [10]. Additionally, the European Association of Urology (EAU) guidelines state that karyotype analysis should be performed in men with azoospermia or oligozoospermia (sperm concentration < 10 million/mL) and a family history of recurrent spontaneous abortions, malformations, or mental retardation [11]. However, other studies have evaluated these cut-offs and questioned whether there might be more effective thresholds to screen for karyotype abnormalities. For example, Dul et al. [12] investigated the incidence of chromosomal abnormalities and showed that azoospermia occurred in 15.4% of infertile men, whereas 0–1, 1–5, 5–20, and more than 20 million sperm/mL were detected in 3.0%, 2.1%, 2.7%, and 2.9% of cases, respectively. These findings suggest that sperm concentration may not be the most effective threshold for recommending karyotype analysis. Current guidelines for oligozoospermia tend to result in over-testing of patients. Additionally, the newly proposed 2021 AUA/ASRM guidelines include recurrent pregnancy loss (≥ 2), and European Society of Human Reproduction and Embryology special interest group study for recurrent implantation failure (≥ 3) during IVF may also be added to these guidelines [10,13].

2. Updated indications for Y-chromosome microdeletion

The AUA, ASRM, and EAU guidelines recommend Y chromosome microdeletion tests for azoospermia or severe oligozoospermia (< 5 million sperm/mL) with elevated FSH or testicular atrophy [10,11].

A recent systematic review and meta-analysis of European and North American studies estimated the prevalence of Y-chromosome microdeletions according to this guideline and noted that the rate of Y-chromosome microdeletions in azoospermia is approximately 8%–12%, whereas those in very severe oligozoospermia (0–1 million sperm/mL), severe oligozoospermia (1–5 million sperm/mL), and sperm concentrations of 5–20 million sperm/mL are approximately 5%, 0.8%, and 0.5%, respectively [14]. These results demonstrate that Y-chromosome microdeletions are much less common than previously estimated in men with more than 1–5 million sperm/mL. In fact, the prevalence of Y-chromosome microdeletions in this population (0.8%) is similar to that for which testing is not recommended (more than 5 million sperm/mL; 0.7%). Newly published data have shown that Y-chromosome microdeletions are rare (i.e., less than 1%); thus, future guidelines may recommend and confirm Y-chromosome microdeletion testing for men when sperm concentrations are less than 1 million sperm/mL (Table 1).

Sex chromosome abnormalities

1. Klinefelter syndrome

Chromosome abnormalities occur when there is an error in cell division following mitosis or meiosis. An abnormal number of chromosomes is referred to as aneuploidy, which occurs when one chromo-

<table>
<thead>
<tr>
<th>Genetic threshold</th>
<th>Current guideline (thresholds)</th>
<th>New threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Karyotype abnormality</td>
<td>AUA</td>
<td>ASRM</td>
</tr>
<tr>
<td>Karyotype abnormality thresholds</td>
<td>&lt; 5 million sperm/mL</td>
<td>&lt; 5 million sperm/mL</td>
</tr>
<tr>
<td>Y chromosome microdeletion thresholds</td>
<td>&lt; 5 million sperm/mL</td>
<td>&lt; 5 million sperm/mL</td>
</tr>
</tbody>
</table>

AUA, American Urological Association; ASRM, American Society for Reproductive Medicine; EAU, European Association of Urology; nonobstructive azoospermia.
some is missing from a pair (monosomy) or there are more than two chromosomes in a pair (trisomy, tetrasomy, etc.). Klinefelter syndrome is associated with small, firm testes with hyalinization of the seminiferous tubules and failure of spermatogenesis. Patients with this syndrome present with clinical features such as tall stature, slightly decreased intelligence, signs of androgen deficiency, and gynecomastia. The karyotype of patients with Klinefelter syndrome is two or more X chromosomes, with 47,XXY being the most common. Symptoms are more severe if three or more X chromosomes are present (e.g., 48,XXXXY or 49,XXXXXY) [15]. Semen analysis of patients with 47,XXY Klinefelter syndrome typically reveals azoospermia, whereas mosaic Klinefelter syndrome (46,XY/47,XXY) is typically accompanied by severe oligozoospermia to azoospermia.

In addition to reproductive issues, patients with Klinefelter syndrome also exhibit general health problems, such as metabolic syndrome, autoimmune diseases, and psychiatric diseases [16]. Notably, age is the most important predictive factor for the success rate of m-TESE in patients with Klinefelter syndrome, and the success rate has been shown to be higher in men younger than 31 years of age [17]. PGT of embryos resulting from intracytoplasmic sperm injection (ICSI) using sperm from m-TESE is the treatment of choice for patients with Klinefelter syndrome.

2. 47,XXY

47,XXY (Jacob syndrome) is a type of sex chromosomal abnormality related to the occurrence of parental non-disjunction at meiosis II, resulting in the production of an extra Y chromosome and acquisition of the 47,XXY karyotype in affected offspring [18]. Most men with the 47,XXY karyotype are phenotypically taller than average and are at an increased risk of learning problems. Men with 47,XXY syndrome can have variable sperm counts, ranging from normozoospermia to azoospermia. An increased incidence of chromosomally abnormal spermatozoa in the semen is the result of a random event during sperm development [19]. Patients with this condition may receive speech therapy or require extra help with schoolwork, and many patients with 47,XXY are unaware that they have a sex chromosome abnormality.

3. 46,XX male syndrome

46,XX male syndrome (de la Chapelle syndrome) is rare disease that occurs in 1 in 20,000 newborns [20]. The phenotype includes azoospermia, but is notably different from that of Klinefelter syndrome, including a small stature and higher incidence rates of cryptorchidism and ambiguous genitalia [21]. Translocation of sex-determining region Y on the X chromosome is responsible for up to 90% of cases of 46,XX male syndrome. The gene is located below the pseudoautosomal region of the short arm of the Y chromosome, and abnormal translocation can occur during meiosis [22].

46,XX male syndrome presents as azoospermia with a pathology of Sertoli cell-only syndrome (SCOS) on testis biopsy and a sperm retrieval rate of 0%. Therefore, patients with 46,XX male syndrome should not undergo m-TESE. A careful endocrine assessment, including an analysis of FSH, luteinizing hormone, and testosterone levels, and monitoring are recommended.

Autosomal abnormalities

1. Translocation

There are two types of translocations: unbalanced and balanced. Robertsonian translocation is the most common form of unbalanced translocation and is found in 0.9%–3.4% of infertile men [23]. Robertsonian translocations occur when two acrocentric chromosomes break near the centromere, resulting in separation of the long and short arms, and have an incidence of 1:1,000 [24]. The long arms fuse to form one large chromosome, and the short arms fuse to form one small chromosome, which is subsequently lost, resulting in 45 chromosomes [25]. The acrocentric chromosomes in Robertsonian translocations include chromosomes 13, 14, 15, 21, and 22. Carriers of a Robertsonian translocation are phenotypically normal because the lost short arms generally contain nonessential genetic information, resulting in a balanced structural chromosome anomaly [26]. However, this chromosome anomaly can cause meiotic disturbances during spermatogenesis and produce sperm with abnormal genetic material. Owing to the increased rate of unbalanced gametes, patients with Robertsonian translocation are at an increased risk of having aneuploid offspring that can potentially result in miscarriage, congenital anomalies, and babies with trisomy syndromes, such as trisomy 21 (Down syndrome) and trisomy 13 (Patau syndrome). Therefore, these patients should undergo genetic counseling and IVF-ICSI with PGT, which may reduce the miscarriage rate by selecting balanced embryos for implantation during IVF-ICSI [27].

2. Inversion

Paracentric inversions occur when the centromere is not included in the inverted segment, whereas pericentric inversions occur when the centromere is included. Although chromosomal inversion is less common than translocation, inversion has been reported to be 13 times more common in infertile men than in the general male population [28]. The majority of inversion carriers are phenotypically normal; however, some people may have a disease secondary to a breakpoint disrupting a gene [29]. The risk of unbalanced gametes in patients with chromosomal inversion depends on the size and location of the inversion, as well as recombination events. Disruptions in meiosis can lead to spermatogenic failure, thereby resulting in infer-
tility. Therefore, male carriers of chromosome inversions may show various findings in semen analysis, such as azoospermia, oligozoospermia, asthenozoospermia, and normozoospermia.

If unbalanced gametes are produced, genetic counseling should be performed to inform patients of the potential for miscarriage, congenital anomalies, and the transmission of genetic abnormalities, which may affect future male infertility. However, in inversion carriers, the need for PGT is still not clear. Because minimal inversion carriers do not harbor recombinant products, they would not be expected to benefit from PGT. Anton et al. [30] proposed a threshold of greater than 100 Mpb for the size of inverted segments as a possible indication of PGT in patients with chromosomal inversion.

3. Deletions

Large chromosome deletions are the least frequent chromosomal abnormalities in infertile men. Carriers of large chromosome deletions are missing a substantial proportion of genetic material. Large autosomal chromosomal deletions may be life-threatening or could result in significant phenotypic abnormalities owing to developmental delays or specific clinical characteristics, including Prader-Willi syndrome, Angelman syndrome, and Williams-Beuren syndrome [31-33]. The clinical characteristics of chromosomal abnormalities are summarized in Table 2.

4. Y-chromosome microdeletion

The incidence of Y-chromosome microdeletion is 10%–15% in infertile men with NOA or severe oligozoospermia [34]. The Y chromosome contains many genes that are important in spermatogenesis and the development of male gonads [35]. The proposed gene region involved in spermatogenesis related to the Y chromosome is named azoospermia factor (AZF); there are three subtypes (AZFa, AZFb, and AZFc). Phenotypes vary depending on the type of AZF region mutation.

5. AZFa deletions

Deletion of the entire AZFa region results in SCOS and azoospermia [36]. The AZFa region contains two genes: USP9Y and DBY (DDX3Y) [37]. A diagnosis of complete deletion of the AZFa region indicates that retrieval of testicular sperm is not feasible, as the sperm retrieval rate in patients with deletion of the AZFa region is 0%. Therefore, m-TESE is contraindicated in patients with complete AZFa deletion.

6. AZFb deletions

The AZFb region contains two main genes: RBMY and PRY [38,39]. The genes in the AZFb region support the maturity of spermatozoa and are required for the efficient progression of sperm through meiosis into spermiogenesis [40]. The histological phenotype in patients with complete AZFb or AZFb+c deletions shows SCOS or maturation arrest, leading to azoospermia [41]. The chance to retrieve sperm from m-TESE is negligible in cases of AZFb deletion. Therefore, m-TESE is contraindicated in patients with complete AZFb deletion.

### Table 2. Clinical characteristics of chromosomal abnormalities

<table>
<thead>
<tr>
<th>Chromosome disorder</th>
<th>Typical karyotype</th>
<th>Semen analysis</th>
<th>Phenotype</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klinefelter syndrome</td>
<td>47, XXY</td>
<td>Azoospermia–severe oligozoospermia</td>
<td>Tall stature, small testis, gynecomastia</td>
<td>Micro-TESE+IVF-ICSI+PGT-SR</td>
</tr>
<tr>
<td>Jacob syndrome</td>
<td>46,XX/47,XXY</td>
<td>Normal to severe OAT</td>
<td>Normal</td>
<td>IVF+PGT-SR</td>
</tr>
<tr>
<td>46 XX male syndrome</td>
<td>46, XX</td>
<td>Normal to azoospermia</td>
<td>Small stature, cryptorchidism</td>
<td>AID or adoption</td>
</tr>
<tr>
<td>Robertsonian translocation</td>
<td>45,XY;rob (14q15q)</td>
<td>Normal to severe OAT</td>
<td>Normal</td>
<td>IVF-ICSI+PGT-SR</td>
</tr>
<tr>
<td>Balanced reciprocal translocation</td>
<td>Various</td>
<td>Normal to azoospermia</td>
<td>Normal</td>
<td>Various depends on SA</td>
</tr>
<tr>
<td>Inversion</td>
<td>[Inv (9)]</td>
<td>Clinically insignificant variant of the normal karyotype</td>
<td>Normal</td>
<td>Various depends on SA</td>
</tr>
<tr>
<td>Deletion</td>
<td>Prader-Willi syndrome (deletion of part of the father’s chromosome 15)</td>
<td>Hypotonia, short stature, obesity</td>
<td>PGT-SR, speech therapy, growth hormone injection</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Angelman syndrome (deletion of part of the mother’s chromosome 15)</td>
<td>Small head, specific facial features, ataxia, happy personality</td>
<td>PGT-SR, anticonvulsant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Williams-Beuren syndrome (chromosome 7 deletion)</td>
<td>Cardiac disease, brain function abnormality</td>
<td>PGT-SR, avoid calcium, vitamin D, cardiologic evaluation</td>
<td></td>
</tr>
</tbody>
</table>

TESE, testicular sperm extraction; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; PGT-SR, preimplantation genetic testing-chromosomal structural rearrangement; AID, artificial insemination by donor; OAT, oligoasthenoteratozoospermia; SA, semen analysis.

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7. AZFc deletions

The AZFc region contains DAZ, GOLGA2LY, and CSPG4LY [42]. Complete AZFc deletion presents as various types of impaired spermatogenesis [43]. Men with AZFc deletions related to the DAZ gene exhibit variable phenotypes ranging from complete azoospermia to mild oligozoospermia [44]. In a few cases, men with the AZFc deletion have conceived multiple children naturally; however, all of the sons of these men have been found to be infertile [45].

Follow-up studies of patients with AZFc deletions have shown that AZFc microdeletions are associated with a progressive decline in sperm concentration and progression of patients from oligozoospermia to azoospermia [46]. These findings suggest that AZFc deletions could further affect spermatogenesis and worsen the overall sperm quality. Therefore, for men with AZFc deletions who have sperm in the semen, samples should be cryopreserved to prevent possible m-TESE in the future. In 2003, Hopps et al. [47] reported a high sperm retrieval rate (75%) in patients with AZFc deletion. m-TESE could be recommended for patients with AZFc deletion, although patients should be informed of the possibility that the deletion may be inherited in their sons.

8. AZFc partial deletions

The influence of partial deletions on spermatogenesis is still unclear. None of the currently identified AZFc partial deletions completely eliminate any gene family; however, some have been shown to reduce the copy number of genes. The most clinically relevant AZFc partial deletion is the gr/gr deletion, which removes nearly half of the genes present in the AZFc region and therefore affects the dosage of multicycopy genes, such as DAZ, CDY1, and BPY2, in this region [48]. Men with this deletion are at a significantly increased risk for oligozoospermia [49].

Importantly, the effects of AZFc partial deletions on patients’ fertility largely reflect ethnic diversity in spermatogenesis. The reason for these differences is unclear, but they may be related to Y haplogroups and deletion subtypes. This may be clinically relevant because this type of deletion could contribute to the etiology of impaired spermatogenesis. Furthermore, couples should be informed that AZFc partial deletion will be transmitted to their sons and could become complete AZFc deletion in the next generation of male offspring [50].

Single-gene mutations related with abnormal sperm count in spermatogenesis

1. Testis-expressed gene 11 (TEX11)

TEX11 is an X-linked meiosis-specific gene that has been found to be essential for spermatogenesis in humans with a phenotype of NOA [51]. The prevalence of TEX11 mutation in men presenting with NOA is 2.4% but can be as high as 15% when specifically considering men with NOA showing no meiotic arrest with SCOS [52]. The identification of TEX11 can be useful in the diagnostic process and genetic counseling of patients with NOA.

2. Testis expressed gene 15 (TEX15)

TEX15 encodes a testis-specific protein that repairs DNA double-stranded breaks [53]. An analysis of testicular histopathology revealed maturation arrest at the primary spermatocyte stage. The TEX15 gene is currently believed to be responsible for spermatogenesis failure.

3. DEAD-box helicase 3 Y-linked (DDX3Y)

DDX3Y encodes a conserved RNA DDX3 helicase specifically expressed in pre-meiotic spermatogonial cells [54]. This gene is mapped within the AZFa region, and deletion of this region causes SCOS. DDX3Y expression is required for human fetal germ-cell proliferation, and DDX3Y deletions have been identified in patients with SCOS or severe hypospermatogenesis [55].

4. Nuclear receptor subfamily 5 group A member 1 (NR5A1)

Among a cohort of infertile patients, NR5A1 mutations were identified in 3.9% of those with azoospermia or cryptozoospermia and in 4.3% of those with severe oligozoospermia [56]. Based on histological analyses, NR5A1 mutations result in SCOS, severe hypospermatogenesis, or partial spermatocyte arrest in patients with NOA.

5. Heat shock factor 2 (HSF2)

HSF2 encodes a protein belonging to the HSF family, which activates target genes under stress conditions. In humans, three synonymous mutations and mutations in HSF2 have been identified in cases of azoospermia with maturation arrest at the spermatocyte stage [57].

6. Doublesex and Mab-3 related transcription factor 1 (DMRT1)

DMRT1 encodes a testis-specific transcription factor that plays a role in testis differentiation. Deletion of chromosome 9p, which includes DMRT1, is known to be associated with 9p deletion syndrome and XY gonadal dysgenesis [58]. Smaller deletions and mutations of this gene have been identified in patients with NOA without XY gonadal dysgenesis [59].

Single-gene mutations related to abnormal sperm motility in spermiogenesis

1. Multiple morphological abnormalities of the sperm flagella and primary ciliary dyskinesia

Multiple morphological abnormalities of the sperm flagella
(MMAF) are defined as asthenozoospermia and teratozoospermia resulting from abnormalities concerning the sperm flagella, including absent, irregular, coiled, bent, or angulated sperm flagella. Mutations in the DNAH1 gene are responsible for 25% of MMAF cases [60]. DNAH1 encodes an axonal inner dynein arm heavy chain, which often lacks the central pair (9+0 structure).

Primary ciliary dyskinesia (PCD) is a rare autosomal genetic disorder that is multisystemic and is characterized by chronic respiratory tract infections, an abnormal position of internal organs, and motility defects resulting from immotile cilia and flagella [61]. Abnormalities in the DNAI1 and DNAH5 genes account for up to 30% of all instances of PCD [62].

Ultrastructural defects, such as a lack of dynein arms, as detectable by electronic microscopy, and an absence of radial spokes are a specific feature of PCD [62]. Flagella abnormalities are associated with an elevated frequency of aneuploidy and poor IVF-ICSI outcomes [63]. The lack of DNAH1 leads to a disorganized axoneme with a 9+0 structure and the absence of the central pair [64]. This structural alteration may be linked to a decreased implantation rate.

**Single-gene mutations related to abnormal sperm morphology in spermiogenesis**

1. **Globozoospermia**

   Globozoospermia is rare, affecting 0.1% of infertile men, and is characterized by round-headed sperm with few acrosomes, which are unable to fertilize oocytes owing to the lack of an acrosome reaction. Mutations in four genes (DPY19L2, ZPBP, PICK1, and SPATA16) have been shown to be associated with globozoospermia [65].

   The most commonly observed genetic defects in patients with globozoospermia are in the DPY19L2 gene. The gene product of DPY19L2 is involved in elongation and acrosome formation [66]. The only fertility option in patients with 100% globozoospermia is IVF-ICSI. Because a lack of acrosome phospholipase is related to oocyte inactivation, artificial oocyte activation with calcium ionophores or electric activation has been proposed for the treatment of patients with complete globozoospermia. Importantly, men with globozoospermia exhibit high rates of sperm aneuploidy and DNA damage, and couples show low pregnancy rates. Screening for the DPY19L2 gene can be considered as a helpful strategy for men with globozoospermia in order to improve the IVF-ICSI and PGT results and family counseling.

2. **Macrozoospermia**

   Macrozoospermia is characterized by large-headed and multi-flagellated spermatozoa. Mutations in AURKC have been shown to be the main genetic cause of sperm macrocephaly, resulting in alterations in meiotic division and tetraploid spermatozoa if both meiotic divisions are affected. Genetic screening of the AURKC gene could reasonably be performed for patients whose sperm contains 30% or more of large-headed spermatozoa. If a mutation is identified, IVF-ICSI is contraindicated because the macroscopic selection of spermatozoa combined with fluorescence in situ hybridization analysis and flow cytometry does not enable a correlation between sperm morphology and the genetic content of the nucleus. IVF-ICSI is not contraindicated in patients without mutations, and PGT can be recommended for those with intermediate aneuploid spermatozoa. The genes related to spermatogenesis and spermiogenesis are listed in Table 3 and Figure 1.

**Treatment of infertile men with chromosomal and genetic abnormalities**

1. **Clomiphene citrate**

   Clomiphene citrate has also shown efficacy when administered prior to m-TESE. An improvement in m-TESE was demonstrated by Hussein et al. [67], who reported that patients receiving therapy with clomiphene citrate had a 20%–25% greater chance of having sperm identified in m-TESE than those not receiving any adjuvant treatment. Clomiphene citrate is generally well-tolerated, and common side effects include gastrointestinal distress, hair loss, gynecomastia, and weight gain. Visual disturbances, such as blurred vision and diplopia, occur in 1.5% of patients [68].

2. **Aromatase inhibitors**

   Aromatase is a cytochrome P450 enzyme that irreversibly converts androgens to estrogens in various tissues, including the testis, liver, brain, and adipose tissue [69]. Aromatase inhibitors can be used in NOA to address the imbalances in testosterone and estrogen levels that affect spermatogenesis. Aromatase inhibitors can selectively increase endogenous testosterone levels without increasing estrogens [70]. The effects of aromatase inhibitors on spermatogenesis have also been investigated. Patients with NOA may be prescribed 2.5 mg of letrozole daily for 4 months, which can convert hypospermatogenesis to normal spermatogenesis, as demonstrated by testis biopsy [71].

3. **m-TESE**

   m-TESE can be used for treatment for NOA with or without genetic diseases, such as Klinefelter syndrome, 47XYY, and AZFc deletions. The contraindications for m-TESE are 46,XX male syndrome and AZFa or AZFb deletions. In those cases, hormone replacement and m-TESE should be absolutely avoided.
Table 3. Genes related to the failure of human spermatogenesis and spermiogenesis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full name</th>
<th>Phenotype</th>
<th>HUGO gene nomenclature ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEX11</td>
<td>Testis expressed gene 11</td>
<td>Spermatocyte maturation arrest</td>
<td>HGNC: 11733</td>
</tr>
<tr>
<td>TEX15</td>
<td>Testis expressed gene 15</td>
<td>Non-obstructive azoospermia</td>
<td>HGNC: 11738</td>
</tr>
<tr>
<td>DDX3Y</td>
<td>DEAD-box helicase 3 Y-linked</td>
<td>Spermatocyte maturation arrest</td>
<td>HGNC: 2699</td>
</tr>
<tr>
<td>NR5A1</td>
<td>Nuclear receptor subfamily 5 group A member 1</td>
<td>Spermatocyte maturation arrest</td>
<td>HGNC: 7983</td>
</tr>
<tr>
<td>HSF2</td>
<td>Heat shock factor protein 2</td>
<td>Spermatocyte maturation arrest</td>
<td>HGNC: 5225</td>
</tr>
<tr>
<td>DMRT1</td>
<td>Doublesex and mab-3 related transcription factor 1</td>
<td>Non-obstructive azoospermia</td>
<td>HGNC: 2934</td>
</tr>
<tr>
<td>DNAI1</td>
<td>Dynein axonemal intermediate chain 1</td>
<td>Asthenozoospermia</td>
<td>HGNC: 2940</td>
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<tr>
<td>DNAH5</td>
<td>Dynein axonemal heavy chain 5</td>
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<tr>
<td>DPY19L2</td>
<td>Dpy-19 like 2</td>
<td>Globozoospermia</td>
<td>HGNC: 19414</td>
</tr>
<tr>
<td>ZPBP</td>
<td>Zona pellucida-binding protein 1</td>
<td>Globozoospermia</td>
<td>HGNC: 15662</td>
</tr>
<tr>
<td>PICK1</td>
<td>Protein interacting with c kinase–1</td>
<td>Globozoospermia</td>
<td>HGNC: 9394</td>
</tr>
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<td>SPATA16</td>
<td>Spermatogenesis-associated protein 16</td>
<td>Globozoospermia</td>
<td>HGNC: 29935</td>
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<tr>
<td>AURKC</td>
<td>Aurora kinase C</td>
<td>Macrozoospermia</td>
<td>HGNC: 11391</td>
</tr>
</tbody>
</table>

HUGO, Human Genome Organisation; HGNC, HUGO Gene Nomenclature Committee.

Figure 1. Biological overview of the genetic factors involved in human spermatogenesis and spermiogenesis. DDX3Y, DEAD-box helicase 3 Y-linked; DMRT1, dynein intermediate chain 1, axonemal; TEX11, testis expressed gene 11; TEX15, testis expressed gene 15; HSF2, heat shock factor protein 2; NR5A1, nuclear receptor subfamily 5 group A member 1; DNAI1, dynein axonemal intermediate chain 1, axonemal; DNAH5, dynein axonemal intermediate chain 5, axonemal; DPY19L2, Dpy-19 like 2; ZPBP, zona pellucida-binding protein 1; PICK1, protein interacting with C kinase–1; SPATA16, spermatogenesis-associated protein 16; AURKC, aurora kinase C. By courtesy of Encyclopaedia Britannica, Inc., copyright 2011; used with permission.
4. Hormone replacement pretreatment before m-TESE

Positive results have been demonstrated for the use of human chorionic gonadotropin (HCG) hormonal injections in patients with NOA undergoing sperm retrieval in a second round of m-TESE. Shinraishi et al. [72] studied therapy with 5,000 IU of HCG three times weekly for 3 months in patients who were previously shown to lack sperm after m-TESE. HCG treatment significantly increased the rate of sperm retrieval at the time of the second round of m-TESE compared with men who did not receive HCG treatment. The sperm retrieval rate was 21% in patients who had undergone HCG treatment. However, no sperm was retrieved in the untreated group [72]. Despite these findings, the results remain inconsistent. For example, in a study of non-mosaic Klinefelter syndrome, Ramasamy et al. [73] found that the sperm retrieval rate during m-TESE did not depend on the preoperative administration of HCG. Additionally, preoperative testosterone levels were found to be a significant predictive factor for the sperm retrieval rate. Patients with testosterone levels greater than or equal to 250 ng/dL had a 22% higher sperm retrieval rate than patients with testosterone levels less than 250 ng/dL.

IVF-ICSI with PGT for chromosomal structural rearrangement

The first report on children born after PGT was published in 1990 by Handsyde et al. [74], who described the use of polymerase chain reaction to detect repetitive Y-sequences for sex determination in families with X-linked diseases. PGT evolved from an experimental procedure in the early 1990s as an alternative to a well-established clinical procedure. Numerous technical advances in the field of single-cell genetic testing and assisted reproduction have led to extensive changes in PGT practice.

PGT involves the biopsy of a single or few cells from in vitro fertilized oocytes or embryos and testing of the biopsied cells for genetic abnormalities [75]. The selective transfer of embryos unaffected by the study conditions offers the advantages of circumventing an invasive prenatal diagnosis and possible therapeutic termination of pregnancy.

PGT for chromosomal structural rearrangement (PGT-SR) is appropriate for patients with chromosome rearrangements and those who are at risk of creating embryos with an incorrect chromosome number or structure. The indications for PGT-SR include sex chromosome abnormalities and an incorrect chromosome number or structure. (47,XXY Klinefelter syndrome, 47,XY Y Jacob syndrome, reciprocal translocation, Robertsonian translocation, or inversion). Many carriers of balanced chromosome rearrangements are healthy and unaware of their carrier status until they try to have children. Carriers of balanced rearrangements are at risk for producing embryos with the incorrect amount of chromosomal material, which typically do not lead to a successful pregnancy. PGT-SR can help identify embryos with the correct amount of chromosomal material that are most likely to lead to a successful pregnancy and healthy live birth.

Reciprocal translocations occur when pieces of genetic material break off from two different chromosomes and swap places. People that carry a balanced translocation can create embryos that have either the same balanced translocation, the unbalanced form of the translocation where there is a gain or loss of chromosomal material, or a completely normal set of chromosomes. If one parent is a carrier of a reciprocal translocation, approximately 80% of the resulting embryos will contain an incorrect amount of genetic material.

Robertsonian translocations occur when two chromosomes join together to form one large chromosome, giving an overall chromosome count of 45 instead of 46. Inversions are chromosome rearrangements that involve only one chromosome. In an inversion, a segment of a chromosome is flipped and reinserted upside down. People with an inversion may create embryos with missing or duplicated segments of chromosomes.

IVF-ICSI and PGT for monogenic disorders

PGT for monogenic disorders (PGT-M) is an increasingly popular technology that allows couples affected by or known to be at risk for a genetic disease to avoid passing on the condition to the next generation. It is used in conjunction with IVF, after which the embryos are biopsied to test for the specific gene mutation before implantation and pregnancy.

Common indications for PGT-M include cystic fibrosis, spinal muscular atrophy, hereditary hemoglobinopathies, and Huntington disease; however, its indications are rapidly expanding in the post-human genome sequencing era. The transmission of genetic urological disorders, such as Alport syndrome, autosomal dominant polycystic kidney disease, and Von Hippel-Lindau syndrome, could also be prevented by IVF-ICSI and PGT-M [76,77]. Many single-gene mutations linked with spermatogenesis (e.g., mutations in X-linked, Y-linked, and autosomal genes) could be detected by PGT-M, enabling the prevention of disease complications (Table 3).

Conclusion

Previously, karyotype analyses and Y-chromosome microdeletion tests were used as effective techniques for detecting the etiology of azoospermia or severe oligozoospermia. However, in recent years, the indications for these tests have been revised to include abnormalities in X-linked and autosomal genes related to genetic infertility, as well as sex chromosome abnormalities and Y-chromosome ab-
normalities. Male infertility related to impaired spermatogenesis is associated with multiple factors and exhibits genetic heterogeneity, and it is necessary to identify additional candidate genes that may be related to male infertility.

With rapid advances in genetic diagnostic tools, such as next-generation sequencing, single genes related to azoospermia, cryptozoospermia, asthenozoospermia, and specific morphological abnormalities have been identified, resulting in a decrease in “idiopathic” male infertility. Personalized hormonal treatment, genetic counseling, and IVF-ICSI with PGT could prevent the transmission of genetic disorders and enable the implantation of normal embryos, thereby improving pregnancy rates in infertile men with genetic diseases. Additionally, cost-benefit analyses of genetic studies should be investigated in future research.

Conflict of interest

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

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References

18. Wong EC, Ferguson KA, Chow V, Ma S. Sperm aneuploidy and...


47. Hopps CV, Mielnik A, Goldstein M, Palermo GD, Rosenwaks Z.


74. Handyside AH, Kontogianni EH, Hardy K, Winston RM. Pregnan-
cies from biopsied human preimplantation embryos sexed by 
75. Zegers-Hochschild F, Adamson GD, Dyer S, Racowsky C, de Mou-
zon J, Sokol R, et al. The International Glossary on Infertility and 
76. Snoek R, Stokman MF, Lichtenbelt KD, van Tilborg TC, Simcox CE, 
Paulussen AD, et al. Preimplantation genetic testing for mono-
77. Obradors A, Fernandez E, Rius M, Oliver-Bonet M, Martinez-Fresno 
M, Benet J, et al. Outcome of twin babies free of Von Hipp-
pel-Lindau disease after a double-factor preimplantation genetic 
diagnosis: monogenetic mutation analysis and comprehensive 
Glucocorticoid therapy in assisted reproduction

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As glucocorticoids are well-known as important regulators of stress and the immune system, their function and clinical use have elicited substantial interest in the field of reproduction. In particular, the effect of glucocorticoid therapy on endometrial receptivity during assisted reproduction, including in vitro fertilization (IVF) cycles, has led to a great deal of interest and controversy. However, previous studies have not been able to provide consistent and reliable evidence due to their small, non-controlled designs and use of different criteria. Considering the potential risk of exposure to glucocorticoids for mothers and fetuses in early pregnancy, the use of glucocorticoids in IVF cycles should be carefully evaluated, including the balance between risk and benefit. To date, there is no conclusive evidence that the use of glucocorticoids improves the pregnancy rate in IVF cycles with unselected subjects, and a further investigation should be considered with a proper study design.

Keywords: In vitro fertilization; Glucocorticoids; Pregnancy rate; Receptivity

Introduction

Achieving pregnancy with assisted reproductive technology can be affected by multifactorial causes. To date, numerous studies have documented ways of improving the outcomes of in vitro fertilization (IVF), the most important assisted reproductive technique clinically used to manage infertility. These efforts include optimizing IVF procedures in terms of ovarian stimulation [1], oocyte retrieval [2], fertilization [3], and the embryo culture system [4]. However, the current technique as relates to the steps from embryo transfer to implantation—the final hurdle to pregnancy—remains difficult to consider as having been optimized [5]. The uterine endometrium is the last barrier to overcome in order to make further progress in assisted reproductive technology [6].

