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폴리트로프 프리필드시린지 • **성상**: 무색 또는 미황색을 띠는 투명한 액이 들어있는 1회용 주사침이 달린 투명한 유리재질의 프리필드시린지 • **원료의약품 및 분량**: 주성분 폴리트로핀(재조합-인산포자극호르몬) 75 IU/0.15 mL, 150 IU/0.3 mL, 225 IU/0.45 mL, 300 IU/0.6 mL • **효능 및 효과**: 다낭과 같은 임상적 상황에서 여성의 불임증 치료 1, 보조생식프로그램(즉, in vitro fertilization/embryo transfer; IVF/ET, gamete intrafallopian transfer; GIFT, zygote intrafallopian transfer; ZIFT, intracytoplasmic sperm injection; ICSI) 실시 중 다수의 난포를 성숙시키기 위한 조절된 난소과자극(controlled ovarian hyperstimulation), 2, Clomiphene citrate로 치료되지 않은 여성의 무배란증 (다낭성난소질환 (PCOD))을 포함하는 WHO 그룹 II에 해당하는 환자들) • **용법 및 용량**: 1, 보조생식술에서 내인성 황체형성호르몬의 급증(LH surge)을 억제하고 황체형성호르몬의 긴장 정도를 조절하기 위해 여러 가지 자극 프로토콜이 사용되어 질 수 있다. 다수난포의 발달을 위한 난소과자극과 배란을 위해 일반적으로 이용되는 방법은 주기의 2일에서 5일째에 시작해서 매일 150 - 300IU를 투여하는 것이다. 치료는 환자의 반응에 따라 용량을 조절하면서 적절한 난포발달이 이루어질 때까지 계속하는(혈청 에스트로겐 농도 모니터링과/혹은 초음파 검사로 평가). 보통 하루에 450IU 이상은 사용되지 않는다. 일반적으로 적절한 난포발달은 평균적으로 치료 10일째에 얻어진다. 최종 난포성숙을 유도하기 위해서는 마지막 이 약 투여로부터 48시간 이내 태반성 성선자극호르몬(hCG)을 최대 10,000IU 단회 투여한다. 2, 무배란증: 이 약 요법의 목표는 hCG 투여후 난자로부터 방출되는 한개의 성숙된 그라프란포를 발달시키는 것이다. 이 약은 매일 투여도 가능하며, 월경중인 환자의 경우 월경주기의 첫 7일 이내에 치료 시작되어야 한다. 치료는 각 환자의 반응에 따라 적절히 변경되어야 하며, 각 환자의 반응은 (초음파로 측정된 난포크기)와/혹은 (에스트로겐 분비)로 측정한다. 일반적으로 사용되는 방법은 75 - 150 IU를 매일 투여하다가 필요한 경우 적절한 반응을 얻기 위해 7일 또는 14일 간격으로 75 IU씩 증량하는 것이다. 1일 최대 용량은 225IU를 넘지 않는다. 치료 4주 후에도 충분한 반응이 얻어지지 않을 경우는 이 치료주기를 중단하고 이 주기보다 더 높은 시작용량으로 치료를 재시작하여야 한다. 최적의 반응이 얻어지면, 마지막 이 약 주사 24 - 48시간 후 태반성성선자극호르몬(hCG)을 250µg 또는 5000IU, 최대 10,000IU까지 투여한다. 환자는 태반성성선자극호르몬 투여일과 그 다음날 성교를 피하도록 추천된다. 태반성성선자극호르몬을 실시할 수도 있다. 단일 과도한 반응이 나타나면 치료를 중단하고 태반성성선자극호르몬을 보류하며, 다음 주기에 치료를 때는 이전 주기에 사용했던 것보다 낮은 용량으로 실시하도록 한다. • **사용상의 주의사항**: 1, 다음 환자의 경우에는 투여하지 말 것 1) 난소, 유방, 자궁, 시상하부나 뇌하수체에 종양이 있는 환자 2) 임신, 수유부 3) 원인불명의 혈소판이 있는 환자 4) 본제의 구성성분에 과민증이 있는 환자 5) 원형성 난소질환 환자 6) 다낭성난소증(PCOD)과 무관한 난소기능이나 확장된 난소가 있는 경우 7) 임신할 수 없는 생식기관의 병력이 있는 경우 8) 임신할 수 없는 자궁의 섬유종(fibroid tumors)이 있는 경우 9) 치료되지 않은 비생식성 내분비질환이 있는 환자(즉 갑상선부신이나 뇌하수체 장애) 2, 다음 환자에게는 신중히 투여할 것 1) 보조생식술, 특히 체외수정(시험관 아기)을 포함하는 중추신경계나 기형이 기형에 자궁 외 임신의 빈도가 증가할 수 있다. 그러므로 초기에 초음파촬영을 통해 자궁 내 임신을 확인하는 것이 중요하다. 2) 난소과자극 난소과자극의 진단은 초음파검사로 확인될 수 있으므로, 이 약 투여시 양제 투여전과 투여 중 규칙적으로 난포성숙에 대한 초음파검사와 혈청 에스트라디올 농도 측정을 실시해야 한다. 바람직하지 않은 난소과자극이 발생되면 난소과자극증후군이 유발될 수 있으므로 신중한 검사를 실시해야 하며, 이 약의 투여를 중단하고 태반성성선자극호르몬(hCG)의 투여도 보류한다. 난소과자극의 초기증후는 하복부통증이며, 오심, 구토, 체중증가가 동반되기도 하며, 드물게 발생하는 심각한 난소과자극의 증상들은 난소의 광범한 증대, 복수증, 흉수증 등이며 파열되기 쉬운 거대난소낭종의 형성이 특징이다. 드물게 발생하는 심각한 난소과자극은 생명을 위협할 수 있으며, 합병증으로 심각한 혈전 색전증이 발생할 수도 있다. 그러나 태반성성선자극호르몬의 투여를 보류하고 적어도 4일간 성교를 금지하면 과도한 에스트로겐 반응이 심각한 난소과자극을 초래하지는 않는다. 다테임신이나 난소과자극증후군(OHSS)의 위험을 최소화하기 위해, 초음파 및 혈청 에스트라디올 농도를 측정한다. 무배란증에서 난소과자극증후군의 위험은 혈청 에스트라디올 농도가 높거나 성숙한 난포가 많을 때 증가한다. 난소의 반응은 개인내(intrapersonal), 개인별(intraindividual)로 변화가 크므로 적절한 난소 반응이 보이도록 추천용량과 투여방법을 잘 준수하고, 주의 깊은 모니터링을 실시하여 난소과자극증후군과 다테임신의 발생을 최소화하도록 한다. • **포장단위**: 1 프리필드시린지 x 자사포장단위 • **저장방법 및 사용기간**: 밀봉용기, 차광냉장(2~8℃보관, 제조일로부터 36개월) • **제조원**: (주) LG화학 ※ 이 약의 최신정보 확인은 의학전문지나(https://medrug.mfds.go.kr/)를 참조하시기 바랍니다. [삭제처 허가사항 2020.08 기준]

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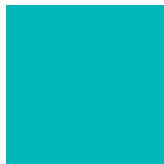


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Aims and Scope

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

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President: Seok Hyun Kim, M.D., PhD.

Editor-in-Chief: Byung Chul Jee, M.D.

Editorial Office

Department of Obstetrics and Gynecology CHA Bundang Medical Center, CHA University 59 Yatap-ro, Bundang-gu, Seongnam-si, Gyeonggi-do, Korea

E-mail: hwas0605@cha.ac.kr Tel: +82-31-727-8701

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E-mail: support@m2-pi.com Tel: +82-2-6966-4930 Fax: +82-2-6966-4945

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대한생식의학회는 난임과 보조생식술, 가임력보존, 생식내분비, 생식면역, 생식유전, 생식 생리 등 생식의학의 기초부터 임상까지 폭넓게 다루며 기초의학자 및 임상의학자들의 학술과 정보 교류의 장이 되어 생식의학의 지경을 넓혀가는 교두보 역할을 하였습니다. 이러한 학술적 교류 이외에도 출산율이 심각하게 감소한 한국 사회에서 저희 대한생식의학회는 저출산이라는 국가적 난제를 극복하는 데에 중요한 역할을 할 것으로 기대합니다.

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다시한번 대한생식의학회가 창립 50주년을 맞이할 수 있도록 노력해주시는 회원 여러분의 열정과 노고에 깊은 감사의 인사를 드립니다.

감사합니다.

2022년 1월

대한생식의학회 회장 김석현

Effects and safety of COVID-19 vaccination on assisted reproductive technology and pregnancy: A comprehensive review and joint statements of the KSRM, the KSRI, and the KOSAR

Ae Ra Han^{1,*}, Dayong Lee^{2,*}, Seul Ki Kim^{3,4}, Chang Woo Choo⁵, Joon Cheol Park⁶, Jung Ryeol Lee^{3,4}, Won Jun Choi⁷, Jin Hyun Jun^{8,9,10}, Jeong Ho Rhee⁶, Seok Hyun Kim³ on behalf of Korean Society for Reproductive Medicine (KSRM); Korean Society for Reproductive Immunology (KSRI); Korean Society for Assisted Reproduction (KOSAR)

¹Department of Obstetrics and Gynecology, CHA Fertility Center, CHA University School of Medicine, Daegu; ²Department of Obstetrics and Gynecology, School of Medicine, Kyungpook National University, Daegu; ³Department of Obstetrics and Gynecology, Seoul National University College of Medicine, Seoul; ⁴Department of Obstetrics and Gynecology, Seoul National University Bundang Hospital, Seongnam; ⁵Department of Obstetrics and Gynecology, Seoul Maria Fertility Hospital, Seoul; ⁶Department of Obstetrics and Gynecology, Keimyung University School of Medicine, Daegu; ⁷Department of Obstetrics and Gynecology, School of Medicine, Gyeongsang National University, Jinju; ⁸Eulji Medi-Bio Research Institute (EMBRI), Eulji University, Daejeon; ⁹Department of Senior Healthcare, BK21 Plus Program, Graduate School of Eulji University, Daejeon; ¹⁰Department of Biomedical Laboratory Science, Eulji University, Seongnam, Republic of Korea

Humanity is in the midst of the coronavirus disease 2019 (COVID-19) pandemic, and vaccines—including mRNA vaccines—have been developed at an unprecedented speed. It is necessary to develop guidelines for vaccination for people undergoing treatment with assisted reproductive technology (ART) and for pregnancy-related situations based on the extant laboratory and clinical data. COVID-19 vaccines do not appear to adversely affect gametes, embryos, or implantation; therefore, active vaccination is recommended for women or men who are preparing for ART. The use of intravenous immunoglobulin G (IVIg) for the treatment of immune-related infertility is unlikely to impact the effectiveness of the vaccines, so COVID-19 vaccines can be administered around ART cycles in which IVIg is scheduled. Pregnant women have been proven to be at risk of severe maternal and neonatal complications from COVID-19. It does not appear that COVID-19 vaccines harm pregnant women or fetuses; instead, they have been observed to deliver antibodies against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) to the fetus. Accordingly, it is recommended that pregnant women receive COVID-19 vaccination. There is no rationale for adverse effects, or clinical cases of adverse reactions, in mothers or neonates after COVID-19 vaccination in lactating women. Instead, antibodies to SARS-CoV-2 can be delivered through breast milk. Therefore, breastfeeding mothers should consider vaccination. In summary, active administration of COVID-19 vaccines will help ensure the safe implementation of ART, pregnancy, and breastfeeding.

Keywords: Assisted reproductive technology; COVID-19; Lactation; Pregnancy; Vaccination

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Corresponding author: **Joon Cheol Park**

Department of Obstetrics and Gynecology, Keimyung University School of Medicine, 1035 Dalgubeol-daero, Dalseo-gu, Daegu, 42601, Republic of Korea
 Tel: +82-53-258-7804 Fax: +82-53-258-4848 E-mail: jcpark@dsmc.or.kr

Co-Corresponding author: **Jung Ryeol Lee**

Department of Obstetrics and Gynecology, Seoul National University Bundang Hospital, 82 Gumi-ro 173beon-gil, Bundang-gu, Seongnam 13620, Republic of Korea
 Tel: +82-31-787-7257 Fax: +82-31-787-4054 E-mail: leejrmd@snu.ac.kr

*These authors contributed equally to this article as first authors.

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Introduction

Coronavirus disease 2019 (COVID-19) is a disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, which is mediated through angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2) on the host cell surface. COVID-19 can be fatal due to systemic inflammation accompanied by the secretion of large amounts of inflammatory cytokines [1]. Since human reproduction, from gametogenesis to delivery, is a dynamic process involving multiple steps of endocrine and immuno-

logical signaling [2-4], and ACE2 and TMPRSS2 are also expressed in various cells of the human reproductive system [5-7], concerns have been raised about the possibility that COVID-19 might have adverse effects on human reproduction. No direct or permanent detrimental effects on the female reproductive system have yet been reported in women infected with COVID-19. However, orchitis and impaired spermatogenesis have been reported in male COVID-19 patients [8].

All the currently approved COVID-19 vaccines act by inducing antibodies against the spike protein, which interacts with ACE2 on the cell surface, and some people have expressed concerns about the possibility that the spike protein might exert harmful effects as a pathogenic protein or a toxin. Due to a lack of information about the possible adverse effects of the COVID-19 vaccines on human reproduction, some couples seeking care at infertility clinics hesitate to receive vaccination [9]. As will be discussed in this review, COVID-19 vaccines have been developed and applied in clinical practice at an unprecedented speed, and the clinical evidence for many questions related to human reproduction is still insufficient. Vaccination is crucial for overcoming the COVID-19 pandemic, and it is very important to provide appropriate guidance for men and women who are preparing for pregnancy or lactating. Healthcare providers working in the field of human reproduction must be well informed on the latest recommendations and research about COVID-19 vaccines. According to this vision, the joint committee members of the Korean Society for Reproductive Immunology, the Korean Society for Reproductive Medicine, and the Korean Society for Assisted Reproduction formed a working group and thoroughly reviewed the representative international guidelines and statements on COVID-19 vaccination and relevant clinical and theoretical evidence about human reproduction [10-13]. Through this process, we provide statements on COVID-19 vaccination in the clinical context of assisted reproductive technologies (ARTs), pregnancy, and lactation.

Should women and men planning to conceive, including ART cycles, receive COVID-19 vaccination?

Since none of the currently approved COVID-19 vaccines, including mRNA vaccines and adenovirus vector vaccines, not contain live viruses, there is no theoretical risk of vaccine-related infection. According to a fact sheet published by the United States Food and Drug Administration, animal studies have demonstrated no increased adverse effects on reproductive outcomes, such as female fertility, embryonic-fetal development, and the postnatal prognosis when mRNA COVID-19 vaccines were delivered before the mating period or during early or late gestation [14]. A recent study showed that mRNA COVID-19 vaccines did not affect subsequent *in vitro* fer-

tilization (IVF) cycle performance from 7 to 85 days after receiving the second vaccine dose, including the number of retrieved mature oocytes, the number of top-quality embryos, and sperm parameters [15]. Although it was only a small-sized study published before being peer-reviewed, a subsequent study also showed the same results [16]. Another study reported no change in sperm parameters before, after, or during COVID-19 vaccination [17]. Accordingly, joint guidance from the International Federation of Fertility Societies and the European Society of Human Reproduction and Embryology advises women who are trying to conceive but are not yet pregnant, that they have the option to “proceed with efforts at conception” and to “seek a COVID-19 vaccination as soon as possible” [11], and the American Society for Reproductive Medicine COVID-19 Task Force re-confirmed their statement, recommending that “there is no reason to delay pregnancy attempts because of vaccination administration or to defer treatment until the second dose has been administered” [12,13].

However, without a clear explanation of the relationship between IVF treatment and the immune reaction from COVID-19 vaccination, clinicians face limitations in counseling patients attempting IVF treatment about the optimal timing of COVID-19 vaccination. In particular, real-world data have yet to be reported for emergent ART cases, such as fertility preservation (FP) just before gonadotoxic treatment or in patients with severe ovarian insufficiency (OI). IVF cycles can usually be electively planned according to patients’ status, and scheduling the timing of vaccination is not difficult. However, in emergent cases, physicians may need to decide to start the ART cycle between the first and second vaccine doses. There is a possible concern about immunological changes resulting from elevated hormonal levels during ART cycles; however, the elevation of estradiol levels is not extremely high in most cases of FP or OI. The embryo transfer schedule is separate from oocyte retrieval in most FP cases, and it can also be delayed in OI cases by embryo cryopreservation. According to animal and human data suggesting no detrimental effects on gametes, physicians should not hesitate to start FP and freeze-all cycles (i.e., cryopreservation of all retrieved oocytes or all fertilized embryos), regardless of the vaccination schedule. The only recommendation is to avoid vaccination 3 days before or after an elective fertility-related procedure such as oocyte retrieval [16]. There is still insufficient evidence as to whether there should be a gap of more than 3 days between the vaccine and the procedure. However, there is a possibility of adverse effects such as fever and generalized pain due to the initial immune response after vaccination, and it may be difficult to differentiate whether side effects are due to vaccination or ART procedures. For this reason, it is recommended to have an interval of approximately 3 days, which does not affect the overall ART schedule.

(1) All women and men planning to conceive are recommended to receive COVID-19 vaccination. (2) Based on current evidence, COVID-19 vaccination does not adversely affect fertility in women or men. (3) Scheduling the ART cycle with consideration of COVID-19 vaccination is helpful when counseling couples visiting infertility clinics. (4) In emergent cases, such as FP just before gonadotoxic treatment, the ART cycle can be started during vaccination after consultation with clinicians.

Should women planning to undergo immunomodulatory or immunosuppressant treatment during the ART cycle receive the COVID-19 vaccine?

Immunomodulatory or immunosuppressant agents are considered in patients with repeated implantation failure or recurrent pregnancy loss with possible immunological etiologies [18,19]. Women on immunomodulatory or immunosuppressant therapies such as anti-tumor necrosis factor agents and high-dose steroids for autoimmune diseases (e.g., rheumatoid arthritis, systemic lupus erythematosus, and poorly controlled asthma) generally visit infertility clinics to have a baby. Although these cases are not common [20,21], physicians can be asked about the possibility of interference between immunomodulatory or immunosuppressant therapies and COVID-19 vaccination during ART procedures.

Antibody-containing blood products, such as intravenous immunoglobulin G (IVIG), interfere with the immune response to some live attenuated vaccines such as measles-mumps-rubella and varicella, but not with the response to inactivated or toxoid vaccines. For inactivated vaccines, administration simultaneously with IVIG injection, or at any time interval before or after IVIG treatment, is allowed [22]. Although no clinical data have yet been published about immune interference between the COVID-19 vaccines and IVIG, mRNA COVID-19 vaccination is theoretically allowed based on these previous data.

Although inactivated non-live vaccines are recommended to be administered to immunosuppressed patients, as for healthy individuals, sometimes a higher dosage or more frequent booster shots may be required [23]. In terms of COVID-19, immunosuppressed patients due to disease or treatment are defined as a clinically vulnerable group and recommended to complete the vaccination schedule as soon as possible [24]. In addition, early and frequent boosters are considered for immunosuppressed individuals according to the results of the OCTAVE trial, which reported failed or reduced generation of protective antibodies after the completion of two doses of COVID-19 vaccines in immunocompromised patients [25]. In this context, the use of immunosuppressive agents such as high doses of

steroids or tacrolimus only for the purposes of ART must be reconsidered in the era of the COVID-19 pandemic.

(1) Theoretically, the administration of IVIG does not interfere with the immune response to mRNA COVID-19 vaccines. (2) Based on current evidence, IVIG administration can be performed at any time during the COVID-19 vaccination schedule. (3) Patients receiving immunosuppressive treatment are recommended to receive a booster shot of the COVID-19 vaccine due to reduced generation of protective antibodies after two vaccine doses. (4) It is not recommended to use immunosuppressants solely for ART without other appropriate indications in the COVID-19 pandemic era.

Is COVID-19 vaccination safe for pregnant women?

Pregnant women have been reported to have a higher risk of developing severe illness from COVID-19 than non-pregnant women. In pregnant women infected with COVID-19, higher rates of maternal complications (e.g., mortality, need for intensive care unit care, mechanical ventilation, cesarean section, preterm delivery, and pre-eclampsia) have been reported, and neonatal and perinatal mortality and morbidity also appear to increase [26-29]. Although very rare, case reports of vertical transmission in the first trimester or third trimester of pregnancy have been reported [30,31]. To protect the vulnerable population of pregnant women, the safety and efficacy of COVID-19 vaccination in pregnant women should be established. Pregnant and lactating women were excluded from the initial clinical trials of COVID-19 vaccines due to safety and liability concerns. To overcome these limitations, clinical trials on the safety of the COVID-19 vaccine in pregnant women are currently underway [32]. However, until the results of these studies are published, clinical judgments must be made based on the previous results of existing vaccines for other viral diseases, animal experiments, and a small number of clinical reports.

It has been reported that the existing mRNA vaccines against influenza virus, rabies virus, and Zika virus are safe during pregnancy and have good immunogenicity profiles [33-35]. Animal developmental and reproductive toxicology studies on mRNA or adenovirus vector vaccines for COVID-19 reported that there were no adverse effects on fertility, maintenance of pregnancy, or embryonic and fetal development [36,37].

In clinical studies of mRNA vaccines or adenovirus vector vaccines against COVID-19, some female participants unintentionally became pregnant. In this minority of women, the miscarriage rates did not differ from those in the placebo group. Therefore, the vaccines do not appear to have a detrimental effect in early pregnancy [38]. When COVID-19-vaccinated pregnant women delivered, placental

examinations showed a similar incidence of decidual arteriopathy, fetal vascular malperfusion, low-grade chronic villitis, or chronic histiocytic inter-villositis as in women who were not vaccinated. This indicates that there is no evidence of a vaccine-derived breakdown in maternal immunologic tolerance to the fetal tissue [39].

The United States Centers for Disease Control and Prevention developed a smartphone-based active-surveillance system (“V-safe”) for the COVID-19 vaccination program. This system allows pregnant women to voluntarily report adverse events after COVID-19 mRNA vaccination. A recent report found no serious vaccine-related adverse events during pregnancy in 35,691 participants [40].

A study found that COVID-19 mRNA vaccines evoked robust humoral immunity in pregnant and lactating women, and the immunogenicity and reactogenicity of vaccination were similar to those observed in non-pregnant women [41]. After vaccination, immunoglobulin G (IgG) antibodies against the SARS-CoV-2 spike protein were observed to cross the placental barrier and approach maternal titers in the fetus within 15 days following the first dose [41,42]. These results support the placental transfer of protective immunoglobulins to neonates by COVID-19 vaccination.

(1) Pregnant women are at increased risk of severe maternal and neonatal complications if they are infected with COVID-19. (2) Pregnant women are recommended to receive COVID-19 vaccination. (3) Based on current evidence, COVID-19 vaccination does not adversely affect pregnancy and neonatal outcomes. (4) COVID-19 vaccination during pregnancy may have a protective effect on the fetus by delivering antiviral immunoglobulins via the placenta.