Endometrial receptivity can be defined as the capacity for endometrial maturation, during which the trophectoderm of the blastocyst can attach to the endometrial epithelial cells and subsequently proceed to invade the endometrial stroma and vasculature [7]. It provides the embryo with the opportunity to attach, invade, and develop in the maternal uterine environment, within the exceptional 3- to 5-day period known as “the window of implantation.” Various strategies for improving endometrial receptivity have been studied, including identifying biomarkers to schedule embryo transfer [8,9], endometrial scratching [10], applying medication or materials to the uterine endometrium [11,12], and even a freeze-all strategy after IVF with controlled ovarian stimulation [13]. However, the strategy that induces optimal endometrial receptivity has not yet been confirmed clinically.

Previous studies have reported that the immune system plays a central role in endometrial receptivity, with resident immune cells modulating the decidual response, epithelial attachment of the embryo, trophoblast invasion, vascular adaptation, and immune tolerance [14-16]. Based on this hypothesis, glucocorticoids have been proposed as a way to improve the embryo implantation rate after IVF and to protect against miscarriage, when administered during embryo implantation through the early placentation phase [17,18]. However, no well-designed clinical studies have offered acceptable conclusions regarding the indications, effectiveness, and safety of adjuvant corticosteroid therapy in IVF cycles. This review aimed to evaluate previous studies on glucocorticoid therapy during IVF cycles and to elucidate weak points to address in future research.
Glucocorticoids and reproduction

Glucocorticoids are essential steroid hormones that regulate diverse cellular functions and are indispensable for maintaining normal physiology by inducing the capacity to respond appropriately to stress through the regulation of metabolic activity, behavior, and even reproduction. Glucocorticoids are synthesized and released by the adrenal cortex under the regulation of the hypothalamus-pituitary-adrenal (HPA) axis, in a pulsatile pattern showing both circadian and ultradian rhythms. Corticotropin-releasing hormone (CRH) and arginine vasopressin are secreted from the parvicellular neurons of the hypothalamus into the pituitary portal circulation, thereby stimulating adrenocorticotropic hormone (ACTH) release from the anterior pituitary gland [19]. ACTH stimulates the adrenal gland to induce steroidogenesis and the production of glucocorticoids. Increased glucocorticoid levels, in contrast, inhibit CRH expression and its secretion, as well as ACTH output by endocrine feedback loops [20]. This rhythmic regulation of glucocorticoid levels is critical for the maintenance of physiological homeostasis and adjustment to acute stress exposure by transiently inducing HPA activity. However, this regulation is not restricted to the HPA axis; instead, it also involves adjustments at the systemic level. The enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) produces cortisol by the enzymatic reduction of cortisone, and the reverse reaction is catalyzed by 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) [21]. Additionally, the glucocorticoid receptor (GR) mediates glucocorticoid activity in a diverse manner, ranging from stimulation to suppression in a cell-specific manner [22-24]. The GR is a member of the nuclear receptor superfamily of ligand-dependent transcription factors and has a modular structure composed of three distinct functional domains [25]. Between the DNA-binding domain and the ligand-binding domain lies a flexible hinge region that provides structural flexibility for genomic interactions and contains a nuclear localization signal [26].

Increased glucocorticoid levels, resulting from either endogenous or exogenous processes, cause various types of reproductive dysfunction via effects in the hypothalamus and pituitary gland within the HPA axis [27]. Recent animal studies suggested that glucocorticoids disturb the hypothalamus-pituitary-ovary axis by direct inhibition of gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus and the synthesis and the release of gonadotropins from the pituitary by suppressing kisspeptin (KISS1) [28,29] and inducing gonadotropin-inhibitory hormone [30,31].

In addition to the effects in the upper neuro-endocrine area, glucocorticoids are known to impact ovarian cyclic physiology and steroidogenesis directly by regulating the functions of granulosa cells, oocytes, cumulus cells, and luteal cells [29,32]. Increased glucocorticoid levels impair the developmental competence of oocytes by triggering apoptosis of granulosa cells [33]. Glucocorticoids differentially induce and repress steroidogenesis in the ovary according to their dose or the stage of follicular development [32,34,35]. The complexity of the relationship between glucocorticoids and ovarian function remains to be elucidated because most previous studies have conducted exposure experiments instead of investigating physiologic functional changes in the ovary.

Early exposure to glucocorticoids blocks estrogen-induced uterine growth, thereby reducing the quantitative capacity of implantation [36,37]. In the endometrium, GR is expressed in stromal, endothelial, and uterine natural killer (uNK) cells [38]. Given that glucocorticoids inhibit angiogenesis [39], reports that the endometrial expression of 11β-HSD1 and GR mRNA is upregulated at menstruation [40], and that the activity of glucocorticoids decreases in response to enhanced 11β-HSD2 levels in the endometrium of women with heavy menstrual bleeding [41], implicate the role of glucocorticoids in the uterine menstrual cycle.

Glucocorticoids and immune modulation

For endometrial receptivity to the semi-allogenic fetus and maintenance of successful pregnancy, various immune cells are recruited and tuned in the microenvironment of the endometrial compartment [42]. Previous studies have revealed the roles of the four main immune cell lineages: uNK cells, dendritic cells (DCs), macrophages, and T-cells. First, uNK cells are known to be important actors in decidual blood vessel modification during the implantation period [43], as well as in uterine arterial modification and optimal placentaion for the development of offspring in early pregnancy [44,45]. Interacting with uNK cells, DCs have been reported to control the adaptive immune compartment and to drive the generation of inducible regulatory T cells to suppress inflammation and mediate immune tolerance of fetal antigens [46,47]. A previous study showed that depletion of uterine DCs resulted in aberrant decidual vascularization and placentaion, leading to impaired implantation [48]. The M2 type of macrophages is required for embryo implantation, ovarian progesterone synthesis, and fetal development [49,50]. It was reported that M2 macrophages inhibit inflammation and contribute to immuno-suppressive function by secreting anti-inflammatory cytokines [51,52]. In interacting with uNK cells and DCs for optimal implantation and successful pregnancy, regulatory T cells regulate vascular adaptation and placental development [53,54]. Some clinical studies suggested evidence that alterations of these immune cells were linked to infertility [55-58]. These immune cells and secreted mediators build on the cyclic immune changes that accompany hormonal fluctuations over the course of every menstrual cycle, particularly af-
ter boosting embryo attachment and trophoblast invasion.

Glucocorticoids could act as potent players in this process due to their potent anti-inflammatory and immunosuppressant actions. Some studies have revealed the effect of exogenous glucocorticoid exposure on these immune cells in the receptivity process [38,59]. uNK cell-mediated cytotoxicity is sensitive to exogenous glucocorticoids by regulation via GR [38,60], and the number of uNK cells in the mid-luteal endometrium decreases [61]. The function of DCs is reported to change in response to glucocorticoids, in terms of their phenotype, maturation, and antigen-presentation [62]. Corticosteroids impair the ability of DCs to activate T cells and shift the balance from cellular (Th1) immunity to humoral (Th2) immunity, and regulatory T cells are induced [63,64]. Corticosteroids shift the macrophage phenotype from pro-inflammatory (M1) to anti-inflammatory (M2), promote phagocytosis, inhibit major histocompatibility complex II expression, block the synthesis of cytokines, prostaglandins, and leukotrienes, and depress tumoricidal and microbicidal activity [63]. Glucocorticoids seem to increase the activity of regulatory T cells and decrease the cytotoxicity of NK cells simultaneously. However, recent studies on immune regulation have suggested that these regulatory activities could be individualized for each respective cell rather than being regulated in the manner of general suppression by glucocorticoids [65,66].

Glucocorticoids and the ovarian response

Some previous studies implied that glucocorticoids have positive effects on the ovarian response to stimulation. One study showed that dexamethasone may influence follicular development and oocyte maturation directly, via 11β-HSD1 in granulosa cells [67], or indirectly, by increasing serum growth hormone and intrafollicular IGF-1 levels [68,69]. The activity of 11β-HSD in ovarian follicular fluid has even been suggested as a predictive marker for IVF outcomes [70]. However, there have been few studies investigating whether glucocorticoid treatment enhances the ovarian response in IVF cycles, and the positive results showing an improved pregnancy rate were mainly reported in old preliminary studies [71]. A previous randomized controlled trial (RCT) showed a lower cycle cancellation rate in IVF cycles of normal responders using oral dexamethasone, but without differences in fertilization, implantation, and pregnancy rates [72]. A recent study suggested that low-dose oral dexamethasone in women with high progesterone levels in the early proliferative phase sensitized the ovary to gonadotropin stimulation, leading to the secretion of less progesterone, and the dexamethasone group showed a higher cumulative live birth rate than the control group [73]. Consequently, to date, the clinical evidence for using glucocorticoids to enhance the ovarian response is limited.

Table 1. Meta-analysis of glucocorticoid therapy in IVF cycles

<table>
<thead>
<tr>
<th>Study</th>
<th>Journal (year)</th>
<th>Included RCT (n, criteria)</th>
<th>Main outcome</th>
<th>Subgroup analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boomsma et al. [74]</td>
<td>Cochrane Database Syst Rev</td>
<td>13 RCTs (1,759 couples, none)</td>
<td>LBR (OR, 1.21; 95% CI, 0.67–2.19; NS; 3 RCTs)</td>
<td>PR per couple in IVF (OR, 1.5; 95% CI, 1.05–2.13; p = 0.02; 6 RCTs)</td>
</tr>
<tr>
<td></td>
<td>(2007)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Boomsma et al. [74]</td>
<td>Cochrane Database Syst Rev</td>
<td>14 RCTs (1,879 couples, none)</td>
<td>LBR (OR, 1.21; 95% CI, 0.67–2.19; NS; 3 RCTs)</td>
<td>PR per couple in IVF (OR, 1.5; 95% CI, 1.05–2.13; p = 0.02; 6 RCTs)</td>
</tr>
<tr>
<td></td>
<td>(2012)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dan et al. [75]</td>
<td>Am J Reprod Immunol (2015)</td>
<td>5 RCTs (828 couples, unexplained recurrent miscarriage)</td>
<td>LBR (OR, 1.58; 95% CI, 1.23–2.02; p = 0.0003; 2 RCTs)</td>
<td>MR (OR, 0.5; 95% CI, 0.31–0.81; p = 0.005; 2 RCTs)</td>
</tr>
<tr>
<td>Kalampokas et al. [76]</td>
<td>Cochrane Database Syst Rev</td>
<td>4 RCTs (416 couples, none)</td>
<td>LBR (OR, 1.08; 95% CI, 0.45–2.58; NS; 2 RCTs)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2017)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Achilli et al. [77]</td>
<td>Fertil Steril (2018)</td>
<td>2 RCTs (202 couples, recurrent pregnancy loss)</td>
<td>OPR (OR, 1.12; 95% CI, 0.75–1.67; NS; 2 RCTs)</td>
<td></td>
</tr>
<tr>
<td>Zhou et al. [78]</td>
<td>Medicine (2021)</td>
<td>3 RCTs (237 couples, anti-thyroid antibody positive)</td>
<td>LBR (OR, 3.19; 95% CI, 1.13–9.04; p = 0.03; 2 RCTs)</td>
<td>MR (OR, 0.62; 95% CI, 0.09–4.32; NS; 3 RCTs)</td>
</tr>
</tbody>
</table>

IVF, in vitro fertilization; RCT, randomized controlled trial; LBR, live birth rate; OR, odds ratio; CI, confidence interval; NS, not significant; PR, pregnancy rate; OPR, ongoing pregnancy rate; MR, miscarriage rate.
Glucocorticoids and embryo implantation

Historically, early experiences of using glucocorticoids in the IVF cycle were for women with positive autoantibodies. With or without low-dose aspirin, some studies reported that glucocorticoid therapy in the peri-embryo implantation period improved the pregnancy rate after IVF in women with positive anti-nuclear antibodies, anti-cardiolipin antibodies, anti-thyroid antibodies, or lupus anticoagulant [17,79,80]. Furthermore, a few studies suggested that glucocorticoid therapy could enhance the IVF pregnancy rate for women without positive autoantibodies [81] and even with unexplained repeated pregnancy loss [82,83]. In contrast to these positive studies, other studies reported that glucocorticoid therapy showed no additional benefit in implantation and pregnancy rates regardless of the dose [84-88]. These discrepant findings concerning the effectiveness of glucocorticoid therapy could be due to inconsistencies in the criteria for recruiting subjects and protocols, including the drugs utilized, dose, and schedule.

Six meta-analyses have been published regarding the effects of glucocorticoids on IVF outcomes [74-78]. The main outcomes are summarized in Table 1. In 2007, the first was published by Boomsma et al. [74], who included 13 studies and found that glucocorticoid therapy led to no significant improvement in the live birth rate (odds ratio [OR], 1.21; 95% confidence interval [CI], 0.67–2.19) or the pregnancy rate (OR, 1.15; 95% CI, 0.76–1.76). In a subgroup analysis including only fresh IVF cycles, they suggested that the pregnancy rate was significantly enhanced (OR, 1.50; 95% CI, 1.05–2.13) in the glucocorticoid treatment groups. In 2012, they reported an updated meta-analysis including 14 studies that showed similar results [74]. Dan et al. [75] reported that prednisolone therapy during IVF cycles improved pregnancy outcomes in women with idiopathic recurrent miscarriage (live birth rate: risk ratio [RR], 1.58; 95% CI, 1.23–2.02; successful pregnancy outcome: RR, 7.63; 95% CI, 3.71–15.69; miscarriage rate: RR, 0.42; 95% CI, 0.28–0.61), in five RCTs. Significant outcomes were not found in a subgroup analysis only including intracytoplasmic sperm injection (ICSI) cycles in their study. In a meta-analysis with four RCTs, Kalampokas et al. [76] reported that there was no conclusive evidence of a difference in the clinical pregnancy rate (OR, 1.69; 95% CI, 0.98–2.90) between glucocorticoid supplementation during ovarian stimulation for IVF or ICSI and the control group. A meta-analysis about the effects of various immunotherapies in IVF cycles for women with recurrent pregnancy loss revealed that prednisolone therapy also showed no significant favorable differences in the pregnancy rate (OR, 1.02; 95% CI, 0.65–1.58) in 2 RCTs [77]. Recently, a meta-analysis of the benefits of glucocorticoid treatment in infertile women with thyroid autoimmune disease during IVF cycles suggested that glucocorticoid therapy showed satisfactory effects on improving the clinical pregnancy (OR, 4.63; 95% CI, 2.23–9.58) and live birth rates (OR, 3.19; 95% CI, 1.13–9.04) in three RCTs [78]. Nonetheless, the efficacy of glucocorticoid therapy in IVF cycles remains to be elucidated, due to the limited number of included studies, as the authors pointed out in their meta-analysis.

Glucocorticoids and potential risk

Most glucocorticoids belong to category C or D according to the United States Food and Drug Administration. This indicates that animal reproduction studies without adequate and well-controlled human data or human data from investigational or marketing experience have shown adverse effects on the fetus, but the potential benefits may warrant use of the drug in pregnant women despite the potential risks. Thus, it is important to consider the benefits and risks of glucocorticoid therapy in IVF cycles, which potentially affect the early pregnancy period. Some animal studies have claimed that exposure to glucocorticoids causes fetal growth retardation, cardiovascular, metabolic, and neuroendocrine disorders, and teratogenic effects [89,90]. Some limited human studies have shown that the use of glucocorticoids spanning the first trimester might be correlated with increases in miscarriage, preterm births, gestational hypertension, and diabetes [91,92]. Although glucocorticoids do not represent a major teratogenic risk in humans, some studies have revealed a possible causal association between cleft lip and palate and the use of corticosteroids during the peri-implantation phase [91,93,94]. Unlike cases in which glucocorticoids must be used due to underlying disease, deliberate risk/benefit analyses should be carried out in cases without preexisting indications for the use of glucocorticoids in IVF cycles.

Conclusions

Glucocorticoids are important regulators of physiologic homeostasis, immune activation, and responses to inflammatory events; therefore, they also play a relevant role in reproduction. The response of endometrial and decidual immune cells required for normal implantation presents a spectrum from normal variation to the status of impaired implantation and affected placentation by corticosteroids. Previous studies have attempted to reveal the effect of glucocorticoid therapy in IVF cycles, showing some possible benefits in patients with autoimmune disease or idiopathic recurrent pregnancy loss. However, most reports were based on small, non-controlled designs with inconsistent criteria, and their conclusions cannot be interpreted as reflecting a scientific consensus. Taken together with the potential maternal and fetal risk, the use of glucocorticoids in IVF cycles should be cautious, and the balance between the risks and ben-
efits should be considered. To elucidate solid indications and a clinical protocol to improve IVF outcomes by using glucocorticoids, further investigation should be considered in properly designed studies.

Conflict of interest

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

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References


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Characteristics and functions of decorin

About 20 years ago, proteoglycan gene families were classified and simplified according to three criteria: their cellular and subcellular location, the overall gene and protein homology, and the presence of specific protein modules within their respective protein cores. In particular, the largest class is formed by a set of extracellular proteoglycans encoded by 25 genes. The first group contains the 4 genes for hyalectan, a major structural component of cartilage, blood vessels, and the central nervous system. The second comprises 18 small leucine-rich proteoglycans (SLRPs). These SLRPs have a multitude of functions, including signaling through various receptors. Many SLRPs are found in the circulation and various body fluids. The third is the SPOCK family of calcium-binding heparan sulfate proteoglycans [1].

The SLRPs expressed in most extracellular matrices (ECMs) generally can act as structural components to maintain tissue architecture. They are involved in range of fundamental biological and physiological functions, including cell adhesion, signal transduction, and the immune response. SLRPs share many biological functions by binding to ECM components, particularly various collagens, receptor tyrosine kinases, and innate immune receptors (Toll-like receptors) on cell surfaces when present in a soluble form [2,3]. They are characterized

Keywords: Decorin; Extracellular matrix; Implantation; Oocyte; Oogenesis; Proteoglycan; Trophoblasts
by a relatively small protein core of 36–42 kDa, including leucine-rich repeats (LRRs) [4]. Individual LRR modules have 20–30 amino acid residues with a highly conserved “LxxLxLxxN” motif. The central LxL part of the module forms the core β strand, with two leucines pointing towards the interior of the protein, making up the hydrophobic core, whereas the variable x residues within the motif are exposed to solvent. Some are involved in interactions with ligands. Asparagines in the motif make continuous hydrogen bonds with backbone carbonyls of neighboring β-strands throughout the entire protein. This extended hydrogen bond network is called the “asparagine ladder.” Therefore, β-strands are more closely packed and assembled into a large β-sheet, making up the entire concave surface of the horseshoe. Variable amino acids, except the conserved β-strands of each LRR module, are surface-exposed. Some of them play important roles in ligand interactions. To prevent the exposure of hydrophobic core of LRR modules, it has two special modules named LRRNT and LRRCT in the N- and C-termini of proteins. These modules do not follow the sequence conservation pattern of LRR modules. They often contain an anti-parallel β-hairpin stabilized by disulfide bridges [5-7].

The 18 SLRP members are grouped into five classes. Classes I–III have the conserved C-terminal cysteine-rich capping motif, a unique feature that has recently been described as the “ear repeat,” appearing as an abnormal pattern of cysteine residues followed by the asparagine residue in consensus sequences. Classes IV and V are non-canonical fragments without the ear repeat [1,2,4]. The sequence alignment and phylogenetic tree of the SLRPs are presented in Figure 1.

Decorin (DCN), which belongs to class I, is produced by a variety of stromal cells in the body, such as fibroblasts in the dermis, cornea, and chondrocytes of cartilage. It may participate in ECM remodeling during the attachment and detachment of the placenta within the course of pregnancy in cows [8]. Those with disrupted DCN genes are viable, but they show fragile skin with markedly reduced tensile strength. As a result, aberrant collagen morphology appears in the

Figure 1. Sequence alignment and phylogenetic tree of small leucine-rich proteoglycans (SLRPs) using known crystal structures of decorin (PDB ID: 1XKU), biglycan (PDB ID: 2FT3), fibromodulin (PDB ID: 5MXX), osteomodulin (PDB ID: 5YQ5), and chondroadherin (PDB ID: 5LFN). (A) Sequence alignment of crystal structures of five SLRPs. Protein sequences were aligned with Clustal Omega (Clustal Omega<Multiple Sequence Alignment<EMBL-EBI) and generated using the ESPript 3.0 program (ESPript 3 [ibcp.fr]). (B) Phylogenetic tree of SLRPs with known crystal structures. (C) Diagram of the crystal structure of Bos taurus decorin rendered with PyMOL v1.8. Vertical arrows indicate β-strands. Coiled ribbons indicate α-helices. Leucine-rich repeats (LRRs) are numbered above the β-strands. N-acetylglucosamine is linked to N182, N233, and N274. Disulfide bridges are shown in green. The terminal LRR Cys capping motif, known as the ear repeat, is highlighted in pink.
skin and tendon with coarser and irregular fiber outlines [9]. Normal DCN expression regulates a wide range of cellular processes including proliferation, migration, apoptosis, and autophagy through interactions with various molecules. However, aberrant expression of DCN has been associated with poor extravillous trophoblast (EVT) invasion of the uterus, which underlies the occurrence of preeclampsia (PE) and intrauterine growth restriction (IUGR) [10].

During pregnancy, placental cells are under tight hormonal control. Among others, they regulate the concentration and activity of specific proteins participating in the ECM remodeling of fetal membranes [11]. Therefore, the proteoglycan DCN plays a variety of roles. The most important role of DCN is that it can regulate cell adhesion mediated by various binding proteins and tissues for fetal formation.

Production of recombinant DCN protein in the laboratory

For functional analysis, DCN should be made by recombinant DNA technology in the laboratory. The whole gene of the human DCN protein was amplified by PCR and cloned into a modified pAcGP67a vector. The cloned DNA of the DCN gene was confirmed by sequencing. Large-scale DNA preparations were then performed to obtain a sufficient amount of transfection-grade plasmid DNA, which was co-transfected with ProGreen (a baculovirus genomic vector) into Sf9 (Spodoptera frugiperda) insect cells. The vector was then generated and amplified by recombination between the cloned vector and viral DNA. The Fc tagged DCN protein was expressed in High Five cells. The culture media of the High Five cells were harvested and supernatants containing secreted DCN-Fc proteins were purified by protein A affinity chromatography. The molecular mass of the DCN protein was calculated to be approximately 37.9 kDa, with an isoelectric point (pI) of 8.61, using the theoretical tool “Compute pI/Mw” (https://web.expasy.org/compute_pi/). The DCN-Fc tag was cleaved by thrombin. Using Superdex 200-size exclusion chromatography (SEC), the cleaved DCN was further purified. It was found to be a monomer, which was validated as a SEC standard component. The purification steps of affinity and SEC were monitored by sodium decyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The above procedure in insect cells allowed the efficient generation of recombinant DCN protein, which was subsequently expressed and purified as shown in Figure 2.

DCN in the ovary and oocyte maturation

The ovary in adults has diverse functions, including follicle growth and maturation, ovulation, regression, and corpus luteum (CL) formation. During this process, bidirectional communication between follicles and the ECM may influence the quality of oocytes in mammals. Many studies have demonstrated that DCN is present in the ECM of normal and tumors ovaries in humans [12,13]. As discussed above, DCN is a secreted proteoglycan that plays a structural role in the ECM. It can interfere with the signaling of multiple growth factors and their receptors [14]. For instance, DCN can directly bind to epidermal growth factor receptor (EGFR), which has many physiological functions in oocyte maturation [15,16]. When DCN binds to EGFR, it causes phosphorylation and activation of EGFR, followed by internalization and downregulation of EGFR signaling, resulting in a chronic blockage of the EGF/EGFR signaling pathway [17-21]. In the ovary, EGF-like molecules, including amphiregulin and epiregulin, with roles in the orchestration of ovulation and subsequent development of the CL in response to the luteinizing hormone surge are produced in a fine-tuned manner [15,22]. Regarding human oocyte maturation, EGF also targets human oocytes to regulate their meiotic maturation [23]. According to a previous report, DCN is present in human and monkey ovarian stroma, follicular theca cells, luteal cells, and the follicular fluid of ovulatory follicles. It has been postulated that DCN can act as a paracrine signaling factor by inhibiting growth factor/growth factor receptor interactions, suggesting that DCN might regulate folliculogenesis and oocyte quality [24]. Peng et al. [25] synthesized full-length cDNA of goat DCN and identified its expression patterns in various tissues and the ovary. They also confirmed that over-expressed DCN not only could promote programmed cell death through a non-mitochondrial apoptosis pathway, but also could enhance cell arrest by p21 upregulation. Notably, the physiological actions of DCN are mediated through multiple signaling pathways, including the PKA, p38 kinase, and PI3K pathways. Kedem et al. [26] recently conducted a prospective study involving 49 patients treated at a local assisted reproductive technology (ART) center. They characterized the in vivo expression of DCN mRNA in mural and cumulus granulosa cells (CGCs). In previous studies, the human chorionic gonadotropin–induced expression of DCN was found to be highly upregulated in pre-ovulatory follicles [27-29]. Interestingly, DCN expression in human CGCs seems to be correlated with maturation stage of the corresponding oocyte. A plausible explanation is that oocytes that arrest at immature stages under controlled ovarian hyperstimulation finally fail to undergo maturation. Their adjacent cumulus cells also fail to express DCN. Another explanation is that downregulated DCN expression in CGCs is one of causative factors for meiotic arrest. Miscommunication between the oocyte and surrounding somatic cells may result in subfertility [26].

Sawada et al. [30] suggested that DCN in the follicular fluid (F-DCN) is a useful biomarker of the quality of oocytes retrieved from the corresponding follicles in ART. They analyzed 130 oocytes of 88 patients treated with ART because of unexplained infertility. In patients with
controlled ovarian stimulation protocols, the median level of F-DCN was slightly higher than that in the serum (S-DCN). However, F-DCN showed a weak negative correlation with S-DCN. They suggested that DCN actively taken into the follicular fluid from blood might play beneficial roles in follicle and oocyte maturation. Regardless of the fertilization method, fertilized eggs showed no significant differences between F-DCN and S-DCN. Interestingly, F-DCN of fertilized oocytes was significantly lower than that of unfertilized oocytes only in patients who underwent intracytoplasmic sperm injection. They also proved that the F-DCN level in intracytoplasmic sperm injection patients had potential to predict fertilization success based on a receiver operating characteristic (ROC) curve analysis. When they established a cut-off level of 34.5 ng/mL for F-DCN based on the ROC curve, they suggested that oocytes from follicles with F-DCN lower than the cut-off level tended to be better than those from oocytes with a high F-DCN [30].

**DCN in the uterus and trophoblast migration**

For successful blastocyst implantation, invasive trophoblast cells should mediate embryonic migration into the decidual matrix, forming abundant networks connecting embryonic tissue to maternal blood vessels. Signaling in pregnancy can induce the differentiation of endometrial stromal cells into decidual cells. Human decidual cells stimulated by steroid hormones can produce 2 SLRPs: DCN and biglycan. The maintenance of pregnancy is guided by the composition and organization of the endometrial ECM in the uterus. Certain pathological conditions that occur during pregnancy, including PE, have been linked to abnormal placental morphology and consequent fetal morbidity. In the uterus of a knockout mouse model, [Figure 2. Production of the recombinant decorin (DCN) protein in insect cells. Recombinant human DCN was generated by a modified pAcGP67a vector. It was purified by affinity chromatography and size exclusion chromatography (SEC). (A) Scheme of affinity chromatography using protein A resin and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel showing the purity of DCN. Lane 1: marker (M); lane 2: flow-through (Ft); lane 3: pre-resin (Pr), which was DCN-Fc bound protein A resin before thrombin treatment; lanes 4-6: elutions (E1-E3) of DCN after thrombin treatment; lane 7: post-resin (Po), which was the remaining resin after elutions of DCN. (B) Elution profiles of SEC and SDS-PAGE gel showing the purity of DCN. For SDS-PAGE analysis, lane 1: marker (M); lane 2-12: fraction numbers of eluted DCN. A) Indicates peak containing DCN.](https://doi.org/10.5653/cerm.2021.05071)
DCN was found to be required for myometrium contraction in a DCN concentration-dependent manner, whereas biglycan exhibited partial compensation for DCN loss [31].

Transforming growth factor (TGF)-β and DCN are produced in the human feto-maternal interface. They play decisive roles in the regulation of trophoblast invasion in the uterus. TGF-β has DCN binding sites, and its activity is controlled by DCN. Lysiak et al. [32] reported that TGF-β and DCN were co-localized in the ECM of first-trimester decidual tissues. They suggested that DCN might inhibit the activity of TGF-β in the ECM of the placenta. In addition, it has been suggested that migration, proliferation, and invasion of EVTs and choriocarcinoma cells are independently regulated by interactions between TGF-β and DCN in decidual tissues [33]. At the human feto-maternal interface, the decidua forms a dense ECM structure that can regulate trophoblast invasion. Experimentally, silencing of KAI1 (a metastasis suppressor) by double-stranded RNA interference reduced expression of DCN, a decidual product implicated in limiting trophoblast invasion. It has been shown that KAI1 is expressed in decidual cells at the feto-maternal interface, where it might participate in the control of trophoblast invasion [34].

**Figure 3.** Schematic diagram showing how decorin acts as a multifunctional molecule during oocyte maturation and embryo implantation. EGF, growth factor receptor; EGFR, epidermal growth factor receptor; TGF, transforming growth factor; PE, preeclampsia; IUGR, intrauterine growth restriction; PROM, premature rupture of membranes.

www.eCERM.org
Several reports have shown that DCN in decidual tissue could regulate migration, proliferation, and invasion of EVTs of the human placenta in a TGF-β-independent manner [33-35]. These functions were differentially mediated by the binding of DCN to various tyrosine kinase receptors, including IGFR1, EGFR, and VEGFR2 [35]. It has been found that the overexpression of DCN in basal decidual cells is associated with a hypo-invasive phenotype and poor endovascular differentiation of trophoblast cells in PE [36]. Supplementation of DCN and knockdown of c-Met can reduce the proliferation and invasion in HTR-8 trophoblast cells. However, induction of autophagy and apoptosis by DCN were not synergistically enhanced by c-Met knockdown. It was found that DCN could promote autophagy and apoptosis mainly through downregulating c-Met/Akt/mTOR activity in human trophoblast cells [10].

Halari et al. [37] found that DCN production increased during the decidualization of human endometrial stromal cells in vitro and early gestation in decidual samples tested ex vivo. Endometrial stromal cell maturation and differentiation into decidual cells are crucial for a normal pregnancy. In their study, depleting DCN in human endometrial stromal cells treated with decidualization stimulation failed to induce morphologically and functionally appropriate maturation and differentiation of decidual cells [37]. Recently, it has been suggested that 2 SLRPs, DCN and biglycan, play important roles in the structural and functional integrity of the placenta and fetal membrane and that their alterations may lead to several pregnancy-related diseases such as repeated implantation failure, PE, IUGR, and premature rupture of membranes [4].

Conclusion

DCN is a multifunctional molecule. Its functions are mediated by a variety of binding events, including receptor-mediated and glycosaminoglycan-mediated binding. At the human feto-maternal interface, TGF-β and DCN play crucial roles in the regulation of trophoblast invasion in the uterus. TGF-β has DCN binding sites. Its activity is controlled by DCN. During pregnancy, orchestrated hormonal control of successful pregnancy should regulate the concentration and activity of specific proteins such as DCN participating in the ECM remodeling of trophoblastic and uterine cells. This review confirms that proteoglycan DCN is an important and multifunctional molecule in the physiological regulation of oocyte maturation and trophoblast migration. The findings discussed herein suggest that recombinant DCN proteins might be useful for substantiating these diverse functions in both animal and in vitro models of oogenesis and implantation (Figure 3).

Conflict of interest

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

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Author contributions

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References


Progress in human ovarian rejuvenation: Current platelet-rich plasma and condensed cytokine research activity by scope and international origin

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**Objective:** As clinicians and patients await consensus on intraovarian platelet-rich plasma (PRP) treatment, this project evaluated contemporary research trends in the literature.

**Methods:** A PubMed/NLM search aggregated all ovarian PRP-related publications (n=54) to evaluate their scope, abstract utility, submission-to-publication interval, journal selected, article processing charge (APC), free reader access to full-text manuscripts, number and nationality of authors, and inclusion of international collaborators. The NIH Clinical Trials database was also audited.

**Results:** Published output on intraovarian PRP has increased consistently since 2016, especially among investigators in Greece, Iran, USA, and Turkey. Between 2013 and 2021, 42 articles met the relevancy criteria, of which 40.5% reported clinical studies, small series, or case reports, 33% described experimental animal models, and 23.8% were opinion/review papers. Only two works included a placebo control group. The submission-to-publication interval (mean±standard deviation) was 130±96 days, there were 5.9±3.2 authors per project, and journals invoiced US $1,613±1,466 (range, $0–$3,860) for APCs.

**Conclusion:** There was no correlation between APC and time to publish (Pearson’s \(r=−0.01\)). Abstract content was inconsistent; sample size and patient age were often missing, yet free full-text “open access” was available for most publications (59.5%). The NIH Clinical Trials portal lists eight registered studies on “ovarian rejuvenation,” of which two are actively recruiting patients, while four have been terminated or have an uncertain status. Two studies have concluded, with results from one posted to the NIH website. PRP and its derivatives for ovarian treatment show early promise, but require further investigation. Research is accelerating and should be encouraged, particularly placebo-controlled randomized clinical trials.

**Keywords:** Fertility; Menopause; Ovary; Platelet-rich plasma; Research

**Introduction**

The pace of intraovarian platelet-rich plasma (PRP) research has increased since the revolutionary work by Pantos et al. in 2016 [1].

Their study was the first to describe a novel PRP application, outlining how a non-pharmacologic method can ameliorate the consequences of ovarian senescence. While hormone replacement therapy (HRT) for menopause and advanced reproductive technologies/in vitro fertilization (IVF) for infertility are well established and familiar [2], the exploration of workable alternatives is important. Successful return of menses after menopause (as a substitute for HRT) and healthy term live births for infertility patients (either with IVF or as unassisted conceptions) following intraovarian PRP have placed this procedure under intense scrutiny, as expected. While intraovarian PRP research remains developmental and nonuniform, a related challenge exists concerning how reports discussing this experimental treatment enter the literature. Against this background, our anal-
ysis addressed these open questions: (1) how have basic science research and experience with intraovarian PRP evolved in recent years, (2) what publishing options are most commonly used by those working in this specific arena, and (3) are there features of the literature that can better meet the needs of the medical authorship community?

Methods

The standard Medical Subject Heading terms “ovarian” AND “platelet rich plasma” were used to conduct a Boolean search on the U.S. NLM PubMed platform to retrieve publications for analysis. Manuscripts accessed with the following terms/key words were also queried: “ovarian,” “ovary,” “PRP,” “platelet rich plasma,” and “rejuvenation.” All results were compared (Supplementary Material 1) and the current analysis was performed on the search that returned the largest set (n = 54). Papers were removed if the subject was outside the scope of reproductive biology (n = 12). Next, the following information was recorded from all remaining abstracts: manuscript type (original data vs. review), veterinary versus human research, sample size, patient age data, the inclusion of a placebo group, the total number of listed authors, the nationality of the first author’s institution, and the availability of free full-text access to the publication. The interval between manuscript submission and publication was also computed from the date information for each article, if given. Article processing charges (APCs) were determined from journal homepages. Information on current clinical study activity in the “ovarian rejuvenation” space was collected from the U.S. NIH Clinical Trials internet portal (www.clinicaltrials.gov) where a similar audit was undertaken. No identifiable patient-level data were included for analysis in this study.

Results

1. Scholarly output

This study considered all NLM publications indexed as related to ovarian PRP from 1983 to 2021, inclusively. Abstracts were collated annually for each full-year sampled except 2021, for which only partial-year data were available. The eligible and excluded journals are compared in Table 1. Only two qualifying papers on ovarian PRP appeared in the NLM literature before 2017, and both involved animal research and were the only research projects to include a placebo control. During the study period, 42 articles met the relevancy criteria, of which 40.5% reported on clinical studies, small series, or case reports, 33% described experimental animal models, and 23.8% were opinion/review papers (Figure 1). Crucially, only two works included a placebo control group and none were randomized clinical

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Table 1. NLM/PubMed journals with “ovarian rejuvenation” publication activity (1983–2021) sorted by criteria, with comparisons in APC and submission-to-publish interval

| APC (USD) | 1,613 ± 1,466 | 2,133 ± 1,248 | 0.31 |
| Interval from submission to publication (day) | 130 ± 96 | 159 ± 54 | 0.35 |

Values are presented as mean ± standard deviation. APC, article processing charge.

*By Student t-test; †Journals publishing multiple works in the designated subject area.
As depicted in Figure 2, the published output on intraovarian PRP has trended upward over time. In this sample, the number of authors per paper (mean ± standard deviation [SD]) was 5.9 ± 3.2 (range, 2–12) and when categorized by the nation of the first author's affiliated institution, 13 nations were represented: China, Egypt, Greece, India, Iran, Italy, Macedonia, Taiwan, Turkey, United Arab Emirates, United Kingdom, Ukraine, and USA. Research deriving from international collaboration—where more than one country appeared in authorship identifiers—accounted for about 21% of published work (n = 9).

The technical aspects of intraovarian PRP publication efforts were also measured, with editorial and publication efficiency calculated from the interval between initial submission and publication. Although these details were absent in 10 of 42 articles (23.8%), where full tracking was possible, the mean ± SD of submission-to-publication interval was 130 ± 96 days (range, 27–431 days). APC data were obtained from each journal's website to determine the cost-to-publish. In this sample, the mean ± SD of APC was US $1,613 ± 1,466 (range, $0–$3,860). While most articles on intraovarian PRP (59.5%) were accessible for free full-text download, publishers of the oldest research (first published in 2013 and 2015) are still collecting access fees to view the full manuscripts. As shown in Figure 3, among the relevant papers there was no correlation between submission-to-publish and APC (Pearson's $r = -0.01$).

2. Clinical trial activity

A U.S. NIH clinical trials registry search for “ovarian PRP or rejuvenation” identified eight studies, of which four were based in the USA, two in Spain, and one each from Greece and Iraq. Four studies examined treatments beyond intraovarian PRP injection, and aimed to ex-
plore intraovarian insertion (by laparoscopy) of autologous bone marrow stem cells or other complex approaches. No study design included a placebo arm. The mean ± SD number of subjects enrolled or planned among all trials was 79.4 ± 64 (range, 3–182), with the eligible patient age ranging from 18 to 60 years.

Discussion

As with any new medical application, the activity of early adopters is often balanced by appropriate mainstream skepticism. This study is the first to describe publishing patterns and measure aggregate output trends on intraovarian PRP from these “lighthouse” researchers. Here a global snapshot of research is provided, both human and veterinary, with documentation of where the research originates, who publishes it, how long it takes to become available, and other aspects. Based on the present findings, the distribution of literature among original clinical work, experimental/animal model activity, and commentary or opinion contributions seems relatively even. Most intraovarian PRP research originates from a handful of countries for now, but the full roster of experts covering this topic actually shows a wider source of global productivity. This shared international interest is also apparent on the U.S. NIH Clinical Trials registry, although this compilation underscores the need for more projects on ovarian rejuvenation.

Several factors identified here might help improve the current situation. Authors of descriptive studies or case reports on intraovarian PRP should include key data in the abstract content, so readers can identify specific facts regarding the population and technique when accessing the abstracts via PubMed. A checklist is now available to meet this need, and adherence to this standard will help clarify future reporting on intraovarian PRP [3]. Academic publishers also have an assignment—they should revisit their toll-collection tendencies and release full texts after 12–18 months into the public domain without charge. The commercial interface between physician-scientists and venues (journals) to disseminate their work will remain relevant given the fast-rising oligopoly of scholarly publishing [4].

As a conspectus of published scholarship on an emerging topic, this analysis has some weaknesses. Unregistered or unpublished activity on intraovarian PRP certainly exists, so the role of publication bias against negative results must be acknowledged. The U.S. NIH Clinical Trials registry also may not give an accurate forecast of research in process, and not all clinical trials are focusing exclusively on PRP. Curiously, no information is available to explain why the status for half of these investigations is either paused or unknown. Furthermore, if the current APCs are not the same as they were when a manuscript was published, this difference would escape detection using this study design. While it is encouraging that each full year’s data reveal progress with increasing activity, further monitoring will be needed to show if this trend continues.

It should be noted that even when a robust, proper RCT for intraovarian PRP does become available, mainstream clinical practice patterns might still resist meaningful change. For example, bias against intrauterine insemination and in favor of IVF as being universally superior has been spotlighted as unfounded, inappropriate, and de-
serving reappraisal [5]. Thus physician training, background, or experience can sometimes outweigh the persuasive gravitas of the RCT. Indeed, this laudable goal is missed by fertility subspecialists who have already admitted much into the clinical arena without RCT support. Perhaps the most conspicuous examples are IVF and intracytoplasmic sperm injection [6,7], both of which became accepted therapeutic mainstays with no RCT validation. Nonetheless, a focus on intraovarian PRP could help enlarge the therapeutic arsenal for women’s health safely, ideally with RCT data. Our analysis suggests this is already underway at numerous research units.

Conflict of interest

ESS and SHW have been assigned a provisional U.S. Patent for process & treatment of ovarian disorders using platelet cytokine derivatives. No other potential conflicts of interest were reported.

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Author contributions

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

Supplementary material

Supplementary material can be found via https://doi.org/10.5653/cerm.2021.04651.

References

The antioxidant roles of L-carnitine and N-acetyl cysteine against oxidative stress on human sperm functional parameters during vitrification

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Objective: Amino acids can protect sperm structure in cryopreservation due to their antioxidant properties. Therefore, the present study aimed to investigate the protective effect of L-carnitine (LC) and N-acetyl cysteine (NAC) on motility parameters, plasma membrane integrity (PMI), mitochondrial membrane potential (MMP), DNA damage, and human sperm intracellular reactive oxygen species (ROS) during vitrification.

Methods: Twenty normal human sperm samples were examined. Each sample was divided into six equal groups: LC (1 and 10 mM), NAC (5 and 10 mM), and cryopreserved and fresh control groups.

Results: The groups treated with LC and NAC showed favorable findings in terms of motility parameters, DNA damage, and MMP. Significantly higher levels of intracellular ROS were observed in all cryopreserved groups than in the fresh group (p ≤ 0.05). The presence of LC and NAC at both concentrations caused an increase in PMI, MMP, and progressive motility parameters, as well as a significant reduction in intracellular ROS compared to the control group (p ≤ 0.05). The concentrations of the amino acids did not show any significant effect.

Conclusion: LC and NAC are promising as potential additives in sperm cryopreservation.

Keywords: Acetylcysteine; Carnitine; Oxidative stress; Spermatozoa; Vitrification

Introduction

Sperm cryopreservation is an effective and useful solution for various conditions such as chemotherapy treatments, donor semen preservation, and infertility surgery that may affect sperm quality [1]. Successful sperm cryopreservation affects the results of infertility treatment [2]. Despite being effective, however, cryopreservation can have adverse effects on sperm functional parameters, as the process of semen cryopreservation can cause the formation of ice crystals, which affect sperm viability [3]. Some research has explored ways to prevent the formation of ice crystals during the process of cryopreservation, and the vitrification technique has been proposed [4]. In the vitrification technique, the processed samples are directly transferred into a liquid nitrogen container [5]. In addition to reducing the damage caused by cryopreservation, this method can be implemented in less time than with conventional methods of sperm cryopreservation, and it is also cheaper [6,7]. It has been found that reactive oxygen species (ROS) are produced during the freeze-thaw process. The unique characteristics of sperm cells, such as a large number of mitochondria and low levels of antioxidants, place sperm at an elevated susceptibility to damage caused by free radicals [8]. The sperm plasma membrane in fresh semen is partially protected against damage by the antioxidant system [8,9-10], but the antioxidant protection of the sperm plasma membrane decreases during cryopreservation; spermatozoa are largely deprived of protection,
and finally, an increase in lipid peroxidation occurs [8]. Researchers have found that ultra-rapid cryopreservation (vitrification) reduces damage to the plasma membrane, motility, mitochondrial membrane integrity, and DNA damage [11]. Amino acids can be used in many ways to increase sperm resistance to cryopreservation cold shock. L-carnitine (LC) is a water-soluble amino acid with an IUPAC name of (3R)-3-hydroxy-4-(trimethylamine)butanoate and a structure derived from the amino acid lysine [8,12]. LC facilitates and increases the entry of long-chain fatty acids into the mitochondria [13]. LC is produced in mammalian epididymal tissue and transported to sperm. Increasing the concentration of LC at the epididymal level causes an increase in sperm motility [14]. Moreover, LC has also been reported to have antioxidant activity [15]. In vivo and in vitro studies have reported that through its antioxidant activity, LC reduces oxidative stress that leads to DNA damage [16,17].

N-acetyl cysteine (NAC) is a thiol-containing compound with strong antioxidant properties. NAC is a precursor to L-cysteine, which plays a role in eliminating free radicals by reacting with ROS. During oxidative stress, the decreased concentration of glutathione is compensated for by the use of NAC as an antioxidant. NAC prevents the sedimentation of membrane proteins in sperm cryopreservation and increases the amount of membrane proteins during cold shock [18]. This amino acid plays an antioxidant role against ROS activity. Numerous studies have documented the effect of NAC in cryopreservation media, leading to improved sperm functional parameters [19]. This study aimed to evaluate the antioxidant effect of NAC and LC on sperm parameters during cryopreservation and to determine the effect of different doses of these amino acids on motility, plasma membrane potential, mitochondrial membrane potential (MMP), intracellular ROS, and DNA damage.

Methods

1. Subject and semen collection
   This study was carried out at the Research Center of Tehran University of Medical Sciences. Twenty normal sperm samples were prepared from the Aban Infertility Center from February 2020 to April 2020. This study was approved by the Ethics Committee of Tehran University of Medical Sciences. Consent was obtained from all participants orally. Samples were obtained from patients by masturbation after 4–6 days of sexual abstinence and kept in sterile cups. Routine sperm parameters were assessed according to the World Health Organization (2010). Sperm motility and concentration were assessed using the CASA system (Sperm Class Analyzer version 5.1; Barcelona, Spain). The inclusion criteria for this study were factors such as volume of 2–6 mL, a concentration of more than $1 \times 10^9$ sperm/mL, and progressive motility of 70%.

2. Vitrification and thawing procedure
   Cryopreservation of sperm samples was performed using the micro-droplet technique. Sperm samples were suspended in human tubal fluid (HTF; Sigma, St. Louis, MO, USA) solution and diluted in a solution containing 0.5 mol/L of sucrose and 5% human serum albumin (HSA, Sigma). Next, 1 and 10 mM LC and 5 and 10 mM NAC (Sigma) were added separately to the previous solution. Finally, a 30-µL drop of the suspension was transferred to a liquid nitrogen container and stored for 1 week. For the warming process, the HTF medium was heated at 37°C for 2 hours. In this phase, the samples were immersed in 5 mL of HTF with 1% HSA. Sperm suspensions were then incubated at 37°C and exposed to 5% CO₂ for 5 minutes. Finally, samples were centrifuged (400 x g, 5 minutes) and the pellets were suspended in 50 µL of HTF [19].

3. Determination of sperm motion characteristics
   To investigate sperm motility, 10 µL of a sample was placed on a Makler slide at 37°C and examined using CASA. The evaluated parameters included motility (%), progressive motility (%), average path velocity (VAP; µm/sec), curvilinear velocity (VCL; µm/s), linearity (LIN; %), and straight-line velocity (VSL; µm/s). Finally, five microscopic fields for 500 spermatozoa were selected to be evaluated.

4. Determination of sperm plasma membrane integrity
   To assess the integrity of the sperm plasma membrane, the hypo-osmotic solution (HOS) test was used. This solution contains 1.35 g of fructose (Merck, Branchburg, NJ, USA) and 0.73 g of sodium citrate (Merck). The HOS solution was diluted with 100 mL of water (osmolality ~190 mOsm/kg). Next, 500 µL of this solution was mixed with 50 µL of the sample at 37°C for 45 minutes, and then 10 µL of the suspension was transferred to a slide. Finally, the samples were evaluated using phase-contrast microscopy (Olympus BX20) [20].

5. Determination of sperm MMP
   A lipophilic cationic dye, JC-1 (T4069; Sigma-Aldrich, USA), was used to investigate MMP. Samples were first centrifuged at 500 x g for 5 minutes, mixed with $1 \times 10^5$ sperm/mL in phosphate-buffered saline (PBS), and 1 mL of this suspension was stained with 1 µg of JC-1 dye. Finally, samples were evaluated using flow cytometry with FL1 (530 nm) and FL2 (585 nm) detectors [21].

6. Determination of sperm DNA damage
   To investigate DNA damage, chromatin was stained using acridine orange. For this purpose, samples were first centrifuged at 5 x g for 5 minutes and were then mixed with Tris-Null-EDTA buffer, which contained 0.15 mM NaCl and 10 mM Tris. Next, 1.2 mL of acridine orange solution and 400 µL of detergent acid solution were added. Finally,
flow cytometry with the FL1 (500–530 nm) and FL2 (620 nm) detectors was used for normal DNA and abnormal DNA, respectively [20].

7. Determination of intracellular ROS
Intracellular ROS levels were evaluated using dihydroethidium. DNA intercalates and emits red fluorescence due to reaction between ethidium bromide and the superoxide anion. Samples were mixed with PBS and a sperm concentration of 1 × 10⁶ sperm/mL was obtained. Next, 1 mL of suspension was added to 10 μL of dihydroethidium solution (Sigma-Aldrich) and incubated at 25°C for 20 minutes. Finally, flow cytometry with the FL2 (525–625 nm) detector was used for the final investigation [19].

8. Flowcytometric analysis
Flowcytometric analysis was performed using the Calibur FACS, with 488-nm excitation of an argon laser. After removing debris, 100,000 sperm cells were assessed through flowcytometry using Cytlogic software version 2.5.1 (Cell Imaging Core, Turku Center).

9. Statistical analysis
The Kolmogorov-Smirnov test was used to confirm the normal distribution of data. One-way analysis of variance and the Tukey test were also used. The statistical analysis was carried out using IBM SPSS ver. 20 (IBM Corp., Armonk, NY, USA). Results are presented as mean ± standard error of the mean. A p-value ≤ 0.05 was considered to indicate statistical significance.

Results
As shown in Table 1, no significant difference was observed in total motility in the LC (1 and 10 mM) and NAC (5 and 10 mM) groups compared to the control group (p ≥ 0.05), but NAC (5 mM) led to a significant increase (p ≤ 0.05). Significantly higher progressive motility and motility characteristics (VSL, VCL, LIN, and VAP) were found in all groups receiving LC and NAC than in the control group. Table 2 shows the results of DNA damage, intracellular ROS, plasma membrane integrity (PMI), and MMP of human frozen-thawed sperm. According to the obtained results, the presence of LC and NAC at both concentrations led to a significant increase in MMP and PMI in comparison to the control group (p ≤ 0.05). LC and NAC significantly reduced DNA damage and intracellular ROS compared to the control group. However, the specific concentrations of LC and NAC

### Table 1. Motility parameters of post-thawed human spermatozoa supplemented with different concentrations of LC and NAC

<table>
<thead>
<tr>
<th>Variable</th>
<th>Fresh-C</th>
<th>Frozen-C</th>
<th>LC 1 mM</th>
<th>LC 10 mM</th>
<th>NAC 5 mM</th>
<th>NAC 10 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility (%)</td>
<td>55.21 ± 15.42</td>
<td>21.33 ± 10.34</td>
<td>25.26 ± 7.51</td>
<td>25.47 ± 7.23</td>
<td>43.17 ± 8.21</td>
<td>29.15 ± 8.33</td>
</tr>
<tr>
<td>Progressive motility (%)</td>
<td>40.52 ± 4.77</td>
<td>18.64 ± 4.22</td>
<td>31.82 ± 4.60</td>
<td>28.60 ± 4.89</td>
<td>30.27 ± 4.86</td>
<td>27.42 ± 4.91</td>
</tr>
<tr>
<td>VCL (µm/sec)</td>
<td>50.16 ± 5.21</td>
<td>31.95 ± 5.65</td>
<td>52.47 ± 5.41</td>
<td>52.18 ± 5.47</td>
<td>52.43 ± 5.77</td>
<td>50.71 ± 6.11</td>
</tr>
<tr>
<td>VSL (µm/sec)</td>
<td>25.12 ± 8.52</td>
<td>12.11 ± 7.73</td>
<td>23.31 ± 8.15</td>
<td>23.91 ± 7.75</td>
<td>25.59 ± 7.23</td>
<td>26.33 ± 7.44</td>
</tr>
<tr>
<td>VAP (µm/sec)</td>
<td>56.32 ± 4.99</td>
<td>35.42 ± 4.74</td>
<td>53.22 ± 5.66</td>
<td>53.45 ± 6.12</td>
<td>57.72 ± 4.83</td>
<td>57.15 ± 5.17</td>
</tr>
<tr>
<td>LIN (%)</td>
<td>13.47 ± 5.49</td>
<td>10.14 ± 5.19</td>
<td>13.50 ± 5.61</td>
<td>13.67 ± 5.12</td>
<td>14.60 ± 5.89</td>
<td>14.88 ± 4.32</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard error of the mean.
LC, L-carnitine; NAC, N-acetyl cysteine; C, control; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; BCF, beat cross frequency.

### Table 2. PMI, DNA damage, intracellular ROS and MMP of post-thawed human spermatozoa supplemented with different concentrations of LC and NAC

<table>
<thead>
<tr>
<th>Variable</th>
<th>Fresh-C</th>
<th>Frozen-C</th>
<th>LC 1 mM</th>
<th>LC 10 mM</th>
<th>NAC 5 mM</th>
<th>NAC 10 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMI</td>
<td>61.5 ± 3.1</td>
<td>29.7 ± 3.5</td>
<td>63.1 ± 3.4</td>
<td>61.3 ± 5.4</td>
<td>66.5 ± 3.1</td>
<td>66.7 ± 3.1</td>
</tr>
<tr>
<td>MMP</td>
<td>60.2 ± 6.4</td>
<td>31.3 ± 6.2</td>
<td>38.6 ± 6.2</td>
<td>39.1 ± 7.0</td>
<td>43.2 ± 7.5</td>
<td>43.2 ± 7.1</td>
</tr>
<tr>
<td>DNA damage</td>
<td>6.2 ± 5.1</td>
<td>9.6 ± 5.5</td>
<td>2.1 ± 1.9</td>
<td>2.9 ± 1.9</td>
<td>3.2 ± 1.5</td>
<td>4.5 ± 1.2</td>
</tr>
<tr>
<td>ROS intracellular</td>
<td>41.4 ± 8.2</td>
<td>62.0 ± 8.1</td>
<td>52.1 ± 8.5</td>
<td>52.6 ± 8.9</td>
<td>51.4 ± 8.3</td>
<td>51.7 ± 8.0</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard error of the mean.
PMI, plasma membrane integrity; ROS, reactive oxygen species; MMP, mitochondrial membrane potential; LC, L-carnitine; NAC, N-acetyl cysteine; C, control.

For intracellular ROS, DNA damage, and MMP, significant differences were observed between the control group and the groups receiving LC and NAC. However, there were no significant differences in these parameters between the control group and the groups receiving the different concentrations of LC and NAC.

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NAC did not show significant relationships with most sperm motility parameters, PMI, DNA damage, intracellular ROS, and MMP. It should be emphasized that there were no differences in the results depending on the concentration of LC, which has been found to be a potent antioxidant capable of improving spermatogenesis.

Discussion

This study investigated the effects of different concentrations of LC and NAC on human sperm parameters during the vitrification process and post-thaw process. ROS products have toxic effects on sperm structure and function in the cryopreservation process. By being converted into hydrogen peroxide, the superoxide anion plays an important role in ROS production. Therefore, by determining the level of intracellular ROS, it is possible to elucidate its effects on sperm structure and function [22]. During the vitrification process, sperm viability and motility are the parameters associated with increased male fertility potential [23]. Antioxidants affect sperm quality and function during the cryopreservation process. Previous studies have emphasized that cryopreservation reduces and even destroys the antioxidant defenses [24,25]. The reduction of antioxidants or inhibition of antioxidant enzymes leads to oxidative stress and, eventually, disruption of membrane fluidity, membrane integrity, impaired sperm motility, and DNA damage during the vitrification process [26]. Oxidative stress, which occurs as a result of ROS accumulation, can have adverse effects on PMI and DNA damage. LC strengthens lipid metabolism, preserves the potential of the plasma membrane, and improves mitochondrial function. However, LC is an apoptosis inhibitor. The antioxidant property that protects the membrane against ROS depends on the oxidation process during which beta-oxidation products are transferred to the mitochondria and finally enter the Krebs cycle [27]. The results of our study confirm that NAC and LC exert a protective function against oxidative stress, but a significant reduction was observed in the level of intracellular ROS in all amino acid-receiving groups at both concentrations. The results of this part of the study are consistent with those of other studies conducted on human and animal sperm, which indicated that lipid peroxidation and intracellular ROS levels are reduced in the presence of NAC and LC [19,28-30]. Evidence indicates that NAC can increase the antioxidant activity of enzymes such as glutathione peroxidase and catalase [31,32]. The reduction of intracellular ROS can thus be explained by the increased activity of antioxidant enzymes. Oxidative phosphorylation in the mitochondria leads to the production of ROS. In the LC and NAC-receiving groups (at both concentrations), the sperm MMP was higher than in the control group. The MMP seems to increase through reductions in intracellular ROS levels. Normal motility is a main characteristic of sperm needed for their physiological activity, and therefore motility can affect fertility outcomes. From the present study, it is inferred that the presence of NAC (10 mM) and LC (1 and 10 mM) in the cryopreservation groups did not improve sperm compared to the control group. One possibility is that the doses of LC and NAC should be adjusted to improve the results in terms of sperm parameters, or the method of administering these amino acids could be changed. These results are inconsistent with studies in the literature showing that LC at both concentrations (1 and 10 mM) can effectively improve sperm motility compared to the control group [19]. However, NAC (5 and 10 mM) and LC (1 and 10 mM) improved sperm motility compared to the control group. This result can probably be attributed to the lack of reduction of the superoxide anion in the presence of NAC and LC at the given concentrations. The motility characteristics (VSL, VCL, VAP and LIN) improved in the NAC and LC-receiving groups compared to the control group. In this study, the results of sperm PMI were similar in the NAC and LC-receiving groups, and NAC and LC caused an increase in PMI compared to the control and fresh groups. This effect was probably associated with the reduced level of the superoxide anion in the presence of these amino acids. The findings from the analysis of DNA damage in this study show that NAC and LC have the ability to prevent DNA damage, as significantly less DNA damage was observed in groups containing these two amino acids in cryopreservation media. This result is inconsistent with that obtained by Banihani et al. [12], who showed that LC did not prevent DNA damage. LC has important effects on sperm metabolism and spermatogenesis [23]. These results are also inconsistent with previous studies on sperm ROM, indicating that cysteine did not prevent DNA damage; this inconsistency can most likely be explained by differences in the consumed doses of amino acids, sperm species (human and animals), test conditions, and chromatin density. However, these results are similar to those reported by Shahverdi, Vatankhah, and Thawanut, who stated that the presence of cysteine in cryopreservation media could reduce DNA damage in buffalo and human sperm [19,33,34]. Despite these inconsistent findings, it can be stated that LC and NAC can probably inhibit DNA damage by affecting the structure and density of DNA. We suggest that more functional parameters, such as sperm acrosome integrity and malondialdehyde levels, should be assessed in the future to elucidate the antioxidant potential of these two amino acids in the vitrification process.

In cryopreservation media, NAC and LC can reduce the level of oxidative stress, their products, intracellular ROS, and DNA damage, and following this reduction, all sperm functional parameters affected and damaged by these products in the cryopreservation media are
recovered. The protective and beneficial effects of these two amino acids were observed for PMI, MMP parameters, and sperm motility characteristics. Thus, NAC and LC can be used to improve sperm function in cryopreservation media.

Conflict of interest

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

Author contributions

Conceptualization: KL, Data curation: FG, Formal analysis: KL, Funding acquisition: KL, Metodology: YK, Project administration: ZN, Visualization: ZN, Writing original draft: KL, Writing review & editing: KL.

References

Adipose tissue-derived mesenchymal stem cells reduce endometriosis cellular proliferation through their anti-inflammatory effects

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Objective: Endometriosis is a chronic debilitating inflammatory condition characterized by the presence of endometrial tissues outside the uterine cavity. Pelvic soreness and infertility are the usual association. Due to the poor effectiveness of the hormone therapy and the high incidence of recurrence following surgical excision, there is no single effective option for management of endometriosis. Mesenchymal stem cells (MSCs) are multipotent stromal cells studied for their broad immunoregulatory and anti-inflammatory properties; however, their efficiency in endometriosis cases is still a controversial issue. Our study aim was to evaluate whether adipose tissue-derived MSCs (AD-MSCs) could help with endometriosis through their studied anti-inflammatory role.

Methods: Female Wistar rats weighting 180 to 250 g were randomly divided into two groups: group 1, endometriosis group; established by transplanting autologous uterine tissue into rats’ peritoneal cavities and group 2, stem cell treated group; treated with AD-MSCs on the 5th day after induction of endometriosis. The proliferative activity of the endometriosis lesions was evaluated through Ki67 staining. Quantitative estimation of interferon γ, tumor necrosis factor-α, interleukin (IL)-6, IL-1β, IL-10, and transforming growth factor β expression, as well as immunohistochemical detection of CD68 positive macrophages, were used to assess the inflammatory status.

Results: The size and proliferative activity of endometriosis lesions were significantly reduced in the stem cell treated group. Stem cells efficiently mitigated endometriosis associated chronic inflammatory reactions estimated through reduction of CD68 positive macrophages and the expression of the proinflammatory cytokines.

Conclusion: Stem cell therapy can be considered a novel remedy in endometriosis possibly through its anti-inflammatory and antiproliferative properties.

Keywords: CD68; Cytokines; Endometriosis; Ki67; Mesenchymal stem cells; Rats

Introduction

Endometriosis is a chronic inflammatory gynecological condition characterized by the presence of ectopic glands and/or endometrial stroma outside the uterine cavity, which may be superficial peritoneal, vaginal, or deep endometriosis [1,2]. It is a benign pathology [3] detected in 20%–50% of women investigated for infertility probably due to the associated adhesions, fibrosis, endocrine abnormalities and immunological disturbances [4]. Dysmenorrhea, dyspareunia and chronic pelvic pain are the usual complaints [5,6]. Endometriosis is considered the major cause of hysterectomy and hospitalization in USA imposing huge economic burden on healthcare system [7].
Chronic intraperitoneal inflammation is a distinct feature of endometriosis [8-10]. T helper and regulatory T (Treg) cell subsets are recruited and activated by endometriosis implants, resulting in an acute inflammatory response [11]. Monocytes/macrophages sustain a state of chronic inflammation [12-18] after acute inflammation has subsided, promoting the development and survival of endometriosis lesions [19-21].

The current endometriosis treatment options are limited to suppressing ovarian function simulating premature menopause or surgical removal of the lesions [22]. Hormonal therapy, androgen, and gonadotropin-releasing hormone, beside non-steroidal anti-inflammatory medications are currently used to treat pain [23]. Hormonal treatment has little to no benefit, with a high rate of endometriosis recurrence [23] and undesirable adverse effects such as hot flushes and genital atrophy [24]. It should only, therefore, be used in conjunction with assisted reproductive technology [11]. Multiple operations may be inevitable [24] to kill or remove the majority of endometriosis implants or restore normal pelvic anatomy [11], however, their role is still controversial. As a result, a novel therapeutic approach for effective management of endometriosis is mandatory.

Mesenchymal stem cells (MSCs) are non-immunogenic cells that can differentiate into a variety of tissue types [25,26]. In inflammatory conditions, they release immunomodulatory, angiogenic, and antiapoptotic factors [25-27]. Their role in cases of endometriosis is still a point of argument [28].

In this context, endometriosis was induced in female rats by autotransplantation uterine implants into their peritoneal cavity. Rats are an excellent experimental model due to their similarity in pathology as well as therapeutic response to human endometriosis [29]. They display human-like symptoms such as decreased fertility and fecundity [29].

The current study has been conducted to investigate the possible mitigating impact of adipose tissue-derived MSCs (AD-MSCs) in endometriosis rat model, as well as their effect on their cytokine profile. Our target was achieved through studying the effect MSCs on endometriosis lesions histopathology, proliferative activity, the expression of CD68 positive macrophages and the proinflammatory cytokines.

**Methods**

1. **Animals and experimental design**

   All procedures were performed in agreement with the ethical principles of Assiut University Animal Care Committee (approval reference No. 17300077) and with the internationally accepted principles for Use and Care of Laboratory Animals. Twenty adult female Wistar albino rats weighting 180 to 250 g, 3 to 6 months of age were purchased from the animal house of Faculty of Medicine, Assiut University. They were housed and bred in a standard animal-grade room with four to five rats in each cage at a 12-hour light/dark cycle with free access to food (laboratory chow) and water ad libitum. The animal room is well-ventilated with temperature ranging from 23°C to 26°C. Estrus cycles were synchronized to estrus phase through furnishing female rat cages with beddings from male rat cages 72 hours before the surgery or tissue collection.