Should lactating women be vaccinated against COVID-19?

An analysis of postvaccination milk samples from women who received mRNA vaccines detected no or little vaccine mRNA was detected [43,44]. DNA sequences for the S protein delivered into cells through adenovirus vector vaccines are transcribed into mRNA, which is translated into the viral S protein. Since this process is similar to that of mRNA vaccines, it can be inferred that adenovirus vector vaccines will theoretically have the same stability as mRNA vaccines.

In breastfeeding women who received the mRNA vaccine, the maternal serum antibody titer was equivalent to that of non-lactating women [45]. After the first dose, anti-spike immunoglobulin A (IgA) and IgG levels in breast milk increased after 1 week. This increase has been reported to persist for more than 6 weeks after the second dose, and the positive effect of delivering antibodies to nursing infants for up to 80 days is likely to be maintained [46–48]. In a study comparing two doses of the Pfizer-BioNTech or Moderna vaccine (mRNA vaccines) with a single dose of the Astra-Zeneca (adenovi-

rus-vector vaccine), both IgA and IgG were observed in breast milk regardless of which commercial vaccine was administered [49]. In contrast, in two studies reported to date, anti-spike IgA and IgG antibodies were not detected in the plasma of infants after their mothers completed two doses of mRNA vaccine [50,51].

In an analysis of maternal interviews and questionnaires about side effects in nursing women who had completed two doses of the mRNA vaccine, some women reported decreased milk supply but returned to normal after 72 hours without any intervention [52]. The study also reported that 4%–8% of women described changes in the color of their milk. In an online survey of 4,455 breastfeeding mothers who received mRNA vaccines, 6% reported a decrease in milk supply, whereas 3.9% reported an increase in milk supply [53]. About 7% of mothers reported adverse effects in their breastfed infants. The most common events were irritability and sleep disturbances of infants (3%). In a cohort study of 180 nursing women who received mRNA vaccines, some reported similar side effects, including irritability in about 10% of infants and poor sleep in about 8% [52]. However, it is unclear whether these common symptoms are directly related to maternal vaccination, and until now, no serious adverse reactions have been reported in infants of breastfeeding mothers vaccinated against COVID-19 [47,51].

(1) Current evidence suggests that COVID-19 vaccination will not harm lactating women or breastfeeding infants. (2) When a lactating woman is vaccinated against COVID-19, antibodies may be secreted in breast milk and delivered to infants to provide protective effects. (3) COVID-19 vaccination is recommended for women who are breastfeeding or planning to start, as the currently known possible benefits outweigh the theoretical risks.

Conclusion

Based on the evidence to date, COVID-19 vaccines do not appear to have detrimental effects on human reproduction, including gametes, embryos, or implantation. Through these observations and analyses of causation, it is recommended that women or men who are preparing for ART be actively vaccinated. The use of IVIG for the treatment of infertility or recurrent miscarriage in patients with possible immune problems is theoretically unlikely to impact the effectiveness of the vaccine, and COVID-19 vaccines can be administered in an ART cycle in which IVIG is scheduled. COVID-19 vaccines do not appear to adversely affect pregnant women or neonates; instead, they deliver antibodies against SARS-CoV-2 to the fetus. Therefore, it is recommended that pregnant women actively take steps to receive the COVID-19 vaccine. When lactating women are infected with COVID-19, their baby can also be exposed to infection, whereas if lactating women are vaccinated against COVID-19, protective anti-

bodies to SARS-CoV-2 can be delivered to the baby via lactation without major adverse effects. Therefore, mothers who are breastfeeding need to consider COVID-19 vaccination. Active COVID-19 vaccination in the reproductive-age population will ensure safe ART, pregnancy, and breastfeeding.

Conflict of interest

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

ORCID

Ae Ra Han	https://orcid.org/0000-0002-5432-548X
Dayong Lee	https://orcid.org/0000-0003-4340-8180
Seul Ki Kim	https://orcid.org/0000-0002-1647-6711
Chang Woo Choo	https://orcid.org/0000-0002-9565-8029
Joon Cheol Park	https://orcid.org/0000-0002-4103-5969
Jung Ryeol Lee	https://orcid.org/0000-0003-3743-2934
Won Jun Choi	https://orcid.org/0000-0002-4887-3201
Jin Hyun Jun	https://orcid.org/0000-0001-9898-4471
Jeong Ho Rhee	https://orcid.org/0000-0002-9280-4561
Seok Hyun Kim	https://orcid.org/0000-0003-0649-3224

Author contributions

Conceptualization: JHJ, JRL, SHK, JCP. Data curation: ARH, DL, JCP. Formal analysis: ARH, DL, JCP. Methodology: JCP. Writing—original draft: ARH, DL. Writing—review & editing: SKK, CWC, WJC.

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The impact of COVID-19 on the male genital tract: A qualitative literature review of sexual transmission and fertility implications

Pierangelo Verrienti, Gianmartin Cito, Fabrizio Di Maida, Riccardo Tellini, Andrea Cocci, Andrea Minervini, Alessandro Natali

Department of Urology, Careggi Hospital, University of Florence, Florence, Italy

The angiotensin-converting enzyme 2 receptor (ACE2) appears to be widely expressed in cells in the testes, predominantly in spermatogonia, Sertoli cells, and Leydig cells, and its co-expression with transmembrane protease serine 2 (TMPRSS2) is essential for the entry of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). For this reason, the male reproductive system could be considered a potential target for SARS-CoV-2, as well as a possible reservoir of infection. However, to date, there is very little evidence about the presence of SARS-CoV-2 in semen and testicular samples. The aim of this paper was to review the current evidence regarding the impact of SARS-CoV-2 on male fertility and sexual health, with a particular focus on reproductive hormones, the presence of the virus in seminal fluid and testis, and its impact on fertility parameters. We found very limited evidence reporting the presence of SARS-CoV-2 in semen and testicular samples, and the impact of SARS-CoV-2 on reproductive hormones and fertility parameters is unclear. The quality of the examined studies was poor due to the small sample size and several selection biases, precluding definitive conclusions. Hence, future well-designed prospective studies are needed to assess the real impact of SARS-CoV-2 on male reproductive function.

Keywords: COVID-19; Fertility; Male; Pathology; Physiology; SARS-CoV-2; Semen analysis; Testis

Introduction

The male genital tract is a target organ for several viral infections, with potential detrimental consequences from the standpoint of individuals, their offspring, and demographics [1]. A new type of coronavirus (severe acute respiratory syndrome coronavirus [SARS-CoV-2] [2]), has rapidly spread among humans, causing a worldwide pandemic. This virus causes a disease known as coronavirus disease 2019 (COVID-19), which is characterized by acute bilateral interstitial pneumonia and severe acute respiratory syndrome, which may lead to death in a short time [2].

In order to contain the spread of this infection and to properly inform the public about appropriate safety measures, it is pivotal to identify all the possible pathways of viral transmission. Besides the typical well-known respiratory route of transmission through droplets, the oral-fecal route due to gastrointestinal viral involvement should also be considered. Moreover, sexual transmission and the immune privilege of the male reproductive tract may play a significant role in the spread of an infectious disease.

Some prior studies explored the potential sexual and vertical transmission of previous epidemic coronaviruses, focusing on possible viral effects on fetuses and newborns in infected women [3,4]. Therefore, it is crucial to provide proper counseling for couples with regard to their sexual behaviors, particularly in asymptomatic or recovered individuals.

Based on our knowledge of other single-stranded RNA viruses, such as Zika and Ebola, which have also been responsible for recent widespread epidemic viral infections [5,6], it could be hypothesized—despite the clear differences between SARS-CoV-2 and the above-mentioned viruses—that the male genitalia could represent

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Corresponding author: **Pierangelo Verrienti**

Department of Urology, Careggi Hospital, University of Florence, Largo Brambilla, 3 50134 Florence, Italy

Tel: +39-0557949203 E-mail: pierangelo.verrienti@gmail.com

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a possible reservoir for SARS-CoV-2, leading to its sexual transmission by asymptomatic or cured men [7]. Indeed, Zika and Ebola viruses are able to cause viremia, overcome the blood-testis barrier, and ultimately become present in semen [5-7]. The presence of Zika virus in human semen has been widely demonstrated for over 188 days after primary infection, even though it is cleared in the serum after the initial viral symptoms subside [8]. As a result, an immune response might be activated, leading to inflammatory processes such as orchitis, resembling what happens in human immunodeficiency virus (HIV), hepatitis B virus (HBV), and mumps infection [9]. However, extensive information about its potential impact on male fertility is currently lacking. Ebola can also persist in bodily fluids during recovery, enabling viral transmission through semen [6]. Preliminary results have shown that male survivors of Ebola virus disease can have virus-positive semen for up to 9 months after the acute infection, as determined by reverse-transcription polymerase chain reaction (RT-PCR) [6]. However, it is unknown with absolute certainty how long the virus can persist in the seminal fluid. Likewise, there are no conclusive data about the medium- or long-term consequences of Ebola virus on male fertility.

In this light, several case series have assessed the possible role of SARS-CoV-2 in male fertility impairment, with inconsistent results; the findings are also difficult to interpret because transmission through droplets can clearly occur during intimate sexual contact [7,10,11]. As such, in the current paper, we attempted to thoroughly explore the impact of SARS-CoV-2 on male fertility and sexual health by conducting a non-systematic review of the literature.

Search strategy

Medline, Embase, and Scopus Library were searched to identify studies published between January 2020 and March 2021 that investigated the impact of SARS-CoV-2 on male fertility. We conducted a non-systematic critical assessment of the current literature focusing on the impact of SARS-CoV-2 on reproductive hormones and fertility parameters, as well as its presence in seminal fluid and the testis. The following string terms were used: ("COVID-19" OR "SARS-CoV-2") AND ("reproductive hormones" OR "fertility" OR "semen" OR "testis"). We report a detailed description of the findings extracted from the included studies.

Pathophysiology and the molecular mechanism of COVID-19 cell entry

The entrance of SARS-CoV-2 into target host cells has been shown to be mediated by the interaction between the surface spike viral

protein (S) and the angiotensin-converting enzyme 2 receptor (ACE2), employing the cellular transmembrane protease serine 2 (TMPRSS2). Therefore, the co-expression of these is needed within the same cell to allow viral entry [12,13]. As a protease, TMPRSS2 is essential for SARS-CoV-2 to penetrate cells, but many cells in the male genital tract do not express these proteins simultaneously with ACE2 [13-15]. However, other authors demonstrated that there was almost no overlapping gene co-expression (< 1%) in the human testicle [12]. The S1 site contains a receptor-binding domain, which then links to ACE2 and facilitates viral entry into the cell. ACE2 is expressed in various organs, including type II alveolar cells of lungs, heart, kidneys, and intestines [16]. In addition, ACE2 seems to be constitutively expressed in the testes, predominantly in spermatogonia, Sertoli cells and Leydig cells [17,18]. Most recently, Vishvkarma and Rajender [19] found the presence of ACE2 transcripts in recent transcriptome sequencing of human spermatozoa, further validating its expression in germ cells. The physiological functions of ACE2 in Leydig cells include the regulation of testosterone production and local balance in interstitial fluid volume by modulating the conversion of angiotensin II to angiotensin I. In the COVID-19 infection process, ACE2 receptors are saturated by binding with the virus, giving rise to the increased availability of angiotensin II, which cannot be converted.

As confirmed by recent studies, the levels of ACE2 transcripts are extremely high in the normal adult testes [13]. Spermatogonia that express ACE2 have higher levels of genes associated with viral reproduction and transmission, but lower levels of genes related to spermatogenesis, than ACE2-negative spermatogonia [20]. This has generated the idea that COVID-19 could have possible implications for the male genital tract, which could be considered a potential target for SARS-CoV-2, as well as a possible reservoir of infection. In this regard, it might be hypothesized that the virus could have a negative influence on male gonads, resulting in spermatogenetic injury and endocrine dysregulation.

In 2006, Xu et al. [11] demonstrated a wide range of histological injuries to germ cells and spermatogenesis, through a complex inflammatory infiltrate, in males who died from SARS-CoV complications, implying a correlation between the disease and subsequent reproductive impairment. SARS-CoV, as well as HIV, HBV, and mumps, can trigger orchitis as a possible complication due to body temperature increase and the virus-induced autoimmune response [9]. Indeed, previous studies, have reported that apoptosis of meiotic germ cells occurs at high temperatures [21]. Therefore, high fevers related to COVID-19 could cause indirect damage to testicular function, leading to temporary sub-fertility.

COVID-19 and male sexual health: the state of the art

1. Impact on reproductive hormones

Several studies have investigated the impact of SARS-CoV-2 on male reproductive hormones (Table 1) [22–24]. Luteinizing hormone and prolactin concentrations appear to be significantly increased in COVID-19-infected men of reproductive age, compared to healthy age-matched controls, while follicle-stimulating hormone and estradiol levels seem to be comparable between these groups [22]. Thus, Leydig cells would seem to be more sensitive to viral attack, with possible implications regarding hypogonadism, while Sertoli cells may be more resistant. Schroeder et al. [23] reported that most patients with COVID-19 had low testosterone and dihydrotestosterone levels. Moreover, total testosterone levels have been shown to be inversely proportional to C-reactive protein (CRP) levels in COVID-19-recovered patients [24]. On the basis of these findings, we could speculate that hypogonadism may be related to the severity of COVID-19 infection, most likely due to a body temperature increase and virus-induced autoimmune response, with consequent apoptosis of meiotic germ cells [9,21]. Moreover, since the co-expression of ACE2 and TMPRSS2 occurs only in a small percentage of prostate epithelial cells, prostate infection is unlikely to explain the dysregulation of steroidogenesis mediated by SARS-CoV-2 infection [13,15,17]. Furthermore, the sexual hormonal alterations observed in SARS-CoV-2 patients may reflect the global stress response [9,21]. However, to date, very few scientific studies have investigated the presence of SARS-CoV-2 in the seminal fluid, or even within the testicles, as reported below.

2. Presence in seminal fluid and testis

A research team focused on this topic analyzed semen samples from a group of 34 men about 1 month after the diagnosis of COVID-19, and reported that viral RNA did not seem to be present [12]. Consistently with this finding, other authors found no presence of viral RNA by RT-PCR in semen or urine samples of patients with laboratory-confirmed SARS-CoV-2 infection, either symptomatic or asymptomatic [25–30]. A possible explanation for this may be the low likelihood of the virus crossing the blood-testis barrier. Indeed, SARS-CoV-2 appears to be present in the blood in only 1% of cases [31], which would suggest a low probability of developing viremia.

Conversely, Li et al. [32] enrolled a cohort of 38 SARS-CoV-2-positive patients for semen testing. Among them, 23 had achieved clinical recovery, while 15 were in the acute stage of infection. Six patients (15.8%) had results positive for SARS-CoV-2, including 4 of the 15 patients (26.7%) in the acute stage of infection and 2 of the 23 patients (8.7%) who were recovering. However, the small sample size makes these findings difficult to generalize universally. Furthermore, the single-center study design and the lack of accurate data about the baseline study cohort might have introduced non-negligible statistical bias, thus meaningfully undermining the reliability of the reported findings.

Another further study explored the presence of SARS-CoV-2 RNA in semen and testicular tissue of 12 men suffering from COVID-19 with symptoms of mild severity. In 10 patients with negative pharyngeal swabs, the presence of viral RNA, tested by RT-PCR, was not found in the seminal fluid. Moreover, a patient with a positive swab was negative for the presence of viral RNA in the semen. Only one

Table 1. Studies investigating the impact of SARS-CoV-2 on male reproductive hormones

Study	Study design	No. of male cases	Age (yr)	SARS-CoV-2 diagnosis ^{a)}	Sex-related hormones analyzed	Sex-related hormone alterations	Correlation with CRP and/or biochemical risk factors	Correlation with disease severity and/or poor prognosis
Ma et al. [22]	Retrospective case-control	81	38 (20–54)	Yes	TT, E2, PG, PRL, LH, FSH, AMH	LH ↑, PRL ↑, T:LH ratio ↓, FSH:LH ratio ↓	T:LH ratio negatively correlated with CRP	T:LH ratio negatively correlated with disease severity
Schroeder et al. [23]	Retrospective single-center cohort	35	62 (31–80)	Yes	TT, cFT, DHY-TT, E2, LH, FSH, PRL, TSH, T3, T4	TT ↓, DHY-TT ↓, LH ↑, E2 ↑	TT negatively correlated with biochemical risk factors	TT negatively correlated with disease severity
Rastrelli et al. [24]	Retrospective single-center cohort	31	21 ^{b)} 63 (55–66) 6 ^{b)} 72 (33–83) 4 ^{b)} 74 (59–85)	Yes Yes Yes	TT, cFT, LH, SHBG	TT ↓, FT ↓, LH ↑	TT negatively correlated with CRP and biochemical risk factors	TT positively correlated with poor prognosis

Values are presented as median (range).

↑, increased levels; ↓, decreased levels; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; CRP, C-reactive protein; TT, total testosterone; E2, 17-β-estradiol; PG, progesterone; PRL, prolactin; LH, luteinizing hormone; FSH, follicle-stimulating hormone; AMH, anti-Müllerian hormone; T, testosterone; cFT, calculated free testosterone; DHY-TT, dihydrotestosterone; TSH, thyroid-stimulating hormone; T3, free triiodothyronine; T4, free thyroxine; SHBG, sex hormone binding globulin; RICU, respiratory intensive care unit; IM, internal medicine unit.

^{a)}SARS-CoV-2 diagnosis with reverse-transcription polymerase chain reaction of nasal/pharyngeal swabs and chest imaging; ^{b)}21: number of patients transferred from RICU to IM, 6: number of patients stable at time of the analysis and maintained in RICU, 4: number of patients worsened their conditions and transferred to ICU.

patient in the acute phase of disease, who died from COVID-19–related complications, underwent a testicular biopsy, which did not demonstrate the presence of the virus in the tissue [33]. In line with these findings, a recent paper by Flaifel et al. [34] analyzed specimens of the testes and epididymis from a series of 10 SARS-CoV-2-positive autopsies. Although the autopsies all showed the presence of SARS-CoV-2 in the respiratory tract, the testicular samples tested by RT-PCR were all negative. However, seven of 10 cases showed significant seminiferous tubular injury, including nuclear fragmentation of spermatocytes, elongation of spermatids, and vacuolization of the Sertoli cells, while 1 case showed an increased mononuclear inflammatory infiltrate (CD8+ dominant) in the interstitial space, compatible with orchitis [34]. In their analysis, the acute morphological changes reported could have been related to oxidative stress and microthrombosis in the testicular vasculature. In addition, the absence of the virus in the testes suggests that direct injury by SARS-CoV-2 infection is unlikely [34].

The characteristics of the studies exploring the presence of SARS-CoV-2 in semen, urine, and testicular tissue are reported in Table 2 [12,22,26-30]. The challenge is to understand whether SARS-CoV-2 can directly infect the testicles, as the main target of the male genital tract, even during the acute phase of the disease. To better understand this issue, it could be useful to have more data from autoptic testicular samples in COVID-19 patients, since we do not know if SARS-CoV-2 is present only in the seminal fluid, binds to spermatozoa, or can even integrate into the cell genome. More recently, Yang et al. [35] examined postmortem testes from 12 COVID-19 patients using RT-PCR. The authors found positive viral detection only in 1 patient with a high viral load. Conversely, concerning morphological changes, the testes from COVID-19 patients exhibited significant seminiferous tubular injuries, reduced Leydig cells, and mild lymphocytic inflammation. Alternatively, hyperthermia, hypoxia, and steroid use could have played a crucial role in testicular damage.

A recent paper by Achua et al. [36] explored the presence of SARS-CoV-2 in the testes of 6 COVID-19-positive autopsies and 3 negative men by hematoxylin and eosin (H&E) histomorphology and transmission electron microscopy (TEM). They reported that 3 (50%) SARS-CoV-2 biopsies had normal spermatogenesis, while the other 3 had impaired spermatogenesis on H&E histomorphology. Moreover, TEM showed SARS-CoV-2 in testis tissue of 1 positive autopsy case and a biopsy obtained from a live patient who was previously diagnosed with SARS-CoV-2 and subsequently seroconverted. In addition, immunofluorescence-stained slides from the positive men demonstrated an association between increased quantitative ACE-2 levels and impairment of spermatogenesis [36]. These findings, especially the inverse association between ACE-2 receptor levels and spermatogenesis, may suggest that the testes could be a target of

SARS-CoV-2, ultimately providing a possible mechanism of post-COVID-19 infertility. However, that study has several limitations, the main ones being the lack of a detailed analysis of seminal parameters and virus detection by RT-PCR, making these results unreliable.

Although the risk of the presence of SARS-CoV-2 in semen appears to be low, future studies need to focus on whether complete viral particles can be observed in semen and the possibility of sexual transmission. Furthermore, the embryology community needs to establish the implications of SARS-CoV-2 for assisted reproductive technology and whether the virus can be removed by sperm washing techniques, as with HIV or hepatitis C virus. Moreover, data about how long the virus remains detectable in the seminal fluid, its ability to actively replicate, and its potential for sexual transmission are still lacking. As such, there are still many open questions to be discussed.

3. Impact on fertility parameters

Due to the reported high expression of ACE2 in seminiferous tubule cells, spermatogonia, adult Leydig cells, and Sertoli cells of the human testis, and TMPRSS2 expression in prostate epithelial cells, SARS-CoV-2 may be involved in the dysregulation of steroidogenesis [17,37]. These findings imply that SARS-CoV-2 infection may have risks for the male reproductive system in terms of impaired spermatogenesis.

In detail, sperm cells express all types of ACEs (1–7) and recent publications reported angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R) expression in sperm, suggesting that sperm cells could act as a direct target of SARS-CoV-2 infection [12,13,16-20,38,39]. In this scenario, SARS-CoV-2 infection would be expected to impact ACE2 activity, leading to an increase in the availability of angiotensin II in sperm cells, stimulating the acrosome reaction [13,18,19,37,38]. This may lead to premature acrosomal exocytosis and sperm senescence [40]. Furthermore, angiotensin II may affect sperm fertilization and motility by stimulating AT1R and AT2R [39].

Although Holtmann et al. [28] in their aforementioned study did not detect SARS-CoV-2 RNA in the semen samples of recovered or acutely infected patients, they found significant impairment of sperm quality (in terms of sperm concentration and motility) in patients with a moderate infection, as compared with men recovered from mild infection and a control group. Similarly, Ma et al. [22,25] reported altered semen quality in four patients (33.3%), who showed decreased sperm concentration and motility and a higher DNA fragmentation index.

A recent paper from Zhang et al. [41] failed to isolate SARS-CoV-2 in prostatic secretions of patients with active disease; however, they described a significant increase of inflammatory markers such as CRP and interleukin-6 in these biologic samples. These results, taken together, suggest a potential impact of SARS-CoV-2 on semen param-

Table 2. Characteristics of the studies exploring the presence of SARS-CoV-2 in semen, urine, and testicular tissue

Study	Study design	No. of total cases	Patient group	Age (yr)	Clinical stage at time of sample collection	Urogenital symptoms or urological disease	No. of cases with semen/urine/tissue samples	Positive semen samples	Positive urine samples	Positive tissue samples	Time between disease onset/diagnosis and samples collection	Correlation with NP swab at the time of collection
Li et al. [32]	Monocentric cohort	38	Recovery, 23 (60.5) Acute stage, 15 (39.5)	NA	Recovery	1 (4.4)	23	2 (8.7)	NA	NA	NA	NA
Ning et al. [30]	Monocentric cohort	112	Mild disease, 40 (35.7) Severe disease, 72 (64.3)	55.5 (23–83)	Recovery	3/112 (2.7) ^{a)}	17	0	NA	NA	27 day (12–64)	Positive NP swab, 9 (52.9)
Holtmann et al. [28]	Monocentric	34	Mild/moderate disease, 18 case	42.2 ± 9.91	Recovery	1/18	18	0	NA	NA	43.5 ± 6.2 day	None
Pan et al. [12]	Case control	34	Control, 14 case	37 (31–49)	Recovery	NA	14	0	NA	NA	47 ± 5.3 day	None
Paoli et al. [26]	Case report	1	Mild disease	31	Recovery	0	1	0	NA	NA	31 day (29–36)	None
Song et al. [33]	Monocentric cohort	13	Mild/moderate disease, 12; severe disease, 1	22–38 67	Recovery Died	0 NA	12 1	0 NA	0 NA	NA	8 day NA	None Positive NP swab, 1
Rawlings et al. [27]	Monocentric cohort	6	NA	28–45	Recovery	0	6	0	NA	NA	12 day (6–17)	Positive NP swab, 3 (50)
Ma et al. [22]	Multicentric case control	12	Mild disease, 1/12 Moderate disease, 11/12	31.5 (25–46)	Recovery	0	12	0 ^{b)}	NA	NA	75.5 day (56–109)	None
Kayaaslan et al. [29]	Monocentric cohort	56	Mild/moderate disease	33.5 (18–54)	Acute stage	0	16	0	NA	NA	1 day (0–7)	Positive NP swab, 5
Yang et al. [35]	Monocentric cohort	12	Severe disease	65 (42–87)	Died	0	10	NA	NA	1	1 hr ^{c)}	NA
Flaifel et al. [34]	Monocentric cohort	10	Severe	49.5 (22–83)	Died	0	10	NA	NA	0 ^{d)}	15 day (7–27)	Positive, 10 ^{e)}

Values are presented as number (%), median (interquartile range), or mean ± standard deviation. All patients were diagnosed with positive RT-PCR results from NP swabs and/or serum IgM/IgG.

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; NP, nasopharyngeal; NA, not applicable; RT-PCR, reverse-transcription polymerase chain reaction; Ig, immunoglobulin.

^{a)}Urogenital symptoms reported only in patients with severe disease; ^{b)}Alteration of semen quality reported in 4 patients (33.3%); ^{c)}Since confirmed death; ^{d)}Morphological alteration of testes reported in 7 patients, 1 case of orchitis detected; ^{e)}RT-PCR on lung autopsies.

eters and ultimately on male reproductive capability. Therefore, given the widespread SARS-CoV-2 pandemic, direct or indirect fertility decline post-COVID-19 seems to be a possible and significant issue, particularly in the hardest-hit countries [42,43]. Given the relevance of these findings, potentially impacting the world's demographics in the near future, we need further and stronger evidence on the real impact of SARS-CoV-2 on male reproductive health.

Conclusion

To date, there is very limited evidence reporting the presence of SARS-CoV-2 in semen and testicular samples. The studies available do not allow us to draw definitive conclusions or exclude the possibility of viral sexual transmission. Despite the small sample size and several selection biases, the male genital tract represents a potential-susceptible organ to viral infection. However, the real impact of SARS-CoV-2 on male reproductive function still remains to be fully determined.

Conflict of interest

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

ORCID

Pierangelo Verrienti	https://orcid.org/0000-0002-7596-5961
Gianmartin Cito	https://orcid.org/0000-0001-7526-4025
Fabrizio Di Maida	https://orcid.org/0000-0003-1885-4808
Riccardo Tellini	https://orcid.org/0000-0003-2189-5352
Andrea Cocci	https://orcid.org/0000-0003-0138-6294

Author contributions

Conceptualization: PV, GC, AN. Data curation: PV, GC, FDM, RT. Formal analysis: PV, GC, RT. Methodology: PV, GC, FDM, RT. Project administration: AC, AM, AN. Visualization: AC, AM, AN. Writing—original draft: PV, GC, FDM. Writing—review & editing: PV, RT, AC, AM, AN.

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Lifetime changes of the oocyte pool: Contributing factors with a focus on ovulatory inflammation

Chan Jin Park^{1,2}, Ji-Eun Oh¹, Jianan Feng¹, Yoon Min Cho¹, Huanyu Qiao¹, CheMyong Ko¹

¹Department of Comparative Biosciences, College of Veterinary Medicine, University of Illinois Urbana-Champaign, Urbana, IL; ²Epivara Inc., Champaign, IL, USA

In mammalian species, females are born with a number of oocytes exceeding what they release via ovulation. In humans, an average girl is born with over a thousand times more oocytes than she will ovulate in her lifetime. The reason for having such an excessive number of oocytes in a neonatal female ovary is currently unknown. However, it is well established that the oocyte number decreases throughout the entire lifetime until the ovary loses them all. In this review, data published in the past 80 years were used to assess the current knowledge regarding the changing number of oocytes in humans and mice, as well as the reported factors that contribute to the decline of oocyte numbers. Briefly, a collective estimation indicates that an average girl is born with approximately 600,000 oocytes, which is 2,000 times more than the number of oocytes that she will ovulate in her lifetime. The oocyte number begins to decrease immediately after birth and is reduced to half of the initial number by puberty and almost zero by age 50 years. Multiple factors that are either intrinsic or extrinsic to the ovary contribute to the decline of the oocyte number. The inflammation caused by the ovulatory luteinizing hormone surge is discussed as a potential contributing factor to the decline of the oocyte pool during the reproductive lifespan.

Keywords: Atretic follicle; Folliculogenesis; Germ cells; Inflammation; Oocytes; Reproductive health

The oocyte pool is the primary determinant of reproductive lifespan

In women of reproductive age, the size of the oocyte pool (the total number of oocytes in the ovaries) is the primary predictor of fertility [1]. It is well established that the oocyte pool can be as large as a few million during the fetal stage, but decreases significantly to a million or less at birth. Here, we report the predicted patterns of

change in the oocyte pool in humans and mice. The human predictions were made based on collective study reports published since 1960 and those that are currently available via PubMed (Figure 1A) [2-8]. Similarly, predictions were also made in mice because they are widely used as animal models for researching human fertility (Figure 1C) [9-18]. Briefly, the predictions in humans indicate that an ovary contains approximately 300,000 oocytes at birth (600,000 per person). The oocyte pool decreases in size throughout the entire reproductive lifespan, down to half by the onset of puberty and one-third by the age of 20. Interestingly, substantial variation in the oocyte pool size is seen by the age of 40 (Figure 1B), but this variation subsequently disappears. Eventually, the human oocyte pool becomes close to zero by the age of 50 years, when most women reach menopause. A similar declining pattern of the oocyte pool is also seen in mice, indicating that there is a regulatory mechanism that is common to both species.

There is a continuing trend worldwide for the current generation of women to plan to bear children at a considerably later time of life than in previous generations. This trend creates a risk that when the time to have a child arrives, the oocyte pool may not be large

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Corresponding author: **Huanyu Qiao**

Department of Comparative Biosciences, College of Veterinary Medicine,
 University of Illinois Urbana-Champaign, Urbana, IL 61802, USA
 Tel: +1-217-300-5397 E-mail: hqiao@illinois.edu

Co-Corresponding author: **CheMyong Ko**

Department of Comparative Biosciences, College of Veterinary Medicine,
 University of Illinois Urbana-Champaign, Urbana, IL 61802, USA
 Tel: +1-217-333-9362 E-mail: jayko@illinois.edu

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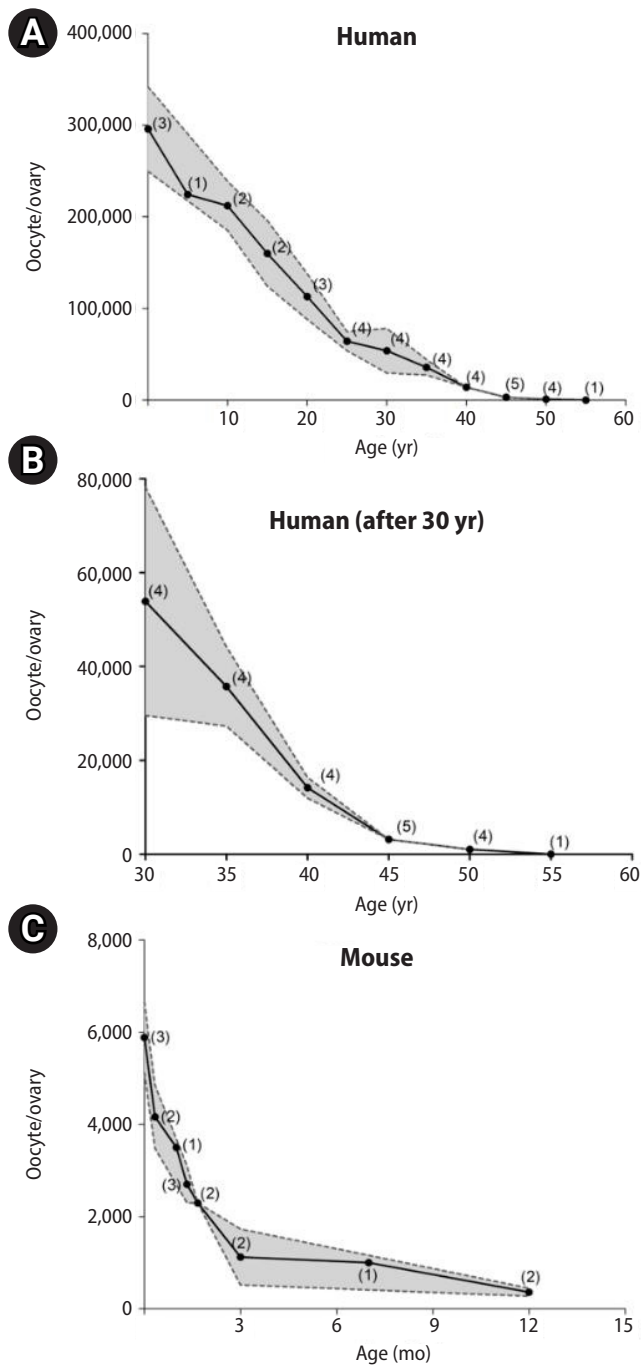


Figure 1. Lifetime changes in the oocyte pool in humans and mice. (A) The graph depicts the cumulative human data from the seven most relevant and reliable published articles that claim that oocyte counting was done in non-pathological ovaries. (B) This is a replica of the human oocyte pool, but only representing the age groups of 30–55 years. (C) The graph contains cumulative data collected from the 10 most relevant and reliable published articles. The reliability of published data was judged by an outlier test. The numbers in the parentheses are the numbers of articles used to calculate the average oocyte numbers in each age group. The gray area represents mean \pm standard error of the mean. Data analysis and graph-plotting were conducted using GraphPad Prism 5 (GraphPad Inc., La Jolla, CA, USA).

enough to support fertility even with help from assisted reproductive technology. No method currently exists for precisely assessing the oocyte pool. Instead, a few methods are used as clinical assessments of the oocyte pool, including measurements of blood levels of anti-Müllerian hormone (AMH) alone or together with follicle-stimulating hormone, the levels of which are affected by the number of follicles in the ovary and the amount of estradiol synthesized by the follicles, respectively [19,20]. AMH is a peptide hormone produced by the granulosa cells of the primary and secondary follicles and released into the bloodstream. Therefore, the blood level of AMH is used as a proxy of the size of the oocyte pool, and this method has been adopted by most *in vitro* fertilization (IVF) clinics to assess the size of their patients' oocyte pool. Low or high blood AMH levels are used as a clinical marker to predict whether ovulation can be induced in an IVF patient or to determine how many oocytes can be retrieved upon inducing ovulation. However, neither AMH nor follicle-stimulating hormone levels are reflective of the actual size of the oocyte pool. Indeed, these levels do not change for the entire fertile lifespan even though the oocyte pool continues to decrease in size; they only begin to change when the oocyte pool becomes so small that not enough primordial follicles can be recruited to the growth phases. Interestingly, in a survey performed with 796 infertility clinics asking which test or factor best predicts fertility, 80% of respondents stated that age was the best predictor [21]. Why age? The oocyte pool decreases nonstop even when a woman's fertility is at its peak or she is in her teens or 20s. As of 2021, no consensus exists regarding the cause(s) of the continued decline of the oocyte pool, but it is believed to be a multifactorial phenomenon. In the following sections, we summarize reported causes of the decline that are backed by experimental or observational data. Additionally, the hypothesis that ovulatory inflammation is a contributing factor to the decline of the oocyte pool will be discussed.

Contributing factors to the decline of the oocyte pool

The peak size of the oocyte pool is established during fetal development, specifically during the transitional period of mid-to-late gestation [22]. Upon reaching the peak, the pool begins to decrease, primarily via a process called oocyte attrition, throughout the rest of the gestational period [23,24]. After birth, the oocyte pool continues to decrease due to a variety of factors including, but not limited to, follicular atresia, meiotic errors, ovulation, environmental toxicants, medical drugs, and possibly some other factors that are yet to be discovered (Figure 2).

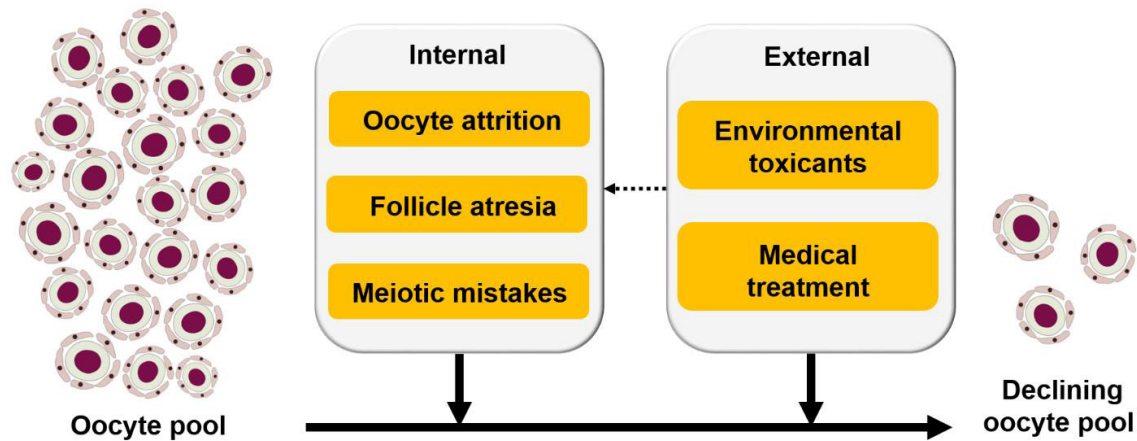


Figure 2. Contributing factors to the decline of the oocyte pool. Internal factors leading to the decline of the oocyte pool include oocyte attrition, follicular atresia, and meiotic errors. External factors, such as environmental toxicants and medical treatments, can either trigger internal factors or directly cause the reduction of the oocyte pool.

1. Perinatal oocyte loss

Immediately prior to or after birth, the ovary loses oocytes via oocyte attrition, which is mediated by immune cells infiltrating the fetal ovaries [25] or apoptosis triggered by DNA breaks [26,27]. Furthermore, autophagy, ferroptosis, and necrosis are also known to cause oocyte demise. Autophagy is a cell recycling pathway that involves the fusion of organelles with lysosomes for degradation [28-30]. Ferroptosis is a type of programmed cell death involving intracellular iron and oxidative phospholipids [22,31]. Necrosis is an inflammatory cell death process that results in swelling and eventually cell rupture [32].

2. Follicular atresia

After birth, a subset of dormant primordial follicles is recruited into a growth phase, which continues throughout the entire reproductive lifespan. Some of them grow and develop into preantral follicles, and eventually release oocytes via ovulation. However, the majority of the recruited follicles die during the growth phase; they eventually degenerate and are removed from the ovary. This phenomenon is called follicular atresia and is often triggered by the death of a granulosa cell in a follicle [22], eventually leading to the death of most granulosa cells and oocytes. Decreased estrogen production in the follicle or a milieu with increased androgen levels is a known cause of the initial death of granulosa cells [33-36]. In addition, erroneous signaling between the oocyte and granulosa cells also causes mis-regulation in the meiotic arrest of oocytes and follicular atresia [37-40].

3. Environmental toxicants

Humans are constantly exposed to environmental toxicants via in-

halation, ingestion, or contact. Recent studies have shown that an alarming number of environmental toxicants can directly or indirectly damage oocytes. For example, oocytes in an ovary that is exposed to cadmium die due to an increased level of reactive oxygen species (ROS), subsequent DNA damage, and mitochondrial disruption in the oocytes [41]. Di(n-butyl) phthalate, an industrial plasticizer and stabilizer, kills oocytes by activating apoptosis pathways upon exposure in animals [42]. Nonylphenol, a commonly used industrial material similar to laundry detergents, has been shown to alter the expression of more than 800 genes in mouse oocytes, increase ROS levels, and eventually eliminate oocytes [43]. Further, a variety of endocrine-disrupting chemicals bind to steroid receptors (e.g., estrogen or progesterone receptors), synergistically disrupt hormonal balance, and directly or indirectly harm oocytes [44-46]. Unlike oocyte attrition and follicular atresia, which are mostly triggered by internal factors, environmental toxicants are from external sources and therefore preventable to some degree.

4. Medical treatments

Multiple drugs are used to cull harmful cells, particularly in cancer patients. These drugs primarily target cell-cycle regulators, making the ovary a vulnerable organ to such medical treatment. When given to a patient, these drugs inevitably impact cells in the ovary because a substantial proportion of ovarian cells undergo cell proliferation (granulosa cells in follicles at various stages) and meiosis (oocytes). Indeed, it is well known that young female cancer patients who receive chemotherapy suffer from premature ovarian insufficiency (POI) [47,48]. Chemotherapeutic drugs such as cyclophosphamide, cisplatin, vincristine, and doxorubicin are all reported to induce POI [49-51]. In addition to targeting cell cycle regulators, some chemo-

therapeutic drugs “burn out” the oocyte pool. For example, cyclophosphamide stimulates follicle activation in oocytes and granulosa cells, thereby pushing an excessive number of primordial follicles into a growth phase and decreasing the oocyte pool [47]. Doxorubicin triggers the DNA-damaging TAp63 α -Cleaved-CASP3 pathway to induce apoptosis in the oocytes of primordial follicles [48]. Radiation therapy is also toxic to oocytes. It increases ROS production in patients, which induces DNA damage either directly or via lipid peroxidation or by increasing the expression of a pro-apoptotic protein, p53 upregulated modulator of apoptosis, in oocytes or granulosa cells [52]. Cryopreservation of oocytes or ovarian tissues has been used to persevere oocytes for later use before chemotherapy [50]. Further, antioxidants such as melatonin are now used to quench ROS before radiation therapy [53].

5. Meiotic errors

Meiotic errors can be caused by environmental toxicants or medical treatments. However, they can also happen by chance during normal oogenesis. Typical meiotic mistakes include failure to repair DNA breaks and defective synapsis [26], abnormal spindle assembly [54], and further chromosome nondisjunction [55]. Surprisingly, 20%–25% of human oocytes obtained in IVF clinics showed aneuploidy [56], and the ratio increases as women become older [57]. To control the quality of the overall oocyte pool, certain meiotic checkpoint mechanisms sacrifice abnormal oocytes and decrease the total oocyte number [54,57]. However, these checkpoints are less stringent in females than in males [57]. Thus, some oocytes with meiotic errors can still be fertilized, leading to chromosome-related diseases/syndromes such as Down syndrome in newborns [58,59]. Nonetheless, oocytes can find a way to efficiently repair DNA double-strand breaks and protect the genetic integrity of offspring if apoptosis is inhibited [60,61].

Ovulatory inflammation: a contributing factor to the decline of the oocyte pool?

Ovulation is the process of releasing oocyte(s) from the ovary. It is triggered by a preovulatory surge of luteinizing hormone (LH) and recurs every reproductive cycle [62]. In the entire reproductive life span, a woman releases only 300–400 oocytes [63], a negligible subset of oocytes compared to the number of oocytes a female is born with. In the previous section, a few well-established causes of oocyte losses were reviewed. In this section, we will discuss another cause that may trigger oocyte loss: the acute inflammation that the ovary experiences at the time of ovulation. When a tissue undergoes acute inflammation, leukocytes quickly infiltrate the tissue and display a series of defensive behaviors with the aim of destroying foreign in-

vaders, such as infectious microorganisms or harmful substances. However, these defensive actions inevitably damage the tissue itself. Overwhelming evidence indicates that the ovary experiences acute inflammation when it undergoes a cascade of ovulatory processes, suggesting that a similar tissue-damaging event may happen in the ovary at the time of ovulation. Therefore, the ovarian cells, including primordial germ cells, may be damaged and eventually removed from the ovary at each ovulation, accounting for a significant portion of oocyte loss during the reproductive lifespan. Herein, we will discuss this possibility.

1. Ovulation is accompanied by acute inflammation

The idea that ovulation is an inflammatory process was originally introduced in the early 1980s by Espey [64], who presented a striking similarity between a general inflammatory process and the events that take place at the time of ovulation. Since then, this idea has gained popularity through extensive observational and experimental data that were recently reviewed by Duffy et al. [65]. The most obvious evidence that the ovary undergoes acute inflammation is that the number and density of proinflammatory leukocytes increase in the ovary immediately after the LH surge [66–69], like any other tissue that undergoes acute inflammation. The LH surge-induced leukocyte infiltration is mediated by pro-inflammatory cytokines and ROS that are either released by ovarian cells or resident leukocytes [64,70–74]. For example, one of the pro-inflammatory cytokines, interleukin 1 beta (IL-1 β), is expressed and secreted from theca cells, granulosa cells, and leukocytes [75,76]. IL-1 β subsequently induces the expression of prostaglandin synthase 2 (PTGS2) in granulosa cells [76]. PTGS2 is a rate-limiting enzyme in the synthetic pathway of prostaglandin E2 (PGE2) [77,78], a pro-inflammatory agent that impacts angiogenesis, blood flow, immune cell function, and tissue remodeling in the ovary [65]. Importantly, locally produced PGE2 stimulates the recruitment of a variety of proinflammatory cells to the ovary in a short time [79], as it does in other inflammatory sites [80], creating acute inflammation in the ovary.