2. **Establishment of the rat model of endometriosis**

   Surgical induction of endometriosis was achieved through autologous transplantation of one of the female rat’s uterine horn onto its intestinal mesentery [30,31]. Briefly, rats were anesthetized with ketamine (90 mg/kg) intraperitoneally (i.p.) and xylazine (10 mg/kg) i.p. Skin on the ventral aspect was cleaned with 70% ethyl alcohol. A lower abdominal midline incision was performed; the left uterine horn was exposed, ligated at cervicouterine junction with silk suture (No. 40) then excised and opened longitudinally. The excised hom was submerged in a sterile small petri dish containing Ham's F-12 medium with 100 U/mL penicillin and 100 pg/mL streptomycin, warmed to 37°C. It was divided into three pieces: each piece was 2 to 3 mm. These cut pieces have been sutured with non-absorbable sutures (Prolene 4/0) onto the intestinal mesentery nearby a branch of the mesenteric arterial arcade. The Abdomen was closed in layers. Animals were observed until full recovery. They were given nalbuphine for postoperative analgesia and were kept on antibiotics for 3 days postoperatively.

3. **Isolation of AD-MSCs**

   MSCs were isolated from adipose tissue (60–100 mL) obtained from lipectomy procedures carried out in plastic surgery department in Assiut University. The samples were washed with 5% antibiotic phosphate buffered saline (PBS) then digested in warm filtered 0.1 collagenase solutions dissolved in PBS in a shaking water bath at 37°C for 60 minutes. The suspension was centrifuged and the supernatant floating fatty layer was discarded. The precipitated cell pellets were re-suspended in freshly prepared complete Dulbecco's modified eagle medium (DMEM) with 10% FBS and 1% penicillin streptomycin; filtered through a 100 μm nylon cell strainer (Falcon; Corning, NY, USA) and cultured in 75 cm² culture flasks. The media was changed after the first 48 hours to remove the non-adherent cells, and then changed every 2–3 days till a confluence of 80%–90% was attained [32].

4. **Differentiation of AD-MSCs**

   For the differentiation analysis, AD-MSCs passage 2 that reached nearly 80% confluence was enzymatically harvested in trypsin-ethylenediaminetetraacetic acid (EDTA 0.25%; Gibco, Amarillo, TX, USA).
AD-MSCs were cultured in 24 well-plate (1 × 10^4 cell/well) in the complete growth medium DMEM low glucose supplemented with 10% FBS. After reaching 100% confluence, the medium was replaced with adipogenic induction medium (adipogenic differentiation medium; R&D Systems, Minneapolis, MN, USA; cat no SC006), a chondrogenic induction medium (chondrogenesis differentiation kit; R&D Systems; cat no. SC006) and osteogenic induction medium (Osteogenesis Differentiation Kit; R&D Systems; Cat no SC006). After 7–21 days incubation (for adipogenesis), 14–21 days (for chondrogenesis) and 14–21 days (for osteogenesis), the cells were observed using an inverted microscope. The cells were fixed in 4% formaldehyde in saline and stained, using oil red O staining for lipid vacuoles in adipocyte, Alcian Blue staining, which is specific for glycosaminoglycan, one of the components in chondrocytes extracellular matrix and Alizarin Red staining which is specific for mineralized matrix expression in osteocytes. The cell were observed and photographed by inverted microscope.

5. Immunophenotyping of AD-MSCs using flowcytometry

AD-MSCs of passage two were trypsinized using 10% trypsin EDTA solution. Then, they were incubated with CD90, CD44, CD45, and CD34 primary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) in 1% bovine serum for 30 minutes. MSCs were washed by PBS, centrifuged for 5 minutes, incubated with the secondary antibody for 30 minutes, washed twice then subjected to fluorescence-activated cell sorting cell analyzer [33].

6. Transplantation of AD-MSCs

The 2 × 10^6 of AD-MSCs were suspended in 500 µL PBS and injected i.p. as a single dose in the stem cell treated group.

7. Animal groups

Rats were randomly assigned into two groups. Group 1: endometriosis group (n = 10); endometriosis was induced through autologous transplantation of one of the female rat’s uterine horn onto its intestinal mesentery [30,31]. After induction, rats were kept without any interference for 4 weeks. Group 2: stem cell treated group (n = 10); rats were injected with AD-MSCs in a dose of 2 × 10^6 on the 5th day after induction of endometriosis. Both groups were scarified 4 weeks after induction of endometriosis.

8. Histopathological studies

At the end of the experiment, rats were sacrificed under general anesthesia through inhalation of ethyl ether. Rats from each group were intracardially perfused by 10% formaldehyde solution. Specimens of ectopic endometriosis lesions from both groups were carefully excised, immersed into 10% formaldehyde, dehydrated, cleared, and embedded in paraffin. Paraffin sections were serially cut from paraffin blocks (5 µm-thick) using a microtome and stained with Hematoxylin and Eosin (H&E) for examination with light microscope [34].

9. Immunohistochemistry studies

Paraffin sections were serially cut from paraffin blocks (5 µm-thick). Antigen retrieval was achieved by boiling the sections with sodium citrate buffer (0.01 mol/L, pH 6) for 9 minutes. Sections were incubated for 10 minutes in 3% hydrogen peroxide to block endogenous peroxidase activity. Immunohistochemical staining was performed by the avidin–biotin immunoperoxidase method. Primary antibodies Ki67 rabbit polyclonal antibody (Novus Biologicals, Centennial, CO, USA; catalog no. NB500-170SS) and CD68 mouse monoclonal antibody (Novus Biologicals; catalog no. NBP2-29406) were used in a dilution of 1:50 and 1:100 respectively. Sections were incubated with the primary antibodies overnight at 4°C. Negative control sections were subjected to the same protocol. Positive control sections for Ki67 were carried out on human breast carcinoma tissue; however rat tonsillar tissue was used for CD68. The UltraVision horseradish peroxidase detection system (Thermo Fisher Scientific, Waltham, MA, USA) was used. The slides were incubated with biotinylated goat antipolyvalent as a secondary antibody at room temperature for 30 minutes then were counterstained with Meyer hematoxylin, dehydrated, and mounted.

10. Morphometric studies

Morphometric studies have been performed using the computer-assisted image analysis (Soft Imaging System, Analysis-2004; Olympus, Tokyo, Japan). The number of Ki67 and CD68 positive cells were counted using ×100 oil immersion lens in five non-overlapping fields in six randomly chosen sections from three different rats from each group.

11. Cytokine expression levels

Rats' macrophages were obtained from peritoneal and endometriosis tissues. Primary peritoneal rat macrophages were isolated as described previously [35]. Briefly, macrophages were collected from the peritoneal cavity of both endometriosis and stem cell treated group by flushing the peritoneal cavity twice with 50 mL of ice-cold PBS. The recovered cells were washed two times, counted, and resuspended in 1 mL TRIZOL reagent (Invitrogen, Waltham, MA, USA). Ectopic endometriosis tissues from both groups were collected and resuspended in 1 mL TRIZOL reagent and RNA was extracted from the samples according to the manufacturer protocol. The reverse transcription was carried out using the high-capacity cDNA reverse transcription kit (Thermo Fisher scientific). Quantitative estimation of interferon-γ (IFNγ), tumor necrosis factor-α (TNF-α), interleukin (IL)-6,

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IL-1β, IL-10, transforming growth factor β (TGF-β) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was carried out using power SYBR green PCR master mix (Applied Biosystems, Waltham, MA, USA). Primers used for the real-time-polymerase chain reaction (PCR) reactions are shown in Table 1. PCR reactions were carried out using the Applied Biosystems 7500 instrument including an initial denaturation step at 94°C for 10 minutes followed by a 43-step cycling procedure (denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds). Cytokine expression levels were normalized to the level of GAPDH as an endogenous control and quantified by the 2^–ΔΔCT method.

12. Statistical analysis
Data were expressed as mean ± standard error of the mean. Unpaired t-test was used for comparing between the treated and non-treated groups. The difference among groups was considered significant for p < 0.05. Statistical tests were carried out using IBM SPSS ver. 25.0 (IBM Corp., Armonk, NY, USA).

Results

1. Macroscopic observation of the endometriosis lesions
The endometriosis lesions, identified by the non-absorbable Prolene suture, were examined in both study groups. Endometriosis group showed implants of ectopic endometrium within the intestinal mesentery detected as hemorrhagic cystic bulge full of fluid and surrounded by adhesions (Figure 1A). Endometriosis colony size was apparently smaller in the stem cell treated group compared to the endometriosis group (Figure 1B), which was documented by statistical analysis (Figure 1C).

2. Microscopic morphology of isolated AD-MSCs
After culturing for 7 days, the isolated MSCs appeared elongated spindle shaped with long cytoplasmic processes and clear elliptical nuclei (Figure 2A).

Table 1. Oligonucleotide primers used for the RT-PCR reactions

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>5'- GAGGAACTGGCAAAAGGACG -3'</td>
</tr>
<tr>
<td></td>
<td>5'- TCAGGGTCCGATTGAAGCA -3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'- AAGGGCTCCCTCTCATCAGT -3'</td>
</tr>
<tr>
<td></td>
<td>5'- GCCTGGTGTTGCTAGCAC -3'</td>
</tr>
<tr>
<td>IL6</td>
<td>5'- CTTGGAAATGAAAAAGATTTGTC -3'</td>
</tr>
<tr>
<td></td>
<td>5'- ACGGAACCTCCAGAAGCAGC -3'</td>
</tr>
<tr>
<td>IL1β</td>
<td>5'- GCACCTTTTCAGAGGAGG -3'</td>
</tr>
<tr>
<td></td>
<td>5'- GCTITCCTCCAGCCCAATG -3'</td>
</tr>
<tr>
<td>IL10</td>
<td>5'- CGAGCGTTGTCATCGATTCAG -3'</td>
</tr>
<tr>
<td></td>
<td>5'- CATGATGCTGGGTGCATGT -3'</td>
</tr>
<tr>
<td>TGFβ</td>
<td>5'- GACCGCAACAACGCAATCTA -3'</td>
</tr>
<tr>
<td></td>
<td>5'- GCAGGAGGTCCACAGATGAC -3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'- CAGGGGCTTTCTTCTTGGT -3'</td>
</tr>
<tr>
<td></td>
<td>5'- GATCTCGCTTGTGAGATGG -3'</td>
</tr>
</tbody>
</table>

RT-PCR, real-time reverse transcription-polymerase chain reaction.

Figure 1. Macroscopic observations of the endometriosis lesions of the study groups. (A) Endometriosis group showing fluid-filled endometriosis lesion at the intestinal mesentery. (B) Stem cell treated group showing smaller endometriosis lesion compared to the endometriosis group. (C) Endometriosis lesion size. Values are presented as mean±standard error of the mean. a)p<0.001.
3. Differentiation of AD-MSCs

The lipid vacuoles were visible in AD-MSCs that had differentiated into adipocytes and were stained with red oil (Figure 2B). Glycosaminoglycans were visible in AD-MSCs which had discriminated into chondrocytes and were stained with Alcian blue (Figure 2C). Calcium deposits were visible in AD-MSCs that had separated into osteocytes and were stained with Alizarin red (Figure 2D).

4. Flowcytometric analysis of AD-MSCs

Expression of MSCs surface markers was evaluated through flowcytometric analysis. CD44 and CD90 markers were highly expressed in AD-MSCs, while CD45 and CD34 markers were weakly expressed (Figure 3).

5. Histopathological results

H&E stained sections from the endometriosis group showed foci

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**Figure 2.** Photomicrographs of adipose tissue-derived mesenchymal stem cells (AD-MSCs). (A) Isolated AD-MSCs after 7 days culture showing 80% confluent. The cells are fibroblast-like shape with fusiform nucleus and tapering ends. (B) AD-MSCs differentiated into adipocytes and stained with Red Oil O. (C) AD-MSCs differentiated into chondrocytes and stained with Alcian blue. (D) AD-MSCs differentiated into osteocytes and stained with Alizarin red.
of multiple endometrial glands and stroma surrounded by mononuclear inflammatory cells (Figure 4A). In sections from stem cells treated group revealed few endometrial glands surrounded by stromal cells (Figure 4B), which was shown in statistical data (Figure 4C). Many hemosiderin-laden macrophages were observed in sections from endometriosis group (Figure 4D) while in stem cells treated group, few macrophages laden with hemosiderin were observed (Figure 4E). Number of hemosiderin-laden macrophages was significantly decreased in the stem cell treated group compared to the endometriosis group (Figure 4F).

6. Immunohistochemistry studies

In the endometriosis group, immunohistochemical staining revealed strong nuclear expression of Ki67 in the epithelial lining of endometrial glands and stromal cells (Figure 5A and B). The stem cell treated group, on the other hand, had a slight positive response to Ki67 in endometrial stromal cells and a negative response in the epithelial lining of glands (Figure 5C-E). Enhanced expression of CD68 in the macrophages located around the endometrial glands was detected in the endometriosis group (Figure 6A). Higher magnification revealed strong CD68 positive cytoplasmic staining (Figure 6B). The expression, however, was hardly identified around the endometrial glands in the stem cell treated group (Figure 6C-E).

7. Cytokine expression levels

TNFα, IL6 and IL1β are pro-inflammatory, while INFγ, TGF-β and IL-
Figure 4. Photomicrographs of endometriosis lesions from the endometriosis group (A, D) and the stem cell treated group (B, E) stained by H&E showing (A) multiple endometrial glands (G), stromal cells (arrows) and multiple blood vessels (bv) in endometriosis group (B) few endometrial glands surrounded by blood vessels. (C) Number of endometrial glands. \(^{a}p<0.001\). (D) Endometriosis group; numerous hemosiderin-laden macrophages (double arrows), (E) stem cell treated group; few hemosiderin-laden macrophages were found (double arrows). (F) Number of hemosiderin loaded macrophage. (C, F) Values are presented as mean±standard error of the mean. \(^{a}p<0.001\).
10 are immunomodulatory cytokines [18,36]. Rats treated with stem cells displayed lower expression levels of the proinflammatory and immunomodulatory cytokines, yet the later were less altered. IL-10 expression was significantly enhanced in stem cell treated group. Stem cell treatment induced marked reduction in IFNγ expression in both macrophages and endometriosis tissue (Figure 7) (mean fold change = 0.1 ± 0.27 and 0.4 ± 0.11 in macrophage and endometriosis tissue of stem cell treated group compared to the endometriosis group, respectively). Likewise, the expression of TNF-α by the peritoneal macrophages isolated from the stem cell treated rats was de-

Figure 5. Photomicrographs of ki67 immune expression in the endometriosis group (A) and the stem cell treated group (B). (A) Endometriosis group showing strong nuclear expression of Ki67 in the epithelial lining of endometrial glands (inset) and stromal cells (arrows; Ki67, ×400). (B) Magnified photo showing the expression of the Ki67 (arrows) in the cells in the endometriosis group (Ki67, ×1,000). (C) Stem cell treated group showing mild immune expression in nuclei of stromal cells (arrows; Ki67, ×400). (D) Magnified photo showing the expression of the Ki67 (arrows) in the cells in the stem cell treated group. (Ki67, ×1,000). (E) Morphometric studies of ki67 expression. Values are presented as mean±standard error of the mean. *p<0.001.
creased (0.1 ± 0.6, p = 0.02) compared to those isolated from the endometriosis group. Lower level of TNF-α expression was observed in endometriosis tissue of the stem cell treated group (0.4 ± 0.2, p = 0.03). Expression of IL-1β was lower (0.5 ± 0.2, p = 0.04) in the endometriosis tissue of the stem cell treated group compared to the endometriosis group. IL-6 expression, on the other hand, was not significantly altered in the stem cell treated group compared to the endometriosis group. The endometriosis tissue and the peritoneal macrophages were tested for the expression of the two anti-inflammatory cytokines; IL-10 and TGF-β. Interestingly, stem cell treated rats demonstrated two fold higher level of IL-10 (2 ± 0.3, p = 0.01); however, TGF-β has not been significantly altered in the endometriosis tis-

Figure 6. Photomicrographs showing immune expression of macrophage marker (CD68) in the endometriosis group (A, B) and the stem cell treated group (C, D). Endometriosis group showing (A) strong positive expression of CD68 in macrophage cells (CD68×400), (B) magnified part of the previous section showing positive expression in the form of brownish cytoplasmic granules (CD68, ×1,000; arrows, CD68 expression). Stem cell treated group showing (C) weak expression of CD68 in macrophage cells (CD68×400), (D) magnified photo showing few numbers of CD68 immunopositive macrophages with pale stained cytoplasmic granules (CD68×1,000). (E) Morphometric studies of CD68 expression. Values are presented as mean±standard error of the mean. *p<0.001.

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IL-10 expression was not affected but TGF-β was lower in the peritoneal macrophages of the stem cell treated group relative to the endometriosis group (0.1 ± 0.2, \(p = 0.01\)).

**Discussion**

At the present study, we used an autologous endometriosis rat model without ovariectomy or any estradiol supplementation in order to mimic the human endometriosis. This model provides suitable and acceptable endometriosis lesions comparable with the moderate stage of human endometriosis. Our study revealed the mitigating effect of AD-MSCs on the endometriosis like lesions as evidenced by their antiproliferative and anti-inflammatory actions. AD-MSCs alleviated endometriosis associated chronic inflammatory reactions through decreased CD68 macrophage infiltration and some of the associated inflammatory cytokines expression.
In our study, endometriosis group showed morphologically cystic implants consistent with other previous studies [37]. Experimental endometriosis detection was based on histopathologic observations of endometrial glands and stroma in the surgical site of endometrial square implantation. Light microscopy revealed ectopic endometrial glands and stroma, aggregating fibroblasts inside the peritoneum adjacent to uterine transplants, as well as mononuclear inflammatory cells and hemosiderin-laden macrophages, in accordance with previous studies [24]. Light and electron microscopic analysis of rat endometriosis demonstrated infiltration with eosinophils, mast cells, plasma cells, lymphocytes, and stromal macrophages of the peritoneum adjacent to implanted uterine tissue [38]. Uterine autotransplantation induced an immune reaction within the peritoneal stroma attached to the endometrial epithelium [38].

Previous theories explored the involvement of the immune system in promoting the development of endometriosis [39]. As macrophages liberating cytokines and growth factors migrate to the site of endometriosis implants were studied to promote its growth [40], increasing evidence supports the concept of endometriosis as a pelvic inflammatory disorder [3]. Women with endometriosis have an increased fluid volume in the peritoneum, with an elevated concentration of activated macrophages, prostaglandins, cytokines or chemokines as macrophage migration inhibitory factor [41], TNF-α [42], IL-1β, IL-6 [43], IL-8 and monocyte chemoattractant protein-1 (MCP-1) [44]. IL-6 is one of the main mediators in the cytokine cascade of endometriosis and its elevated levels correlate with the disease activity [43]. TNF-α stimulates the expression of matrix metalloproteinase and inhibits their inhibitors’ expression, hence contributing to the invasion of endometriosis and its extracellular matrix remodeling [45,46]. Both TNF-α and IL-8 concentrations in peritoneal fluid have been reported to correlate with the size and number of active endometriosis lesions [47]. Levels of proinflammatory cytokines IL-1, IL-6, TNF-α, TGF-β, and vascular endothelial growth factor are synonymous with severity of endometriosis and presence of adhesion [48,49]. Furthermore, TGF-β is a major driver of fibrosis in endometriosis [50]. As a result, targeting proinflammatory cytokines as a therapeutic strategy has been suggested. Because of its influence on dorsal root neurons, the pelvic inflammatory environment may contribute to the pathophysiology of pain perception [3,51].

MSCs have been suggested as a treatment modality for inflammatory [52] and autoimmune diseases as graft versus host disease, multiple sclerosis, and Crohn disease [54]. The anti-inflammatory role of MSCs became more apparent as the events initiating inflammation have been defined [55-58] due to their ability to sense the changing levels of inflammation in their microenvironment and respond accordingly [59]. Our study showed decreased the size of the endometriosis lesion as well as decreased number of endometrial glands which are consider as landmarks of endometriosis.

Decreased expression of IFNy and TNF-α in the activated peritoneal macrophages and endometrium tissue of the stem cell treated group. Stem cell treatment induced enhanced expression of IL-10 in endometrial tissue and TGF-β suppression in the activated macrophages of the peritoneum. MSCs are turned on by signals from injured tissues to secrete anti-inflammatory factors [60]. The production of proinflammatory cytokines by M1 macrophages or activated T cells may activate MSCs and stimulate release of mediators that distort the differentiation of monocytes toward an anti-inflammatory profile and eventually toward M2 macrophages [61]. M2 polarized macrophages generate IL-10 and facilitate the emergence of Tregs specialized in suppression of T cell-mediated immune responses [62] leading to amplification of the anti-inflammatory response [62]. In addition CCL18, a factor produced by M2 macrophages, promotes the generation of Tregs in conjunction with TGF-β [63]. TNF-α and other proinflammatory cytokines from resident macrophages activate MSCs to secrete the multifunctional anti-inflammatory protein; TNF-α-stimulated gene/protein 6 (TSG-6) [60]. The TSG-6 lowers nuclear factor-κB signaling in the resident macrophages modulating the cascade of proinflammatory cytokines [64]. Furthermore, TNF-α, nitric oxide, and probably other damage-associated molecular patterns from injured tissues and macrophages activate MSCs to secrete prostaglandin E2 [60] that converts macrophages to the M2 phenotype [60]. MSCs may produce anti-inflammatory effects by enhancing expression of the anti-reactive oxygen species protein stanniocalcin-1 as well [65].

Despite previous research suggesting that AD-MSCs may aid in the maintenance and growth of ectopic endometrial tissue [52], our results documented that AD-MSCs treated group showed small-sized endometrial glands surrounded by stromal cells with lesser inflammatory cellular infiltration compared to the endometriosis group. Our findings are consistent with the study that showed that transplanted menstrual blood MSCs improved fertility substantially by increasing the synthesis of angiogenic and anti-inflammatory factors [66]. Human endometrial MSCs were successfully applied for Asherman’s syndrome treatment in the rat model indicated through the higher pregnancy outcome and litter size compared with those
received autologous rat bone marrow cells [66].

Macrophages are the key cells contributing to the local inflammatory response in endometriosis [67]. Monocytes are recruited to the endometriosis lesion by the chemotactic chemokine MCP-1 then transformed into mature macrophages [67]. The hemosiderin loaded macrophage were significantly decreased via treatment with MSCs which was confirmed by immunohistochemical staining using CD68.

CD68 is a glycoprotein surface marker expressed on the circulating and tissue macrophages [68]. Our study showed high CD68 expression in ectopic endometrium in the endometriosis group however, very faint expression was observed around the endometrial glands in the stem cell treated group. There was a statistically significant difference between the values of CD68 for endometriosis of the ovaries, endometriosis of the pelvis, adenomyosis, endometriosis of the abdominal wall compared to normal endometrium [69]. The maximum frequency of endometriosis-associated immune cell infiltrates as macrophages (CD68+) was observed in peritoneal and ovarian endometriosis [70].

Ki67 represents a nuclear protein associated with cell division as it is present during all active phases of the cell cycle (G1, S, G2 and mitosis), however absent in G0. It represents a marker of cell proliferation [71]. In our study, many epithelial cells of endometriosis group showed Ki67 positive nuclear staining with immunoreactivity in some proportion of cells in the cytoplasm as well. Stem cell treated group showed mild positive reaction in endometrial stromal cells and negative reaction in the glands. Positivity of the Ki67 proliferation marker is directed toward increased aggressiveness of endometrial ectopic tissue being directly proportional to the size of endometriosis foci [69]. The expression of this marker is low in cases of moderate endometriosis and increased in severe ones [72]. Women aged 36–47 with regular menstrual cycle, demonstrated a significant increase of Ki67 H score in the stroma of eutopic endometrium of those affected by endometriosis suggesting an enhancement of the proliferative processes in this location [73].

MSCs could be efficiently used as an adjuvant therapy for mitigating the inflammatory component of endometriosis which consequently mediate the antiproliferative effect on endometriosis cells. Further studies are still required to enclose MSCs in a beneficial regimen that could alleviate endometriosis distressing manifestations keep fertility and decrease the incidence of recurrence.

Conflict of interest

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

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Author contributions

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References


Administration of red ginseng regulates microRNA expression in a mouse model of endometriosis

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Objective: Red ginseng (RG) exerts anti-inflammatory, anti-proliferative, and immunomodulatory effects on endometriosis through the regulation of microRNA (miRNA) expression. It may also ameliorate endometriosis by affecting the expression of multiple miRNAs simultaneously, rather than acting on a single miRNA at a given time. Since studies on the overall effects of RG on endometriosis via the regulation of miRNA expression are lacking, the current study aimed to explore the global effect of RG on miRNA expression in a mouse model of endometriosis.

Methods: To establish the mouse model, the uterine horn of donor mice was implanted into the lateral side of the recipients' peritoneum, followed by vehicle or RG treatment for 8 weeks.

Results: To confirm the effects of RG on the established mouse model, the size of the implanted uterus was measured; it was found to be lower in mice from the RG group than in mice from the control group. miRNA expression profiles in the implanted uterus of the mouse model of endometriosis after vehicle or RG administration were analyzed using microarray technology. Thereafter, seven candidate miRNAs and 125 candidate genes (miRNA targets) were identified through a bioinformatics analysis.

Conclusion: The present findings suggest that RG regulates the expression of multiple miRNAs and mRNAs, thereby alleviating endometriosis in a mouse model of the disease.

Keywords: Endometriosis; Medical informatics; MicroRNAs; Panax; Red ginseng

Introduction

Endometriosis is a commonly occurring (affecting 6%–10% of women in the United States) chronic inflammatory disease, characterized by the formation of endometrial-like tissue outside the uterus, and associated with dysmenorrhea, dyspareunia, chronic pelvic pain, and infertility [1]. Sampson’s theory, suggesting retrograde menstruation via the fallopian tubes into the peritoneal cavity to implant on the surface of pelvic organs, is the most widely accepted hypothesis regarding the origin of ectopic endometrial tissue [2]. However, the exact cause of endometriosis is not fully understood. Since the incidence of retrograde menstruation is similar in women with and without endometriosis, numerous other mechanisms underlying endometriosis have also been proposed, such as coelomic metaplasia, altered immunity, and epigenetic modifications [3-5].

Recent research has focused on the contribution of epigenetic mechanisms, including DNA methylation, histone acetylation, and changes in co-regulators, such as microRNAs (miRNAs), to the development of endometriosis [6-8]. In particular, miRNAs, which are involved in mRNA silencing and post-transcriptional regulation of gene expression, have been reported to be aberrantly expressed in patients with endometriosis [9-11]. A recent prospective case-control study using miRNA microarray and quantitative polymerase chain reaction analyses of serum samples from women with or without endometriosis reported abnormally regulated miRNA expression.
in women with endometriosis, thus suggesting the possibility of a novel non-invasive diagnostic test for the early detection of endometriosis [10-12].

Panax ginseng, which has been used in traditional medicine for centuries [13], is being actively studied for its medical effectiveness in modern clinical research. Among its various types, red ginseng (RG; P. ginseng Meyer) is prepared by steaming and drying fresh P. ginseng to prevent its rapid decay. During this processing, the properties of ginsenosides are chemically transformed, resulting in a pharmacological efficacy different from that of the original P. ginseng [14-16]. Recent studies have proven that RG exerts various medical effects, including anti-inflammatory, antioxidant, and immunomodulatory effects, along with epigenetic regulation, in various diseases [16-18]. Moreover, RG exerts anti-tumor effects in endometriosis by inhibiting angiogenesis and cell migration in vitro and in vivo [19,20].

Because endometriosis occurs spontaneously only in humans and some non-human primates, an increasing number of studies have attempted to establish models for endometriosis using small laboratory animals, especially rodents, by transplanting pieces of endometrial tissue to ectopic sites [21]. In particular, animal models are often used to assess endometriosis to confirm the efficacy of drugs in vivo or to characterize epigenetic changes resulting from various interventions [22-24]. These animal models of induced endometriosis have proven to be useful in the evaluation of the mechanisms underlying the pathogenesis of this complex disease [21].

We previously reported that ginsenoside Rg3 of RG decreases the fibrotic and invasive nature of endometriosis by modulating miRNA-27b expression in a mouse model of endometriosis [19]. Although we previously investigated the regulatory effect of RG on miRNA-27b, we now hypothesize that RG may ameliorate endometriosis by simultaneously regulating the expression of multiple miRNAs, rather than that of a single miRNA at a time. Hence, in this study, we analyzed the expression levels of various miRNAs isolated from a mouse model of endometriosis after RG administration and performed bioinformatics analyses to investigate the global effect of RG on endometriosis in vivo.

Methods

1. Ethics statement and animal care

This study was approved by the Institutional Animal Care and Use Committee and the Institutional Committee on Animal Care of Gangnam Severance Hospital, Yonsei University Health System (approval No. 3-2015-0007). All animal experiments were performed in accordance with the animal research: reporting of in vivo experiments (ARRIVE) guidelines [25]. The mice used herein were purchased from Orient-Bio Laboratory Animal Co., Ltd. (Seongnam, Korea). They had ad libitum access to food and water, and were housed in a polycrylic cage under controlled conditions at 24°C ± 1°C, with 50% ± 5% humidity and a 12-hour day/night cycle.

During the surgical establishment of the endometriosis model, the mice were placed in a Harvard-type isoflurane chamber, and 3.5%-4% isoflurane (Hana Pharmaceutical, Seoul, Korea) was administered in conjunction with air for anesthesia induction. Thereafter, 1.5%-3% isoflurane was administered to maintain anesthesia in mice. Their breathing was carefully observed, and in cases of respiratory depression, oxygen levels were increased in the chamber because isoflurane anesthesia is rapidly recoverable [26]. To prevent infection, 10 mg/kg cefazolin sodium (Korea United Pharm, Seoul, Korea) was intravenously administered before the procedure. After all animal experiments, the mice were euthanized. To decrease pain and animal distress, the experimental animals were treated with 6%-7% isoflurane until 2 minutes after breathing cessation, and euthanasia was performed through cervical dislocation.

2. Mouse model of endometriosis and RG administration

A mouse model of endometriosis was established using 6-week-old C57b/6 female mice (n = 9) having a similar weight (28 ± 4 g) as described previously [11]. In brief, each donor mouse (n = 3) was sacrificed following an anesthetic overdose and its uterus was harvested. The “Y”-shaped uterus was cut in half, and each uterine horn was dissected to expose the endometrium. The recipient mouse was anesthetized, and each uterine horn was transplanted into the corresponding lateral side of the recipient’s peritoneum using Vicryl 3-0 sutures. Before transplantation, a cytological evaluation of vaginal smears from virgin female mice was used to determine the current estrous stage. The peritoneum and skin were closed with Vicryl 3-0 sutures and staplers, and the postoperative condition of the mice was examined. RG extract, provided by Korea Ginseng Corporation, was diluted in PBS, as described previously [19]. The experimental animals were randomly divided into two groups—vehicle and RG—each comprising three mice. The RG group was administered 0.2 mg/g RG extract by oral gavage once a day; similarly, the control vehicle group was administered an equivalent amount of water. RG administration was initiated 1 day after transplantation and was continued for 8 weeks. Thereafter, all mice were sacrificed for endometrial tissue retrieval. The area of endometrial implants was measured based on the length and width of each lesion and calculated using ImageJ (version 1.41o, Java 1.6.0_10, Wayne Rasband; US National Institutes of Health, Bethesda, MD, USA).

3. Histological examination

The endometrial implants were collected, fixed in 10% formalin-acetic acid, and embedded in paraffin for histopathological ex-
amination. Sections of paraffin-embedded tissue were stained with Masson trichrome. To quantify the severity of fibrosis in stained tissue sections, staining intensity was measured using ImageJ, and the severity of fibrosis was evaluated according to the Ashcroft method [27].

4. Isolation of miRNAs from endometrial implants

Total RNA was isolated using TRIzol (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) and purified using the RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. RNA quality and quantity were estimated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA), respectively.

5. Affymetrix miRNA arrays

The Affymetrix GeneChip miRNA 4.0 array process was carried out according to the manufacturer’s protocol (Affymetrix, Santa Clara, CA, USA). Approximately 1,000 ng of RNA was labeled with the FlashTag Biotin RNA Labeling Kit (Genisphere, Hatfield, PA, USA). The labeled RNA was then quantified, fractionated, and hybridized to the miRNA microarray chip according to the standard procedures recommended by the manufacturer. The labeled RNA was heated to 99°C for 5 minutes and then to 45°C for another 5 minutes. RNA-array hybridization was performed in an Affymetrix Fluidics Station 450 with agitation at 60 rotations per minute for 16 hours at 48°C. The chips were washed and stained using a GeneChip Fluidics Station 450 (Affymetrix) and then scanned with an Affymetrix GCS 3000 scanner. Signals were quantified using the Affymetrix GeneChip Command Console (AGCC) software.

6. Bioinformatics prediction

The predicted target genes of candidate miRNAs were determined using three bioinformatics prediction tools: TargetScan v6.2 (http://www.targetscan.org/mamm_31/), miRmap (http://mirmap.ezlab.org/), and miRDB (http://www.mirdb.org/ miRDB/). The selection criteria were as follows: correlation > 0.99 or <-0.99 and a p-value < 0.05. The genes that overlapped in all three databases were selected for further functional analyses. The miRNAs and predicted mRNA target genes were then subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses using David v6.7 (http://david.abcc.ncifcrf.gov/) online. The predicted target gene list of differently expressed miRNAs obtained as described above was entered into the Cytoscape software (version 3.4.0), and the connections between miRNAs and their predicted gene targets were visualized.