2. Ovulatory inflammation and tissue damage

Acute inflammation causes oxidative stress and DNA damage in the resident cells, and this occurs in the ovary when it undergoes ovulatory processes [81–84]. As the ovary repeats ovulation, the ovarian tissue, including oocytes, may also be damaged by proinflammatory cells via engulfment and the proteases that the immune cells release [85]. Importantly, inflammatory cells and their secretions are primarily localized and execute their inflammatory actions around the blood vessels in the ovulatory follicles and medullary region. However, primordial follicles are sequestered in the cortex, the least

vascularized region in the ovary. Therefore, presumably, the primordial follicles localized close to medulla, but not those far away from the inflammatory sites and in the cortex, may be damaged and lost at the time of ovulation (Figure 3).

In support of the suggested role of inflammatory cells in decreasing the oocyte pool, recent studies have pointed out that a pathological level of ovarian inflammation induces POI, which is caused by an accelerated decline of the oocyte pool [86-89]. For instance, wom-

en with chronic systemic inflammation caused by an autoimmune disease (e.g., Addison disease) or by pathologically elevated production of inflammatory cytokines have a significantly higher likelihood of developing POI [87,90-93].

In addition to its direct impact on oocytes or follicles, inflammation may indirectly accelerate the decline of the oocyte pool by inducing fibrosis (deposition of connective tissue), which is generally observed in aged ovaries [94,95]. Indeed, fibrosis is a part of the natural aging

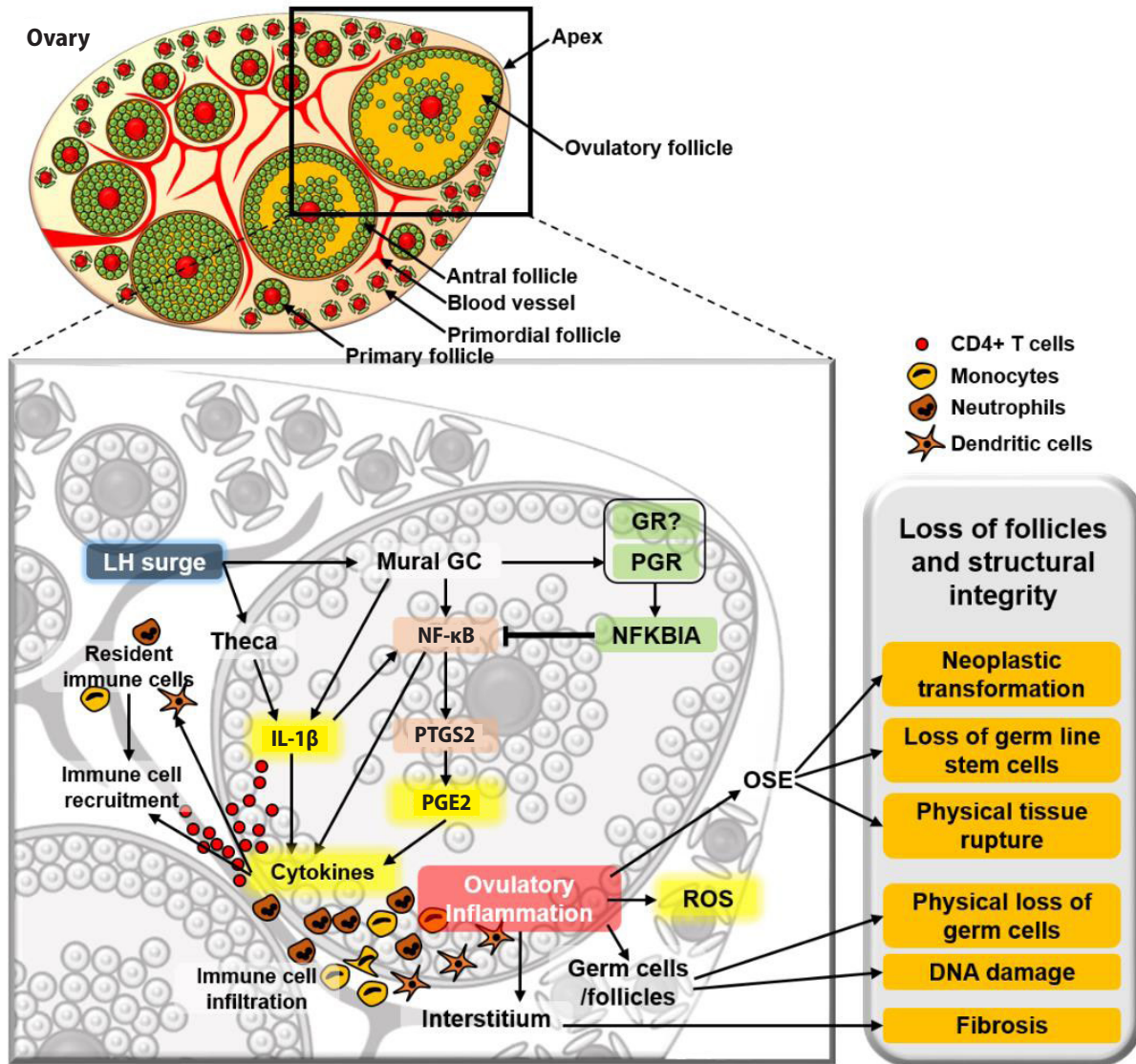


Figure 3. Ovulatory inflammation and the oocyte pool. The luteinizing hormone (LH) surge stimulates theca and mural granulosa cells (GCs) to produce prostaglandin E2 (PGE2) and interleukin 1 beta (IL-1β), which then trigger them and resident immune cells to produce inflammatory cytokines. These cytokines increase pro-inflammatory immune cells in the ovary by facilitating their infiltration into the ovarian tissue from the circulating bloodstream, causing localized inflammatory situation in the ovary. As a result, germ cells and follicles may get damaged directly by the proinflammatory cells or indirectly via reactive oxygen species (ROS) produced by the immune cells or ovarian cells. The repeated ovulatory inflammation itself and physical tearing of the ovarian surface epithelium (OSE) result in both the loss of germ cells and neoplastic transformation. NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; GR, glucocorticoid receptor; PGR, progesterone receptor; NFKBIA, NF-κB inhibitor alpha.

process in the ovary and likely contributes to the decline of the oocyte pool, as fibrous tissues may interfere with normal ovarian dynamics, as well as impair follicular survival, recruitment, and growth (Figure 3).

3. Polycystic ovarian syndrome, contraception, and the oocyte pool

If it is true that ovulatory inflammation accounts for the loss of a significant portion of oocytes, and therefore is a contributing factor to the decline of oocyte pool, one can predict that the oocyte pool decreases more slowly and therefore the reproductive lifespan would be longer in women whose ovulation frequency or number is less than that of average women. Women with polycystic ovarian syndrome (PCOS) do not ovulate at all or have a much lower ovulation frequency than those without the syndrome. As a result, women with PCOS experience a significantly lower level of ovulatory inflammation. Indeed, women with PCOS have a larger ovarian pool and exhibit a slower rate of follicular atresia than infertile eumenorrheic women [96–98]. Another example of less ovulation can be seen in women who take contraceptive pills. Unfortunately, there is no direct evidence that taking contraceptive pills may delay the oocyte-pool decline. However, it is well established that women who take contraceptive pills have a lower probability of developing ovarian cancer than those who do not [99–101], likely because the ovarian tissues have less exposure to ovulatory inflammation.

In summary, the literature clearly shows that the causes of the continuous decline of the oocyte pool throughout the entire reproductive lifespan are multifactorial. Some factors are intrinsic to the ovary (oocyte attrition, meiotic errors, follicular atresia) and unavoidable, while others are external and preventable (environmental toxicants, drugs). In addition, here, we discuss an additional potential intrinsic factor: ovulatory inflammation. The question remains of why females are born with excess oocytes. Might it be to ensure that they maintain fertility for a sufficient period when the ovary continues to lose oocytes due to the aforementioned reasons? Males are born without sperm in their gonads. Instead, their gonads contain germ cell stem cells, from which sperms are continuously produced for a few decades in humans, with the stem cells serving a reservoir for continued production of the male germ cells. The female gonads, however, do not contain germ cell stem cells; instead, ovarian egg production has to rely on the finite number of oocytes with which they are born. Therefore, having an excess number of oocytes at the time of birth may be a mechanism to ensure egg production for an extended period and long-term fertility in humans.

Conflict of interest

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

ORCID

Chan Jin Park	https://orcid.org/0000-0001-9008-1964
Ji-Eun Oh	https://orcid.org/0000-0003-3352-9608
Jianan Feng	https://orcid.org/0000-0001-6987-1187
Yoon Min Cho	https://orcid.org/0000-0003-4278-8775
Huanyu Qiao	https://orcid.org/0000-0003-0966-8077
CheMyong Ko	https://orcid.org/0000-0002-0963-3731

Author contributions

Conceptualization: CMK. Data curation: CJP, JEO, JF, YMC. Formal analysis: CJP, JEO, JF, YMC. Funding acquisition: CJP, HQ, CMK. Methodology: JEO, YMC, CMK. Project administration: HQ, CMK. Visualization: CJP, JF, JO, YC. Writing—original draft: all authors. Writing—review & editing: CJP, HQ, CMK.

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Effects of astaxanthin supplementation in fertilization medium and/or culture medium on the fertilization and development of mouse oocytes

Chonthicha Tana¹, Pareeya Somsak², Waraporn Piromlertamorn¹, Usanee Sanmee^{1,2}

¹Division of Reproductive Medicine, Department of Obstetrics and Gynecology, Faculty of Medicine, Chiang Mai University, Chiang Mai; ²CMEEx Fertility Center, Center of Medical Excellence, Chiang Mai University, Chiang Mai, Thailand

Objective: We investigated the effect of supplementing fertilization medium and/or culture medium with astaxanthin (AST) on the two phases of *in vitro* fertilization: gamete fertilization and embryo development.

Methods: Mouse cumulus-oocyte complexes were divided into four groups with 5 μM AST added to the fertilization medium (group 3, n=300), culture medium (group 2, n=300), or both media (group 4, n=290). No AST was added to the control group (group 1, n=300).

Results: The fertilization rate was significantly higher ($p < 0.001$) in the groups using AST supplemented fertilization medium (group 3, 79.0%; group 4, 81.4%) than those without AST (group 1, 56.3%; group 2, 52.3%). The blastocyst rate calculated from the two-cell stage was significantly lower ($p < 0.001$) in the groups using AST-supplemented embryo culture medium (group 2, 58.0%; group 4, 62.3%) than in those without AST (group 1, 82.8%; group 3, 79.8%). The blastocyst rate calculated from the number of inseminated oocytes was highest in group 3 (189/300, 63.0%) and lowest in group 2 (91/300, 30.3%) with statistical significance compared to other groups ($p < 0.001$). There were significantly higher numbers of cells in the inner cell mass and trophectoderm, as well as significantly higher total blastocyst cell counts, in group 3 than in the control group.

Conclusion: An increased blastocyst formation rate and high-quality blastocysts were found only in the fertilization medium that had been supplemented with AST. In contrast, AST supplementation of the embryo culture medium was found to impair embryo development.

Keywords: Astaxanthin; Culture media; Embryo development

Introduction

Physiologic levels of reactive oxygen species (ROS) are essential for gamete function and embryo development. *In vivo*, the female reproductive tract produces antioxidants to maintain an optimal level of ROS [1]. In contrast, in assisted reproductive technology (ART), the

environment surrounding gametes and embryos lacks this natural antioxidant system. Furthermore, the *in vitro* fertilization (IVF) process and culture environment can create an incidental build-up of ROS. When ROS production exceeds the cellular defense system, oxidative stress occurs, causing oxidative damage to lipids, proteins, and DNA, resulting in defective gametes and poor-quality embryos [2]. To maintain optimal ROS levels during the IVF process and prevent oxidative stress development, excess ROS must be continuously counteracted. Several antioxidant agents have been used to supplement media with the aim of improving *in vitro* embryo production, such as acetyl-L-carnitine, N-acetyl-L-cysteine, α-lipoic acid, selenium, vitamin E, and vitamin C [3-7].

In this study, we focused on astaxanthin (AST), a powerful antioxidant of the xanthophyll carotenoid group. AST is a red, lipid-soluble pigment extracted mainly from *Haematococcus pluvialis* algae [8]. It has 10 times more active free radical scavengers than other carot-

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Corresponding author: **Usanee Sanmee**

Division of Reproductive Medicine, Department of Obstetrics and Gynecology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand
 Tel: +66-53-935555 Fax: +66-53-936112-3 E-mail: usanee.s@cmu.ac.th

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enoids (zeaxanthin, lutein, canthaxanthin, β -carotene) and 100 times more than α -tocopherol [9]. AST has been widely utilized in dietary supplements that claim to possess anti-cancer, anti-diabetes, anti-aging, and anti-inflammatory properties. However, very few studies have been done on the use of AST supplements in laboratory media. We investigated the effect of AST supplementation in fertilization medium and/or embryo culture medium according to the two phases of the IVF process (gamete fertilization and embryo development), in order to observe the effect of AST on the fertilization rate, the blastocyst formation rate, and the blastocyst cell numbers.

Methods

1. Animals

The Animal Ethics Committee of the Faculty of Medicine, Chiang Mai University approved the use of mice in our study (protocol No. 47/2020). The investigators were competent in the use and care of animals for research and were certified by the Institute of Animals for Scientific Purposes Development, National Research Council of Thailand.

Male and female mice bred at the Institute of Cancer Research were obtained from the National Animal Institute, Mahidol University, Bangkok, Thailand. All mice were kept in an optimized environment in the animal husbandry unit at the Faculty of Medicine, Chiang Mai University. The room had adequate ventilation, was kept at a temperature of $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and 60%–70% humidity, and had controlled 12-hour light/dark cycles. The mice were left undisturbed for 7 days to minimize the transportation stress effect. The international and national guidelines for ethical conduct in the care and use of animals for research were followed.

2. Collection of cumulus-oocyte complexes

For superovulation, 5- to 9-week-old female mice were injected intraperitoneally (IP) with 10 units of pregnant mare's serum gonadotropin (Sigma, St. Louis, MO, USA). Forty-eight hours later, the mice received IP injections of 10 units of human chorionic gonadotropin (Pregnyl, Merck, NJ, USA). Sixteen hours after the second injection, the mice were killed by dislocation of the cervical vertebrae. The peritoneal cavity was exposed and the two oviducts were aseptically removed and placed in Earle's Balanced Salts Solution (Biological Industries, Kibbutz Beit Haemek, Israel), containing 0.5% bovine serum albumin (Sigma). Cumulus-oocyte complexes (COCs) were removed from the oviduct and separated into four groups for the experiment. COCs from each mouse were distributed to all four experimental groups. The goal was to reduce the potential variations among oocytes from different mice.

3. Experimental design

AST (Sigma; extracted from *H. pluvialis* algae) was dissolved in dimethyl sulfoxide at a concentration of 0.0005%. In preliminary trials, different concentrations of AST (1, 2.5, 5, 10, and 20 μM) were examined. The addition of 5 μM AST provided the best blastocyst formation rates in mouse oocytes. Therefore, a 5 μM concentration of AST was used in this study. The basic mediums used in this study were fertilization medium (G-IVF; Vitrolife, Sydney, Australia), cleavage medium (G1-plus; Vitrolife), and blastocyst medium (G2-plus; Vitrolife).

To assess the effect of AST supplementation in the gamete fertilization medium and/or embryo culture medium, four experimental groups were studied: group 1: no AST supplement was added to the fertilization medium or to the culture medium; group 2: the culture medium was supplemented with 5 μM AST; group 3: the fertilization medium was supplemented with 5 μM AST; group 4: both fertilization and culture mediums were supplemented with 5 μM AST.

4. In vitro fertilization and embryo culture

To obtain sperm, 10- to 12-week-old male mice were killed by dislocation of the cervical vertebrae. The epididymis was excised, the tissue was nicked to release the sperm, and the sperm were placed in 1 mL of G-IVF (Vitrolife). The suspensions were allowed to capacitate for 30 minutes in the incubator at 37°C , 6% CO_2 , 5% O_2 , and 89% N_2 . The spermatozoa were transferred to each experimental group of COCs in 50 μL drops of G-IVF (Vitrolife) under mineral oil (Irvine Scientific, Santa Ana, CA, USA) with or without AST, as assigned, for insemination at a final motile sperm concentration of 2.5×10^5 /mL. Two hours later, fertilized oocytes were transferred to the culture in groups of 10 in 10 μL drops of G1-plus (Vitrolife) under mineral oil (Irvine Scientific) with or without AST, as assigned. The fertilization rate was determined the next day by counting the number of two-cell embryos. Seventy-two hours post-insemination, the embryos were transferred to G2-plus (Vitrolife) under mineral oil (Irvine Scientific) with or without AST, as obtained in G1-plus, and cultured in similar conditions for 48 hours. Embryo development was assessed daily under an inverted microscope until reaching 120 hours of embryo culture growth. The mouse blastocysts were classified as early, partial, full, expanding, hatching and hatched blastocysts, using the human blastocyst development criteria proposed by Gardner et al. [10].

5. Differential staining of the inner cell mass and trophectoderm cells

At 120 hours of embryo culture growth, differential staining was performed on all expanding, hatching, and hatched blastocysts, using the protocol described by Pampfer et al. [11]. Briefly, the blastocyst with intact zona was placed in a 0.5% pronase solution (Sigma P8811) for 10 minutes to remove the zona pellucida. The zona-free

blastocysts were then washed in calcium and magnesium-free phosphate-buffered saline (PBS; Gibco, Waltham, MA, USA). Washed blastocysts were exposed to rabbit anti-mouse antibody (Sigma M5774; concentration 1:50) for 30 minutes at 37°C, then washed in calcium and magnesium-free PBS, and transferred into a solution containing: (1) guinea pig complement serum (Sigma S1639; concentration 1:4), (2) 10 µg/mL Hoechst 33342 (Sigma H1399), and (3) 20 µg/mL propidium iodide (Sigma P4170) for 30 minutes at 37°C. The blastocysts were washed, placed on a glass slide, and allowed to air dry. The slides were covered with coverslips and mounted with glycerol. The cell numbers were counted using a fluorescence microscope (Nikon E600) with an excitation filter of 330-385 nm and a barrier filter of 400 nm, and then analyzed using LUCIA Cytogenetics FISH software (Laboratory Imaging, Prague, Czech Republic). The inner cell mass (ICM) nuclei were stained blue with Hoechst, while trophoblast (TE) nuclei were stained red with propidium iodide.

6. Statistical analysis

The statistical analysis was performed using SPSS ver. 16 (SPSS Inc., Chicago, IL, USA). The fertilization rates and blastocyst formation rates among the four experimental groups were compared using the chi-square test. The mean numbers of ICM, TE, and total cells, as well as the ICM-to-TE ratio, were compared by one-way analysis of variance (ANOVA) when the data distribution was normal, or the Kruskal-Wallis test when normality could not be confirmed. *P*-values < 0.05 were considered to indicate statistical significance.

Results

1. Fertilization rates

A total of 1,190 COCs were included in the study. The COCs were divided into four groups with AST added to the fertilization medium (group 3, *n* = 300), culture medium (group 2, *n* = 300), and both media (group 4, *n* = 290) and the control (group 1, *n* = 300) did not have AST supplementation in either medium. The fertilization rate was significantly higher in the groups with AST supplementation of the fertilization medium (group 3, 79.0%; group 4, 81.4%) than in those without AST in the fertilization medium (group 1, 56.3%; group 2, 52.3%; *p* < 0.001) (Figure 1).

2. Embryo development

The rate of blastocyst formation calculated from two-cell embryos was significantly lower in the groups with AST supplementation of embryo culture medium (group 2, 58.0%; group 4, 62.3%) than those without AST in the culture medium (group 1, 82.8%; group 3, 79.8%; *p* < 0.001) (Figure 2A). The blastocyst rate, calculated from the number of inseminated oocytes, was highest in group 3 (189/300, 63.0%)

and lowest in group 2 (91/300, 30.3%) with statistical significance compared to other groups (*p* < 0.001) (Figure 2B). The number of embryos that reached various stages of blastocyst development after 120 hours of culture growth is shown in Table 1. The proportion of hatching and hatched blastocysts was significantly lower in group 4 than in groups 1 and 3 (51.0%, 68.6%, and 65.6%, respectively, *p* < 0.05).

3. Blastocyst cell numbers

There were significantly higher numbers of cells in the ICM and TE, as well as significantly higher total cells in the expanding, hatching, and hatched blastocysts in group 3 than in group 1 (Table 2), while the numbers of cells in the ICM and TE, as well as the total number of cells in the blastocysts, in groups 2 and 4 were comparable to group 1. The ICM-to-TE ratio was not significantly different among the four groups (ANOVA, *p* = 0.102).

Discussion

Oxidative stress had a toxic effect on the gametes and embryos during IVF and embryo development. The high ROS levels resulted in impaired oocyte quality, loss of sperm membrane integrity, sperm

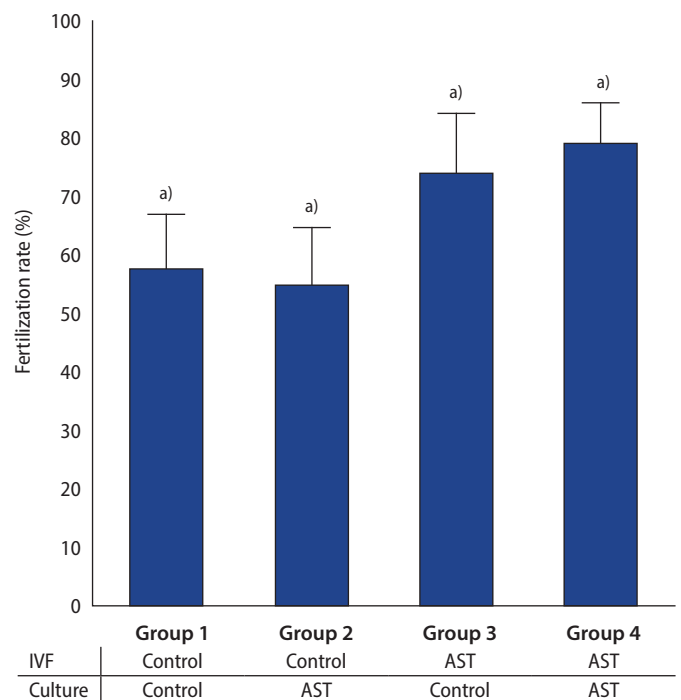


Figure 1. Effect of astaxanthin (AST) supplementation of fertilization medium and/or culture medium on the fertilization rate of mouse oocytes. The experiments were performed in six replicates and the results are presented as mean ± standard deviation. IVF, *in vitro* fertilization. ^{a)}Chi-square test, *p* < 0.001.

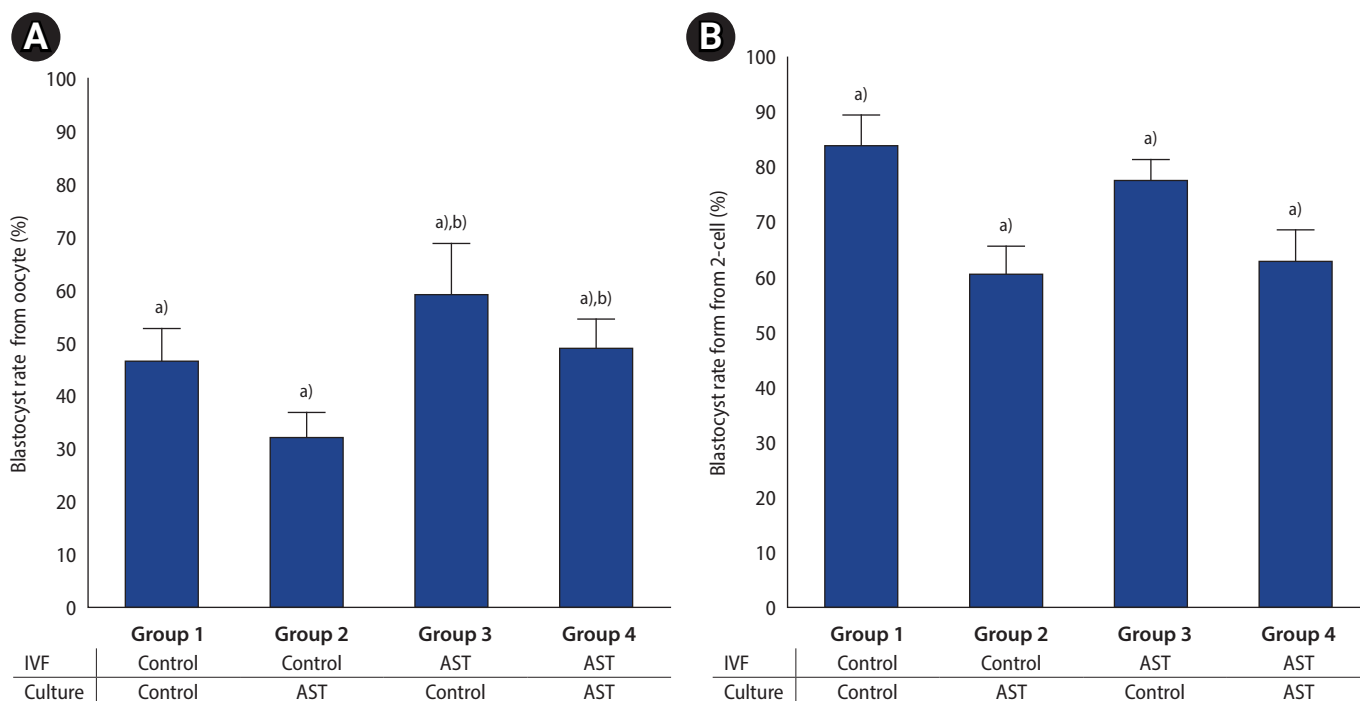


Figure 2. Effect of astaxanthin (AST) supplementation of fertilization medium and/or culture medium on blastocyst formation rate. The experiments were performed in six replicates and the results are presented as mean±standard deviation. (A) Blastocyst rate from two-cell embryos. (B) Blastocyst rate from oocytes. IVF, *in vitro* fertilization. Chi-square test: ^{a)} $p < 0.001$, ^{b)} $p = 0.012$.

Table 1. Blastocyst formation after 120 hours of culture growth, using different conditions of fertilization medium and culture medium

Variable	Group 1	Group 2	Group 3	Group 4	p-value
No. of blastocyst	140	91	189	147	-
EB to B	16 (11.4) ^{a)}	16 (17.6)	33 (17.5)	43 (29.3) ^{a)}	0.001
FB to ExB	28 (20.0)	26 (28.6)	32 (16.9)	29 (19.7)	0.157
HB to HtB	96 (68.6) ^{b)}	49 (53.9)	124 (65.6) ^{c)}	75 (51.0) ^{b,c)}	0.004
Blastocyst rate per oocyte	140/300 (46.7) ^{d),e)}	91/300 (30.3) ^{d),f),g)}	189/300 (63.0) ^{b),e),f)}	147/290 (50.7) ^{b),g)}	< 0.001
Blastocyst rate per two-cell embryo	140/169 (82.8) ^{d),e)}	91/157 (58.0) ^{d),f)}	189/237 (79.8) ^{f),g)}	147/236 (62.3) ^{e),g)}	< 0.001

Values are presented as number (%). Group 1 (IVF: control; culture: control), Group 2 (IVF: control; culture: AST), Group 3 (IVF: AST; culture: control), Group 4 (IVF: AST; culture: AST).

EB, early blastocyst; B, partial blastocyst; FB, full blastocyst; ExB, expanding blastocyst; HB, hatching blastocyst; HtB, hatched blastocyst; IVF, *in vitro* fertilization; AST, astaxanthin.

Chi-square test: ^{a)} $p = 0.001$, ^{b)} $p = 0.012$, ^{c)} $p = 0.032$, ^{d),e),f),g)} $p < 0.001$, same letter means a statistically significant pair.

Table 2. Numbers of cells in the ICM, TE, total cell number, and the ICM-to-TE ratio in four experimental groups

Variable	Group 1 (n = 103)	Group 2 (n = 58)	Group 3 (n = 121)	Group 4 (n = 77)	p-value
ICM	18.56 ± 4.46 ^{a)}	18.10 ± 5.08	21.03 ± 6.39 ^{a)}	20.55 ± 5.30	0.041
TE	62.11 ± 19.43 ^{b)}	55.97 ± 13.15 ^{c)}	73.57 ± 21.80 ^{b),c)}	65.17 ± 18.25	0.001
Total cell	80.41 ± 22.38 ^{d)}	73.81 ± 17.01 ^{e)}	94.32 ± 26.53 ^{d),e)}	85.55 ± 21.94	0.001
ICM/TE	0.31 ± 0.08	0.32 ± 0.08	0.29 ± 0.07	0.32 ± 0.07	0.102

Values are presented as mean±standard deviation. The experiments were performed in six replicates. Group 1 (IVF: control; culture: control), Group 2 (IVF: control; culture: AST), Group 3 (IVF: AST; culture: control), Group 4 (IVF: AST; culture: AST).

ICM, inner cell mass; TE, trophectoderm; IVF, *in vitro* fertilization; AST, astaxanthin.

Kruskal-Wallis test: ^{a)} $p = 0.034$, ^{b)} $p = 0.005$, ^{c)} $p < 0.001$; Analysis of variance: ^{d)} $p = 0.002$, ^{e)} $p = 0.001$.

DNA fragmentation, diminished embryo quality, and reduced blastocyst development rates [12]. The exogenous conditions causing this supraphysiologic level of ROS included high oxygen concentration, culture medium composition, energy source, changes in pH and temperature, visible light exposure, and the manipulation of gametes/embryos [13]. Strategies to overcome the oxidative stress of the ART process by reducing incidental production of ROS include: (1) decreasing oxygen tension to 5% to more closely match the oxygen concentration of the oviduct and uterus (2% to 8%), (2) the use of light filters, (3) controlling the stability of pH and temperature, (4) the addition of metal chelators in the culture medium, and (5) adjusting techniques for handling, preparing, and manipulating the gametes/embryos during the ART procedure [13]. Another method to address incidental ROS production was increasing the free radical scavenger to detoxify the excess ROS, by adding antioxidants to the IVF process and culture system [3].

An antioxidant is a scavenging enzyme or molecule that converts ROS to water. Antioxidants are classified as enzymatic or non-enzymatic. Enzymatic antioxidants include superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. Non-enzymatic antioxidants are also known as synthetic or dietary antioxidants (e.g., vitamin C, vitamin E, β -carotene, selenium, zinc, taurine, and glutathione) [14]. AST is a carotenoid nutrient with a unique molecular structure containing conjugated double bonds, and hydroxyl ($-OH$) and keto ($C=O$) groups, which contribute to its high antioxidant properties [15]. AST spans itself across the cell membrane by using its polar end groups to overlap the polar boundary zones of the membrane. In this way, it scavenges free radicals both within the membrane's hydrophobic interior and along its hydrophilic boundaries [16]. AST has been widely used as a dietary supplement due to its anti-inflammatory, antioxidant, anti-aging, anti-diabetes, and anti-cancer properties. Utilization of AST in the reproductive field has been limited, especially as an *in vitro* supplement in the IVF medium during the ART process. Previous studies found that AST supplementation in the *in vitro* maturation medium increased oocyte maturation rate, and subsequent fertilization and blastocyst development rates [17,18]. However, the addition of AST has not been studied in IVF media. Therefore, this study focused on the effect of AST supplementation in IVF media by studying both the gamete fertilization medium and the embryo culture medium.

This study demonstrated that AST supplementation in the fertilization medium had a positive effect on the fertilization rate of mouse oocytes. However, the addition of AST into the culture medium had a detrimental effect on embryo development, resulting in decreased blastocyst formation rates. Therefore, considering the entire IVF process, supplementation with AST in the fertilization medium alone gave the best results for blastocyst development. More-

over, the resulting blastocysts were of higher quality, as measured by the proportion of hatching and hatched blastocysts and the blastocyst cell numbers. Routine supplementation of AST in all mediums of the IVF process should be avoided. Our study showed that the positive effect of an increased fertilization rate was diminished by poor embryo development rates, resulting in the same blastocyst rate as the control group (no AST supplementation in all steps), but with an even lower proportion of hatching and hatched blastocysts. Therefore, the routine addition of AST in all steps of the IVF process can have negative consequences.

There is a higher likelihood of oxidative stress occurring *in vitro* than *in vivo*. However, it remains unclear the extent to which the IVF process itself contributes to increased ROS levels. Moreover, the different steps of embryo production involve different levels of ROS. Therefore, the need for antioxidants varies depending on the stage of embryo production. Gamete fertilization in conventional IVF process appears to produce the highest levels of supraphysiologic ROS. The exact conditions under which natural fertilization takes place cannot be recreated in the laboratory setting. During natural fertilization, approximately 200 spermatozoa reach the egg in the Fallopian tube, but during IVF the number of spermatozoa inseminated into the drop of egg-containing medium is many times higher. ROS is generated during the normal aerobic metabolism of spermatozoa and oocytes, immature spermatozoa, dead spermatozoa, and leukocytes. This natural level of ROS is less than the amount generated by the large number of inseminated spermatozoa in conjunction with the lack of the reproductive system's natural antioxidant defense mechanism. Excess ROS results in oocyte damage, sperm destruction, and loss of the ability to fertilize the oocyte. Sperm plasma membranes are composed of polyunsaturated fatty acids, which are particularly vulnerable to the lipid peroxidation process. Moreover, spermatozoa lack cytoplasmic enzymes and antioxidant mechanisms, making them susceptible to oxidative damage and allowing them to act as ROS carriers [19]. In practice, it is important to minimize ROS damage by using a lower concentration of inseminated sperm and minimizing the period of sperm-oocyte co-incubation. The addition of antioxidants to reduce excess ROS and minimize oxidative damage is a viable option. Our study showed a significant increase in the fertilization rate of mouse oocytes after supplementing the fertilization medium with AST. Moreover, reducing oxidative damage to gametes led to the formation of a higher number of blastocysts and high-quality embryos.

In contrast, our study showed that AST supplementation in the culture medium impaired embryo development, with a significant decrease in the blastocyst formation rate when compared to the control group. The negative result may be because the embryo culture conditions did not produce ROS at supraphysiologic levels, or it

could be from an excessive supplemental dose of AST. A low level of ROS is important for normal embryo development [20]. Excess antioxidant supplementation can exert pro-oxidant activity, thereby inducing oxidative stress deleterious to the gamete/embryo [21]. Therefore, the use of antioxidants should be limited to conditions that have a known likelihood of oxidative stress. Moreover, antioxidant potency and concentrations need to be carefully defined so that only the optimal amount is used to counteract the excess ROS, while maintaining a physiologic level of ROS.

Maintaining pro-oxidant/antioxidant equilibrium with antioxidant supplementation is difficult because of differences in ROS levels at each stage of development and because there is no standardized value for ROS. Moreover, the optimal concentration of antioxidants is important because antioxidants can have negative as well as positive effects, depending on the concentration of the supplement in the medium. Therefore, before supplementing the *in vitro* medium with an antioxidant, one should ask whether the supplement is indicated, and if the answer is “yes,” what concentration should be used. These issues remain subjects for further research.

In conclusion, AST supplementation in the fertilization medium alone resulted in the highest blastocyst formation rate and high-quality blastocysts. However, AST supplementation in the embryo culture medium was found to impair embryo development.

Conflict of interest

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

ORCID

Chonthicha Tana	https://orcid.org/0000-0002-3341-4811
Pareeya Somsak	https://orcid.org/0000-0002-4203-0297
Waraporn Piromlertamorn	https://orcid.org/0000-0003-3203-390X
Usanee Sanmee	https://orcid.org/0000-0001-6775-8607

Author contributions

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A comparison of different O-antigen serogroups of *Escherichia coli* in semen samples of fertile and infertile men

Ali Nabi^{1,2}, Mohammad Bagher Khalili³, Gilda Eslami⁴, Mahmood Vakili⁵, Fatemeh Anbari^{2,6}, Alireza Toriki^{3,7}

¹Andrology Research Center, Yazd Reproductive Sciences Institute, ²Department of Reproductive Biology, Faculty of Medicine, ³Department of Microbiology, School of Medicine, ⁴Research Center for Food Hygiene and Safety, ⁵Community Medicine, Monitoring of Health Research Center, School of Medicine, and ⁶Research and Clinical Center for Infertility, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd; ⁷Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

Objective: Male genital tract infections have been associated with infertility, and *Escherichia coli* has drawn increasing attention as an important bacterium in this context. This investigation aimed to characterize and compare the distributions of O-antigen serogroups of *E. coli* in the semen samples of fertile and infertile men.

Methods: In this case-control study, semen samples were collected from 618 fertile and 1,535 infertile men. The *E. coli*-positive samples were evaluated in terms of concentration, morphology, viability, and motility parameters according to the World Health Organization 2010 guidelines. Finally, different serogroups of *E. coli* were identified by multiplex polymerase chain reaction targeting the O-antigen variations of the bacterium.

Results: The prevalence of *E. coli* among fertile men was significantly higher than among infertile men ($p < 0.001$). The sperm morphology, viability, and motility in the *E. coli*-positive fertile group were significantly higher than in the *E. coli*-positive infertile group ($p < 0.001$). *E. coli* O6 was the most prevalent serogroup found in both groups. However, there was no significant difference in the frequency of different serogroups of *E. coli* between the two groups ($p = 0.55$).

Conclusion: Despite the higher prevalence of *E. coli* among fertile men, *E. coli* had more detrimental effects on semen parameters in infertile men. There was no significant difference in *E. coli* serogroups between the fertile and infertile groups.

Keywords: *Escherichia coli*; Male infertility; Semen; Serogroups

Introduction

Infertility is a worldwide health problem that causes an emotional impact in about 15% of cases. On average, male factor infertility is responsible for 50% of reproductive problems [1]. There is consider-

able variation in the causes of male infertility, including anatomical problems, hormonal disorders, genetic defects, infections, psychological conditions, and lifestyle factors. However, 25%–30% of couples have idiopathic infertility, meaning that the cause of infertility remains unknown [2,3].

Urogenital tract infections (UTIs) are the main cause of infertility in male partners [4]. Studies have reported that UTIs are responsible for 8%–35% of male infertility cases [5,6]. Sexually transmitted diseases have negative effects on both spermatogenesis and sperm transfer from the testes to the ejaculation ducts [7,8]. In some cases, an infection may create an obstruction in the reproductive tract, thereby causing infertility [9].

Several microorganisms have been isolated from the male reproductive tract in infertile individuals. Microorganisms can cause infer-

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Corresponding author: **Alireza Toriki**

Department of Microbiology, School of Medicine, Shahid Sadoughi University of Medical Sciences, Javan Blvd., Safayeh, Yazd 8916978477, Iran

Tel: +98-91-3250-8432 Fax: +98-91-3250-8432 E-mail: ar.torki@yahoo.com

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tility by various mechanisms, including sperm agglutination, immobilization of the spermatozoa by direct binding or production of immobilizing factors, interactions with the immune system and induction of chronic inflammation, alteration of the function of accessory sex glands, defects in sperm function, and a decreased proportion of spermatozoa with normal morphology [10,11].

Among bacterial species, *Escherichia coli* is the most common agent of male reproductive tract infections; therefore, it is the most prevalent microorganism isolated from urine and seminal fluid of patients with reproductive tract disorders. It is hypothesized that the presence of *E. coli*, as is the case for many other bacteria, may decrease sperm concentration, motility, and viability [12,13]. Although several studies have investigated the role of *E. coli* in male infertility, some reported there was no association between bacteria and male infertility [14,15]. According to a previous study, 174 O-serogroups have been identified for *E. coli*, that some of them are pathogenic [16]. The O1, O2, O4, O6, O16, O25, and O75 serogroups were predominantly found in uropathogenic *E. coli* [17].

No previous studies have compared different serogroups of *E. coli* in semen from infertile and fertile men. For this purpose, this study aimed to investigate the prevalence of *E. coli* and to identify *E. coli* serogroups in the semen of fertile and infertile men. In this study, serogroup identification was based on multiplex polymerase chain reaction (PCR), which is more accurate than serologic detection [16].

Methods

1. Study population

In this case-control study, 618 fertile and 1,535 infertile men with male factor infertility who were referred to the Yazd Infertility Research Center from April 2018 to October 2019 were voluntarily enrolled. All patients were diagnosed as fertile or infertile by a urologist according to the World Health Organization (WHO) 2010 guidelines [17], and then they were screened for the presence of *E. coli* in their semen. Participants who had used antibiotics within 2 weeks prior to the semen sampling, smokers, varicocele, and samples with mixed infections were excluded. All participants provided written informed consent, the confidentiality of the identity and clinical information of the participants was guaranteed, and the ethical committee of the Research and Clinical Center for Infertility, Yazd, approved the study proposal (IR.IUMS.FMD.REC1396.9323133001).

2. Sperm analysis

Semen samples were collected by masturbation into sterile tubes; the abstinence period for all participants was 2–5 days. Following liquefaction at 37°C for 20 minutes, the sperm morphology, concentration, viability, and motility parameters (including progressive mo-

tility, non-progressive motility and immotility), were evaluated according to the WHO guidelines for the examination and processing of human semen samples [18].

3. Isolation of *E. coli* from semen

To isolate *E. coli*, the semen samples were streaked on plates containing eosin methylene blue using a sterile calibrated loop, and incubated at 37°C for 24–48 hours [19]. The *E. coli* colonies were phenotypically identified by Gram staining, followed by biochemical tests, including catalase, oxidase, lysine decarboxylase, sulfide-indole-motility, methyl red–Voges-Proskauer, triple sugar iron, and Simmons citrate.

4. DNA extraction

A colony of the isolated *E. coli* was grown in 3 mL of tryptic soy broth at 37°C for 24 hours. Then, 1 mL of the bacterial suspension added into a sterile 1.5 mL micro-tube, centrifuged at 5,000 × *g* for 10 minutes, and washed three times by re-suspending the bacterial pellet in 1 mL of sterile phosphate-buffered saline and centrifuged at 6,000 × *g* for 4 minutes. The cells were lysed with 450 µL of lysis buffer (20 mM Tris, 25 mM EDTA, and 100 mM NaCl) and 50 µL of sodium dodecyl sulfate (10%) followed by incubation at 56°C for 20 minutes. Then, 300 µL of NaCl was added, mixed, and centrifuged at 12,000 × *g* for 10 minutes. The supernatant was equally transferred into two 1.5 mL micro-tubes, and 1 mL of cold absolute ethanol was added, mixed, and centrifuged at 12,000 × *g* for 15 minutes. Cold 70% ethanol (300 µL) was added, mixed, and centrifuged at 12,000 × *g* for 3 minutes. The supernatant was discarded, and the DNA precipitate was dissolved in 100 µL of sterile distilled water at 56°C for 10 minutes and kept at –20°C.

5. Multiplex PCR

For the molecular characterization of 14 serogroups (categorized as group 1 and group 2) of the isolated *E. coli*, multiplex PCR was carried out using specific primers (Table 1), as previously described [16]. First, 1 µL of each pair of primers (0.5 µL of forward primer and 0.5 µL of reverse primer) of group 1 were mixed in a separate micro-tube; the same was done for the primers of group 2. The assay was then performed in a final volume of 20 µL consisting of 2 µL of template DNA, 10 µL of PCR master mix (Taq DNA polymerase 2X Master Mix Red, Ampliqon, Denmark), 7 µL of the primer pool (group 1 or group 2), and 1 µL of diethylpyrocarbonate-treated water (ddH₂O). The thermal profile of the reaction for both groups was one cycle of primary denaturation at 94°C for 300 seconds, followed by 40 cycles of denaturation of DNA at 94°C for 60 seconds, primer annealing at 58°C for 60 seconds, extension at 72°C for 90 seconds, and final extension at 72°C for 300 seconds. The PCR products were detected by

Table 1. Primers used for multiplex PCR assays for 14 serogroups of the *Escherichia coli* isolated from the semen of fertile and infertile men

Serogroup	Specific gene	Genebank accession No. or reference	Primer name	Primer sequence (5'-3')	Amplicon size (bp)	Concentration in multiplex PCR (μM)
Group 1						
<i>E. coli</i> O1	Wzx	GU299791	wl-14632	(F) GTGAGCAAAGTGAATAAGGAACG	1,098	0.05
			wl-14633	(R) CGCTGATACGAATACCATCCTAC		
<i>E. coli</i> O6	Wzy	AJ426423	wl-14646	(F) GGATGACGATGTGATTTGGCTAAC	783	0.07
			wl-14647	(R) TCTGGGTTTCTGTGTATGAGGC		
<i>E. coli</i> O7	Wzx	AF125322	wl-14648	(F) CTATCAAATACCTCTGCTGGAATC	610	0.12
			wl-14649	(R) TGGCTTCGAGATTAACCTATTCTC		
<i>E. coli</i> O8	orf469	AB010150	wl-14652	(F) CCAGAGGCATAATCAGAAATAACAG	448	0.12
			wl-14653	(R) GCAGAGTTAGTCAACAAAAGGTCAG		
<i>E. coli</i> O16	Wzx	AAC31631	wl-14654	(F) GGTTTCAATCTCACAGCAACTCAG	302	0.13
			wl-14655	(R) GTTAGAGGGATAATAGCCAAGCGG		
<i>E. coli</i> O21	Wzx	EU694098	wl-14676	(F) CTGCTGATGTCGCTATTATTGCTG	209	0.12
			wl-14677	(R) TGAAAAAAGGGAAACAGAAGAGCC		
<i>E. coli</i> O75	Wzy	GU299795	wl-17413	(F) GAGATATACATGGGAGGTAGGCT	511	0.07
			wl-17414	(R) ACCCGATAATCATATTCTCCCAAC		
Group 2						
<i>E. coli</i> O2	Wzy	GU299792	wl-14636	(F) AGTGAGTTACTTTTTAGCGATGGAC	770	0.07
			wl-14637	(R) AGTTTAGTATGCCCTGACTTTGAA		
<i>E. coli</i> O4	Wzx	AY568960	wl-14642	(F) TTGTTGCGATAATGTGCATGTTCC	664	0.07
			wl-14643	(R) AATAATTTGCTATACCCACACCCCTC		
<i>E. coli</i> O15	Wzy	AY647261	wl-14672	(F) TCTTGTAGAGTCATTGGTGATCG	183	0.08
			wl-14673	(R) ATAAAACGAGCAAGCACACCACACC		
<i>E. coli</i> O18	Wzx	GU299793	wl-14656	(F) GTTCGGTGGTTGGATTACAGTTAG	551	0.08
			wl-14657	(R) CTAATATCATCCTCACTGACCACG		
<i>E. coli</i> O22	Wzx	DQ851855	wl-14660	(F) TTCATTGTGCCACTACTTTCCG	468	0.08
			wl-14661	(R) GAAACAGCCCATGACATTACTACG		
<i>E. coli</i> O25	Wzy	GU299796	wl-14666	(F) AGAGATCCGCTTTTATTGTTTCGC	230	0.08
			wl-14667	(R) GTTCTGGATACCTAACGCAATACCC		
<i>E. coli</i> O83	Wzx	GU299797	wl-14668	(F) GTACACCAGGCAAACCTCGAAAG	362	0.08
			wl-14669	(R) TTCTGTAAGCTAATGAATAGGCACC		

The serogroups were divided into two groups according to a previous study [18]. PCR, polymerase chain reaction; F, forward; R, reverse.

electrophoresis on 1% agarose gel containing DNA Green Viewer (P7890; ParsTous Biotechnology, Mashhad, Iran), using a 50 kbp DNA ladder (PR901633; Fermentas, Stanford, CA, USA) and under an ultra-violet Benchtop transilluminator (LTF00205; Bodensee, Baden-Württemberg, Germany). The amplified products were sequenced for final confirmation.