7. Raw data preparation and statistical analysis

Raw data were extracted automatically via the Affymetrix data extraction protocol using the software provided by the AGCC. The CEL file import, miRNA level RMA+DABG-all analysis, and results export were performed using Affymetrix Expression Console software. Array data were filtered using probe-annotated species. Comparative analysis between the test and control samples was conducted using the independent t-test and fold changes, in which the null hypothesis was that no difference existed between the groups. The false discovery rate was controlled by adjusting the p-value using the Benjamini-Hochberg algorithm. All statistical tests and visualization of differentially expressed genes were conducted using the statistical language R v3.1.2. (www.r-project.org).

Results

1. RG administration attenuated the symptoms of endometriosis

After 8 weeks of RG administration, the mice were sacrificed and their endometrial lesions were analyzed. All transplanted endometrial lesions were found in the peritoneum of the sacrificed mice. The mean areas of lesions in the vehicle (n = 3/3) and RG (n = 3/3) groups were 0.22 ± 0.03 cm² and 0.14 ± 0.03 cm², respectively. The mean area of endometrial implants was lower in the RG group than in the vehicle group (p = 0.043) (Figure 1, Table 1). A histological analysis was carried out to evaluate the effects of RG administration on the endometrial lesions, and a reduction of the mean area and fibrosis scores were observed in the implants of RG group (p < 0.001) (Figure 1, Table 1).

2. RG administration altered miRNA expression in the mouse model of endometriosis

miRNA array data were analyzed to detect the expression profiles of miRNAs in endometrial implants isolated from the mice in the two groups to examine whether RG administration could alter the miRNA expression profile. Seven miRNAs were observed to be differentially expressed (fold change ≥ 1.5) in the RG group compared to the control group (Figure 2). Expression data were normalized using the me-

Table 1. Area of endometrial implants and severity of fibrosis after RG administration

<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>Area of endometrial implants (cm²)</th>
<th>Fibrotic area (%)</th>
<th>Fibrosis score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>0.23</td>
<td>42.91</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>0.23</td>
<td>41.41</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>0.21</td>
<td>32.34</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>RG</td>
<td>0.17</td>
<td>34.53</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>RG</td>
<td>0.16</td>
<td>35.06</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>RG</td>
<td>0.14</td>
<td>15.07</td>
<td>1</td>
</tr>
</tbody>
</table>

RG, red ginseng.
Figure 1. Results from the mouse model of endometriosis. (A) The circles indicate endometrial implants in the mouse model from the control group (vehicle) and the red ginseng (RG) group (red ginseng extract 0.2 mg/g) after 8 weeks of treatment. (B) The endometrial implants of mice from the RG group were significantly smaller than those of mice from the control group. (C) Masson trichrome staining of endometrial implants from both control and RG groups (scale bar=20 μm). (D) The staining intensity of samples from the RG group was significantly lower than that of samples from the control group. Control vs. RG, a) \( p < 0.05 \) and b) \( p < 0.01 \), n=3 per group.

Figure 2. Heat map of microRNA (miRNA) profiling after the different treatments. Hierarchical clustering shows seven significantly regulated miRNAs in the red ginseng (RG) and control groups. A total of six miRNAs were downregulated and one was upregulated in the control group compared to the RG group. The color code in the heat maps is linear, with yellow and blue indicating the lowest and highest expression.

Median normalization method, after which the differentially expressed miRNAs between two samples were filtered based on fold change. The miRNA array results, obtained after normalization, and the calculated fold change values are listed in Table 2. The significant difference in the expression profiles between the two groups suggested that RG administration altered the expression of miRNAs in the mouse model of endometriosis.

3. GO and KEGG analyses of miRNAs
To determine the potential effect of differentially expressed miR-
Table 2. miRNA array ratios obtained following normalization and the calculation of fold change values

<table>
<thead>
<tr>
<th>miRNA name</th>
<th>Fold change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mmu-miR-19b-3p</td>
<td>1.619866</td>
<td>0.048138688</td>
</tr>
<tr>
<td>Mmu-miR-7669-3p</td>
<td>–2.25369</td>
<td>0.046730352</td>
</tr>
<tr>
<td>Mmu-miR-6978-5p</td>
<td>–2.42313</td>
<td>0.038811474</td>
</tr>
<tr>
<td>Mmu-miR-7034-5p</td>
<td>–3.27481</td>
<td>0.04796048</td>
</tr>
<tr>
<td>Mmu-miR-8113</td>
<td>–3.44458</td>
<td>0.025400705</td>
</tr>
<tr>
<td>Mmu-miR-7058-5p</td>
<td>–4.17534</td>
<td>0.023996733</td>
</tr>
<tr>
<td>Mmu-miR-6391</td>
<td>–7.57483</td>
<td>0.049712946</td>
</tr>
</tbody>
</table>

miRNA, microRNA.
tein kinase (MAPK), Axon, PI3K-Akt, and chemokine signaling pathways (Figure 3).

4. Integrated analysis of miRNAs and mRNAs

Because each miRNA has multiple potential mRNA targets, a single mRNA can be regulated by multiple miRNAs. The 7 miRNAs observed to be differentially expressed were further analyzed, and their 125 predicted target genes were screened (miRNA-target gene cutoff: TargetScan context score < –0.6; visualization tool: Cytoscape 3.7.1). The results demonstrated that two genes, Hif3a and Scrt2, were co-regulated by at least two miRNAs (Figure 4).

Discussion

This study investigated the molecular network of miRNA and mRNA expression that modulates critical processes in a mouse model of endometriosis following RG administration. To clarify the influence of miRNAs and mRNAs on the mouse model, a group of mice was treated with RG and the differences in miRNA expression between these mice and control mice were evaluated by microarray analysis. Along with bioinformatics-based predictions, RG administration was found to alter the expression profile of multiple miRNAs in the endometriotic lesions. Among them, seven miRNAs and 125 candidate genes were involved in the main microRNA-mRNA regula-

Figure 4. Relationships among microRNAs (miRNAs) and their predicted gene targets. The relationships of seven miRNAs with their 125 predicted target genes are represented here.

https://doi.org/10.5653/cerm.2021.04392
This study successfully established a mouse model of endometriosis and accompanying fibrosis, as confirmed by Masson trichrome staining. RG administration significantly reduced the size of the endometrial implants compared to that in the control group. As is commonly observed, alterations in the morphology and size of various organs are accompanied by changes in gene expression. One of our previous studies suggested that ginseng induced apoptosis in endometrial cells derived from patients with endometriosis via the regulation of miR-21-5p expression [28]. In another study, we reported that RG administration changed the fibrotic properties of human endometrial stromal cells by regulating miR-27b-3p levels in vivo [19].

The results from the miRNA array analysis in this study revealed seven miRNAs to be differentially expressed in the RG group compared to the control group. Through GO and KEGG analysis, we identified the molecular miRNA-mRNA expression network that regulated protein binding, metabolic pathways (including Ras, Rap1, MAPK, Axon, and PI3K-Akt), and chemokine signaling pathways in the mouse model of endometriosis following RG administration. In fact, most of these pathways have already been reported to play an important role in endometriosis. Increased activation of the Ras/Raf/MAPK pathway is known to be associated with the enhanced proliferation and survival of eutopic endometrial cells (from patients with endometriosis) in vitro [29]. Many reports have already suggested that the PI3K/Akt/mTOR pathway is associated with the development of endometriosis. In vivo studies have shown that the mTOR/AKT inhibitor temsirolimus prevents deep infiltrating endometriosis, which is the most severe form of endometriosis [30,31]. Moreover, ginsenoside Rg3 has been reported to inhibit the angiogenesis associated with endometriosis through the vascular endothelial growth factor receptor-2-mediated PI3K/Akt/mTOR signaling pathway in a rat model [20]. RG administration is thought to have reduced the growth of ectopic endometriotic tissue by modulating the expression of miRNAs targeting the PI3K/Akt/mTOR pathway in this animal-based study.

As mentioned above, endometriosis is associated with altered immunity, suggesting that impaired immune function may contribute to the development of this disease. Instead of acting as scavengers to eliminate ectopic endometrial cells, activated macrophages and circulating monocytes in women with endometriosis appear to promote the disease by secreting growth factors and cytokines (interleukin [IL]-1, IL-6, and IL-8, tumor necrosis factor, regulated on activation, normal T cell expressed and secreted, and vascular endothelial growth factor) that stimulate the proliferation of ectopic endometrial tissue and inhibit their scavenging functions [32-34]. Based on the results of this study, RG may affect proliferation and mitosis of ectopic endometrial cells of mice, and seems to act on the scavenging mechanism by which endometriosis is eliminated from the body. There is sufficient evidence for alterations in both cellular and humoral immunity in women with endometriosis, thus implying that endometriosis is the result of dysregulated chemotaxis [35].

Ginseng has been extensively reported to maintain the homeostasis of the immune system and enhance resistance to inflammation and microbial attacks through the regulation of the immune system [36]. Rg3, one of the major components of RG, has been reported to induce immunogenic cell death in tumor cell lines (B16F10 melanoma cells) by suppressing pro-angiogenic (tumor necrosis factor-α) and immunosuppressive cytokine (transforming growth factor-β) secretion, as well as interferon-gamma production from Rg3-treated tumor cells [37]. In another study, RG extract was shown to inhibit the apoptosis of cells from a pancreatic β-cell line (MIN6N8), possibly via the reduction of nitric oxide and reactive oxygen species production, inhibition of p53/p21 expression, and inhibition of cleavage of caspases and poly(ADP-ribose) polymerase; this suggests the RG-mediated inhibition of cytokine-induced apoptosis in pancreatic β-cells [38]. Collectively, RG may regulate the expression of several miRNAs involved in the Ras/Raf/MAPK and PI3K/Akt/mTOR pathways and immune system regulation in a mouse model of endometriosis.

In this study, an integrated analysis of miRNAs and mRNAs demonstrated that two genes, HIF3α and SCRT2, were co-regulated by at least two miRNAs (Figure 4). Neither the genes nor the pathways containing the genes have been reported to be associated with endometriosis in the previous literature. The relationships between the two genes and endometriosis are not yet understood; however, such a relationship seems reasonable, owing to the tissue-specific nature of miRNA expression [39]. For example, the HIF3α gene is known to exhibit high miRNA-related expression levels in the bone, vessels, and prostate [40-42]; however, thus far, there are very few reports of its expression in the endometrium.

This study has several strengths. It describes the successful establishment of a mouse model of endometriosis and reports the global effects of RG on the miRNA-mRNA expression network in the model. Furthermore, various miRNA target genes were evaluated through bioinformatics analyses. These results suggest that RG potentially influences endometriosis via various target genes. However, this study has some limitations. First, considering the limitations of animal-based studies, it is difficult to interpret these findings as indicative of the efficacy of RG among endometriosis patients. Second, because the experiment included only a few animals, to minimize the sacrifice of experimental animals, there may be some differences among the miRNAs and mRNAs that show altered expression following RG treatment when compared to experiments performed using a large number of animals. Third, there are limitations in applying the results of this study to humans directly, because the mouse model of...
endometriosis was established in different ways from endometriosis development in the human body. Moreover, miRNAs of mice (Mmu-miR) were analyzed as outcomes in this study. Nevertheless, it is known that genetic variation is similar between endometriosis in mouse models and endometriosis in humans. For example, when a gonadotropin-releasing hormone agonist was administered in an endometriosis animal model, the expression of matrix metalloproteinases and matrix metalloproteinase inhibitors, which are known to affect the pathogenesis of human endometriosis, was also changed [43]. Due to the similarity between endometriosis in murine models and humans, endometriosis murine models have been used in many studies as a test-bed for potential therapeutics including melatonin [44], doxycycline [45], etanercept (an anti-tumor necrosis factor-α antibody) [46], and gene therapy delivered via polymeric micelles [47].

In conclusion, RG administration may be considered to have significant effects on reducing the size of endometrial implants besides regulating miRNA expression levels in a mouse model of endometriosis. Functional analysis indicated that altered miRNA expression is involved in multiple pathways, including the Ras/Raf/MAPK and PI3K/Akt/mTOR pathways, and immune system regulation; targeting these pathways may eventually alleviate the symptoms of endometriosis, thereby inhibiting progression of the disease.

**Conflict of interest**

This study was financially supported by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI18C2047). The authors declare that they have no conflict of interest. The funders played no role in the study design, the collection, analysis, and interpretation of data, and the writing of the manuscript, which were completely the responsibilities of the authors.

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**Author contributions**

Conceptualization: SC, JHL. Formal analysis: SC, JHL. Investigation: JHP, BHW, WI. Writing—original draft: JHL. Writing—review & editing: all authors.

**References**

17. Lee JH, Min DS, Lee CW, Song KH, Kim YS, Kim HP. Ginsenosides from Korean Red Ginseng ameliorate lung inflammatory respons-


mass index [2]. Two main enzymes are involved in the synthesis of VD [3]. Hepatic 25-hydroxylase converts VD to 25-hydroxyvitamin D (25OHD, 25-hydroxycholecalciferol; the major circulating form of VD), and renal 1-alpha-hydroxylase converts 25OHD to the active form 1,25-dihydroxyvitamin D3 (1,25(OH)2D3, 1,25- hydroxycholecalciferol, calcitriol, VD3) [4]. CYP2R1 is the most important 25-hydroxylase, and CYP27B1 is the key 1-alpha-hydroxylase. The active form VD3 is the ligand for the vitamin D receptor (VDR), which is present in many female reproductive organs, such as the ovary, endometrium, fallopian tube, and placenta [3]. VDR is present particularly in granulosa cells of the ovarian follicles; therefore, it is believed that VD plays an essential role in ovarian follicular development [3,5,6].

In a human granulosa cell culture model, VD3 supplementation in vitro enhanced 3-beta-hydroxysteroid dehydrogenase activity and progesterone production, but did not affect follicle-stimulating hormone (FSH)-induced aromatase activity and estradiol production [5].

Impact of vitamin D3 supplementation on the in vitro growth of mouse preantral follicles

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Objective: We investigated the impact of vitamin D3 (VD3) supplementation during mouse preantral follicle culture in vitro and the mRNA expression of 25-hydroxylase (CYP2R1), 1-alpha-hydroxylase (CYP27B1), and vitamin D receptor (VDR) in mouse ovarian follicles at different stages.

Methods: Preantral follicles were retrieved from 39 BDF1 mice (7–8 weeks old) and then cultured in vitro for 12 days under VD3 supplementation (0, 25, and 50 pg/mL). Follicular development and the final oocyte acquisition were assessed. Preantral follicles were retrieved from 15 other BDF1 mice (7–8 weeks old) and cultured without VD3 supplementation. Three stages of mouse ovarian follicles were obtained (preantral, antral, and ruptured follicles). Total RNA was extracted from the pooled cells (from 20 follicles at each stage), and then reverse transcriptase-polymerase chain reaction was performed to identify mRNA for CYP2R1, CYP27B1, and VDR.

Results: The survival of preantral follicles, rates of antrum formation and ruptured follicles (per initiated follicle) and the number of total or mature oocytes were all comparable among the three groups. Both CYP2R1 and CYP27B1 were expressed in antral and ruptured follicles, but not in preantral follicles. VDR was expressed in all three follicular stages.

Conclusion: VD3 supplementation in vitro (25 or 50 pg/mL) did not enhance mouse follicular development or final oocyte acquisition. Follicular stage-specific expression of CYP2R1, CYP27B1, and VDR was observed.

Keywords: 1Alpha-hydroxylase; 25-Hydroxylase; Antral follicle; Calcitriol receptors; Cholecalciferol; Preantral follicle
In women with polycystic ovary syndrome, VD supplementation was beneficial for follicular development, with a higher number of dominant follicles and a more regular menstrual cycle [7].

In human in vitro fertilization (IVF) cycles, several studies have reported an association between higher serum or follicular fluid (FF) 25OHD concentrations and the clinical pregnancy rate; however, other studies have reported contradictory findings [3,6]. Furthermore, it was reported that FF 25OHD levels were inversely related to embryo quality [8,9]. Therefore, there are insufficient data to reach a definitive conclusion regarding the effect of VD supplementation for VD-depleted women undergoing IVF [6]. A few experiments have reported that VD3 supplementation had a direct effect on ovarian follicular development. In goats, VD3 supplementation in vitro during granulosa cell culture decreased the level of reactive oxygen species [10]. In rhesus macaques, VD3 supplementation in vitro increased preantral follicle survival up to the antral follicle stage [11,12].

However, no animal model studies have yet investigated the impact of VD3 supplementation in vitro on the entire folliculogenesis process, up to oocyte acquisition. We investigated whether VD3 supplementation in vitro affected follicular development and final oocyte acquisition in a mouse model of preantral follicle culture. We also investigated whether 25-hydroxylase, 1-alpha-hydroxylase, and VDR were expressed in the three stages of mouse ovarian follicles. If there VD3 supplementation is beneficial for follicular development, a rationale will be provided to recommend sufficient VD intake to improve IVF results.

Methods

1. Impact of VD3 supplementation in vitro on mouse preantral follicle development

Female 7- to 8-week old BDF1 mice ( Orient Co., Seoul, Korea) were nurtured under 12-hour day and a 12-hour night conditions at 23°C and fed ad libitum. The Institutional Animal Care and Use Committee (IACUC) of Seoul National University Bundang Hospital approved the experiment (No. BA1903-267/014-01). After 1 week of adaptation, 39 mice were killed by cervical dislocation and bilateral ovaries were obtained. They were collected in 1 mL of L-15 medium (WelGENE, Daegu, Korea) supplemented with 0.4% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA). From the ovaries, intact preantral follicles were isolated via mechanical tearing by a 1-mL tuberculin syringe [13] and randomly divided into three groups.

Preantral follicles were cultured in a growth medium containing alpha-minimum essential medium (WelGENE), 5% fetal bovine serum (Gibco, Paisley, UK), 10 mIU/mL recombinant FSH (Merck-Serono, Geneva, Switzerland), 1% insulin-transferrin-selenium mixture (Sigma-Aldrich), and 1% penicillin-streptomycin mixture (Sigma-Aldrich). All follicles were cultured in 96-well plates (BD BioCoat; BD Falcon, Franklin Lakes, NJ, USA) at 37°C in 5% CO2 for 10 days. Medium changes were conducted every other day, and follicle survival and antrum formation were assessed. If granulosa cells appeared to be dark and fragmented, they were considered to be dead.

After 10 days of culture in the growth medium, the follicles were transferred to maturation medium and cultured for another 16 hours at 37°C in 5% CO2 to obtain ruptured follicles. The maturation medium was composed of 1.5 IU/mL human chorionic gonadotropin (Merck-Serono), and 5 ng/mL recombinant mouse epidermal growth factor (Sigma-Aldrich) was added to the growth medium. During the 12 days of culture, supplementation with 0, 25, and 50 pg/mL of active-form VD3 (1,25(OH)2D3, Sigma-Aldrich) was performed.

Oocytes were harvested from the finally ruptured (ovulated) follicles. Surrounding cumulus cells were removed by treatment with 0.3% hyaluronidase (Sigma-Aldrich) and gentle pipetting. Oocytes were classified into five categories: germinal vesicle (GV), GV breakdown, metaphase II (MII), degenerated, or dead. If a polar body was present in the perivitelline space, the oocytes were considered to be MII oocytes. Fragmented or dark oocytes were classified as degenerated. If the oocytes were not enclosed by granulosa cells, they were classified as dead. The analysis was performed by one skilled embryologist to minimize inter-observer variability.

2. Isolation of mRNA, cDNA synthesis, and reverse transcriptase-polymerase chain reaction

From 15 mice, preantral follicles were isolated and cultured in growth medium for 10 days and then transferred to maturation medium for 16 hours, as described above (without VD3 supplementation). Twenty follicles in each stage of folliculogenesis (preantral, antral, and ruptured follicles) were pooled, and the total RNA was extracted using the Dynabeads protocol (Dynabeads mRNA DIRECT Kit; Ambion, Oslo, Norway). The mRNA of CYP2R1 (encoding 25-hydroxylase), CYP27B1 (encoding 1-alpha-hydroxylase), and VDR was transcribed into cDNA using a PrimeScript first-strand cDNA Synthesis Kit (Takara, Kusatsu, Japan) according to the manufacturer’s instructions. Each gene was amplified from cDNA using the reverse transcription system: 95°C for 1 minute followed by 35 cycles of 95°C for 15 seconds, 62°C for 15 seconds, and 72°C for 15 seconds. The final extension was at 72°C for 3 minutes. Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out with a StepOne-Plus real-time PCR system with TaqMan probes (Applied Biosystems, Foster City, CA, USA) in a 20-μL reaction volume containing 10 μL of Applied Biosystems TaqMan Universal PCR Master Mix I (Cat. no. 4427788), 2 μL of cDNA, and 6 μL of RNase-free water. The specific primers are listed in Table 1, and all were purchased from Integrated DNA Technology (Coralville, IA, USA). The amplified RT-PCR products were transcribed into cDNA using a PrimeScript first-strand cDNA Synthesis Kit (Takara, Kusatsu, Japan) according to the manufacturer’s instructions. Each gene was amplified from cDNA using the reverse transcription system: 95°C for 1 minute followed by 35 cycles of 95°C for 15 seconds, 62°C for 15 seconds, and 72°C for 15 seconds. The final extension was at 72°C for 3 minutes. Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out with a StepOne-Plus real-time PCR system with TaqMan probes (Applied Biosystems, Foster City, CA, USA) in a 20-μL reaction volume containing 10 μL of Applied Biosystems TaqMan Universal PCR Master Mix I (Cat. no. 4427788), 2 μL of cDNA, and 6 μL of RNase-free water. The specific primers are listed in Table 1, and all were purchased from Integrated DNA Technology (Coralville, IA, USA). The amplified RT-PCR products were
were purified and separated using 2% agarose gel electrophoresis. The assays were repeated three times per sample.

3. Statistical analysis

Statistical analyses were performed using IBM SPSS ver. 25.0 (IBM Corp., Armonk, NY, USA). The Fisher exact test was used to compare the rates among the groups. Numerical data were compared using the Kruskal-Wallis test. If the value was significant, the Mann-Whitney U-test with the Bonferroni correction was used for further analysis. A p-value less than 0.05 was considered to indicate statistical significance.

Results

The overall outcomes of in vitro growth of preantral follicles and the percentage of MII oocytes are presented in Table 2. Among the three VD3-supplemented groups (0, 25, and 50 pg/mL), the survival of preantral follicles and the rates of antrum formation or ruptured follicles (per initiated follicle) were all comparable. The number of total oocytes per initiated follicle (80.2%, 81.0%, and 76.2%) and mature oocytes per initiated follicle (24.6%, 27.8%, and 23.0%) were also similar among the three VD3-supplemented groups. Both CYP2R1 and CYP27B1 were expressed in antral and ruptured follicles, but not in preantral follicles (Figure 1). VDR was expressed in all three follicular stages.

Discussion

In the present work, the expression of mRNA for 25-hydroxylase (CYP2R1) and 1-alpha-hydroxylase (CYP27B1) was observed both in antral follicles and ruptured follicles, but not in preantral follicles. However, mRNA for VDR was expressed in preantral follicles as well as antral and ruptured follicles. It is known that VDR is expressed in mouse ovarian follicles [14]. In that study, however, the follicular stage-specific expression of VDR was not assessed. We obtained preantral follicles as well as antral and ruptured follicles separately, through mouse preantral follicle culture, and confirmed that VDR was expressed in all three stages. Our research is significant because we observed the follicular stage-specific expression of VDR.

Table 2. Outcomes of in vitro growth of mice preantral follicle under vitamin D supplementation

<table>
<thead>
<tr>
<th>Variable</th>
<th>Vitamin D supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 pg/mL</td>
</tr>
<tr>
<td>No. of preantral follicles</td>
<td>126</td>
</tr>
<tr>
<td>No. of follicles survived at day 10 (% per initiated follicle)</td>
<td>124 (98.4)</td>
</tr>
<tr>
<td>No. of follicles with antrum formation (% per initiated follicle)</td>
<td>113 (89.7)</td>
</tr>
<tr>
<td>No. of follicles with spontaneous rupture (% per initiated follicle)</td>
<td>104 (82.5)</td>
</tr>
<tr>
<td>No. of total oocytes (% per initiated follicle)</td>
<td>101 (80.2)</td>
</tr>
<tr>
<td>No. of degenerated oocytes</td>
<td>2</td>
</tr>
<tr>
<td>No. of GV oocytes</td>
<td>27</td>
</tr>
<tr>
<td>No. of GVBD oocytes</td>
<td>43</td>
</tr>
<tr>
<td>No. of MI oocytes</td>
<td>31</td>
</tr>
<tr>
<td>% Per initiated follicle</td>
<td>24.6</td>
</tr>
<tr>
<td>% Per total oocytes</td>
<td>30.7</td>
</tr>
</tbody>
</table>

All parameters were similar among the three groups. GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase II.

Table 1. Gene primer sequences and their conditions for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product size (bp)</th>
<th>Accession number</th>
<th>Annealing temperature (°C)</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2R1</td>
<td>F: 5'-TAGATGAGATGGATCAAGTGCAAA-3'</td>
<td>226</td>
<td>NM_177382.4</td>
<td>62</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CATTTGATTTCCTGCAAGTGCTC-3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP27B1</td>
<td>F: 5'-CTCTCTTGGAGCCTACGATACTGTT-3'</td>
<td>265</td>
<td>NM_010009.2</td>
<td>62</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>R: 5'-TGATCAGTGTAGTTATAGGGAGACTA-3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VDR</td>
<td>F: 5'-AAGCCACTTTTACCTTGGATGAC-3'</td>
<td>273</td>
<td>NM_009504.4</td>
<td>62</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GTAGGTTCTCGAGTGTGTTGAGTAG-3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RT-PCR, reverse transcriptase-polymerase chain reaction; F, forward; R, reverse.
Bieche et al. [15] observed that most of the CYP2 family, including CYP2R1 (except CYP2F1), were expressed at variable levels in the human ovary. In that study, however, follicular stage-specific expression of the CYP2 family was not assessed. In the macaque, VDR, CYP2R1, and CYP27B1 were expressed in preantral and small antral follicles [12]. In contrast, we did not observe the expression of CYP2R1 and CYP27B1 mRNA in mouse preantral follicles. The reason for this difference is largely unknown. However, the absence of the two enzymes in mouse preantral follicles does not mean that VD3 has no effect on the growth of mouse preantral follicles. Based on our observations, since VDR is expressed in mouse preantral follicles, we can assume that VD3 can act on preantral follicles in mice. Overall, VD3 is expressed in preantral follicles, and CYP2R1 and CYP27B1 are expressed in antral follicles in mice; therefore, the intrinsic VD regulating system is present in the antral follicles.

Nonetheless, VD3 supplementation in vitro (25 or 50 pg/mL) did not enhance the full development of mouse preantral follicles (just up to the ruptured follicle stage) or oocyte acquisition in our study. In a macaque experiment, Xu et al. [11] observed that 25 pg/mL VD3 supplementation in vitro improved preantral follicle survival at week 2, but 100 pg/mL supplementation in vitro did not. Instead, 100 pg/mL VD3 supplementation in vitro led to a larger diameter of antral follicles at week 5. Thus, they argued that VD locally acts on primate follicular development, in a dose- and stage-dependent manner.

The optimal supplemental concentration of VD3 for preantral follicle culture in animals is unknown. Usually, in humans, a serum level of 25OHD (major circulating VD) higher than 30 ng/mL is regarded as replete, and serum level of 25OHD less than 20 ng/mL is regarded as deficient [16]. In the study by Potashnik et al. [17], the average level of 25OHD was 9.1 ± 1.8 ng/mL in human FF and 16.9 ± 1.9 ng/mL in human serum; in contrast, the average level of active VD3 was 22.3 ± 3.4 pg/mL in human FF and 48.5 ± 8.7 pg/mL in human serum at the time of oocyte pickup. Although there have been no reports regarding the appropriate level of active VD3 in mouse FF, we assumed that the 2 VD3 concentrations in our experiment (25 or 50 pg/mL) were sufficient.

In humans, three meta-analyses have reported that women undergoing IVF who have deficient or insufficient 25OHD levels have a lower live birth rate than women who have sufficient 25OHD levels [18-20]. However, the results from individual studies are inconsistent. For example, in a review by Pacis et al. [21], one study demonstrated a negative relationship between VD status and assisted reproductive technology (ART) outcomes, while two studies showed no association; the remaining five studies concluded that ART outcomes improved after VD supplementation. Furthermore, no association of serum 25OHD levels with IVF outcomes, in terms of the clinical pregnancy rate and live birth rate, has yet been reported [22,23]. Moreover, intake of calcitriol (an active form of VD) did not enhance the clinical pregnancy rate of IVF in women with 25OHD deficiency [24]. Therefore, there is no consensus on whether serum 25OHD levels are related to the IVF pregnancy rate, or whether VD supplementation could improve the IVF pregnancy rate in women with 25OHD deficiency.

Measurement of various steroid hormone levels or genes related to the VD3 metabolic pathway after VD3 supplementation would help to elucidate the role of VD3 in folliculogenesis. In a recent study, Makieva et al. [25] found that oral VD supplementation altered the hormonal milieu of FF and the transcriptomic profile of luteinized granulosa cells in women with 25OHD deficiency. They found upregulation of VDR, glutathione-S-transferase A3 (GSTA3), and the interleukin-21 receptor, and downregulation of prostaglandin-endoperoxide synthase 2 (PTG2S2), Kruppel-like factor 4 (KLF4), transient receptor potential cation channel subfamily C member (TRPC4), vascular endothelial growth factor, retinoid X receptor beta, and advanced glycation end-product specific receptor by oral VD supplementation. The mechanistic pathway of folliculogenesis after VD3 supplementation in vitro should be further investigated in animal models.

In conclusion, in the current study, we found follicular stage-specific expression of VDR, CYP2R1, and CYP27B1 in mice. Unfortunately, VD3 supplementation in vitro (25 or 50 pg/mL) was not helpful for the full development of preantral follicles and the resultant oocyte acquisition. Further studies are needed to verify whether VD3 supplementation in vitro is effective for the stage-specific development of mouse preantral follicles.

Conflict of interest

Byung Chul Lee is the editor-in-chief of the journal, but he was not involved in the peer reviewer selection, evaluation, or decision process of this article. No other potential conflicts of interest relevant to this article are reported.

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tronation: all authors. Writing-original draft: YJS, YHH, BCJ. Writing-review & editing: YJS, YHH, BCJ.

References

Effects of prematuration culture with a phosphodiesterase-3 inhibitor on oocyte morphology and embryo quality in *in vitro* maturation

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**Objective:** The study assessed the developmental potential of germinal vesicle (GV) oocytes subjected to *in vitro* maturation (IVM) after prematuration culture with cilostamide (a phosphodiesterase-3 inhibitor) and the impact of cilostamide exposure on the morphology of meiosis II (MII) oocytes and subsequent embryo quality.

**Methods:** In total, 994 oocytes were collected from 63 patients. Among 307 GV oocytes, 140 oocytes were selected for the experimental group and 130 oocytes for the control group. The denuded GV-stage oocytes were cultured for 6 hours with cilostamide in the experimental group and without cilostamide in the control group. After 6 hours, the oocytes in the experimental group were washed and transferred to fresh IVM medium. The maturational status of the oocytes in both groups was examined at 26, 36, and 48 hours. Fertilization was assessed at 18 hours post-intracytoplasmic sperm injection. Embryo quality was assessed on days 3 and 5.

**Results:** In total, 92.1% of the oocytes remained in the GV stage, while 6.4% converted to the MI stage (*p* < 0.01) after cilostamide exposure. In both groups, more MII oocytes were observed at 36 hours (25.8% vs. 21.5%) than at 26 hours (10.8% vs. 14.6%) and 48 hours (13% vs. 7.9%) (*p* > 0.05). With the advent of cilostamide, blastocyst quality was better in the experimental group than in the control group (*p* < 0.05).

**Conclusion:** Cilostamide effectively blocked nuclear maturation and promoted cytoplasmic growth. Prematuration culture with cilostamide enabled synchronization between cytoplasmic and nuclear maturity, resulting in better blastocyst outcomes.