6. Statistical analysis

For the statistical analysis, the two-sample proportional test, Mann-Whitney *U*-test, and Student *t*-test were performed using IBM SPSS ver. 20 (IBM Corp., Armonk, NY, USA). The Student *t*-test (independent sample *t*-test) was used to compare sperm parameters between the fertile and infertile groups. The normality of the data distribution was checked using the Kolmogorov-Smirnov test. A *p*-value

< 0.05 was considered to indicate statistical significance.

Results

The two groups were matched for demographic characteristics. The mean ages of the *E. coli*-positive fertile (ECPF) and *E. coli*-positive infertile (ECPI) groups were 31.0 ± 2.8 and 33.8 ± 6.2 years, respectively. *E. coli* was isolated at a significantly higher frequency in the semen of fertile men than in that of infertile men ($p < 0.001$); 80 out of the 618 fertile patients (12.9%) had *E. coli* in their semen, comprising the ECPF group, while 80 out of the 1,535 infertile men (5.2%) had *E. coli* in their semen, comprising the ECPI group. The sperm parameters of the ECPF and ECPI groups are compared in [Table 2](#). The frequencies of normal morphology, motility, and viability

Table 2. Comparisons of the sperm parameters between the ECPF and ECPI groups

Variable	ECPF (n = 575)	ECPI (n = 1,725)	p-value
Concentration ($\times 10^6$ /mL)	106.2 \pm 53.2	98.8 \pm 51.7	0.58
Normal morphology (%)	7.3 \pm 3.4	2.4 \pm 2.5	< 0.001 ^{a)}
Progressive motility (%)	61.3 \pm 9.4	20.6 \pm 5.3	< 0.001 ^{a)}
Non-progressive motility (%)	10.1 \pm 5.7	9.2 \pm 7.1	< 0.001 ^{a)}
Immotile (%)	28.6 \pm 7.6	70.2 \pm 9.3	< 0.001 ^{a)}
Viability	42.6 \pm 6.3	15.4 \pm 3.5	< 0.001 ^{a)}

Values are presented as mean \pm standard deviation.

ECPF, *Escherichia coli*-positive fertile; ECPI, *E. coli*-positive infertile.

^{a)}A p-value ≤ 0.05 was considered to indicate a statistically significant difference between the ECPF and ECPI groups.

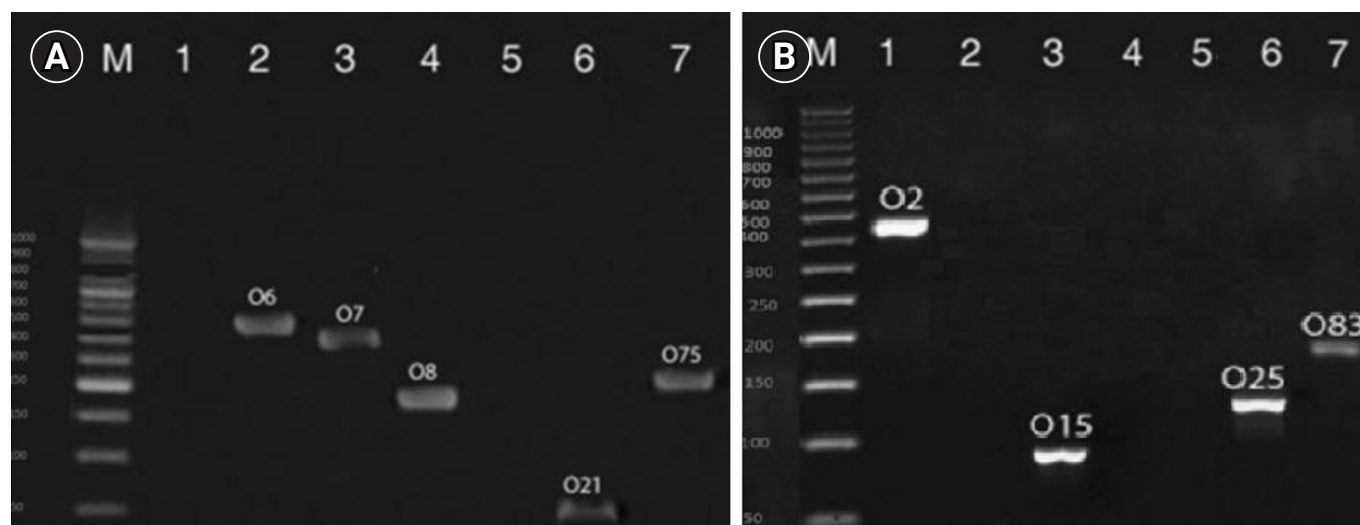


Figure 1. Results of multiplex polymerase chain reaction (PCR) divided into group 1 (A) and group 2 (B) for identification and comparison of serogroup distributions of *Escherichia coli* in the semen of the *E. coli*-positive fertile and *E. coli*-positive infertile groups.

of the sperm in the ECPF group were significantly higher than those in the ECPI group ($p < 0.001$). There was no significant difference in sperm concentration between the two groups.

Multiplex PCR was carried out in order to identify and compare the serogroup distributions of the *E. coli* isolated from the ECPF and ECPI groups (Figure 1). As shown in Table 3, the O2, O6, O7, O8, O15, O21, O25, O75, and O83 serogroups were detected in the ECPF group, and the O2, O6, O7, O8, O15, O25, and O75 serogroups were found in the ECPI group. O6 was the most prevalent serogroup found in both the ECPF (44.8%) and ECPI (50%) groups. The O8, O25, and O75 serogroups were the second most prevalent serogroups in the ECPF group, while O25 and O75 were the second most prevalent serogroups in the ECPI group (Table 3). However, there was no significant difference in the frequencies of different serogroups of *E. coli* between the ECPF and ECPI groups ($p > 0.05$).

Discussion

It has been accepted that reproductive bacterial infections are associated with male infertility [20]. Some studies have suggested that bacterial infections can induce inflammation or create changes in the biological or physiological function of the male reproductive system [21-23]. Filipiak et al. [24] found no statistically significant relationship between the presence of *E. coli* in semen and reduced sperm motility and morphology in infertile men. In contrast, Puerta Suarez et al. [25] reported that *E. coli* could significantly decrease sperm motility. However, the putative detrimental effect of *E. coli* on the seminal fluid and male infertility remains controversial [26,27]. In the present study, we evaluated the prevalence of *E. coli* isolated from semen samples of fertile and infertile men. To the best of our knowledge, this was the first study to compare the prevalence of different serogroups of *E. coli* in the semen of infertile and fertile men using multiplex PCR, which can detect serogroups of *E. coli* with high

Table 3. Comparisons between the serogroups of the ECPF and ECPI groups

Serogroup	ECPF	ECPI	p-value
O1	0	0	-
O2	2 (3)	4 (6.25)	0.37
O4	0	0	-
O6	30 (44.8)	32 (50)	0.55
O7	5 (7.5)	4 (6.25)	0.78
O8	8 (11.9)	4 (6.25)	0.26
O15	4 (6)	4 (6.25)	0.95
O16	0	0	-
O18	0	0	-
O21	1 (1.5)	0	0.33
O22	0	0	-
O25	8 (11.9)	8 (12.5)	0.92
O75	8 (11.9)	8 (12.5)	0.92
O83	1 (1.5)	0	0.33
Total	67 (100)	64 (100)	-

Values are presented as number (%). A p -value ≤ 0.05 was considered to indicate a statistically significant difference between the ECPF and ECPI groups; using this criterion, there was no significant difference in the serogroups of *Escherichia coli* between the two groups. The statistical analysis was conducted using the Mann-Whitney test.

ECPF, *Escherichia coli*-positive fertile; ECPI, *E. coli*-positive infertile.

specificity and sensitivity. The principal limitation of this technique is that some samples were non-typeable, meaning that we could not detect the serotype with our primers. Furthermore, collecting this number of samples to analyze using multiplex PCR required a considerable investment of time and money.

Our results showed that the prevalence of *E. coli* in the semen of fertile men was significantly higher than in that of infertile men. Although the prevalence of *E. coli* was low in infertile men, the detrimental effect of *E. coli* on semen quality was more severe in this group. This finding is similar to previous clinical evidence showing that bacteria isolated from the genital tracts of normozoospermic men had no effect on semen quality; however, in infertile men, an impairment of sperm parameters was seen due to the diminished antioxidant capacity of sperm [28]. However, in line with our study, previous findings have confirmed that bacterial contamination of the semen samples of fertile men did not compromise the sperm quality, while in infertile men, it is possible that bacteria further deteriorated the overall quality of the seminal plasma [29]. Salisu et al. [30] reported that *E. coli* constituted one of the main microorganisms in the semen samples of infertile men, and also they concluded that high levels of *E. coli* had negative effects on sperm parameters, including sperm count, motility, and morphology.

Our results showed that sperm parameters such as motility, morphology, and viability were significantly impaired in infertile patients

who tested positive for *E. coli* in comparison with the semen samples from healthy fertile men that tested positive for *E. coli*. Because both groups tested positive for *E. coli*, it can be inferred that *E. coli* may have a greater impact on infertile men due to etiology of male infertility. It has been suggested that *E. coli* can decrease male fertility potential by several mechanisms, including reduction of sperm parameters [31], defects in spermatogenesis, and reduction of the secretory capacity in the male accessory glands [32].

Infections of the accessory glands and male reproductive tract are harmful for sperm maturation. Epididymitis is a common urogenital disease in men between 18 and 50 years (the optimal age for fertility) [33]. Interestingly, *E. coli* has been found to be the most common cause of epididymitis and prostatitis [34]. Lang et al. [35] reported that alterations in spermatogenesis and defects in sperm structure occurred in almost 60% of patients with acute epididymitis.

The mechanism of the effect of *E. coli* on male fertility remains unknown, but it has been generally accepted that *E. coli* is the most common bacterium causing genital tract infections [36]. Fraczek et al. [37] reported that *in vitro* contact of *E. coli* with ejaculated spermatozoa can severely damage sperm membrane stability and mitochondrial activity. Defects in sperm membrane and activity were responsible for reducing male fertility potential. Another study evaluated the mechanism of the effects of *E. coli* on sperm and suggested that several soluble factors and adhesion molecules secreted by *E. coli* may be responsible for defects in sperm function, causing infertility in men [22].

It is noteworthy that microorganisms affect male infertility through various mechanisms, depending on the host immune system, age, sexual activity, and genetic background. Studies have reported that some microorganisms, including *Streptococcus viridans*, *E. coli*, *Enterococcus faecalis*, and *Staphylococcus aureus* together or alone, were harmful to male fertility potential [38,39]. In the present study, we only focused on *E. coli* as the most important bacterium in semen; other microorganisms were not evaluated.

Additionally, in the present study, molecular O-serotyping of the *E. coli* isolates revealed slight differences in the serotype distribution between fertile and infertile men; however, these differences were not statistically significant between the two groups. *E. coli* O6 was the most common isolate in semen samples of both fertile and infertile men. This finding was similar to that of Lloyd et al. [40], who found the O6 serogroup in the semen samples. Furthermore, Boguen et al. [12] reported that the O1, O2, and O4 serogroups were the most prevalent serogroups of *E. coli* in the semen. Boguen et al. [41] reported that serogroups affected neither sperm viability nor mitochondrial membrane potential in infertile men. In that study, the O4 strain decreased sperm motility; however, we did not detect the O1 and O4 serogroups in the semen of either fertile or infertile men.

Based on the findings of this study, despite the higher prevalence of *E. coli* in the semen of fertile men than in that of infertile men, the distribution of *E. coli* subgroups did not show any significant difference between the two study groups. In this study, O6 was the most common serogroup of *E. coli* in both groups. In addition, it seems that *E. coli* may be responsible for changes in sperm parameters including motility, morphology, and viability. More studies are suggested to clarify this issue in the future.

Conflict of interest

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

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Author contributions

Conceptualization: AN, MBK. Funding acquisition: AN, AT. Methodology: GE, AT, MV. Project administration: AN, AT. Writing—original draft: all authors. Writing—review & editing: FA.

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Correlations between abnormalities of morphological details and DNA fragmentation in human sperm

Hiep Tuyet Thi Nguyen¹, Hong Nhan Thi Dang¹, Thai Thanh Thi Nguyen¹, Trung Van Nguyen¹, Thuan Cong Dang², Quoc Huy Vu Nguyen³, Minh Tam Le^{1,3}

¹Center for Reproductive Endocrinology and Infertility, Departments of ²Histology, Embryology, and Pathology and ³Obstetrics and Gynecology, Hue University of Medicine and Pharmacy, Hue University, Hue, Vietnam

Objective: As the associations of sperm DNA fragmentation with morphology have not been examined in detail, this study aimed to investigate the relationship between abnormalities of morphological details and DNA integrity in human sperm.

Methods: In this cross-sectional study, men from infertile couples were enrolled at Hue Center for Reproductive Endocrinology and Infertility, Vietnam. Conventional semen parameters, including morphological details, were analyzed following the World Health Organization 2010 criteria. Sperm DNA fragmentation was evaluated using a sperm chromatin dispersion assay. The relationships and correlations between semen parameters, sperm morphology, and the type of halo sperm and the DNA fragmentation index (DFI) were analyzed.

Results: Among 130 men in infertile couples, statistically significant differences were not found in the sperm halo type between the normal and abnormal sperm morphology groups. The percentage of round-head spermatozoa was higher in the DFI >15% group (16.98%±12.50%) than in the DFI ≤15% group (13.13% ±8.82%), higher values for amorphous heads were found in the DFI >15% group, and lower values for tapered heads were observed in the DFI ≤15% group; however, these differences were not statistically significant. Small-halo sperm and the DFI were positively correlated with round-head sperm ($r=0.243, p=0.005$ and $r=0.197, p=0.025$, respectively).

Conclusion: The rate of general sperm morphological abnormalities in semen analysis was not related to sperm DNA integrity. However, round sperm heads were closely associated with sperm DNA fragmentation.

Keywords: Anatomy and histology; DNA fragmentation; Infertility; Male; Spermatozoa

Introduction

Infertility is a reproductive health problem that affects approximately 15% of reproductive-aged couples worldwide; male factors

contribute as a unique cause in approximately 30% of infertility cases [1]. Male factor infertility results from pre-testicular, testicular, or post-testicular abnormalities. However, in some cases, no reason for diminished sperm quality can be identified [2]. Conventional semen analysis is routinely performed according to standard criteria in the World Health Organization (WHO) guidelines and is accepted as the first step in evaluating male fertility [3]. Further, sperm DNA fragmentation (SDF) tests have recently gained interest as a way to assess sperm quality in the context of recurrent miscarriage, unexplained infertility, varicocele, or recurrent implantation failure after *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) [2].

During spermiogenesis, when spermatids transform into spermatozoa, the chromatin of sperm cells can be damaged by endogenous and exogenous factors [4,5]. Due to chromatin compaction, the substantial reduction in cell cytoplasm leads to a limited self-repair pro-

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Corresponding author: **Minh Tam Le**

Department of Obstetrics and Gynecology, Hue University of Medicine and Pharmacy, Hue University, 6 Ngo Quyen st, Hue, Viet Nam

Tel: +84-23-4626-9696 Fax: +84-23-4382-2173

E-mail: leminhtam@hueuni.edu.vn

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cess for fragmentation [5]. Severe DNA fragmentation may occur after spermatogenesis is completed, during the time in the epididymis and other ducts. Lifestyle-related risk factors (e.g., alcohol consumption and smoking, or toxic and hot environments) may also lead to increased concentrations of oxygen free radicals, thereby increasing the levels of sperm DNA damage [6-8]. SDF is correlated with apoptosis and dysfunction of sperm mitochondrial membrane potential, which is in turn negatively related to motility and normal morphology [9].

A high percentage of abnormal sperm, especially abnormalities of structural details, may result from a defective mechanism associated with spermatogenesis and/or sperm maturation. Abnormal sperm morphology is associated with a decrease in routine semen parameters [10] as well as signs of sperm damage (e.g., DNA fragmentation levels) and increased concentrations of reactive oxygen species (ROS) [11].

To date, the relationship between sperm morphology and SDF remains unclear. Furthermore, very few results have been obtained from fresh or prepared samples using different techniques, and sperm morphology has not been assessed in detail [12-14]. Additional information on the detailed characteristics of sperm morphology and information regarding sperm cells may be helpful for predicting sperm quality and advising patients. Therefore, the goal of this study was to determine the relationship between abnormalities of morphological details and DNA integrity of human spermatozoa using the sperm chromatin dispersion (SCD) assay.

Methods

1. Study design

A cross-sectional study was conducted at the Center for Reproductive Endocrinology and Infertility, Hue University of Medicine and Pharmacy, Vietnam, from April 2020 to October 2020. The inclusion criteria were men from infertile couples, with semen analysis and SCD test results. The exclusion criteria were patients with retrograde ejaculation, severe oligospermia (under 1 million/mL), azoospermia, sperm from cryopreservation, or sperm retrieved by surgery. Men with general urogenital infections or a history of inguinal hernia were also excluded. This study was approved by the Ethics Committee of Hue University of Medicine and Pharmacy (No. 678a/QĐ-ĐHYD). All patients agreed to participate in this study and signed an informed consent form.

2. Semen analysis

Fresh semen samples were obtained and tested in accordance with the 2010 WHO standards [3]. Sperm motility was analyzed manually using phase-contrast microscopy at $\times 400$ magnification; sperm viability was assessed by eosin staining. For morphological as-

essment, after staining with Giemsa, the morphology of the sperm head shape, acrosomal region, sperm neck, midpiece, tail, and cytoplasmic droplets was determined under a microscope at $\times 1,000$ magnification, according to the WHO 2010 guidelines. Head defects included abnormal head shapes and vacuolation, abnormal acrosomal areas, double heads, or any combination thereof. Neck and midpiece defects included a folding neck, an irregular thick, thin or a sharply bent midpiece. Principal piece defects included short, multiple, broken, smooth hairpin bends, sharply angulated bends, an irregular width, coiled structure, or any combination thereof. Excess residual cytoplasm (ERC) was recorded when the cytoplasm was larger than one-third of the sperm head size [3]. The images of abnormal sperm were taken using an Olympus CX41 microscope (Cebu, Philippines) and Infinity 1 Lumenera software (Ottawa, Canada) and shown in Figure 1. Based on the morphology results, the samples were separated into normal (normal morphology rate $\geq 4\%$) and abnormal (normal morphology rate $< 4\%$) morphology groups.

3. SCD test

All fresh semen samples were tested for fragmented DNA using the Halosperm HT-HS10 system (Halotech DNA, Madrid, Spain). A 20- μL sperm sample was added to 40 μL of low-melting agarose and mixed evenly. Subsequently, an 8- μL aliquot was placed on a slide and refrigerated at 4°C for 10 minutes to solidify the agarose. The slide was then immersed respectively in an acid denaturant for 7 minutes and lysis solution for 25 minutes. After that, the slide was rinsed in distilled water for 5 minutes, dehydrated in ethanol baths at concentrations of 70% (2 minutes) and 100% (2 minutes), and air-dried. Giemsa staining was used to stain the sperm at the end. The slides were examined under phase-contrast microscopy at $\times 1,000$ magnification. The DNA fragmentation index (DFI) was estimated as the sum of DNA fragmentation spermatozoa per 500 spermatozoa. Spermatozoa without DNA fragmentation included sperm cells with a large halo (halo width equal to or higher than the diameter of the core) or medium halo (halo size between large and small). Spermatozoa with fragmented DNA included any sperm cells with small halos (halo width equal to or smaller than one-third of the diameter of the core) or no halo, as well as those that were degraded (no halo and presenting a core that was irregularly or weakly stained). To prevent bias, the slide observations were recorded and then assessed by two experienced andrologists (NTH and DTHN). The images of sperm halos were taken using an Olympus CX41 microscope and Infinity 1 Lumenera software, as shown in Figure 2. The normal DFI group was defined as those with a DFI $\leq 15\%$, while a DFI $> 15\%$ was considered abnormal.