**Keywords:** *In vitro* maturation; Intracytoplasmic sperm injection; Germinal vesicle oocyte; Phosphodiesterase-3 inhibitor

**Introduction**

The term *in vitro* maturation (IVM) refers to the maturation of immature oocytes retrieved from antral follicles with minimal or no gonadotropin stimulation [1]. Developed as early as 1935 by Pincus and Enzmann, this technique—although it seemed promising—did not come to the limelight like conventional *in vitro* fertilization (IVF). IVM was initially done in natural cycles and with the advent of better protocols, the option of follicle-stimulating hormone (FSH) priming along with luteinizing hormone (LH)/human chorionic gonadotrophin (hCG) triggering has been advocated to enhance oocyte competence [2]. Irrespective of FSH/LH/hCG priming, the outcomes of IVM for oocytes with regard to fertilization/implantation rates have been bleak in comparison with conventional stimulation cycles [3]. The suboptimal embryo quality obtained from IVM oocytes could be due to asynchrony between nuclear and cytoplasmic maturation at the cellular level [4]. Nuclear maturation comprises re-initiation of meiosis in oocytes arrested at prophase I, which is heralded by the loss of the nucleolar membrane (germinal vesicle [GV] breakdown) and ends in the completion of meiosis I with a concomitant expulsion of the first polar body [5]. Cytoplasmic maturation encompasses
all the events at the organelle level along with various epigenetic modifications that prepare oocytes for subsequent fertilization and embryo development [6]. Furthermore, cytoplasmic maturation cannot be quantified by events as nuclear maturation and is therefore poorly understood. One approach that has been postulated to enhance oocyte competence is to promote nuclear and cytoplasmic synchrony by delaying nuclear maturation while simultaneously allowing cytoplasmic maturation to manifest [7]. This might possibly lead to the development of good-quality embryos. In vivo, cyclic adenosine monophosphate (cAMP) is the main meiotic inhibitor that keeps the oocyte in an arrested state (prophase I) until the hours preceding ovulation. The cAMP generated in granulosa cells is also transported via gap junctions [8,9]. The maintenance of an elevated intra-oocyte cAMP level maintains meiotic arrest and keeps the oocyte in its dormant state. Synchrony can therefore be achieved by a prematuration culture that maintains higher levels of cAMP and inhibits the meiotic cycle within the oocyte in vivo [10,11]. Levels of cAMP are maintained by inhibiting the enzyme-phosphodiesterase (PDE) or by upregulating adenyl cyclase. Although nuclear and cytoplasmic maturation are stepwise processes, their coordination is essential for the development of competent oocytes. Various studies have shown that about 5% to 20% of oocytes retrieved in controlled ovarian stimulation (COS) cycles are immature [1,12]. Some of these oocytes have the potential for spontaneous maturation during in vitro culture, and are used for intracytoplasmic sperm injection (ICSI). However, immature oocytes are usually discarded due to the possibility of an increased rate of miscarriages or risk of anomalies [13,14]. In patients with a cohort of asynchronized follicles, where there is a higher proportion of immature oocytes, the use of these oocytes is essential to increase the number of injectable oocytes for ICSI and eventually to generate more embryos for transfer. Studies have explored various prematuration cultures of immature oocytes, probing the use of PDE-3 inhibitors (PDE-3Is) at an ideal concentration and duration of exposure [15]. The main challenge in developing IVM culture systems is to adopt the ideal culture conditions required in a particular stage of oocyte dependency [16]. PDE-3 is located in oo-
culture systems is to adopt the ideal culture conditions required in a

Methods

1. Ethical approval

The study was conducted at a tertiary care reproductive medicine unit in India. It was approved by the Institutional Review Board and the Nitte Central Ethics Committee (NU/CEC/2018/0191 NU/CEC/2020/2069). Informed consent was obtained from patients prior to the laboratory access of oocytes and sperm.

2. Study population and oocyte source

In total, 994 oocytes were collected from 63 patients, of which 307 were GV oocytes. Thirty-seven morphologically abnormal GV oocytes were excluded from the study. The present study comprised 270 viable GV oocytes obtained from women aged 25–35 years, who underwent COS for IVF/ICSI at Milann Fertility Center from April 2018 to March 2020. Patients with a minimum of four GV oocytes were included in the study. Only one treatment cycle per patient was incorporated in the study.

3. Stimulation protocol

COS was started on day 2 or 3 of the menstrual cycle after a baseline transvaginal ultrasound scan (Voluson P6 with a 4-8 MHz vaginal probe) and hormonal assessment (estradiol \[E_2\], progesterone, and LH if deemed necessary). Gonadotropins included either recombinant FSH, (Recagon, Organon, Ess, The Netherlands; Gonal F, Merck Serono, Modugno, Italy) or human menopausal gonadotropin (Menopo; Ferring Leciva, As, Czech Republic). The starting dose was calculated based on age, body mass index, and antral follicle count, and ranged from 112.5 to 187.5 IU daily for 4 days. The ovarian response to stimulation was monitored on the fifth day of stimulation with transvaginal ultrasound scan and serum \[E_2\], LH, and progesterone measurements, and the dose of gonadotropins was adjusted accordingly. A gonadotropin-releasing hormone antagonist (0.25 mg; Cetrotide, Merck, Darmstadt, Germany) was given daily subcutaneously based on a flexible antagonist protocol when the dominant follicle was \( \geq 12–14 \) mm in diameter or the serum \( E_2 \) level was \( > 350–400 \) pg/mL until the day of triggering. Triggering was performed when at least 3 follicles reached \( \geq 17 \) mm in diameter. Oocytes were aspirated transvaginally 35–36 hours post-triggering under intravenous sedation and ultrasound guidance. A single-lumen oocyte retrieval needle (Vitrolife, Vaxtra Frolunda, Sweden) was used.

4. Laboratory protocol

1) Preparation of cilostamide

The PDE-3I cilostamide (Cayman Chemical, Ann Arbor, MI, USA) was used for prematuration culture. A stock solution was prepared by dissolving cilostamide in crystalline form with dimethyl sulfoxide.
A final concentration of 1 μM cilostamide was added to the prematuration culture with the intention of achieving efficient meiotic inhibition and maximum reversibility of inhibition.

2) Preparation of cumulus-oocyte complexes

Cumulus-oocyte complexes (COCs) were incubated in the culture medium (G-IVF-PLUS, Vitrolife) covered with mineral oil (Ovoil, Vitrolife) at 37°C in 6% CO₂ for 2–3 hours. Cumulus cells were removed after exposure to G-Mops (3-N-morpholino-propane sulfonic acid) buffered medium containing 80 IU/mL hyaluronidase (Hyase, Vitrolife) for 30 seconds with the help of a glass Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, VA, USA). The nuclear status of the denuded oocytes was then assessed. Oocytes that were observed to have released the first polar body were considered mature and used for ICSI. Immature oocytes with a prominent nucleus (GVs) were assigned to the study and taken up for the prematuration culture.

5. Experimental setup
1) Prematuration culture

The denuded GV-stage oocytes of each patient were distributed into two groups (experimental and control group) and subjected to two different culture conditions. All immature oocytes were cultured individually in 25-μL drops of medium overlaid with oil in a humidified atmosphere at 37°C in 6% CO₂. The oocytes in the experimental group were placed in an IVM medium (Oocyte Maturation Medium; Cooper Surgical/SAGE, Trumbull, CT, USA) supplemented with 5 mg/mL serum protein substitute and FSH/LH (75 mIU/mL for each; Serono, Mississauga, ON, Canada) supplemented with 1 μM cilostamide, while the oocytes in the control group were placed in an IVM medium without cilostamide. After culturing these GV-stage oocytes for 6 hours [20], the cilostamide was removed from the experimental group. These oocytes were then transferred to inhibitor-free IVM medium and cultured for 42 hours.

2) Maturation of oocytes

The oocytes were then classified as GV, MI, or MII. ICSI was performed on MII oocytes, and the GV oocytes were cultured up to 48 hours.

3) Semen samples for ICSI

Semen samples were collected at the time of oocyte retrieval. After liquefaction, a simple preparative method, such as the swim-up or double density gradient method, was used to prepare semen samples for the ICSI procedures [21,22]. Since the oocytes did not mature synchronously during culture, the semen samples were frozen in multiple vials and thawed accordingly for further insemination.

4) Intracytoplasmic sperm injection

Morphologically normal spermatozoa were injected into the GV-converted MII oocytes. Following ICSI, these two groups (experimental and control groups) of injected oocytes were then incubated in 25-μL drops of single-step medium (GTL; Vitrolife) for further development.

5) Fertilization assessment

Fertilization was assessed at 18 hours post-ICSI. Fertilization was determined by the appearance of two distinct pronuclei and two polar bodies.

6) Embryo culture system

The fertilized oocytes were cultured for 120 hours. Embryo development was observed at 24-hour intervals, which were continued until day 5 (120 hours). On days 3 and 5, the embryos were scored as per the Istanbul consensus [23]. Embryo grading was performed and validated by expert embryologists.

6. Statistical analysis

Data were analyzed using IBM SPSS ver. 23 (IBM Corp., Armonk, NY, USA). The differences between the treatment groups were analyzed using the chi-square test and when appropriate, the two-tailed Fisher exact test. A p-value < 0.05 was considered to indicate statistical significance.

Results

The present study comprised 270 GV oocytes, of which 140 oocytes were selected for the experimental group and 130 oocytes for the control group (Figure 1). The quality and morphology of the GV oocytes were similar in both the experimental and control groups (p > 0.05).

1. Blocking of nuclear maturation by prematuration culture with cilostamide

The GV oocytes in the experimental group were exposed to 1 μM cilostamide for 6 hours. The control group was free from cilostamide. After 6 hours of culture in cilostamide, it was observed that 92.1% of oocytes remained in the GV stage and only 6.4% of oocytes had converted to the MI stage. However, in the control group, 20.8% of GV oocytes converted to the MI stage. A statistically significant result was observed between the experimental and control groups (p < 0.01) (Table 1). It was shown that cilostamide was effective at blocking nuclear maturation and promoting cytoplasmic growth. The number of MI oocytes showed a statistically significant difference between the experimental and control groups (p < 0.001) (Table 1).
2. Culture system of GV oocytes after 6 hours: progression of meiosis at 26, 36, and 48 hours in oocytes subjected to prematuration culture with cilostamide (1 μM) followed by IVM culture

The GV oocytes were then cultured in IVM medium (without cilostamide) and were observed at 26, 36, and 48 hours (Table 2). The experimental and the control groups showed no significant differences at 36 hours and 48 hours, although a significant difference was found for GV oocytes at 26 hours (p = 0.05). The experimental group showed the highest number of MII oocytes at 36 hours (25.8%), followed by 48 hours (13%) and 26 hours (10.8%). The control group also showed the maximum number of MII oocytes at 36 hours (21.5%), followed by 26 hours (14.6%) and 48 hours (7.9%). There was no statistically significant difference between the experimental and control groups (p > 0.05). For the 57 oocytes that matured from 140 GV oocytes in the experimental group and the 48 oocytes that matured out of 130 GV oocytes in the control group, the maturation rate was 41% and 37%, respectively. The present study also observed an increased number of degenerated oocytes at 48 hours in the control group as compared to the experimental group (Table 2).

3. Morphological assessment of MII oocytes

Cytoplasmic abnormalities such as increased granulations, the presence of vacuoles, refractile bodies, smooth endoplasmic reticulum clusters, organelle clustering, and dark cytoplasm were found in the MII oocytes of both the experimental and control groups (Figure 2). However, the morphological features were similar in both groups (p > 0.05). All the MII oocytes were then injected with morphologically normal sperm.

4. Fertilization of oocytes

The total number of MII oocytes obtained was 57 in the experimental group and 48 in the control group. Fertilization was confirmed with the appearance of two distinct pronuclei and two polar bodies in 35 oocytes (61.4%) in the experimental group and 22 oocytes (45.8%) in the control group. However, there were no statistically sig-

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Table 1. Efficacy of cilostamide (1 μM) in maintaining the meiotic arrest of GV oocytes in the experimental group as compared to the control group

<table>
<thead>
<tr>
<th>Oocyte</th>
<th>Experimental group (n = 140)</th>
<th>Control group (n = 130)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GV</td>
<td>129 (92.1)</td>
<td>103 (79.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MI</td>
<td>9 (6.4)</td>
<td>27 (20.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Degenerated</td>
<td>2 (1.4)</td>
<td>0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Values are presented as number (%). The p<0.05 is considered to indicate statistical significance.
GV, germinal vesicle; MI, meiosis I.

Table 2. Progression of meiosis at 26, 36, and 48 hours of IVM culture of oocytes subjected to prematuration culture with cilostamide (1 μM) followed by IVM culture

<table>
<thead>
<tr>
<th>Variable</th>
<th>GV E</th>
<th>C</th>
<th>p-value</th>
<th>MI E</th>
<th>C</th>
<th>p-value</th>
<th>MI E</th>
<th>C</th>
<th>p-value</th>
<th>Degenerated E</th>
<th>C</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>After 26 hr (E = 138, C = 130)</td>
<td>103 (74.6)</td>
<td>83 (63.8)</td>
<td>0.05</td>
<td>17 (12.3)</td>
<td>24 (18.5)</td>
<td>0.1</td>
<td>15 (10.8)</td>
<td>19 (14.6)</td>
<td>0.3</td>
<td>3 (2.2)</td>
<td>4 (3)</td>
<td>0.6</td>
</tr>
<tr>
<td>After 36 hr (E = 120, C = 107)</td>
<td>61 (50.8)</td>
<td>49 (45.8)</td>
<td>0.4</td>
<td>23 (19.1)</td>
<td>27 (25.2)</td>
<td>0.2</td>
<td>31 (25.8)</td>
<td>23 (21.5)</td>
<td>0.4</td>
<td>5 (4.2)</td>
<td>8 (7.5)</td>
<td>0.2</td>
</tr>
<tr>
<td>After 48 hr (E = 84, C = 76)</td>
<td>36 (42.9)</td>
<td>34 (44.7)</td>
<td>0.8</td>
<td>28 (33.3)</td>
<td>25 (32.9)</td>
<td>0.9</td>
<td>11 (13)</td>
<td>6 (7.9)</td>
<td>0.2</td>
<td>9 (10.7)</td>
<td>11 (14.5)</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Values are presented as number (%).
IVM, in vitro maturation; GV, germinal vesicle; MI, meiosis I; MI, meiosis II; E, experimental group; C, control group.
significant differences between the two groups ($p > 0.05$) (Figure 1).

5. Cleavage of embryos

The fertilized oocytes in the experimental ($n = 35$) and control ($n = 22$) groups were further cultured. After 27 hours, the embryo culture showed a higher frequency of cleavage in the experimental group (88.6%) than in the control group (77.3%), although the results were statistically insignificant ($p = 0.2$). Similar results were observed on day 2 and day 3 of embryo culture with regard to cleavage ($p > 0.05$). However, optimal cleavage was more frequent in the experimental group (40%) than in the control group (27.3%), although this difference did not reach statistical significance ($p = 0.33$) (Table 3).

Even though the cleavage rate was similar in both groups, the proportion of good-quality embryos (i.e., those with no apparent morphological abnormalities and few anucleate fragments) obtained was higher in the experimental group than in the control group.

The experimental group showed the highest number of grade 1 embryos, followed by grade 2 and grade 3, whereas the control group showed the highest number of grade 2 embryos, followed by grade 1 and grade 3. However, the distribution of grades 1, 2, and 3 among the experimental and control groups was similar ($p > 0.05$) (Figure 3).

6. Hourly cleavage status of embryos

The number of good-quality embryos was higher in the experimental group than in the control group, although this difference did not reach statistical significance. Considering the optimal time required to reach the cleavage stage, culturing for 26 hours and 36 hours resulted in the maximum number of embryos, whereas prolonged culture up to 48 hours did not give any advantage in terms of grade 1 embryos (Table 4).

Figure 4 shows the results of the development of human blastocysts from IVM GV oocytes. The present study yielded a total of 13 blastocysts in the experimental group and 4 blastocysts in the control group.

![Figure 2. Morphological evaluation of cytoplasmic abnormalities of mature oocytes following in vitro maturation culture (meiosis II [MII]). G, granulations; V, vacuoles; RB, refractile bodies; SER, smooth endoplasmic reticulum clusters; CP, central pitting; OC, organelle clustering; DC, dark cytoplasm.](https://doi.org/10.5653/cerm.2021.04413)

Table 3. Cleavage status of injected MII oocytes cultured in IVM medium

<table>
<thead>
<tr>
<th>Cleaved embryo</th>
<th>Experimental (n = 35)</th>
<th>Control (n = 22)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of fertilized oocytes (%)</td>
<td>Arrested</td>
<td>No. of fertilized oocytes (%)</td>
</tr>
<tr>
<td>No. of cleaved 2 PN (&gt; 27 hr)</td>
<td>31 (88.6)</td>
<td>4</td>
<td>17 (77.3)</td>
</tr>
<tr>
<td>No. of 4-cell embryos on day 2</td>
<td>28 (80)</td>
<td>3</td>
<td>13 (59.1)</td>
</tr>
<tr>
<td>No. of 8-cell embryos on day 3</td>
<td>23 (65.7)</td>
<td>5</td>
<td>9 (40.9)</td>
</tr>
<tr>
<td>No. of embryos with optimal cleavage</td>
<td>14 (40)</td>
<td></td>
<td>6 (27.3)</td>
</tr>
</tbody>
</table>

MII, meiosis II; IVM, in vitro maturation; PN, pronuclei.
Furthermore, we observed a statistically significant difference in the number of blastocysts obtained between the experimental and control groups ($p < 0.05$).

**Discussion**

The complexities around the mechanisms leading to final oocyte maturation remain largely unresolved, which may be why the technique of IVM lacks a precise rationale. The present study was based on the hypothesis that COS can lead to an acceleration of meiotic progression in oocytes, wherein the cytoplasmic maturation often lags behind nuclear maturation [24]. Studies have also shown that the extrusion of the polar body appears much earlier in denuded oocytes; this is deficient in cumulus cells due to the loss of gap junctions, which provide the oocyte with cAMP to maintain dormancy. Therefore, oocytes relieved from the inherent inhibitory effect on meiosis can proceed with nuclear maturation at a faster rate [25]. It has been shown that asynchrony between nuclear and cytoplasmic maturation can cause a negative impact on IVM outcomes. Pre-maturation culture has emerged as a potential method to prolong nuclear maturation and also allows adequate cytoplasmic competence to manifest. Incomplete cytoplasmic maturity can result in multinucleation and aneuploidy, thereby impairing embryo development [8]. However, to date, no consensus exists on the IVM culture system or which, if any, additives are beneficial.

**Table 4.** Embryonic development of MII oocytes on day 3 subjected to prematuration culture with cilostamide (1 μM) followed by IVM culture

<table>
<thead>
<tr>
<th>Day 3 embryo grading</th>
<th>26 hr</th>
<th>36 hr</th>
<th>48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E (n = 7)</td>
<td>C (n = 3)</td>
<td>E (n = 12)</td>
</tr>
<tr>
<td>8CG1</td>
<td>4 (57.1)</td>
<td>1 (33.3)</td>
<td>7 (58.3)</td>
</tr>
<tr>
<td>8CG2</td>
<td>2 (28.6)</td>
<td>2 (66.7)</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td>8CG3</td>
<td>1 (14.3)</td>
<td>0</td>
<td>1 (8.3)</td>
</tr>
</tbody>
</table>

Values are presented as number (%). Grade 1 (G1), good embryos with less than 10% fragmentation, equal blastomeres, and no multinucleation; grade 2 (G2), fair embryos with 10%–25% fragmentation; grade 3 (G3), poor embryos with severe fragmentation (>25%), unequal blastomeres, and multinucleation.

MII, meiosis II; IVM, *in vitro* maturation; E, experimental group; C, control group.

**Figure 3.** Grading of day 3 embryos (as per the Istanbul consensus) following *in vitro* maturation culture.

**Table 5.** Development of blastocysts from MII oocytes subjected to prematuration culture with cilostamide (1 μM) followed by IVM culture

<table>
<thead>
<tr>
<th>Variable</th>
<th>26 hr</th>
<th>36 hr</th>
<th>48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E (n = 7)</td>
<td>C (n = 3)</td>
<td>E (n = 12)</td>
</tr>
<tr>
<td>Grade 1</td>
<td>1, 1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Grade 2</td>
<td>1, 2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Grade 3</td>
<td>2, 1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2, 2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3, 2</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3, 3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cell arrest</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
</tbody>
</table>

Blastocysts are graded according to the stage of development, using grades of the inner cell mass (1–3; good to poor) based on the number of cells, compaction, and adhesion, and grades of the trophectoderm (1–3; good to poor) based on the number of cells and the cohesiveness of the epithelium.

MII, meiosis II; IVM, *in vitro* maturation; E, experimental group; C, control group.
nance of an elevated intra-oocyte cAMP level can keep meiosis in a dormant state. Hence, there is a delay in nuclear maturation, which allows time for cytoplasmic maturation. cAMP levels can be elevated within the oocyte with the help of kinase inhibitors (roscovitine or butyrolactone) [30] or by protein synthesis inhibitors (cycloheximide or puromycin) [31]. As these enzymes can act on both oocytes and granulosa cells, they may impair granulosa cell-oocyte interactions and future embryogenesis. Therefore, in the present study, we used the PDE-3i cilostamide, the action of which is oocyte-specific.

While using cilostamide, the reversibility of meiotic inhibition is an important consideration. A higher concentration of cilostamide can harm the reversibility of meiotic inhibition and spindle structure. Hence, unduly extending the period between transcriptional inactivation and initiation of meiotic maturation may reduce the developmental competence of immature oocytes [20]. Thus, in the present study we used denuded GV oocytes that were exposed to 6 hours of prematuration culture.

As compared to previous studies [32], the present study showed effectiveness in blocking nuclear maturation with 1 μM cilostamide for 6 hours. There was a statistically significant increase in the number of oocytes that remained in the GV stage, as compared to those that progressed towards M1, between the experimental and control groups (92.1% vs. 79.2%, \( p < 0.01 \)). At the end of meiotic arrest, a higher proportion of GV-stage oocytes with the surrounded-nucleolus chromatin configuration [33] indicates a morphological parameter of transcriptional repression. This transformation from a diffused or non-surrounded nucleolus configuration to a compacted or surrounded-nucleolus shape is crucial for successful embryonic development [34]. The results were comparable to those reported in previous studies [20,35]. In our study, we used SAGE medium (Cooper Surgical) to induce oocyte maturation. It has shown proven efficacy for maturing oocytes retrieved from patients with polycystic ovarian syndrome, as well as for the maturation of immature oocytes aspirated prior to treatment of ovarian malignancy [36,37].

The shorter duration of IVM culture is also an independent predictor of better oocyte and embryo quality. The outcome after with-
drawal of the inhibitor is still a debatable topic. Son et al. [38] showed that embryos derived from late-maturing oocytes (> 48 hours of IVM) were of suboptimal quality in comparison with oocytes maturing between 24 hours and 30 hours of IVM. Therefore, it can be inferred that prolonged blockage of meiotic progression can result in poor-quality embryo outcomes. After the removal of cilostamide blockage, the GV oocytes were cultured in IVM medium and were observed at 26 hours, 36 hours, and 48 hours. A higher number of MII oocytes was observed at 36 hours in the experimental group (25.8%) than in the control group (21.5%). Previous studies have followed several approaches concerning the time of culture for IVM, but there has been no consensus on the optimal maturation time for GV oocytes in IVM cycles in the literature so far [39,40]. We found no significant difference in the fertilization rates among MII oocytes with or without cilostamide exposure (p = 0.3). However, the study by Nogueira et al. [32] showed an increased fertilization rate and the study by Jee et al. [20] reported a paradoxical reduction in the fertilization rate.

A previous study by Xie et al. [40] showed that the rates of cleavage and blastocyst development in oocytes maturing at 48 hours after IVM (experimental, 72.2%; control, 19.0%) were significantly lower than in GV oocytes matured 24 hours after IVM (experimental, 91.5%; control, 50.4%). The present study also showed comparable results after 48 hours of IVM culture. From a metabolic perspective, successful IVM of an immature oocyte results from the facilitation of an appropriate environment for the oocyte to attain its developmental competence. COCs require glucose in the medium for metabolism to take place. Cumulus cells possess a substantial capacity for metabolizing glucose, which further produces carboxylic acids and adenosine triphosphate (ATP). The tricarboxylic acid cycle utilizes carboxylic acids within the oocyte. The developmental competence of the oocyte during IVM determines the generation of ATP by the COC. ATP, which is generated by oxidative phosphorylation, also forms the substrate for the production of cAMP. Meiotic inhibition during follicle development is maintained by cAMP. Even in the absence of cumulus cells, immature oocytes can be matured in vitro by the projections of cumulus cells embedded in the zona pellucida. These projections of cumulus cells provide inhibitory and stimulatory signals to the oocytes [41]. Therefore, it is evident that cumulus-free immature human oocytes can mature in IVM medium and also have the potential for early embryonic (cleavage and blastocyst) development.

Furthermore, we observed a statistically insignificant increase in embryo grading in the experimental group, when compared to the control group. However, surprisingly, we found a statistically significant increase in blastocysts in the experimental group (22.8% of MII oocytes injected) as compared to the control group (8.3%). Hence, narrowing the nuclear-cytoplasmic asynchrony with cilostamide plays an instrumental role in obtaining more blastocysts from IVM GV oocytes and could be used as an effective tool in increasing oocyte competence during IVM culture.

In conclusion, the present study provides insights into the IVM culture system for maturing GV oocytes. The developmental competence of oocytes can be enhanced through an improved understanding of the mechanisms that regulate meiotic competence, arrest, and resumption. Further studies are needed to understand the mechanisms through which meiosis is regulated in human oocytes and how oocyte-derived factors influence zygotic genome activation and embryonic developmental competence in human oocytes.

**Conflict of interest**

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

**Acknowledgments**

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**Author contributions**

Conceptualization: MAC, PKS, KAR. Data curation: MAC, MA. Formal analysis: PKS, KAR. Methodology: MAC, PKS. Project administration: AR. Visualization: MAC, MA. Writing—original draft: MAC. Writing—review & editing: MAC, AR, PKS, MA, KAR.

**References**

6. Bassham S, Canestro C, Postlethwait JH. Evolution of develop-


Comparison of embryonic competence and clinical outcomes between early and late cumulus cell removal for in vitro fertilization

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Objective: The impact of early mechanical removal of cumulus cells on fertilization and embryonic development is not yet precisely known. This study aimed to investigate the effects of early and late cumulus cell removal on fertilization, polyspermy, embryonic development potential, blastocyst development, and clinical outcomes.

Methods: A prospective study was conducted of patients who underwent in vitro fertilization between September 2019 and October 2020. Sibling oocytes were randomly allocated after insemination to early cumulus cell removal at 6 hours (group I) and late cumulus cell removal at 16–18 hours (group II). If total fertilization failure (TFF) was determined to have occurred at early cumulus cell removal, rescue intracytoplasmic sperm injection (ICSI) was performed. Fertilization, embryonic development, and pregnancy outcomes were compared.

Results: A total of 912 oocytes were assigned to group I (458 oocytes) and group II (454 oocytes). Fertilization, polyspermy, embryo quality, and pregnancy outcomes were not significantly different between both groups. Rescue ICSI enabled fertilization of 79.2% of the TFF oocytes.

Conclusion: Early cumulus cell removal at 6 hours had no significant difference in fertilization, polyspermy, embryo development, or obstetric and perinatal outcomes compared to late removal. Early cumulus cell removal combined with early rescue ICSI may have the potential to help couples with TFF.

Keywords: Cumulus cell; In vitro fertilization; Embryo development

Introduction
Cumulus cells play a significant role in supporting oocytes and fertilization. In natural conception, cumulus-enclosed oocyte and spermatozoa meet together at the ampulla of the uterine tube. Cumulus cells enhance capacitation, sperm binding and penetration of the zona pellucida, passage through the perivitelline space, binding and fusion to the oolemma, activation of the cortical reaction, and formation of the male pronucleus. They can also provide nutrients, hormones, and glycosaminoglycans, which are essential for the growth and development of embryos. Removal of the cumulus cells at this moment can markedly decrease fertilization. In conventional in vitro fertilization (IVF), co-incubation of the cumulus-oocyte complex and sperm for 16–18 hours can imitate natural conditions, but prolonged exposure time may generate high levels of reactive oxygen species produced by cumulus cells and excess spermatozoa, resulting in impaired embryo development and vitality [1,2]. Rescue intracytoplasmic sperm injection (ICSI) of unfertilized oocytes in such a situation has shown disappointing results [3]. Nevertheless, it is very important to determine how to improve treatment outcomes. In current practice, short co-incubation of the cumulus-oocyte complex with sperm and early cumulus cell removal (6 hours) is challenging [4-7].

The impact of early mechanical denudation of cumulus cells on fertilization and embryonic development is not yet precisely known. Early cumulus cell removal has been claimed to yield better embryo quality [4,5], but some studies showed comparable results [6,7] and

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Early cumulus cell removal. This study aimed to investigate the effects of early cumulus cell removal compared to late cumulus cell removal on fertilization, polyspermy, embryonic development potential, blastocyst development, and clinical outcomes.

Methods

1. Study design and patient selection

This prospective randomized clinical study was carried out at Buddha Chinaraj Hospital between September 2019 and October 2020. The study was registered with the Thai Clinical Trial Registry (TCTR 20190817001) and approved by the Research Ethics Committee of Buddha Chinaraj Hospital Medical School (IRB 066/62). Patients who attended the infertility clinic and had indications for assisted reproduction were invited to join the study and provided written informed consent after enrollment. The inclusion criteria were women undergoing their first IVF treatment cycle aged 20–38 years old, who had at least 6 retrieved oocytes and whose partners had normal semen parameters. The etiologies of infertility included ovulatory dysfunction, tubal disease, unexplained infertility, and endometriosis. The oocytes were randomly allocated into two groups: early cumulus cell removal (group I), in which cumulus cells were removed 6 hours after insemination, and late cumulus cell removal (group II), in which cumulus cells were removed 16–18 hours after insemination. Rescue ICSI was conducted when total fertilization failure (TFF) was expected to occur in the early cumulus cell removal group 6 hours after insemination. Embryo transfers were selected randomly in the two groups in terms of whether the transferred embryos originated from the early or late cumulus cell removal group.

2. Controlled ovarian hyperstimulation and oocyte retrieval

Controlled ovarian hyperstimulation was started with a gonadotropin-releasing hormone agonist for down regulation in the mid-luteal phase of the previous cycle. Follicle-stimulating hormone and/or human menopausal gonadotropin in individually adjusted doses were administered after pituitary desensitization. If at least three follicles were ≥ 18 mm, 5,000–10,000 IU of human chorionic gonadotropin was injected to induce ovulation. The oocytes were then retrieved with a single-lumen needle under transvaginal ultrasound guidance 36–38 hours later.

3. Sperm preparation and insemination

Semen samples were gathered by masturbation in the morning on the same day of oocyte collection following 3–5 days of sexual abstinence. Sperm concentration, motility, and morphology were examined under a light microscope based on the World Health Organization criteria (fifth edition, 2010). Gradient centrifugation was used for sperm preparation. Active motile spermatozoa were harvested for insemination, with 20,000–50,000 spermatozoa contained in 50 μL of insemination medium.

4. Cumulus cell removal

The oocytes were transferred to a new sperm-free medium after 4 hours of co-incubation. In group I, the cumulus cells were mechanically removed at 6 hours post-insemination using a denuding pipette (Flexipet; Cook, Brisbane, Australia) with an inner diameter of 140 μm under an inverted microscope. Fertilization was considered to have occurred when a second polar body was present, and TTF was deemed to have occurred when in the absence of a second polar body in any of the mature oocytes. Rescue ICSI was done if none of the oocytes showed early fertilization after insemination for 6 hours. In group II, the cumulus cells were removed at 16–18 hours after insemination.

5. Fertilization assessment and embryo culture

The developmental competence of zygotes was evaluated after 96–120 hours. Normal fertilization was defined as the presence of 2 pronuclei and polyspermy as the presence of ≥ 3 pronuclei. Embryos were placed in cleavage medium during days 1–3 after fertilization, followed by blastocyst medium during days 4–5. Embryo morphology was assessed on day 5. The blastocysts were assigned a score based on the Gardner system, with high-quality blastocysts having scores of ≥ 4 BB [10]. The surplus high-scoring blastocysts were cryopreserved for future transfers.

6. Clinical and birth outcomes

Embryo transfer took place on day 5 under ultrasound guidance. The number of embryos was limited to one blastocyst to reduce the risk of multiple pregnancies. Progesterone was started on day 3 after oocyte retrieval for luteal support. The implantation rate was defined as the number of gestational sacs divided by the number of embryos that were transferred. The fetal heartbeat demonstrated by ultrasoundography was considered to be clinical pregnancy at 5 weeks after embryo transfer. Information was gathered on ongoing pregnancy at 20 weeks and the birth outcomes. Premature delivery was defined as a baby born before 37 weeks.
7. Statistical analyses

Continuous data were compared using the Student t-test, and proportional data were compared with the chi-square test and the Fisher exact test. A p-value < 0.05 was considered to indicate statistical significance. The analyses were performed using SPSS ver. 13.0 (SPSS Inc., Chicago, IL, USA). A sample size calculation found that at least 240 oocytes in each arm would be satisfactory for a 10% difference in maturation rates between oocytes in the two groups, given a type I error of 5% (two-tailed) and a type II error of 20%.

Results

A total of 85 patients were eligible for the study. Seven patients were excluded due to TTF, and rescue ICSI was performed (Figure 1). The remaining 78 patients were 32.8 ± 2.6 years old. Thirty-three patients (42.3%) had tubal factor fertility, 8 (10.2%) had endometriosis, 10 (12.8%) had polycystic ovary syndrome, and 27 (34.7%) had unexplained infertility (Table 1). In total, 912 oocytes were randomly allocated to early cumulus cell removal (group I, 458 oocytes) and late cumulus cell removal (group II, 454 oocytes). The details of fertilization and embryo development are summarized in Table 2. No significant differences were observed in fertilization, cleavage rate, and embryo quality. Polyspermy was higher in group I than in group II (9.0% vs. 5.9%, respectively), but the difference did not reach statistical significance.

Embryo transfer was conducted in 78 patients, of whom 40 patients obtained embryos from group I and 38 patients from group II. There were no significant differences between the two groups in terms of age, and the number of oocytes. Implantation rates, clinical pregnancy, ongoing pregnancy, and live birth rate were not significantly different between the patients with embryos transferred from the early and late cumulus cell removal groups (Table 3). No twin pregnancies occurred in this study.

Notably, seven patients in the early cumulus cell removal group had TTF, and rescue ICSI was carried out. Nineteen of 24 oocytes (79.2%) had normal fertilization without detected polyspermy and three embryos were transferred in three cycles leading to one single pregnancy with an uneventful course of gestation and delivery of a normal newborn (Table 4). However, in the late cumulus cell removal group, four patients had simultaneous fertilization in the sibling oocytes of the same patients, the embryos were transferred with a suc-

Table 1. Baseline characteristics of the 78 patients who participated in the study

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>30.4 ± 3.7</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.5 ± 2.6</td>
</tr>
<tr>
<td>Etiology of infertility</td>
<td></td>
</tr>
<tr>
<td>Tubal factor</td>
<td>33 (42.3)</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>8 (10.2)</td>
</tr>
<tr>
<td>PCOS</td>
<td>10 (12.8)</td>
</tr>
<tr>
<td>Unexplained infertility</td>
<td>27 (34.7)</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation or number (%). PCOS, polycystic ovarian syndrome.

Table 2. Outcomes of oocyte and embryo development using early and late cumulus cell removal

<table>
<thead>
<tr>
<th>Variable</th>
<th>Early removal (group I)</th>
<th>Late removal (group II)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature oocyte</td>
<td>458</td>
<td>454</td>
<td>-</td>
</tr>
<tr>
<td>Two pronuclei</td>
<td>375 (81.9)</td>
<td>373 (82.2)</td>
<td>0.913</td>
</tr>
<tr>
<td>Polyspermy</td>
<td>41 (9.0)</td>
<td>27 (5.9)</td>
<td>0.111</td>
</tr>
<tr>
<td>Cleavage</td>
<td>320 (85.3)</td>
<td>302 (81.0)</td>
<td>0.112</td>
</tr>
<tr>
<td>Blastocyst formation</td>
<td>178 (55.6)</td>
<td>156 (51.7)</td>
<td>0.323</td>
</tr>
<tr>
<td>High quality blastocyst</td>
<td>87 (27.1)</td>
<td>71 (23.5)</td>
<td>0.294</td>
</tr>
</tbody>
</table>

Values are presented as number (%).

*Chi-square test.

Figure 1. Consolidated standards of reporting trials (CONSORT) flowchart. ICSI, intracytoplasmic sperm injection.
successful singleton, and there was a normal course of pregnancy with breech presentation, followed by delivery of a normal infant by cesarean section (Table 5).

Discussion

There are inconsistent reports on the outcomes of early cumulus cell removal. Some studies have shown that early cumulus cell removal has fertilization and clinical pregnancy rates similar to late removal at 20 hours [6,7]. Another study demonstrated that early cumulus removal after 4 hours of insemination resulted in low numbers of available embryos [8]. However, a meta-analysis [11] showed that early cumulus cell removal was associated with a significant increase in the implantation rate and clinical pregnancy rate. The ability to draw a clear conclusion may be hindered by differences in study designs and populations. In the present study, sibling oocytes were randomly allocated into two groups to minimize the confounding between patients, and the findings indicated that early cumulus cell removal had no significant difference in fertilization, polyspermy, cleavage of embryos, clinical pregnancy, and ongoing pregnancy, comparable with the findings of previous studies [4,5,12-16].

Conventionally, cumulus cells are recognized as essential for oo-

Table 3. Pregnancy outcomes according to early and late cumulus cell removal

<table>
<thead>
<tr>
<th>Variable</th>
<th>Early removal (group I)</th>
<th>Late removal (group II)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>40</td>
<td>38</td>
<td>-</td>
</tr>
<tr>
<td>Mean age (yr)</td>
<td>32.2 ± 2.7</td>
<td>33.3 ± 2.5</td>
<td>0.066(^a)</td>
</tr>
<tr>
<td>No. of embryos transferred</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Implantation rate</td>
<td>19/40 (47.5)</td>
<td>19/38 (50.0)</td>
<td>0.995(^b)</td>
</tr>
<tr>
<td>Clinical pregnancy rate</td>
<td>18/40 (45.0)</td>
<td>17/38 (44.7)</td>
<td>0.838(^b)</td>
</tr>
<tr>
<td>Ongoing pregnancy rate</td>
<td>16/40 (40.0)</td>
<td>15/38 (39.5)</td>
<td>0.854(^b)</td>
</tr>
<tr>
<td>Live birth rate</td>
<td>14/40 (35.0)</td>
<td>14/38 (36.8)</td>
<td>0.947(^b)</td>
</tr>
<tr>
<td>Premature delivery rate</td>
<td>2/16 (12.5)</td>
<td>1/15 (6.7)</td>
<td>0.999(^c)</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>2,930 ± 266</td>
<td>3,040 ± 250</td>
<td>0.059(^d)</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation or number (%).
\(^a\)Independent t-test; \(^b\)Chi-square test; \(^c\)Fisher exact test.

Table 4. Details of embryonic competence and clinical outcomes in seven patients who had rescue ICSI

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Oocyte</th>
<th>Fertilization</th>
<th>Cleavage</th>
<th>Blastocyst</th>
<th>Implantation</th>
<th>Clinical pregnancy</th>
<th>Birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(^a)</td>
<td>28</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2(^a)</td>
<td>30</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3(^b)</td>
<td>31</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4(^b)</td>
<td>31</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5(^b)</td>
<td>32</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6(^b)</td>
<td>34</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7(^b)</td>
<td>37</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

ICSI, intracytoplasmic sperm injection.
\(^a\)Patients 1, 2, and 6 had a rescue ICSI-derived embryo transferred. Patient 2 became pregnant and delivered a normal newborn; \(^b\)Patients 3, 4, 5, and 7 did not have a rescue ICSI-derived embryo transferred.

Table 5. Details of embryonic competence and clinical outcomes of late cumulus removal oocytes in four patients who had TFF of sibling oocytes of early cumulus removal

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Oocyte</th>
<th>Fertilization</th>
<th>Cleavage</th>
<th>Blastocyst</th>
<th>Implantation</th>
<th>Clinical pregnancy</th>
<th>Birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>31</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4(^a)</td>
<td>31</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5(^b)</td>
<td>32</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>37</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

TFF, total fertilization failure.
\(^a\)Patient 4 had a pregnancy with breech presentation, and the infant was delivered by cesarean section; \(^b\)Patient 5 had two polyspermic-fertilized zygotes...
cyte development and the natural fertilization process, but become less important after ICSI because embryos can develop normally without cumulus cells. This fact implies that the early removal of cumulus cells may not affect embryonic development. Moreover, Nagy et al. [17] found that oocytes were fertilized 2-4 hours after exposure to spermatozoa and the second polar body was extruded by approximately 90% into perivitelline space by 6 hours [6]. If this event is observed during early cumulus cell removal, rescue ICSI can be performed, resulting in higher fertilization rates and optimal embryos compared with rescue ICSI after late cumulus cell removal (20 hours) [6]. Interestingly, the time-course of fertilization in early rescue ICSI has a similar pattern to those oocytes that undergo ICSI at the normal time of fertilization, allowing the embryos to be obtained in synchronized development with the endometrium [6]. In this study, cumulus cells were dissected at 6 hours and rescue ICSI was conducted in seven cases of TFF, which obtained a satisfactory fertilization rate (79.2%); thus, mean ICSI after early cumulus cell removal (6 hours) could rescue most of the unfertilized oocytes. Therefore, early cumulus cell removal in conjunction with rescue ICSI provides an additional benefit by alleviating cycle cancellation in patients with TFF. Nevertheless, it is possible that injecting fertilized eggs can delay extrusion of the second polar body (3.2%) [9]. To avoid such an event, rescue ICSI can be postponed to 9 hours after insemination [12]. Remarkably, polyspermy after rescue ICSI was not found even when performed at 6 hours in our study. Despite the potential advantage of early cumulus cell removal and rescue ICSI, the demand of embryologists to cover the additional work during the extra period must be considered [6,11].

This study found a trend toward a higher polyspermy rate, albeit without statistical significance, in early cumulus cell removal (9.0%) than in late removal (5.9%), which aligns with previous reports [7,15]. It is unclear whether early cumulus cell removal can affect polyspermy. Cumulus cells are tougher and more difficult to remove at the earlier time point than at the later time point. However, at an early period after insemination, the oocytes are also more vulnerable due to their active spindles and microtubules [18,19]. Thus, they may have more susceptible ability to withstand the additional mechanical force created by the denuding pipette during cumulus cell removal [16]. Additionally, repeated mechanical stress can also have adverse effects on the integrity of the zona pellucida and may reduce the protective mechanism against polyspermic fertilization [16]. Of particular note, unstable culture conditions or an excessive number of sperm in the culture medium can also affect abnormal fertilization through different pathways [12]. These possible mechanisms may contribute to an increase in polyspermy. However, a meta-analysis showed that the use of a denuding pipette during cumulus cell removal was not harmful to the clinical pregnancy or implantation rate [11].

Our results showed that early cumulus cell removal had comparable obstetric and prenatal outcomes with late cumulus cell removal, in agreement with Liu et al. [14]. However, recent studies [11,13] reported higher rates of premature delivery, twins, and low-birthweight newborns in patients who underwent early cumulus cell removal. They proposed that the process during removal of cumulus cell at the early time point could possibly alter spindle integrity and impair cell division more than late removal, which might give rise to twins or poor fetal growth [20]. In addition, inappropriate mechanical forces during cumulus cell removal can cause epigenetic changes associated with low birth weight [21,22]. Further studies should be conducted to elucidate this interesting issue.

The present study showed that early cumulus cell removal at 6 hours after insemination had no significant difference in fertilization, polyspermy, embryo development, obstetric and perinatal outcomes. Early cumulus cell removal combined with early rescue ICSI may have the potential to help couples with TTF.

Conflict of interest

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

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References

6. Chen C, Kattera S. Rescue ICSI of oocytes that failed to extrude the
Anorexigenic peptide (leptin, obestatin, nesfatin-1) levels and their impact on assisted reproductive technology treatment outcomes in patients with polycystic ovary syndrome

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Objective: In this study we aimed to assess anorexigenic peptide levels in patients with or without polycystic ovary syndrome (PCOS) and their effects on assisted reproductive treatment (ART) outcomes.

Methods: A prospective case-control study was conducted in a tertiary care university-based ART clinic. Eighty-three patients were included in the study. The PCOS group included 41 patients, and the non-PCOS group included 42 controls. The 2003 Rotterdam criteria were used for PCOS patient selection. The ART indications in the non-PCOS group were tubal factor or unexplained infertility. Venous blood samples were taken on the third day of the menstrual cycle to determine the serum anorexigenic peptide levels. The enzyme-linked immunosorbent assay method was used for laboratory analyses.

Results: In the PCOS group, serum obestatin levels were significantly lower than in the control group, but serum anorexigenic peptide levels were similar in PCOS patients with or without clinical pregnancy. Ovarian hyperstimulation syndrome (OHSS) was diagnosed only in PCOS patients, and the obestatin levels of OHSS patients were significantly lower than those of other PCOS patients.

Conclusion: Baseline anorexigenic peptide levels did not affect the clinical pregnancy rate in ART cycles. Obestatin may play a role in the pathophysiology of OHSS. This possibility should be confirmed in further research.

Keywords: Assisted reproductive technique; Leptin; Nesfatin; Obestatin; Polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) is a complex clinical and biochemical condition with signs of ovulatory dysfunction and/or hyperandrogenism during adolescence, followed by infertility due to anovulation. Approximately 5% to 10% of reproductive-age women have been diagnosed with PCOS [1]. Stein and Leventhal defined PCOS in 1935; thousands of articles on PCOS are now published annually, but the exact mechanism of the syndrome remains unknown.

Brain and peripheral tissues control reproductive function [2], and anorexigenic peptides provide communication between these two systems [3]. Leptin, obestatin, and nesfatin-1 are examples of anorexigenic peptides that may have a relationship with PCOS pathophysiology [4,5]. Leptin is an important peptide for signal transmis-
sion between the adipose tissue and the reproductive system [5]. The true effect of PCOS on serum leptin levels is unknown, as some researchers found no difference and others found higher leptin levels in PCOS patients than in healthy controls [3,6]. The granulosa and theca cells of ovarian follicles have leptin receptors, and elevated leptin levels can affect follicular growth [7]. Obestatin, a newly discovered hormone, is mainly synthesized by gastrointestinal organs [8]. Reductions in appetite and nutrient intake are its most prominent physiological effects [9]. However, the effects of obestatin on reproductive physiology are being investigated, and its exact role in PCOS pathophysiology is not known [10]. The central nervous system, especially the hypothalamus, is the primary source of nesfatin-1 [11], although insulin-synthesizing pancreatic beta-cells also synthesize nesfatin-1. As a result of interactions with pancreatic beta-cells, nesfatin-1 may play an important role in the regulation of insulin production [12]. As is the case with obestatin, firm conclusions about the effect of PCOS on serum nesfatin-1 levels are not possible because the results of studies comparing PCOS subjects with controls are scarce [4,13].

In light of the inconsistent results that have been reported on this topic, we aimed to assess serum anorexigenic peptide levels in patients with PCOS in this study and to determine their effects on assisted reproductive treatment (ART) treatment outcomes.

Methods

A case-control study was conducted among couples with primary infertility who were admitted for an ART cycle at the outpatient infertility center of our department. The study was conducted between March 2016 and December 2016. The patients were informed about the study protocol and all participants provided written informed consent. The local ethical committee of Ankara University School of Medicine approved the study protocol (No. 11-456-15). The Code of Ethics of the World Medical Association (Declaration of Helsinki) was carefully followed throughout the entire study period.

The inclusion criteria for the study were an age between 18 and 40 years, as well as a baseline follicle-stimulating hormone level between 3 and 12 IU/L. The exclusion criteria were a history of bariatric surgery, diagnosis of endometriosis with laparoscopy, a history of ovarian surgery, a chromosomal abnormality in the female partner, endocrine disorders related to female infertility, and male factor infertility. The case group comprised patients who were diagnosed with PCOS according to the Rotterdam criteria [14] and scheduled for an ART cycle. The control group consisted of patients who were scheduled for ART either due to tubal factor infertility or unexplained infertility.

Recombinant follicle-stimulating hormone (Gonal-F, Merck-Serono, Istanbul, Turkey) was started after venous blood sampling to determine serum anorexigenic peptide levels. Gonadotropin dose adjustment was done according to age, body mass index (BMI), and the results of ovarian reserve tests. A gonadotropin-releasing hormone antagonist (Cetrotide, Merck-Serono) was generally added to the ovarian stimulation protocol (0.25 mg/day) on the sixth day of stimulation or when the leading follicle was ≥13 mm. When at least 3 follicles were ≥18 mm, final oocyte triggering was performed with a subcutaneous injection of 250 mg of recombinant human chorionic gonadotropin (Ovitrelle, Merck-Serono). Oocyte pick-up was performed under transvaginal ultrasound guidance 35–36 hours after the final oocyte trigger. All embryo transfers were performed with day 3 embryos. The number of transferred embryos was in accordance with national embryo transfer regulations. The luteal phase was supported with vaginal micronized progesterone (90 mg/day, Crinone 8% gel; Merck-Serono).

Clinical pregnancy was defined as the presence of an intrauterine fetal heartbeat at 6 gestational weeks. The implantation rate was calculated separately for each woman as the number of gestational sacs divided by the transferred embryos, multiplied by 100. The live birth rate was the primary outcome measure. Peripheral blood samples were obtained from the brachial vein after overnight fasting on day 3 of the menstrual cycle to determine serum anorexigenic peptide levels. Blood samples were drawn into tubes containing ethylene-diamine-tetraacetic acid (EDTA) 2Na (1 mg/mL) and aprotonin (500 U/mL). Samples were immediately centrifuged at 3000 × g for 10 minutes at 4°C and then stored at –80°C until the day when enzyme-linked immunosorbent assay (ELISA) was performed. Serum leptin, obestatin, and nesfatin-1 analyses were done with commercial ELISA kits (DIAsource Leptin ELISA Kit [sensitivity, 0.1 ng/mL; intra-assay coefficient of variability [CV], < 13.3%; inter-assay CV, < 10.2%]; KAP2281, Belgium; SunRed Biotechnology human obestatin ELISA kit [sensitivity, 0.1 mg/mL; intra-assay CV, < 10%; inter-assay CV, < 15%], 201-12-0971, China; and SunRed Biotechnology Human Nesfatin-1 ELISA kit [sensitivity, 0.113 mmol/L; intra-assay CV, < 10%; inter-assay CV, < 12%], 201-12-4341, China).

SPSS ver. 15.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. The Kolmogorov-Smirnov test was performed to test the normality of the distribution of the variables. According to distribution of each variable, either the Student t-test or the Mann-Whitney U-test was calculated. Categorical variables were compared using the chi-square test. A p-value of < 0.05 was considered to indicate statistical significance.

Results

In the final analysis, 83 patients were eligible for the study. The
study group included 41 patients with PCOS and the control group included 42 patients with either tubal factor infertility or unexplained infertility. The groups were similar in terms of baseline and demographic parameters except for baseline luteinizing hormone (LH) levels (Table 1). The features of the ovarian stimulation cycles are presented in Table 1. The clinical pregnancy rate per embryo transfer was comparable between the PCOS (35.1%) and control (35.7%) groups.

Table 1 also presents the serum anorexigenic peptide levels of both groups. The obestatin concentration was found to be significantly lower in the PCOS group (169.02 pg/mL; range, 15.42–358.98 pg/mL) than in the control group (224.51 pg/mL; range, 22.20–437.79 pg/mL) (p = 0.04). The median serum leptin concentrations in the PCOS and control groups were 26.15 ng/mL (range, 4.60–88.50 ng/mL) and 27.20 ng/mL (range, 1.90–122.90 ng/mL), respectively (p = 0.530). The nesfatin-1 concentrations in the PCOS and control groups were 100.47 mmol/L (range, 13.70–411.51 mmol/L) and 144.35 mmol/L (range, 26.99–450.82 mmol/L), respectively (p = 0.096).

Additionally, anorexigenic peptide levels were similar in PCOS patients with and without clinical pregnancy (Table 2). The mean levels of leptin, nesfatin-1, and obestatin in patients with clinical pregnancy were 21.21 ± 12.74 ng/mL, 96.89 ± 72.49 mmol/L, and 136.97 ± 86.92 pg/mL, respectively. The mean levels of leptin, nesfatin-1, and obestatin in patients without clinical pregnancy were 27.25 ± 17.71 ng/mL, 77.50 ± 66.08 mmol/L, and 188.51 ± 110.05 pg/mL, respectively.

In the PCOS group, 4 embryo transfers were postponed due to the development of early ovarian hyperstimulation syndrome (OHSS). The mean obestatin level was significantly lower in patients who had both PCOS and OHSS than in patients with only PCOS (93.68 ± 49.33 pg/mL vs. 169.97 ± 98.33 pg/mL, p = 0.04). However, the limited number of patients with OHSS limited the statistical power. We also

Table 1. Demographic characteristics and baseline variables of the study population

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n = 42)</th>
<th>PCOS (n = 41)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>29.0 ± 3.7</td>
<td>27.7 ± 3.6</td>
<td>0.690</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.7 ± 5.0</td>
<td>24.8 ± 4.2</td>
<td>0.552</td>
</tr>
<tr>
<td>Duration of infertility (yr)</td>
<td>6.5 ± 4.6</td>
<td>5.0 ± 3.2</td>
<td>0.100</td>
</tr>
<tr>
<td>FSH (mIU/mL)</td>
<td>7.4 ± 3.2</td>
<td>6.2 ± 2.0</td>
<td>0.462</td>
</tr>
<tr>
<td>LH (mIU/mL)</td>
<td>4.5 ± 2.1</td>
<td>7.5 ± 4.8</td>
<td>0.008</td>
</tr>
<tr>
<td>Duration of ovarian stimulation (day)</td>
<td>9.9 ± 1.9</td>
<td>10.4 ± 2.2</td>
<td>0.191</td>
</tr>
<tr>
<td>Total dose of gonadotropins (IU)</td>
<td>2,689 ± 871</td>
<td>2,351 ± 735</td>
<td>0.254</td>
</tr>
<tr>
<td>Maximum E2 concentration (pg/mL)</td>
<td>2,304 ± 1,628</td>
<td>2,977 ± 1,821</td>
<td>0.197</td>
</tr>
<tr>
<td>No. of follicles ≥ 17 mm on day of hCG</td>
<td>3.4 ± 2.0</td>
<td>5.3 ± 3.0</td>
<td>0.114</td>
</tr>
<tr>
<td>Endometrial thickness on day of hCG (mm)</td>
<td>10.0 ± 2.0</td>
<td>10.0 ± 3.1</td>
<td>0.326</td>
</tr>
<tr>
<td>No. of oocytes retrieved</td>
<td>7.2 ± 3.7</td>
<td>12.4 ± 6.2</td>
<td>0.002</td>
</tr>
<tr>
<td>No. of MII oocytes</td>
<td>6.5 ± 3.9</td>
<td>11.1 ± 5.2</td>
<td>0.005</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>63.6 ± 30.4</td>
<td>68.1 ± 25.9</td>
<td>0.234</td>
</tr>
<tr>
<td>No. of day 3 grade A embryos</td>
<td>4.0 ± 2.5</td>
<td>7.9 ± 2.9</td>
<td>0.006</td>
</tr>
<tr>
<td>Clinical pregnancy rate</td>
<td>15 (35.7)</td>
<td>13 (35.1)</td>
<td>0.950</td>
</tr>
<tr>
<td>OHSS</td>
<td>0</td>
<td>4 (9.52)</td>
<td>0.040</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>27.20 (1.90–122.90)</td>
<td>26.15 (4.60–88.50)</td>
<td>0.530</td>
</tr>
<tr>
<td>Nesfatin-1 (mmol/L)</td>
<td>144.35 (26.99–450.82)</td>
<td>100.47 (13.70–411.51)</td>
<td>0.096</td>
</tr>
<tr>
<td>Obestatin (pg/mL)</td>
<td>224.51 (22.20–437.79)</td>
<td>169.02 (15.40–358.98)</td>
<td>0.040</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviation, number (%), or median (range). PCOS, polycystic ovary syndrome; BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, estradiol; hCG, human chorionic gonadotropin; MII, metaphase II; OHSS, ovarian hyperstimulation syndrome.

Table 2. Anorexigenic peptide levels in PCOS patients with and without clinical pregnancy

<table>
<thead>
<tr>
<th>Variable</th>
<th>Clinical pregnancy (+) (n = 13)</th>
<th>Clinical pregnancy (–) (n = 28)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin (ng/mL)</td>
<td>21.21 ± 12.74</td>
<td>27.25 ± 17.71</td>
<td>0.458</td>
</tr>
<tr>
<td>Nesfatin-1 (mmol/L)</td>
<td>96.89 ± 72.49</td>
<td>77.50 ± 66.08</td>
<td>0.601</td>
</tr>
<tr>
<td>Obestatin (pg/mL)</td>
<td>136.97 ± 86.92</td>
<td>188.51 ± 110.05</td>
<td>0.328</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviation. PCOS, polycystic ovary syndrome.
compared the baseline and ovarian stimulation characteristics of these patients with OHSS-free PCOS patients, as shown in Table 3, and BMI was the only factor that showed a significant difference between these groups (21.7 ± 1.2 kg/m\(^2\) vs. 26.4 ± 5.5 kg/m\(^2\), \(p = 0.005\)).

### Discussion

In this study, we aimed to assess the levels of anorexigenic peptides in patients with PCOS and their effects on ART outcomes. In PCOS patients, only obestatin levels were found to be significantly lower than in controls. Baseline anorexigenic peptide levels did not show any effect on ART outcomes in our study. The most interesting finding of the study may have been that, in the PCOS group, four patients were diagnosed with early OHSS, and their baseline serum obestatin levels were significantly lower than the rest of the PCOS group.

The hormones secreted by the gastrointestinal system and the adipose tissue play important roles in the management of the reproductive axis via autocrine, paracrine, and endocrine effects. Recently, Comninos et al. [2] reviewed the effects of seven gastrointestinal system-derived hormones and five adipose tissue-derived hormones on the reproductive system. According to them, these hormones can be used exogenously to solve reproductive problems.

Leptin is the most investigated anorexigenic peptide in the literature. In our study, no significant difference in serum leptin levels was found between patients with PCOS and the control group. This finding is also supported by other studies [3,15]. The effects of leptin on ART outcomes are unclear. High leptin levels can increase the oocyte maturation and fertilization rates, but can also affect the pregnancy rate negatively. High leptin levels can cause intra-follicular hypoxia due to the suppression of steroidogenesis [16-18]. In support of the latter findings, women with low leptin levels have been reported to have better ART outcomes [19,20]. In our study, although we found lower leptin levels in women who achieved clinical pregnancy, the difference was statistically insignificant between groups.

Vascular endothelial growth factor (VEGF) is an important mediator in the pathogenesis of OHSS. Leptin levels showed positive correlations with follicular-fluid VEGF levels in previous studies, but 2 studies did not show any difference in baseline serum leptin levels between patients who did and did not develop OHSS [21,22]. Our findings are also similar to those. In the PCOS group, four patients were diagnosed with early OHSS. However, their baseline serum leptin levels were similar to those of the rest of the PCOS group.

In animal studies, insulin-secreting beta-cells and cells secreting pro-nesfatin-1 (the precursor of nesfatin-1) were found in the same location in the pancreas [12]. Hyperinsulinemia is a common clinical finding in PCOS [13], and hyperinsulinemia can impair beta-cell function. As their location in the pancreas is the same, nesfatin-1 secretion might also be affected by hyperinsulinemia. This theory could explain the low nesfatin-1 levels in PCOS patients [23,24], which was also observed in our study. Considering the effects of nesfatin-1 on gonadotropin synthesis in the pituitary gland, a decrease in central nesfatin-1 levels may also increase serum LH levels in women with PCOS [25].

In a previous study, researchers evaluated the effect of nesfatin-1 levels on ART outcomes. No relationships were found between the fertilization rate, pregnancy rate, and nesfatin-1 levels. However, patients with higher intrafollicular fluid nesfatin-1 levels were found to have a higher number of oocytes and good-quality embryos available for cryopreservation [26]. In our study, we did not find a relationship between serum nesfatin-1 levels and the number of mature oocytes retrieved. However, we found higher serum nesfatin-1 levels in patients who achieved clinical pregnancy, but the difference was statistically insignificant. Larger studies, particularly focusing on his-

### Table 3. Comparison of the demographic characteristics, ovarian reserve test results, cycle parameters, and anorexigenic peptide levels in PCOS patients who were diagnosed with OHSS

<table>
<thead>
<tr>
<th>Variable</th>
<th>OHSS (+) (n = 4)</th>
<th>OHSS (–) (n = 37)</th>
<th>(p)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>28.8 ± 2.6</td>
<td>29.3 ± 3.9</td>
<td>0.769</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>21.7 ± 1.2</td>
<td>26.4 ± 5.5</td>
<td>0.005</td>
</tr>
<tr>
<td>FSH (mIU/mL)</td>
<td>6.5 ± 1.3</td>
<td>7.3 ± 2.4</td>
<td>0.667</td>
</tr>
<tr>
<td>LH (mIU/mL)</td>
<td>10.3 ± 3.1</td>
<td>7.2 ± 2.2</td>
<td>0.262</td>
</tr>
<tr>
<td>No. of antral follicle counts</td>
<td>22.2 ± 3.4</td>
<td>19.5 ± 2.8</td>
<td>0.080</td>
</tr>
<tr>
<td>Duration of ovarian stimulation (day)</td>
<td>9.8 ± 2.5</td>
<td>10.6 ± 2.2</td>
<td>0.554</td>
</tr>
<tr>
<td>Total dose of gonadotropins (IU)</td>
<td>2,756 ± 1,068</td>
<td>2,313 ± 617</td>
<td>0.476</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>33.60 (12–88.50)</td>
<td>25.90 (4.60–73.95)</td>
<td>0.706</td>
</tr>
<tr>
<td>Nesfatin-1 (mmol/L)</td>
<td>55.95 (30.41–101.23)</td>
<td>106.60 (13.70–451.11)</td>
<td>0.133</td>
</tr>
<tr>
<td>Obestatin (pg/mL)</td>
<td>93.68 (15.40–154.91)</td>
<td>169.97 (26.44–358.98)</td>
<td>0.040</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviation or median (range). PCOS, polycystic ovary syndrome; OHSS, ovarian hyperstimulation syndrome; BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone.
Ghrelin, which was first discovered in 1999, affects luteal cell function by reducing progesterone synthesis and shifting the balance between luteotropic and luteolytic factors [27]. Obestatin, which has the same origin as ghrelin, antagonizes the effects of ghrelin [8]. Obestatin can have effects on ovarian remodeling. The first study evaluating the effects of obestatin on the reproductive system was reported in 2008. In that study, granulosa cells were exposed to obestatin, and levels of proliferation markers (cyclin B1, MAP kinase) and apoptotic peptides (BAX, caspase 3) increased in granulosa cells after obestatin exposure [28]. Low obestatin levels may play a role in the development of the ultrasonographic findings of PCOS by negatively affecting ovarian morphology and the ovarian microenvironment. In our study, patients with PCOS had significantly lower serum levels of obestatin than the control group.

OHSS is a dreaded possibility for clinicians dealing with ART, since it can cause life-threatening complications. In the PCOS group, basal obestatin levels in patients who developed OHSS were significantly lower than in those who did not. Romani et al. [29] reported that the exposure of luteal cells to obestatin reduced VEGF release, which is responsible for capillary permeability during OHSS. Obestatin may act as a protective agent against OHSS. Future studies with larger cohorts are urgently needed to clarify whether obestatin has a protective effect against OHSS.

The most important strength of our study was the systematic exploration of individual anorexigenic peptide parameters. In addition, to the best of our knowledge, this is the first study to show a significant association between decreased serum obestatin levels and OHSS. However, only four patients developed OHSS, which makes the statistical significance of the difference between groups questionable. Further studies are needed to clarify our findings.

The main limitation of this study is the lack of measurements of anorexigenic peptide levels in the follicular fluid and the fact that correlations between serum and follicular fluid levels were not determined. We measured serum anorexigenic peptide levels before commencing exogenous gonadotropins. These medications can alter serum anorexigenic peptide levels. If serum anorexigenic peptide level measurements were also done during the ovarian stimulation period, the effects of serum anorexigenic peptide levels could have been evaluated more precisely. This is another limitation of our study.

In conclusion, according to the results of our study, serum obestatin levels are significantly lower in patients with PCOS. However, neither obestatin nor the other anorexigenic peptides showed significant effects on ART outcomes. Furthermore, obestatin levels were significantly lower in PCOS patients who developed OHSS, suggesting a possible prophylactic effect of normal obestatin levels against OHSS.

Conflict of interest

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

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References

7. Cioffi JA, Van Blerkom J, Antczak M, Shafer A, Wittmer S, Snodgrass HR. The expression of leptin and its receptors in pre-ovula-


Association between the serum estrone-to-estradiol ratio and parameters related to glucose metabolism and insulin resistance in women with polycystic ovary syndrome

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Objective: We aimed to evaluate associations between the ratio of serum estrone (E1) to estradiol (E2) and parameters related to serum glucose metabolism and insulin resistance in women with polycystic ovary syndrome (PCOS).

Methods: In total, 133 women between the ages of 18 and 35 diagnosed with PCOS were enrolled in this study. All participants with PCOS underwent blood tests to determine hormonal and biochemical metabolic parameters and a standard 2-hour 75-g oral glucose tolerance test. They were divided into two groups according to the serum E1-to-E2 ratio: group 1 (E1/E2 ratio <2.0) and group 2 (E1/E2 ratio ≥2.0).