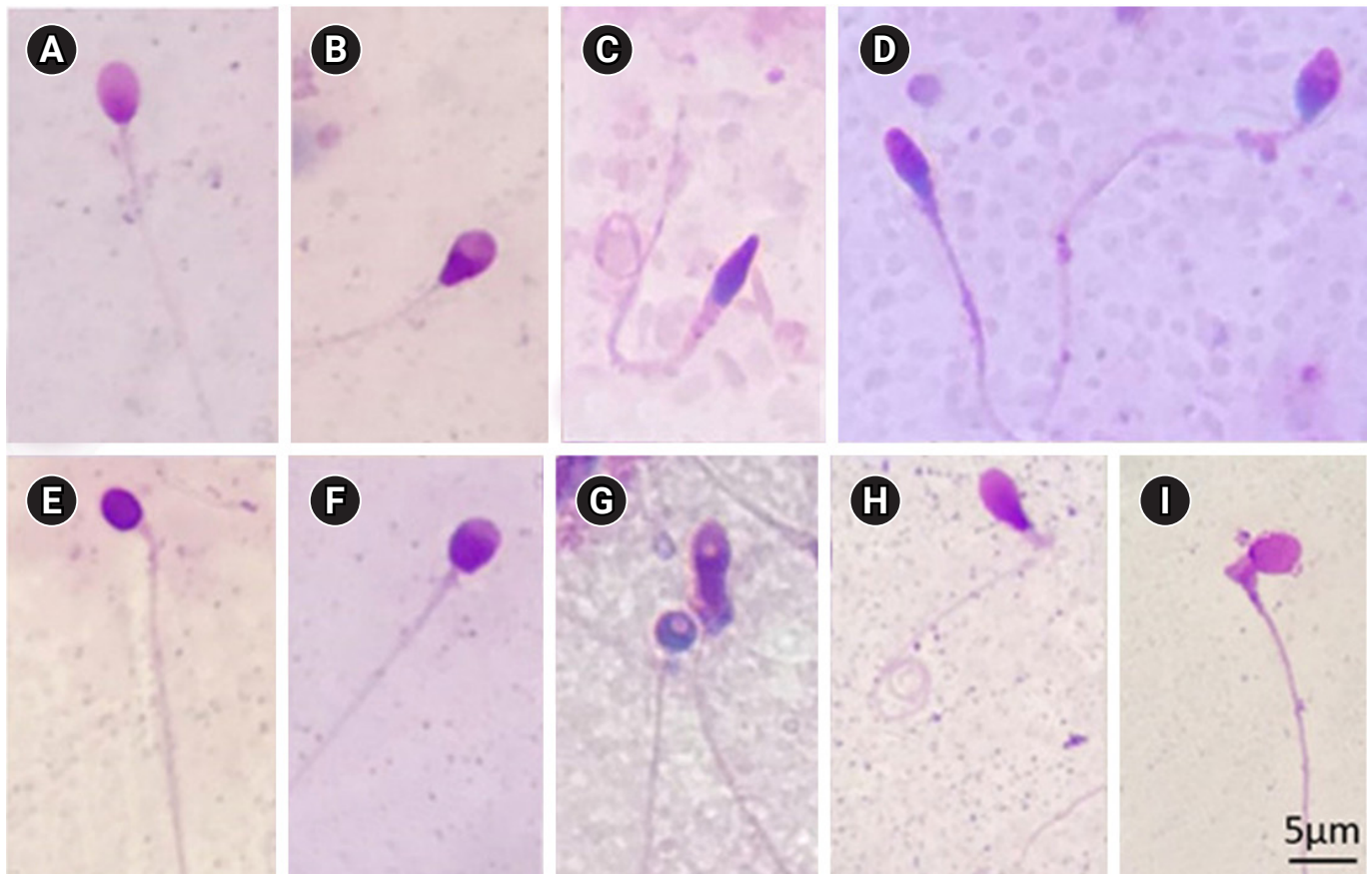


Figure 1. Images of abnormal sperm. (A) Normal sperm cell. (B) Pyriform-head sperm cell. (C) Sperm cell with a tapered head, thick insertion midpiece, and coiled tail. (D) Tapered-head sperm cell and an amorphous-head sperm cell. (E) Round-head sperm cell without an acrosome. (F) Sperm cell with a small acrosomal area. (G) Round-head sperm cell with vacuolation and an amorphous-head sperm cell with vacuolation and excess residual cytoplasm. (H) Pyriform sperm head with bent midpiece and coiled tail. (I) Sperm cell with a bent neck and excess residual cytoplasm greater than one-third of the head. (Giemsa stain, x1,000)

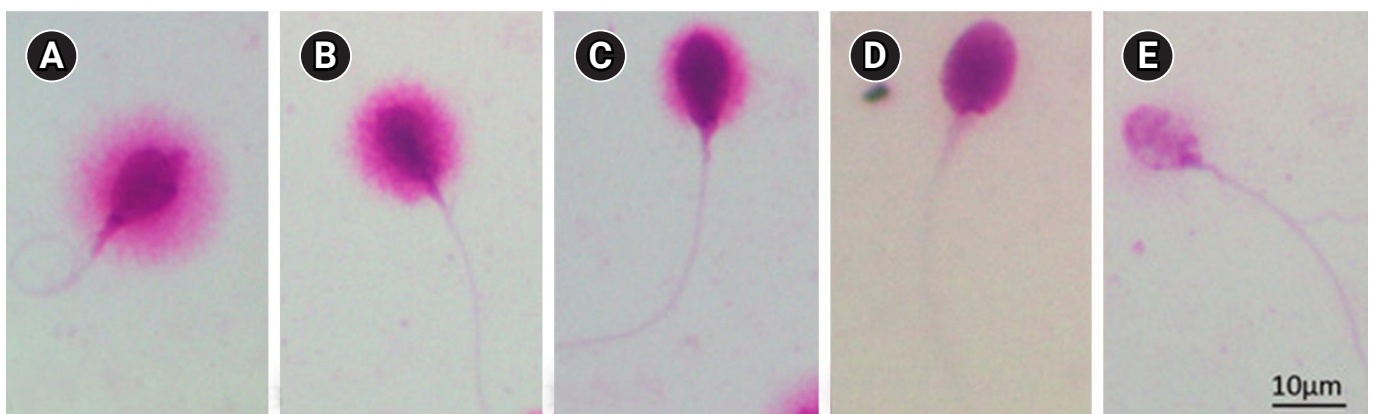


Figure 2. Classification of human sperm DNA fragmentation using the Halosperm test. The normal group included (A) sperm with a large halo and (B) sperm with a medium halo; while the abnormal group included (C) sperm with a small halo, (D) sperm without a halo, and (E) degraded sperm. (Giemsa stain, x1,000)

4. Statistical analysis

All numerical data were presented as mean \pm standard deviation. Frequencies were expressed as percentages, and the mean values were compared between two groups using analysis of variance. The Pearson correlation coefficient (r) was used to determine the association between values of sperm cells and the DFI. Differences between values were considered statistically significant at $p < 0.05$. IBM SPSS ver. 20.0 (IBM Corp., Armonk, NY, USA) was used for statistical analyses.

Results

In total, 130 semen samples from 130 infertile men were collected for this study. As shown in Table 1, they ranged in age from 25 to 51 years old (mean, 34.16 \pm 5.72 years). The following results were obtained for semen parameters: semen volume, 2.33 \pm 0.88 mL; pH, 7.31 \pm 0.47; sperm progressive motility (PR), 28.55% \pm 9.88%; concentration, 34.35 \pm 18.34 $\times 10^6$ /mL; vitality, 83.02% \pm 9.06%; normal morphology, 4.75% \pm 3.06%; and sperm DFI, 34.57% \pm 22.42%.

Statistically significant differences were found between the normal and aberrant morphology groups in terms of PR (31.35% \pm 9.13% vs. 24.73% \pm 9.66%, $p = 0.000$), sperm concentration (38.40 \pm 18.08 $\times 10^6$ /mL vs. 28.84 \pm 17.37 $\times 10^6$ /mL, $p = 0.003$), viability rate (84.81% \pm 5.31% vs. 80.58% \pm 12.11%, $p = 0.018$), abnormal head rate (85.20% \pm 7.73% vs. 91.96% \pm 7.27%, $p = 0.00$), pyriform rate (18.41% \pm 10.44% vs. 12.56% \pm 10.74%, $p = 0.002$), and ERC rate (0.73% \pm 1.43% vs. 3.20% \pm 4.69%, $p = 0.000$). Amorphous morphology was the most common head abnormality, at 29.66% \pm 13.46%; however, only a trend towards significance was observed between the two morphological groups ($p = 0.051$). Regarding the characteristics of SDF, no statistically significant differences in the halo characteristics were found between the normal and abnormal morphology groups.

Table 2 shows the results for the relationship of semen characteristics with the two groups (DFI \leq 15% and DFI $>$ 15%); the results revealed no statistically significant difference between the two groups in terms of routine semen parameters and abnormalities of morpho-

Table 1. Comparison of semen parameters and sperm DFI between normal and abnormal sperm morphology groups

Characteristics	Total (n = 130)	Normal morphology (n = 75)	Abnormal morphology (n = 55)	p-value
Semen parameter				
Volume (mL)	2.33 \pm 0.88 (1–5)	2.33 \pm 0.82	2.35 \pm 0.97	0.907
pH	7.31 \pm 0.47 (6–9)	7.27 \pm 0.45	7.36 \pm 0.53	0.286
PR (%)	28.55 \pm 9.88 (0–56)	31.35 \pm 9.13	24.73 \pm 9.66	0.000
Concentration (10^6 /mL)	34.35 \pm 18.34 (2–80)	38.40 \pm 18.08	28.84 \pm 17.37	0.003
Viability (%)	83.02 \pm 9.06 (8–93)	84.81 \pm 5.31	80.58 \pm 12.11	0.018
Sperm morphology				
Normal morphology (%)	4.75 \pm 3.06 (0–14)	6.77 \pm 2.43	2.00 \pm 0.94	0.000
Abnormal head (%)	88.06 \pm 8.23 (58–99)	85.20 \pm 7.73	91.96 \pm 7.27	0.000
Amorphous (%)	29.66 \pm 13.46 (4–72)	27.69 \pm 11.45	32.35 \pm 15.50	0.051
Tapered (%)	18.59 \pm 15.72 (0–71)	13.21 \pm 12.15	25.93 \pm 17.11	0.000
Round head (%)	16.03 \pm 11.79 (1–60)	17.17 \pm 11.72	14.47 \pm 11.82	0.198
Pyriform (%)	15.94 \pm 10.92 (0–49)	18.41 \pm 10.44	12.56 \pm 10.74	0.002
Small acrosomal area (%)	7.32 \pm 8.23 (0–34)	6.64 \pm 6.94	8.24 \pm 9.703	0.301
Vacuolated (%)	2.84 \pm 5.11 (0–34)	3.47 \pm 6.03	1.98 \pm 3.35	0.077
Neck and midpiece defects (%)	40.96 \pm 15.00 (12–81)	39.43 \pm 13.08	43.05 \pm 17.19	0.193
Tail defects (%)	22.24 \pm 11.28 (2–80)	21.68 \pm 9.94	23.00 \pm 12.94	0.529
ERC (%)	2.15 \pm 3.87 (0–23)	0.73 \pm 1.43	3.20 \pm 4.69	0.000
Sperm DNA fragmentation				
DFI (%)	34.57 \pm 22.42 (6–90.6)	34.16 \pm 23.34	35.12 \pm 21.32	0.812
Big halo (%)	27.82 \pm 28.45 (0–90.20)	27.71 \pm 28.68	27.98 \pm 28.40	0.958
Medium halo (%)	37.60 \pm 19.65 (2.8–79.6)	38.12 \pm 19.69	36.89 \pm 19.75	0.728
Small halo (%)	17.85 \pm 12.98 (2.4–54.6)	17.26 \pm 12.39	18.65 \pm 13.83	0.548
Without halo (%)	11.86 \pm 11.80 (0.8–58.6)	12.41 \pm 13.06	11.12 \pm 9.90	0.542
Degraded (%)	4.85 \pm 4.04 (0–20.4)	4.49 \pm 3.78	5.33 \pm 4.35	0.241

Values are presented as mean \pm standard deviation (range) or mean \pm standard deviation. DFI, DNA fragmentation index; PR, progressive motility; ERC, excess residual cytoplasm.

Table 2. Comparison semen parameters and detailed sperm morphology in two groups of sperm DFI

Characteristics	DFI ≤ 15% (n= 32)	DFI > 15% (n= 98)	p-value
Volume (mL)	2.45 ± 0.82	2.30 ± 0.903	0.385
pH	7.42 ± 0.42	7.27 ± 0.484	0.111
PR (%)	29.16 ± 7.58	28.35 ± 10.55	0.638
Concentration (10 ⁶ /mL)	32.16 ± 15.89	35.07 ± 19.09	0.437
Viability (%)	84.66 ± 7.00	82.49 ± 9.60	0.242
Normal morphology (%)	5.09 ± 2.57	4.64 ± 3.20	0.471
Abnormal head (%)	87.69 ± 9.86	88.18 ± 1.67	0.768
Amorphous (%)	27.13 ± 11.48	30.49 ± 4.00	0.221
Tapered (%)	22.53 ± 20.19	17.31 ± 13.83	0.180
Round-head spermatozoa (%)	13.13 ± 8.82	16.98 ± 12.50	0.059
Pyriform (%)	16.53 ± 11.48	15.74 ± 10.78	0.725
Small acrosomal area (%)	6.56 ± 6.34	7.56 ± 8.77	0.553
Vacuolated (%)	2.50 ± 4.95	2.95 ± 5.19	0.668
Neck and midpiece defects (%)	39.94 ± 13.11	41.30 ± 15.61	0.658
Tail defects (%)	22.66 ± 8.50	22.10 ± 12.08	0.810
ERC (%)	2.00 ± 3.35	2.20 ± 4.046	0.797

Values are presented as mean ± standard deviation.

DFI, DNA fragmentation index; PR, progressive motility; ERC, excess residual cytoplasm.

Table 3. The correlation between the classification of halo and semen parameters

Characteristics	Big halo	Medium halo	Small halo	Without halo	Degraded	DFI
Volume						
<i>r</i>	0.081	-0.033	-0.122	0.044	-0.148	-0.074
<i>p</i> -value	0.360	0.713	0.167	0.620	0.092	0.402
pH						
<i>r</i>	0.189 ^{a)}	-0.144	-0.045	-0.150	-0.053	-0.114
<i>p</i> -value	0.031	0.103	0.611	0.089	0.546	0.195
PR						
<i>r</i>	-0.027	0.170	0.000	-0.104	-0.332 ^{b)}	-0.115
<i>p</i> -value	0.761	0.053	0.999	0.237	0.000	0.194
Concentration						
<i>r</i>	0.041	0.024	-0.077	-0.018	-0.099	-0.072
<i>p</i> -value	0.647	0.789	0.383	0.836	0.261	0.414
Vitality						
<i>r</i>	0.044	0.150	0.014	-0.301 ^{b)}	-0.205 ^{a)}	-0.188 ^{a)}
<i>p</i> -value	0.618	0.088	0.875	0.000	0.019	0.033

DFI, DNA fragmentation index; PR, progressive motility.

^{a)}The correlation is significant at the 0.05 level; ^{b)}The correlation is significant at the 0.01 level.

logical details. Table 3 presents the relationship between DNA fragmentation characteristics and semen parameters. A weak positive correlation was found between large halos and semen pH. Further, the absence of a halo was negatively correlated with viability ($r = -0.301, p = 0.000$), and the incidence of degraded sperm was also negatively correlated with the rate of PR ($r = -0.332, p = 0.000$) and viability ($r = -0.205, p = 0.019$); in general, the DFI was negatively

correlated with the viability of spermatozoa ($r = -0.188, p = 0.033$).

The correlations between the classification of halo types and abnormal morphology are shown in Table 4. Round-head spermatozoa had a positive correlation with small-halo sperm and the DFI ($r = 0.243, p = 0.005$ and $r = 0.197, p = 0.025$), respectively. There were no correlations between other halo characteristics and abnormal sperm morphology.

Table 4. The correlation between the halo types and detailed sperm morphology abnormalities

Characteristics		Big halo	Medium halo	Small halo	Without halo	Degraded	DFI
Normal morphology	<i>r</i>	0.039	-0.033	0.030	-0.026	-0.136	-0.021
	<i>p</i> -value	0.660	0.712	0.734	0.768	0.124	0.814
Abnormal head	<i>r</i>	-0.104	0.116	0.003	0.004	0.150	0.031
	<i>p</i> -value	0.237	0.188	0.977	0.963	0.089	0.729
Tapered	<i>r</i>	0.106	-0.039	-0.159	-0.040	0.070	-0.100
	<i>p</i> -value	0.231	0.659	0.071	0.655	0.428	0.258
Pyriform	<i>r</i>	-0.055	0.141	-0.017	-0.053	-0.093	-0.054
	<i>p</i> -value	0.537	0.109	0.850	0.547	0.293	0.538
Round-head spermatozoa	<i>r</i>	-0.125	-0.044	0.243 ^{a)}	0.091	0.046	0.197 ^{b)}
	<i>p</i> -value	0.157	0.618	0.005	0.303	0.600	0.025
Amorphous	<i>r</i>	-0.108	0.140	0.002	0.025	0.000	0.014
	<i>p</i> -value	0.223	0.112	0.985	0.781	0.997	0.875
Vacuolated	<i>r</i>	-0.009	-0.028	-0.030	0.065	0.107	0.036
	<i>p</i> -value	0.922	0.749	0.733	0.464	0.228	0.686
Small acrosomal area	<i>r</i>	-0.013	-0.005	0.063	-0.053	0.066	0.020
	<i>p</i> -value	0.887	0.954	0.479	0.550	0.453	0.817
Neck and midpiece defects	<i>r</i>	-0.010	0.115	-0.139	0.032	-0.140	-0.089
	<i>p</i> -value	0.914	0.191	0.115	0.718	0.111	0.314
Tail defect	<i>r</i>	0.054	-0.036	-0.107	0.083	-0.100	-0.036
	<i>p</i> -value	0.545	0.681	0.227	0.346	0.257	0.684
ERC	<i>r</i>	-0.001	-0.084	0.083	0.020	0.087	0.075
	<i>p</i> -value	0.995	0.339	0.345	0.819	0.323	0.398

DFI, DNA fragmentation index; ERC, excess residual cytoplasm.

^{a)}The correlation is significant at the 0.01 level; ^{b)}The correlation is significant at the 0.05 level.

Discussion

Sperm morphology is the most relevant parameter in conventional semen analysis for predicting fertilization potential [11,15], a prerequisite factor for the successful use of assisted reproductive technology (ART) [16,17]. Our results indicated that abnormal sperm mainly showed changes in head morphology, and that amorphous-head and tapered spermatozoa were more common in the abnormal morphology group. Amorphous heads, tapered heads, and microheads were also reported to predominate abnormal sperm morphology in previous studies [18,19]. However, the relationship between the level of SDF and semen parameters has remained unclear. In some studies, the DFI was associated with some semen parameters such as sperm viability [20], motility [21,22], concentration, and normal morphology [13,22]. However, other studies indicated that DFI was not correlated with conventional fresh semen parameters [12,23]. In this study, no significant difference in semen parameters was found between the groups with a DFI ≤ 15% and a DFI > 15%. However, there was a negative correlation between the viability rate and SDF.

Men with teratozoospermia may show significantly higher rates of ROS production, denatured DNA, and fragmented DNA [18]. Jaku-

bik-Uljasz et al. [13] reported that the DFI was positively correlated with the teratozoospermia index, the proportion of sperm with head, midpiece, and tail abnormalities, and sperm with ERC. Even in pellet swim-up samples, 14% of morphologically abnormal spermatozoa showed fragmented DNA [12]. SDF primarily occurs by defective maturation from spermatids to mature spermatozoa, abortive apoptosis within the testis, and ROS throughout the male reproductive tract [24,25]. Abnormal spermatozoa arise due to failed apoptosis and/or failure to repair DNA strand breaks that appear during early spermatogenesis. Spermatogonia marked for apoptosis escape this process, leading to possible anomalies that may result in abnormal sperm morphology [26]. Therefore, the relationship between sperm morphology and the SDF level is predictable. However, the details regarding the types of anomalies closely related to SDF are still unclear. Our results showed no correlation of DFI or halo type with abnormal sperm morphology in general, except for a positive correlation between small halos and the DFI in round-head sperm cells.

The occurrence of round-head sperm cells, known as globozoospermia, is a severe male infertility disorder that usually causes low fertilization and pregnancy rates [27,28]; furthermore, it has recently been found to show a relationship with the outcome of ART cycles (ART) [29,30]. Globozoospermia can result from mutations, deletion

of gene products associated with the Golgi apparatus in cells, formation of the acroplaxome, or attachment of the acrosome to the nucleus in the sperm head [31]. Our results confirmed a relationship between round-head sperm and SDF, highlighting that round-head sperm were positively correlated with the incidence of small halos and DFI in the SCD test; further, the rate of round-head spermatozoa tended to be higher in the high-DFI group (DFI > 15%). Dam et al. [32] observed sperm cells by electron microscopy and discovered that in partial globozoospermia, the sperm head has less condensed chromatin, part of the acrosome, and mitochondria, and that round-head sperm are acrosome-less, with disturbed chromatin compaction. This evidence indicates that improperly compacted chromatin is related to round-head spermatozoa, suggesting that more DNA damage is present in globozoospermia [32]. Moreover, using the dUTP nick-end labeling assay and chromomycin A3 staining, another study found that the percentages of SDF and protamine-deficient spermatozoa were higher in men with globozoospermia than in fertile men [33]. Clearly, DNA fragmentation appears to be a contributor to round-head sperm cells.

Although SDF has been recognized as a supporting tool for male fertility assessment by leading research groups, studies using different assays have resulted in inconsistent conclusions [2,24]. Furthermore, the DFI cut-off values are not identical in previous studies. A DFI threshold of 15% was proposed in some recent studies that evaluated the association between the DFI and sperm morphology of infertile patients [12], embryological or clinical IVF/ICSI outcomes [34], blastocyst culture [35], or embryo development [36]. This cut-off value of 15% was also selected for treatment of SDF by micronutrient supplementation [37]. The threshold of > 15% as an abnormal DFI selected in this study should be specifically considered when drawing a conclusion. A major strength of our study is the detection of the relationship of round-head sperm with SDF and the classification of halo types. However, our conclusions were drawn from results obtained using the SCD technique alone, which constitutes a limitation of our study. Thus, further studies using different techniques should be performed to confirm this relationship.

In conclusion, the rate of sperm morphological abnormalities in semen analysis was not related to sperm DNA integrity. However, a detailed assessment of sperm morphology revealed an association with SDF with the classification of halos and the DFI using the SCD assay. Specifically, round-head sperm were found to have a strong relationship with SDF and the DFI, and a higher percentage of round-head sperm was observed in the group of men with higher DFI. Therefore, in routine semen analysis, the detailed characteristics of abnormal sperm morphology should be described, together with a SDF assay for a better assessment of male fertility potential.

Conflict of interest

Hiep Tuyet Thi Nguyen was funded by Vingroup Joint Stock Company and was supported by the Domestic Master/ PhD Scholarship Program of Vingroup Innovation Foundation (VINIF), Vingroup Big Data Institute (VINBIGDATA), code VINIF. 2020.TS.44. No other potential conflicts of interest relevant to this article were reported.