Results: In the comparative analysis, the waist-to-hip ratio (WHR) was the only clinical variable that was significantly different between the two groups. Patients with a higher E1/E2 ratio showed higher fasting insulin levels, homeostasis model for insulin resistance, and postprandial glucose level at 2 hours (PPG2). In a correlation analysis, only PPG2 was significantly related to the serum E1/E2 ratio. However, after controlling for the confounding effects of body mass index (BMI) and WHR, fasting glucose was also significantly correlated with the serum E1/E2 ratio.

Conclusion: Women with PCOS with a higher serum E1/E2 ratio were found to be more likely to show higher fasting insulin and postprandial glucose levels. Significant correlations were found between the serum E1/E2 ratio and both fasting and postprandial serum glucose levels after adjusting for BMI and WHR in women with PCOS.

Keywords: Estradiol; Estrone; Glucose; Insulin resistance; Polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) is regarded as the most common endocrine disorder in women of reproductive age [1,2]. The prevalence of PCOS in women of reproductive age is approximately 3%–10%. PCOS is one of the major causes of infertility with anovulation, and it is present in approximately 25%–30% of patients with infertility [1].

The common characteristic clinical features of PCOS are amenorrhea or oligomenorrhea, inappropriate hormonal secretion (including hyperandrogenism), and abnormal metabolic status [1-3]. Hormonal imbalances besides hyperandrogenism in PCOS include increased luteinizing hormone levels, a reversed ratio of luteinizing hormone to follicle-stimulating hormone, increased anti-Müllerian hormone levels, mild prolactin elevation, increased inhibin and estrone (E1) levels, and a reversal of the ratio of E1 to estradiol (E2) (E1/E2 ratio) [1,2]. Common metabolic problems associated with PCOS are glucose intolerance, type 2 diabetes mellitus (T2DM), central obesity, insulin resistance, and hyperinsulinemia [1-6].

Insulin resistance and hyperinsulinemia are the cardinal factors involved in the pathogenesis of PCOS [7,8], and the reported preva-
lence of insulin resistance in women with PCOS varies depending on race, ethnicity, and nationality [9-14]. Obese women with PCOS are characterized by insulin resistance, which increases the risk of metabolic and cardiovascular diseases [15]. In obese patients with PCOS, insulin resistance is higher than in non-obese patients with PCOS [16], but even in non-obese patients, insulin resistance is higher than that of controls [17,18]. Insulin resistance is defined as a state of sub-normal to abnormal glucose utilization and homeostasis under normal concentrations of insulin production, and PCOS with obesity commonly amplifies the degree of these metabolic abnormalities [19,20]. Several assays can be used to evaluate insulin resistance, including the hyperinsulinemnic clamp, homeostasis model for insulin resistance (HOMA-IR), fasting glucose-to-insulin ratio (GIR), quantitative insulin sensitivity check index (QUICKI), and the results of oral glucose tolerance testing (OGTT) [2,21,22]. The Endocrine Society Clinical Practice Guidelines [21] recommend the use of OGTT to screen adolescents and adult women with PCOS, who are at high risk for impaired glucose tolerance and T2DM. The OGTT is a standard diagnostic tool for impaired glucose tolerance and T2DM [2,21].

Elevated serum E1 levels and the resulting reversal of the E1/E2 ratio are common abnormal hormonal characteristics in patients with PCOS [1,2,6,23]. In women with PCOS, aromatase and 17-β hydroxysteroid dehydrogenase activities are increased in cumulated peripheral fat cells along with increased peripheral aromatization and weight gain. E2 levels remain in the follicular phase range without mid-cycle level changes; on the contrary, E1 levels increase because of peripheral aromatization in response to increased androstenedione levels [24-26]. In a chronic hyperestrogenic state with reversal of the E1/E2 ratio, hormonal status and outcomes are unopposed by progesterone [1,23].

Several studies have explored the relationship between hormonal characteristics and insulin resistance-related parameters in women with PCOS [27-30]. A reversed E1/E2 ratio is a distinctive hormonal characteristic of PCOS; however, to our knowledge, no study has been conducted to evaluate the relationships between an increased E1/E2 ratio and parameters related to glucose and insulin metabolism in PCOS. The aim of the present study was to evaluate whether the serum E1/E2 ratio is related to other metabolic parameters associated with insulin resistance in women with PCOS.

Methods

1. Subjects

This study was approved by the Institutional Review Board of Inje University Haeundae Paik Hospital (IRB No. 129792-2014-035), and patient’s informed consent in this study was waived by the IRB. All patients were newly diagnosed with PCOS at 18–35 years of age from January 2010 to December 2013 at the above-mentioned university hospital. The patients were diagnosed with PCOS on the basis of the 2003 Rotterdam criteria. All patients who met at least two of the three criteria, including (1) oligo-anovulation, (2) biochemical and/or clinical signs of hyperandrogenism (e.g., hirsutism, acne, androgenic alopecia), and (3) ultrasonographically identified polycystic ovarian morphology (PCOM) of at least 1 ovary, were diagnosed with PCOS after excluding other diseases or etiologies [31]. Using the transvaginal or transrectal ultrasound approach, PCOM was defined as an ovarian volume of over 10 cm³ and/or the presence of over 12 follicles (2–9 mm in size). Pelvic ultrasonography (through the vagina or rectum) for assessing PCOM was conducted in the early follicular phase using a Voluson LOGIQ S7 (GE Ultrasound Korea, Seongnam, Korea) equipped with a transvaginal probe with a frequency range of 3.6–9 MHz, and all ultrasound examinations were conducted by the same reproductive endocrinologist. Oligo-anovulation was estimated on the basis of menstrual history and the presence of amenorrhea or oligomenorrhea. Amenorrhea was defined as a menstrual cycle interval of over 90 days without menstruation, and oligomenorrhea was defined as an interval of over 35 days. The most common clinical sign of hyperandrogenism is the presence of hirsutism, which was identified using a modified Ferriman-Gallwey score > 6, based on a previous study of hirsutism in Korean women [32]. Biochemical hyperandrogenism was confirmed by elevated serum androgen concentration beyond the 95% confidence limits in the control group of a previous study (total testosterone > 0.68 ng/mL and/or free testosterone > 1.72 pg/mL) [9,32]. Patients with a previous history of diagnosed diabetes, thyroid disease, hyperprolactinemia, or ovarian surgery were excluded. Patients who were taking oral contraceptives with or without prescriptions within the last 6 months and anti-diabetic drugs, including insulin sensitzers, were also excluded. All patients were divided into two groups based on the serum E1/E2 ratio: group 1 (E1/E2 ratio < 2.0) and group 2 (E1/E2 ratio ≥ 2.0).

2. Clinical and biochemical measurements

All clinical variables of the study participants were assessed when they first visited the outpatient department. Blood samples for biochemical laboratory analyses were taken from all subjects in the early follicular phase after overnight fasting. Serum E1 was measured using the Dsl-8700 Estrone ELISA kit (Beckman Coulter, Brea, CA, USA) and serum E2 was measured using Elecsys Estradiol II (Roche, Indianapolis, IN, USA) [23]. Serum insulin and glucose levels were analyzed using an Elecsys Insulin assay (Roche) and an L-Type GluI device (Wako, Osaka, Japan), respectively. Cholesterol and triglyceride levels were measured using Pureauto S (Sekisui, Tokyo, Japan), and serum high-density lipoprotein and low-density lipoprotein levels were
measured using Cholestest (Sekisui) [30]. Both intra- and inter-assay coefficients of variation for all assays were below 8%.

3. Assessment of insulin resistance

After overnight fasting, serum glucose and insulin levels were checked. Glucose levels at 60 minutes and 120 minutes after glucose ingestion during a 2-hour 75-g OGTT were measured. The fasting glucose level and postprandial glucose level at 2 hours (PPG2) were analyzed using an L-Type GluI device (Wako, Osaka, Japan). The insulin resistance parameters included HOMA-IR, QUICKI, and GIR. The GIR was calculated by dividing the glucose value (mg/dL) by the insulin value (µU/mL); HOMA-IR was calculated as fasting glucose (mg/dL) × fasting insulin (µU/mL)/405; and QUICKI was calculated as 1/[log(insulin value (µU/mL))+log(glucose value (mg/dL))].

4. Statistical analysis

All values are expressed as mean ± standard deviation. All statistical analyses were performed using SPSS ver. 18.0 (SPSS Inc., Chicago, IL, USA). The paired t-test was used to compare clinical and biochemical parameters, including hormonal and glucose and insulin metabolism-related parameters, between the two groups categorized by the E1/E2 ratio. The correlations between the serum E1/E2 ratio and insulin resistance-related parameters were analyzed with Pearson correlation coefficients, and partial correlation coefficients were used after adjusting for body mass index (BMI) and the waist-to-hip ratio (WHR). In all analyses, p-values < 0.05 were considered to indicate statistical significance.

Results

The mean E1/E2 ratio in group 1 (n = 74) was 1.31 ± 0.42 and that in group 2 (n = 59) was 3.23 ± 1.40. The comparisons of anthropometric parameters and serum hormonal levels, including E1 and E2, of the two groups are shown in Table 1. Among various clinical parameters, the WHR was the only parameter that was significantly different between the two groups (p = 0.010). As shown in Table 2, the

Table 1. Comparison of clinical characteristics between the two groups defined according to the serum E1-to-E2 ratio in women with polycystic ovary syndrome

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group 1 (n = 74)</th>
<th>Group 2 (n = 59)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>27.35 ± 5.69</td>
<td>26.54 ± 5.29</td>
<td>0.402</td>
</tr>
<tr>
<td>Parity</td>
<td>0.23 ± 0.54</td>
<td>0.20 ± 0.61</td>
<td>0.792</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>162.35 ± 5.18</td>
<td>160.85 ± 5.36</td>
<td>0.103</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>55.67 ± 14.10</td>
<td>57.78 ± 12.65</td>
<td>0.371</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>21.10 ± 5.22</td>
<td>22.31 ± 4.60</td>
<td>0.165</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.78 ± 0.06</td>
<td>0.81 ± 0.06</td>
<td>0.010</td>
</tr>
<tr>
<td>E1/E2 ratio</td>
<td>1.31 ± 0.42</td>
<td>3.23 ± 1.40</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation. Group 1, E1/E2 < 2.0; group 2, E1/E2 ≥ 2.0. A p-value was obtained by paired sample t-test. E1, estrone; E2, estradiol.

Table 2. Comparison of insulin resistance-related parameters between the two groups defined according to the serum E1-to-E2 ratio in women with polycystic ovary syndrome

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group 1 (n = 74)</th>
<th>Group 2 (n = 59)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting insulin (µU/mL)</td>
<td>6.87 ± 5.85</td>
<td>9.60 ± 8.43</td>
<td>0.047</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>91.48 ± 20.28</td>
<td>94.05 ± 19.36</td>
<td>0.468</td>
</tr>
<tr>
<td>PPG2 (mg/dL)</td>
<td>103.97 ± 29.07</td>
<td>120.91 ± 56.09</td>
<td>0.034</td>
</tr>
<tr>
<td>HOMA-IR (fasting)</td>
<td>1.57 ± 1.36</td>
<td>2.46 ± 2.93</td>
<td>0.044</td>
</tr>
<tr>
<td>GIR (fasting)</td>
<td>18.81 ± 9.98</td>
<td>16.46 ± 11.76</td>
<td>0.243</td>
</tr>
<tr>
<td>QUICKI (fasting)</td>
<td>0.37 ± 0.04</td>
<td>0.36 ± 0.05</td>
<td>0.089</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>170.86 ± 28.41</td>
<td>178.22 ± 31.92</td>
<td>0.167</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>89.55 ± 78.11</td>
<td>105.20 ± 97.98</td>
<td>0.313</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>58.46 ± 12.30</td>
<td>58.31 ± 14.75</td>
<td>0.947</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>95.65 ± 25.18</td>
<td>98.19 ± 31.09</td>
<td>0.615</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation. Group 1, E1/E2 < 2.0; group 2, E1/E2 ≥ 2.0. A p-value was obtained by paired sample t-test. E1, estrone; E2, estradiol; PPG2, postprandial glucose level at 2 hours; HOMA-IR, homeostasis model assessment of insulin resistance; GIR, glucose-to-insulin ratio; QUICKI, quantitative insulin sensitivity check index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
fasting insulin level, HOMA-IR, and PPG2 were significantly higher in group 2 (E1/E2 ratio ≥ 2.0) than in group 1.

In the correlation analysis, only PPG2 was significantly correlated with the serum E1/E2 ratio (Table 3). However, after adjusting for BMI and WHR (as anthropometric parameters known to be closely related to insulin resistance), postprandial and fasting glucose levels were significantly correlated with the serum E1/E2 ratio.

Discussion

A reversed E1/E2 ratio is a distinctive hormonal characteristic of PCOS [1,2]. The authors recently reported that serum E1 levels and the E1/E2 ratio were correlated with blood androgen levels, and in particular, the E1/E2 ratio was significantly correlated with the serum free testosterone level \( r = 0.260, p = 0.003 \) [23]. Insulin resistance and hyperinsulinemia are the cardinal factors involved in the pathogenesis of PCOS, which is associated with a high risk of glucose intolerance and T2DM [1-6,9,19-21]. However, to our knowledge, studies evaluating the relationship between increased serum E1 levels and parameters related to glucose and insulin metabolism are still lacking. Moreover, no previous study has evaluated the correlation between an increased E1/E2 ratio and the parameters related to insulin resistance in PCOS. This may be due to the high cost of commercial kits for determining E1 and the need for more complex laboratory techniques other than hormonal assays [23]. To the best of our knowledge, this is the first study conducted to evaluate the association between the serum E1/E2 ratio and parameters related to insulin and glucose metabolism in women with PCOS, and the results of our study suggest that the serum E1/E2 ratio is significantly related to both fasting and postprandial glucose levels in women with PCOS, after adjusting for confounding anthropometric factors.

Both fasting and postprandial glucose levels are major factors involved in insulin resistance, but the site of insulin resistance is known to be different between patients with abnormal fasting glucose and those with abnormal postprandial glucose levels according to previous clinical studies [33-37]. Individuals with impaired fasting glucose mainly show hepatic insulin resistance with normal muscle insulin sensitivity; on the contrary, those with impaired glucose tolerance typically show muscle insulin resistance with normal hepatic insulin sensitivity [34]. In the present study, the serum E1/E2 ratio was significantly related to both fasting and postprandial serum glucose levels after adjusting for BMI and WHR, suggesting that the E1/E2 ratio is related to both muscle insulin resistance and hepatic insulin resistance. Increased levels of androgenic precursors in theca cells induce the increased production of androstenedione, which is converted by 17β-hydroxysteroid dehydrogenase to testosterone or E1 by aromatization [6,24,26]. Aromatase and 17β-hydroxysteroid dehydrogenase activities occur in fat cells and ovarian theca cells; thus, weight gain leads to increased peripheral aromatization of androstenedione [6,24]. In PCOS, increased production of androstenedione with increased peripheral aromatization triggers an increase in serum E1 levels, which is frequently accompanied by weight gain and an increase in the WHR. In the present study, the WHR was significantly different between the two groups categorized on the basis of the E1/E2 ratio, which is partially consistent with the previous studies [6,24,26]. However, there was no significant difference in BMI between the two groups, which was contrary to what we had hypothesized.

In conclusion, women with PCOS with higher serum E1/E2 ratios were more likely to show higher fasting insulin and postprandial glucose levels. The serum E1/E2 ratio was significantly related to fasting and postprandial serum glucose levels after adjusting for BMI and WHR in women with PCOS. On the basis of this result, the serum E1/E2 ratio may be a feasible hormonal marker that reflects the status of

Table 3. Correlations of insulin resistance-related parameters with the serum E1-to-E2 ratio in women with polycystic ovary syndrome

<table>
<thead>
<tr>
<th>Variable</th>
<th>( r )</th>
<th>( p )-value</th>
<th>( r' )</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting insulin (μU/mL)</td>
<td>0.154</td>
<td>0.095</td>
<td>0.143</td>
<td>0.162</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>0.123</td>
<td>0.170</td>
<td>0.270</td>
<td>0.007</td>
</tr>
<tr>
<td>PPG2 (mg/dL)</td>
<td>0.251</td>
<td>0.005</td>
<td>0.308</td>
<td>0.002</td>
</tr>
<tr>
<td>HOMA-IR (fasting)</td>
<td>0.161</td>
<td>0.081</td>
<td>0.192</td>
<td>0.060</td>
</tr>
<tr>
<td>GIR (fasting)</td>
<td>−0.101</td>
<td>0.278</td>
<td>−0.037</td>
<td>0.192</td>
</tr>
<tr>
<td>QUICKI (fasting)</td>
<td>−0.156</td>
<td>0.092</td>
<td>−0.119</td>
<td>0.246</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>0.114</td>
<td>0.197</td>
<td>0.146</td>
<td>0.141</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>0.085</td>
<td>0.336</td>
<td>0.065</td>
<td>0.511</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>0.040</td>
<td>0.649</td>
<td>0.085</td>
<td>0.389</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>0.037</td>
<td>0.672</td>
<td>0.095</td>
<td>0.339</td>
</tr>
</tbody>
</table>

r, Pearson correlation coefficient; \( r' \), partial correlation coefficient adjusted by body mass index and waist-to-hip ratio.

E1, estrone; E2, estradiol; PPG2, postprandial glucose level at 2 hours; HOMA-IR, homeostasis model assessment of insulin resistance; GIR, glucose-to-insulin ratio; QUICKI, quantitative insulin sensitivity check index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
insulin and glucose metabolism in women with PCOS; however, additional studies are needed to corroborate our results so that they can be applied clinically.

Conflict of interest

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

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Author contributions

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

References

23. Chun S. Relationship between early follicular serum estrone level and other hormonal or ultrasonographic parameters in women with polycystic ovary syndrome. Gynecol Endocrinol 2020;36:143–7.


30. Chun S. 1-h Postprandial glucose level is related to the serum anti-Müllerian hormone level in women with polycystic ovary syndrome. Gynecol Endocrinol 2015;31:815–8.


Delayed postpartum regression of theca lutein cysts with maternal virilization: A case report

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Theca lutein cysts are rare, benign lesions responsible for gross cystic enlargement of both ovaries during pregnancy. This condition is also termed hyperreactio luteinalis. Elevated human chorionic gonadotropin (hCG) levels or states of hCG hypersensitivity seem to promote these changes, which in up to 30% of patients produce clinical signs of hyperandrogenism. Given the self-limiting course of theca lutein cysts, which are subject to spontaneous postpartum resolution, conservative treatment is the mainstay of patient management. Described herein is a rare case of theca lutein cysts with maternal virilization that failed to regress by 9 months after childbirth. Surgical intervention was eventually undertaken, necessitated by adnexal torsion.

Keywords: Hyperandrogenism; Hyperreactio luteinalis; Ovarian torsion; Theca lutein cyst; Virilism

Introduction

Theca lutein cysts are rare, benign, and typically bilateral cystic lesions producing grossly enlarged ovaries during pregnancy. In the realm of pathology, the term hyperreactio luteinalis is applied [1,2]. Although the pathogenesis is unclear, elevated levels of human chorionic gonadotropin (hCG) or hypersensitivity to prolonged hCG exposure may provoke exaggerated ovarian responses and cyst formation, triggering hyperandrogenism [3]. These derangements generally resolve spontaneously within months after delivery, regardless of ovarian size. In singleton pregnancies, pertinent publications consist chiefly of case reports and accounts detailing spontaneous resolution between 6 and 12 weeks postpartum [4-9]. Herein, we present a rare case of theca lutein cyst with virilization that failed to regress by 9 months after childbirth. Emergency laparoscopic surgery was eventually required due to adnexal torsion.

Case report

This study was approved by the Institutional Review Board at Severance Hospital (IRB No. 4-2019-0268) and adhered to the principles stipulated by the Declaration of Helsinki. The board members waived patient consent, provided there was no disclosure of identifiable personal information.

A 33-year-old woman (primigravida) was referred to our tertiary hospital for bilateral multilocular ovarian cysts (right: 17.0 × 8.9 × 16.2 cm; left: 11.0 × 5.8 × 14.4 cm) that developed at week 12 of gestation (Figure 1A). Prior to week 10, ultrasound studies of both ovaries were normal. The patient claimed to have regular menstrual cycles and de-
nied use of ovulation-inducing drugs. Deepening of the voice by week 21 was the sole indicator of virilization. At week 38 (+4 days), she delivered a male infant (weight, 3,230 g; Apgar score, 7–8) via cesarean section. Both cysts remained unchanged at childbirth, conferring no apparent fetal virilization.

At 10 weeks postpartum, the ovarian dimensions were still sizable (right: 25.3 × 19.1 cm; left: 10.0 × 6.1 cm) on computed tomography (CT) imaging (Figure 1B). Her voice continued to deepen, and excessive hair growth had begun in a male-pattern distribution. There were marked elevations of serum total testosterone (7.542 ng/mL), free testo-

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**Figure 1.** (A) Sonograms of multilocular ovarian cysts (right and left) discovered early in pregnancy (12 weeks). (B) Computed tomography (CT) views of the same cysts, 10 weeks postpartum. (C) CT image showing a smaller left ovary (LO) and enlarged right ovary (RO) with twisted pedicle (arrows), 9 months postpartum. (D) Operative photos of the shrunken left ovary, the enlarged right ovary with a twisted pedicle, and the right ovary after torsion release and cyst enucleation. (E) Gross findings of the right ovary (left), with a benign denuded wall and mildly edematous stroma (H&E sections; original magnification, ×40; inset ×200). (F) Sonograms of normally appearing ovaries 10 weeks after surgery.
and the free androgen index (FAI) was high (32.1). However, serum dehydroepiandrosterone sulfate was within the normal range. The serum total hCG level was still elevated (23 mlU/mL), with suppressed levels of luteinizing hormone (LH; < 0.1 mIU/mL) and follicle-stimulating hormone (0.6 mIU/mL). The estradiol concentration was 94.9 pg/mL (Table 1). Various tumor markers, including carcinoembryonic antigen, α-fetoprotein, cancer antigen (CA) 125, and CA 19-9, were within the respective normal ranges.

To lower androgen levels and slow progression of virilization, oral contraceptives (ethinyl estradiol [20 μg] and drospirenone [3 mg]), were administered, along with spironolactone (50 mg/day). After 3 months of treatment, serum androgen levels had declined but remained elevated: testosterone, 3.14 ng/mL; free testosterone, 3.62 pg/mL; 17-hydroxyprogesterone, 11.67 ng/mL; and FAI, 5.2. Serum total hCG was also much lower (6 mlU/mL) (Table 1), with unchanged ovarian dimensions on ultrasound. Having curbed the virilization and reduced androgen levels, we discontinued the anti-androgenic regimen.

Six months after delivery, serum testosterone, free testosterone, and 17-hydroxyprogesterone levels had normalized (0.084 ng/mL, 0.89 pg/mL, and 2.16 ng/mL, respectively), and the serum total hCG level was < 0.2 mlU/mL. The cystic ovaries had finally regressed (right: 11.2 × 10.7 cm; left: 4.1 × 4.1 cm), albeit more so on the left (Table 1).

Seventeen days later, the patient presented to the emergency room with intermittent, severe abdominal pain. Torsion of the enlarged right ovary was evident on CT (Figure 1C), calling for emergency laparoscopic surgery. During the procedure, we encountered a sizeable cyst (up to 10.0 cm) of the twisted right ovary (Figure 1D). We released the torsion and enucleated the cyst, which later proved benign. Its denuded lining and mildly edematous stroma are visible in Figure 1E. Eight weeks after surgery, theca lutein cysts of both ovaries were undetectable by ultrasound (Figure 1F), and all signs of virilization (e.g., deepening of the voice, excessive hair growth) had noticeably improved.

### Discussion

Theca lutein cysts are common causes of maternal hyperandrogenism [10]. Because LH and hCG are almost structurally identical, exaggerated follicular responses to the LH-like effects of hCG promote hypertrophy and luteinization of theca cells, inducing hyperandrogenism [11]. Consequently, up to 30% of patients will experience temporal balding, deepening of the voice, clitoromegaly, hirsutism, or acne [10]. However, fetal masculinization or virilization seldom oc-

---

**Table 1.** Postpartum chronology of shifting hormonal levels and ovarian cyst regression

<table>
<thead>
<tr>
<th>Variable</th>
<th>2 mo</th>
<th>4 mo&lt;sup&gt;a&lt;/sup&gt;</th>
<th>6 mo&lt;sup&gt;b&lt;/sup&gt;</th>
<th>9 mo</th>
<th>13 mo/2 mo PO</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total T (ng/mL)</td>
<td>7.542</td>
<td>3.140</td>
<td>0.942</td>
<td>0.084</td>
<td>0.084–0.481</td>
<td>0.084–0.481</td>
</tr>
<tr>
<td>SHBG (nmol/L)</td>
<td>80.4</td>
<td>210.1</td>
<td>114.0</td>
<td>82.3</td>
<td>6–152</td>
<td></td>
</tr>
<tr>
<td>FAI</td>
<td>32.6</td>
<td>5.2</td>
<td>2.9</td>
<td>0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free T (pg/mL)</td>
<td>13.38</td>
<td>3.62</td>
<td>2.31</td>
<td>0.89</td>
<td>1.33</td>
<td>0.00–3.09</td>
</tr>
<tr>
<td>17-OHP (ng/mL)</td>
<td>35.5</td>
<td>11.67</td>
<td>5.16</td>
<td>2.16</td>
<td>0.78</td>
<td>0.11–1.08 (F) 0.95–5.0 (L)</td>
</tr>
<tr>
<td>ThCG (mIU/mL)</td>
<td>23</td>
<td>6</td>
<td>2</td>
<td>&lt; 0.2</td>
<td></td>
<td>&lt; 1</td>
</tr>
<tr>
<td>DHEAS (ug/dL)</td>
<td>226.7</td>
<td></td>
<td></td>
<td></td>
<td>25.9–460.2</td>
<td></td>
</tr>
<tr>
<td>LH (IU/L)</td>
<td>&lt; 0.1</td>
<td>3.3</td>
<td></td>
<td></td>
<td>1.9–12.5 (F)</td>
<td></td>
</tr>
<tr>
<td>FSH (IU/L)</td>
<td>0.6</td>
<td>9.0</td>
<td></td>
<td></td>
<td>2.5–10.2 (F)</td>
<td></td>
</tr>
<tr>
<td>E2 (pg/mL)</td>
<td>94.9</td>
<td>39.3</td>
<td></td>
<td></td>
<td>19.5–144.2 (F)</td>
<td></td>
</tr>
<tr>
<td>AMH (ng/mL)</td>
<td>2.03</td>
<td></td>
<td></td>
<td></td>
<td>0.58–8.13</td>
<td></td>
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<tr>
<td>Ovarian size (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>25.3 × 19.1</td>
<td>16.6 × 7.5</td>
<td>11.2 × 10.7</td>
<td>6.4 × 4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>10.0 × 6.1</td>
<td>8.2 × 3.4</td>
<td>4.1 × 4.1</td>
<td>4.1 × 3.1</td>
<td></td>
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</tr>
</tbody>
</table>

Normal hormonal ranges (assay specifications) pertain to healthy nonpregnant women. PO, postoperative; T, testosterone; SHBG, sex hormone-binding globulin; FAI, free androgen index; 17OHP, 17-hydroxyprogesterone; F, follicular phase; L, luteal phase; ThCG, total human chorionic gonadotropin; DHEAS, dehydroepiandrosterone sulfate; LH, luteinizing hormone; FSH, follicle-stimulating hormone; E2, estradiol; AMH, anti-Müllerian hormone.

<sup>a</sup>1 month after medical intervention; <sup>b</sup>3 months after medical intervention.

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curs, as in patients with luteoma or androgen-producing ovarian tumors [1]. Our patient delivered a male fetus with no untoward effects of high androgen exposure in utero. Although there have been few case reports of virilized female fetuses [12,13], female fetuses born in mothers with virilization could show the effects of high androgen exposure including labioscrotal fusion and clitoromegaly. However, exposure to high androgen levels after 12 weeks of gestation generally does not produce labial fusion [12,13]. Besides the effect of the timing of androgen exposure, the lack of androgen-related fetal manifestations is also likely due to several protective mechanisms, including a rise in maternal sex hormone-binding globulin concentration, progesterone competition for androgen receptors or androgen activation in target tissues, and placental androgen aromatization [1].

This patient experienced some virilization during pregnancy (deepened voice, week 21) that worsened after childbirth (hirsutism, 10 weeks postpartum). Theca lutein cysts usually regress following delivery, but these persisted, causing marked and increasingly problematic androgen elevations. We thus prescribed oral contraceptives and spironolactone to lower androgen levels and slow the progressive effects. Androgen levels declined after 3 months of this regimen, despite unchanged ovarian status. Three months later, they had normalized spontaneously, along with gradual cystic regression. Unfortunately, surgical intervention was necessary to release right-sided adnexal torsion. It is likely that conservative treatment would have otherwise sufficed. Five months after laparoscopic enucleation of a right ovarian cyst, both ovaries appeared normal on ultrasound.

Theca lutein cysts are diagnosable clinically based on the characteristic spoke-wheel sonographic sign and physiological flow of color Doppler velocimetry [14]. Biochemical markers and magnetic resonance imaging may be helpful in differentiating theca lutein cysts from other disorders, especially malignancies [8]. Histologically, luteinized theca cells line the cyst walls, and the luteinized stromal cells appear edematous [3,4]. In most cases, such cysts are asymptomatic incidental findings discovered through routine sonography or cesarean section. Large ovarian cysts may cause abdominal discomfort, dyspnea, or abdominal pain due to torsion; peritonitis related to hemorrhage/rupture or mass effects; or overt virilization [9]. Past studies have reported natural regression of theca lutein cysts at 6–12 weeks postpartum [4–9]. Compared to previous examples, in the present case, the theca lutein cyst regressed gradually, but persisted until 9 months after delivery. To the best of our knowledge, this is the case of virilizing theca lutein cyst that persisted for the longest time after delivery.

Both of the patient’s ovaries showed spoke-wheel signs on ultrasound studies performed early in pregnancy (12 weeks). Having no history of ovulation-inducing drugs, theca lutein cystic change was favored rather than ovarian hyperstimulation syndrome as a diagnostic possibility. The inordinate persistence of cysts 2 months after delivery compelled us to consider other virilizing tumors in the patient: particularly Krukenberg, mucinous cystic, Brenner, and endodermal sinus tumors; serous cystadenoma; and dermoid cyst [15]. CT and tumor marker studies ruled out these disorders, with the denuded epithelium rendering cystadenoma indistinguishable on histologic grounds. Nonetheless, the left ovarian cyst spontaneously regressed, and there was similar shrinkage on the right, entirely compatible with theca lutein cysts.

Given their relation to hCG, theca lutein cysts tend to arise in conjunction with multiple pregnancies, gestational trophoblastic disease, or choriocarcinoma [2]. This case involved a singleton pregnancy and lacked any basis for such disorders. Levels of hCG also peak during early pregnancy (~100,000 mIU/mL), declining substantially thereafter (to ~30,000 mIU/mL) and persisting until term. Following delivery, they fall rapidly and reach normal levels for nonpregnant women by 3 weeks postpartum [16]. In our patient, hCG remained elevated (23 mIU/mL at 2 months postpartum, failing to normalize until 9 months after delivery. This curious regressive delay may be attributable to LH/hCG receptor-mediated mechanisms that prolong hCG elevations.

Considering that breastfed infants appear not to be adversely affected by maternal testosterone therapy [17], maternal hyperandrogenism is not a contraindication of lactation. However, due to the inhibitory effect of androgen on milk production, whereas lactogenesis in normal pregnancies begins between 30 and 40 hours postpartum, high maternal levels of testosterone and hCG can delay lactogenesis for as long as 31 days. It has also been reported that testosterone levels of approximately 3 ng/mL or less are required for successful milk production [1]. In this case, however, the patient breastfed until 2 months after delivery, even though her total serum testosterone was over 7 ng/mL.

The majority of theca lutein cysts have been successfully managed with a conservative approach; nevertheless, prior research found that 36.2% of patients underwent surgery, of whom 23.8% had an acute complication (e.g., ovarian torsion, pain, or hemoperitoneum) and the remaining 76.2% were suspected of having a malignancy [9]. Although there will always be a role for surgical exploration in atypical cases or cases complicated by torsion or rupture-associated hemorrhage, management of theca lutein cysts must be based on a conservative approach, mitigating undue surgical and reproductive morbidity [1]. In the present case, the virilization symptoms gradually improved with the decrease of androgen levels and subsided without surgery around 9 months after delivery. In addition, since the theca lutein cysts gradually regressed before surgery, they could be successfully managed with a conservative approach if adnexal torsion did not occur.
Herein, we presented a rare case of virilizing theca lutein cysts that did not readily regress after childbirth. Ultimately, adnexal torsion necessitated emergency laparoscopic surgery. Even if the resolution is delayed (i.e., > 9 months postpartum), it appears that theca lutein cysts may be managed conservatively and surgical intervention could be reserved for acute complications.

Conflict of interest

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

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References

I. ABOUT THE JOURNAL

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

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

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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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