ORCID

Hiep Tuyet Thi Nguyen	https://orcid.org/0000-0002-8019-9608
Hong Nhan Thi Dang	https://orcid.org/0000-0003-3872-5456
Thai Thanh Thi Nguyen	https://orcid.org/0000-0001-9995-9948
Trung Van Nguyen	https://orcid.org/0000-0003-3363-5064
Thuan Cong Dang	https://orcid.org/0000-0002-2657-6791
Quoc Huy Vu Nguyen	https://orcid.org/0000-0002-4744-7059
Minh Tam Le	https://orcid.org/0000-0001-6225-3108

Author contributions

Conceptualization: HTTN, MTL. Data curation: HTTN, HNTD, TTTN, TVN. Formal analysis: HTTN, HNTD. Methodology: MTL, HTTN. Project administration: MTL, HTTN. Visualization: MTL, HTTN. Writing—original draft: HTTN, HNTD, TTTN, TVN, MTL. Writing—review & editing: all authors.

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Comparison of the effects of coenzyme Q10 and Centrum multivitamins on semen parameters, oxidative stress markers, and sperm DNA fragmentation in infertile men with idiopathic oligoasthenospermia

Ahmed T Alahmar¹, Rajender Singh²

¹Department of Medical Physiology, College of Medicine, University of Babylon, Hillah, Iraq; ²CSIR-Central Drug Research Institute, Lucknow, India

Objective: Oxidative stress and sperm DNA fragmentation (SDF) have been linked to idiopathic male infertility (IMI). Various antioxidants have been tried to improve semen parameters and fertility potential in IMI patients, but with inconsistent results. The study aimed to compare the effects of coenzyme Q10 (CoQ10) and Centrum multivitamins on semen parameters, seminal antioxidant capacity, and SDF in infertile men with idiopathic oligoasthenospermia (OA).

Methods: This prospective controlled clinical study involved 130 patients with idiopathic OA and 58 fertile controls. The patients were divided randomly into two groups: the first group received CoQ10 (200 mg/day orally) and the second group received Centrum multivitamins (1 tablet/day) for 3 months. Semen parameters, CoQ10 levels, reactive oxygen species (ROS), total antioxidant capacity (TAC), catalase, SDF, and serum hormone levels (follicle-stimulating hormone, luteinizing hormone, testosterone, and prolactin) were compared at baseline and after 3 months.

Results: Both CoQ10 and Centrum improved sperm concentration and motility, but the improvement was greater with Centrum therapy ($p < 0.05$). Similarly, both therapies improved antioxidant capacity, but TAC and catalase improvement was greater ($p < 0.01$ and $p < 0.001$ respectively) with CoQ10, whereas ROS ($p < 0.01$) and SDF ($p < 0.001$) improvements were greater with Centrum administration. Centrum therapy was associated with reduced serum testosterone ($p < 0.05$).

Conclusion: In conclusion, both CoQ10 and Centrum were effective in improving semen parameters, antioxidant capacity, and SDF, but the improvement was greater with Centrum than with CoQ10. Therefore, Centrum—as a source of combined antioxidants—may provide more effective results than individual antioxidants such as CoQ10 in the treatment of infertile men with idiopathic OA.

Keywords: Centrum; Coenzyme Q10; Idiopathic oligoasthenozoospermia; Oxidative stress

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Corresponding author: **Ahmed T Alahmar**

Department of Medical Physiology, College of Medicine, University of Babylon, Hillah, Iraq 51001 Iraq

Tel: +964-78-0818-0900 E-mail: ahmed.t.alahmar@gmail.com

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Introduction

Infertility is defined as the failure to achieve pregnancy after 12 months or more of regular unprotected intercourse [1]. Infertility is a global health issue affecting approximately 48 million couples worldwide [2], and male factors are considered to be the contributor in approximately 50% of infertile couples [3]. The etiology of male infertility is complex and includes underlying factors such as cryptorchidism, varicocele, genital tract infections, genetic mutations, immunological, endocrine disorders, systemic diseases, and environmental

factors [4]. However, in 30%–50% of all male infertility cases, the underlying cause of semen abnormalities remains unknown, leading to their classification as idiopathic male infertility (IMI) [5]. Oxidative stress (OS) is also a contributing factor in a significant number of infertile men. OS is defined as the distortion of the prooxidant-antioxidant balance, resulting in elevated levels of reactive oxygen species (ROS) [6]. Increased levels of seminal ROS have been found in 30%–80% of men with infertility [7]. Excessive ROS could be due to immature sperm, white blood cells, testicular torsion, cryptorchidism, varicocele, aging, infection, tumors, radiation, chemotherapy, environmental toxins, smoking, alcohol use, or systemic disease [8]. An increase in OS levels can lead to sperm plasma membrane damage due to lipid peroxidation, reduced sperm motility, reduced fertilization, and sperm DNA fragmentation (SDF) [9].

Spermatozoa possess limited-capacity antioxidant defense mechanisms that protect against ROS-induced damage [10]. Intrinsic protection is mediated by enzymatic antioxidants such as superoxide dismutase, catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase, and non-enzymatic antioxidants including urate, carnitine, glutathione, coenzyme Q10 (CoQ10), and vitamins C and E [11]. A low total antioxidant capacity (TAC) in infertile men has been attributed to a significant reduction in both enzymic and non-enzymic antioxidants [12]. A reduction in the antioxidant defense and OS may also result in SDF, which has been associated with abnormal semen parameters, reduced fertilization, and conception rates, as well as increased rates of malignancies and neurological disorders in offspring [13].

CoQ10, which is a component of the mitochondrial respiratory chain, exerts antioxidant effects [14]. CoQ10 deficiency has been associated with lower sperm motility, count, and male infertility, while CoQ10 supplementation has been shown to improve sperm count and sperm morphology in infertile men [15]. We and others have reported that CoQ10 therapy increased antioxidant capacity in men with idiopathic oligoasthenospermia (OA) [16–18]. However, studies exploring the impacts of CoQ10 on seminal antioxidant capacity and SDF in men with idiopathic OA are limited. Other studies have tried combinations of multiple antioxidants to treat male infertility and have reported variable effects on semen parameters, antioxidant capacity, and SDF [19,20]. Furthermore, there is a lack of agreement on the type, dose, or the use of the individual or combined antioxidants in IMI [17]. Therefore, the present study aimed to compare the effects of CoQ10 and Centrum multivitamins (containing 26 vitamins and minerals) on semen parameters, OS markers, and SDF in infertile men with idiopathic OA.

Methods

This prospective clinical study was conducted with a 3-month follow-up period. One hundred and thirty patients with idiopathic OA and 58 fertile men as the control group were enrolled at the Fertility Clinic, Babyl, Iraq, from August 2018 to February 2019. A comprehensive fertility assessment was performed for all participants. Nine patients did not complete the study and were excluded. Patients were allocated randomly to one of two groups (each containing 65 patients). The first group received a daily dose of 200 mg of CoQ10 (in the form of ubiquinol) (AMS, Woodinville, WA, USA) as a single oral dose for 3 months [21]. The second group received Centrum multivitamins (Pfizer, New York, NY, USA) as 1 tablet per day orally containing 26 vitamins and minerals for 3 months. Semen analysis findings, seminal CoQ10 levels, ROS, TAC, CAT, SDF, and serum hormone levels (follicle-stimulating hormone [FSH], luteinizing hormone [LH], testosterone, and prolactin) levels were compared at baseline and after 3 months. The primary endpoint was improvement in semen parameters, and the secondary endpoint was improvement in seminal antioxidant markers. The sample size was calculated using 80% power and a 5% level of error, yielding 54 for each group.

The study was approved by the Research Ethical Committee of the University of Sumer (EC/2018/8879). All the participants provided an informed consent before enrollment. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

1. Eligibility criteria

Fertile controls had a history of fathering a child in the last 2 years, with normal seminal fluid analysis findings and normal female fertility assessment. The patients included in the study had a history of infertility of 1 year or more without the use of contraception. OA was defined according to the World Health Organization (WHO) 2010 criteria [22]. Patients were excluded if they had genital infections, azoospermia, cryptorchidism, varicocele, testicular trauma or surgery, an endocrine disorder, systemic disease, relevant medications, smoking, alcohol, recent administration of antioxidants, and female factor infertility.

2. Semen analysis

Semen samples were collected by masturbation following a period of abstinence of 2–3 days. The semen sample was collected in a special container, followed by incubation at 37°C until semen liquefaction, and then semen analysis was performed within 1 hour following the WHO manual criteria (fifth edition, 2010) [22]. Two semen analyses were performed at baseline and after 3 months, and mean

values were analyzed as the results of both analyses. All semen analysis tests were performed by the same investigator (ATA) to ensure data consistency.

3. Measurements of seminal CoQ10 concentrations

Seminal CoQ10 levels were measured using high-performance liquid chromatography (HPLC) using an ultraviolet (UV) detector at 275 nm and calculated using a published method [23]. Reverse-phase HPLC with UV detection using coenzyme Q9 as the internal standard was utilized to obtain seminal CoQ10 levels.

4. Seminal ROS measurements

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

5. Measurement of seminal TAC and CAT

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

6. Chromatin dispersion test

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

7. Hormonal assays

Venipuncture was performed to collect blood samples (5 mL) using clean, plain labeled tubes. The samples were allowed to clot, and centrifugation was performed at 3,000 rpm for 10 minutes for analysis of hormones. Serum FSH, LH, testosterone, and prolactin levels were estimated using enzyme-linked fluorescent assays with the

Mini Vidas system (bioMérieux, Marcy l'Etoile, France).

8. Statistical analysis

IBM SPSS ver. 24 (IBM Corp., Armonk, NY, USA) was used for the data analysis. The results were expressed as mean \pm standard deviation. Data normality was assessed using Kolmogorov-Smirnov test. The paired *t*-test was used to compare pre- and post-treatment values. The unpaired *t*-test was used to compare means between independent groups. Pearson correlation coefficients were calculated to evaluate the correlations of seminal fluid parameters with CoQ10 levels and SDF. A false discovery rate (FDR) correction for multiple comparisons was performed using the Benjamini–Hochberg procedure. A *p*-value lower than 0.05 was considered to indicate statistical significance.

Results

Semen parameters in patients were significantly lower than in the control group. Both CoQ10 and Centrum improved sperm concentration ($p < 0.05$), progressive motility ($p < 0.01$), and total motility ($p < 0.05$); however, sperm motility showed better improvement with Centrum therapy ($p < 0.05$) (Table 1).

Infertile patients showed poorer seminal antioxidant status than the fertile control group (Table 2). Seminal CoQ10 levels were significantly lower in infertile men than in controls and significantly increased following CoQ10 therapy (FDR $p < 0.01$). CoQ10 and Centrum therapy both reduced ROS and DNA fragmentation and improved TAC and CAT activity, but the reduction in ROS (FDR $p < 0.01$) and CAT (FDR $p < 0.001$) was significantly lower with Centrum therapy and the improvement of TAC (FDR $p < 0.01$) was significantly higher with CoQ10 treatment (Table 2).

Patients had higher FSH, LH, and prolactin and lower testosterone levels than controls. Centrum therapy resulted in a decrease in serum testosterone in patients ($p < 0.05$) (Figure 1). Correlations were also found between total sperm motility and SDF ($r = -0.51$, $p = 0.002$) and between total sperm motility and seminal CoQ10 levels ($r = 0.42$, $p = 0.007$) (Table 3).

Discussion

With an increasing body of evidence linking OS and SDF to male infertility, antioxidant supplementation has been recommended for the treatment of IMI [6]. Both individual and combined antioxidant treatments have been attempted in men with IMI [26]. Recent systematic reviews have examined several studies and reported that antioxidants exert beneficial effects on semen parameters, antioxidant status, and fertility potential in men with IMI [26–28]. However,

Table 1. Semen parameters in fertile and infertile men before and after administration of CoQ10 and Centrum

Variable	Fertile control (n=58)	Patient before CoQ10 (n=65)	Patient after CoQ10	Patient before Centrum	Patient after Centrum
Age (yr)	36.4 ± 15.2	33.6 ± 14.1		32.4 ± 13.6	
Infertility duration (yr)		6.4 ± 5.2		7.1 ± 6.4	
Volume (mL)	3.1 ± 1.6	3.3 ± 1.8	3.5 ± 1.6	3.6 ± 2.0	3.6 ± 1.7
Concentration (million/mL)	50.4 ± 27.3	8.9 ± 5.1 ^{c)}	10.6 ± 6.4 ^{a),c)}	9.8 ± 5.7 ^{c)}	11.8 ± 7.0 ^{a),c)}
Progressive motility (%)	46.1 ± 9.7	20.8 ± 8.4 ^{c)}	25.7 ± 12.5 ^{b),c)}	22.9 ± 9.3 ^{c)}	30.2 ± 13.6 ^{b),c),d)}
Total motility (%)	63.5 ± 12.6	28.8 ± 8.2 ^{c)}	35.1 ± 14.6 ^{a),c)}	31.6 ± 9.2 ^{c)}	41.4 ± 16.2 ^{a),c),d)}
Normal morphology (%)	6.3 ± 2.9	2.7 ± 2.1 ^{c)}	3.3 ± 1.6 ^{c)}	2.5 ± 1.3 ^{c)}	3.7 ± 1.6 ^{c)}

Values are presented as mean ± standard deviation.

CoQ10, coenzyme Q10.

^{a)}vs. patients before CoQ10, $p < 0.05$; ^{b)}vs. patients before CoQ10, $p < 0.01$; ^{c)}vs. fertile control group, $p < 0.001$; ^{d)}vs. patients after CoQ10, $p < 0.05$.

Table 2. Seminal plasma CoQ10, oxidative stress markers, and sperm DNA fragmentation levels in fertile and infertile men before and after administration of CoQ10 and Centrum

Variable	Fertile control	Patient before CoQ10	Patient after CoQ10	Patient before Centrum	Patient after Centrum
CoQ10 level (ng/mL)	56.2 ± 38.5	41.6 ± 29.8 ^{d)}	76.9 ± 26.3 ^{b),e)}	38.9 ± 27.6 ^{d)}	40.2 ± 28.1 ^{d),g)}
ROS ($\times 10^4$ RLU/min/20 million spermatozoa)	0.07 ± 0.03	3.52 ± 1.29 ^{e)}	2.68 ± 1.31 ^{c),e)}	2.8 ± 0.96 ^{e)}	2.08 ± 1.04 ^{c),e),f)}
TAC (mmol/L)	1.12 ± 0.21	0.73 ± 0.36 ^{d)}	0.92 ± 0.4 ^{b),d)}	0.56 ± 0.24 ^{d)}	0.73 ± 0.3 ^{c),d),f)}
Catalase (U/mL)	12.45 ± 2.49	8.42 ± 2.21 ^{e)}	9.8 ± 2.06 ^{b),e)}	6.72 ± 1.75 ^{e)}	7.8 ± 1.4 ^{b),e),g)}
Sperm DNA fragmentation (%)	13.2 ± 3.8	35.2 ± 6.4 ^{e)}	32.1 ± 7.9 ^{a),e)}	28.3 ± 5.1 ^{e)}	25.7 ± 4.1 ^{b),e),g)}

CoQ10, coenzyme Q10; ROS, reactive oxygen species; TAC, total antioxidant capacity; FDR, false discovery rate.

^{a)}vs. patients before CoQ10, FDR $p < 0.05$; ^{b)}vs. patients before CoQ10, FDR $p < 0.01$; ^{c)}vs. patients before CoQ10, FDR $p < 0.001$; ^{d)}vs. fertile control group, FDR $p < 0.01$; ^{e)}vs. fertile control group, FDR $p < 0.001$; ^{f)}vs. patients after CoQ10, FDR $p < 0.01$; ^{g)}vs. patients after CoQ10, FDR $p < 0.001$.

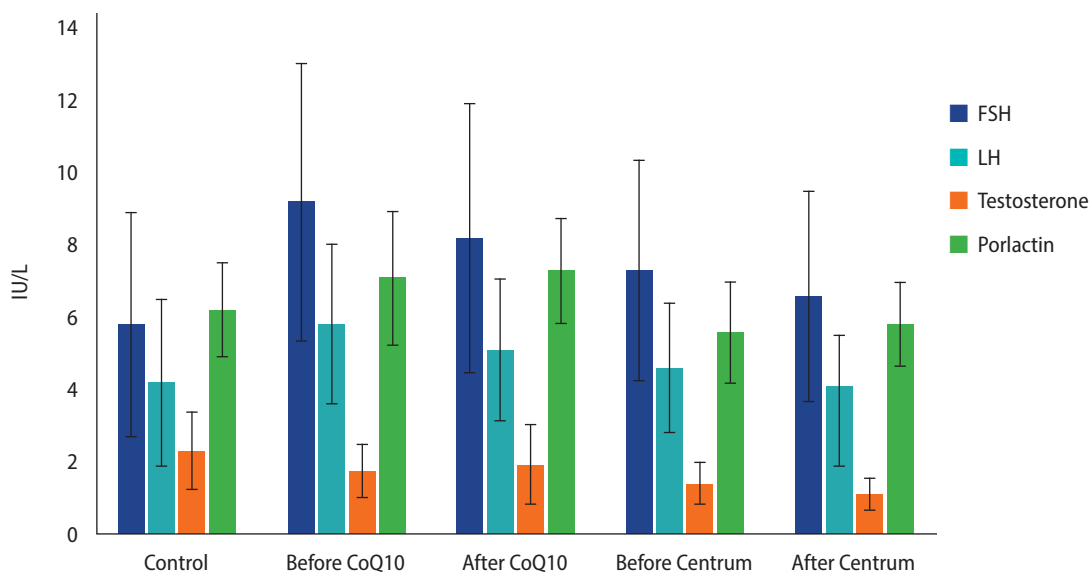


Figure 1. Hormones in fertile and infertile men before and after administration of coenzyme Q10 (CoQ10) and Centrum. FSH, follicle-stimulating hormone; LH, luteinizing hormone.

Table 3. Correlations between SDF, CoQ10 levels, and semen parameters in infertile subjects after CoQ10 treatment

Variable	<i>r</i> (<i>p</i> -value)		
	Concentration	Total motility	Normal morphology
SDF	-0.07 (0.34)	-0.51 (0.002)	-0.14 (0.24)
CoQ10	0.23 (0.16)	0.42 (0.007)	0.11 (0.26)

r: Pearson correlation coefficient.

SDF, sperm DNA fragmentation; CoQ10, coenzyme Q10.

there is a lack of consensus on the type, dosing, target group, and the use of individual or combined antioxidants in IMI [27]. To our knowledge, this study is the first to compare the effects of CoQ10 and Centrum on semen parameters, seminal antioxidant capacity, and SDF in men with idiopathic OA.

In our study, both CoQ10 and Centrum improved sperm concentration, progressive motility, and total motility; however, sperm motility showed better improvement with Centrum therapy. A randomized, double-blind, placebo-controlled trial of 212 men with idiopathic oligoasthenoteratospermia (OAT) who received CoQ10 (300 mg/day) for 26 weeks reported improvements in sperm concentration and motility post-therapy [29]. Balercia et al. [21,30], in two studies of 82 men with idiopathic asthenospermia treated with CoQ10 (200 mg/day) for 6 months, also confirmed higher sperm progressive and total motility and an increase in seminal CoQ10 level after treatment. Furthermore, our recent systematic review and another systematic review including three randomized clinical trials in infertile men who received CoQ10 therapy confirmed improvements in sperm concentration and motility in these men, although there was no increment in pregnancy rates [31,32]. Another study, however, reported no improvement in semen parameters in men with idiopathic OAT following CoQ10 therapy [33].

We could not find studies on the effects of Centrum on semen parameters or antioxidant status or in comparison with the effects of CoQ10 therapy. However, many studies have tried combinations of multiple antioxidants in men with IMI and reported improvement in 1 or more semen parameters [34,35] and in the pregnancy rate [36]. One study reported no improvement in semen parameters or pregnancy rates after combined antioxidant treatment [37]. As expected, infertile men had lower semen parameters than fertile controls. The improvement in semen parameters observed in our study could be attributed to the antioxidant properties of CoQ10 and Centrum multivitamins, which counteract OS and its detrimental effects on sperm in men with idiopathic OA [6]. Nevertheless, a comparison with the results of the aforementioned studies is challenging due to the heterogeneity of design, antioxidants, doses, and treatment duration.

Seminal CoQ10 levels were significantly lower in infertile men than

in controls and significantly increased following CoQ10 therapy. CoQ10 and Centrum therapy both reduced ROS and DNA fragmentation and improved TAC and CAT activity, but the reduction in ROS and SDF was significantly lower with Centrum therapy and the improvement of TAC and CAT was significantly higher with CoQ10 treatment. Our results are consistent with previous studies that demonstrated lower seminal antioxidant capacity [12] and higher SDF in infertile men than in controls [38]. Our previous studies on men with idiopathic OA and OAT treated with CoQ10 (200 mg/day) for 3 months demonstrated improvements in semen parameters, ROS, TAC, CAT, and GPx, as well as a reduction in SDF following CoQ10 therapy [13,18,39,40]. Our findings are consistent with other studies showing that CoQ10 therapy resulted in improvement in antioxidant capacity and reduced SDF levels in infertile men [13,41]. Our study also demonstrated a correlation between seminal CoQ10 and SDF levels and sperm total motility. This finding aligns with our previous studies, which have also shown correlations between CoQ10 levels, SDF, and sperm motility [13,18]. Other studies have also reported similar correlations in men with IMI [42,43]. We could not find studies on the effect of Centrum on SDF, but several studies have explored the impact of different combinations of antioxidants in IMI and reported reductions in SDF levels [44,19]. Other studies, however, reported no alterations in SDF levels after antioxidant treatment [45,46]. CoQ10 treatment in our study resulted in decreased FSH and LH levels, but this change was not statistically significant, and there was a reduction in serum testosterone with Centrum treatment. The reduction in serum testosterone after Centrum therapy was an unexpected finding, which might have been due to the direct effects of 1 or more of the 26 antioxidants in Centrum on seminiferous tubules. This possibility requires further investigation. A previous study of men with idiopathic OAT treated with CoQ10 (300 mg/day) reported reductions in FSH, LH, and inhibin levels [29].

The improvement in antioxidant capacity and reduction in SDF level detected in our study after CoQ10 and Centrum therapy could be due to lower antioxidant capacity in infertile men and the antioxidant properties of CoQ10 and Centrum multivitamins [12]. These properties may counteract OS, increase seminal antioxidant defense, and reduce OS-induced SDF and therefore may enhance fertility potential in men with idiopathic OA [14]. The greater improvement after Centrum therapy could have been due to the synergistic antioxidant action of the 26 combined antioxidants. Comparisons of the studies discussed above are also limited by the heterogeneity of studies' designs and the different combinations of antioxidants used. The correlations between semen parameters and antioxidant capacity and SDF may establish the foundation for the use of oral antioxidants, including CoQ10, in the treatment of infertile men with IMI and idiopathic OA to enhance their pregnancy outcomes [7]. Fur-

Furthermore, these measures could also be used as diagnostic biomarkers for male fertility and pregnancy outcomes. The limitations of our study include the small sample size and the lack of long-term follow-up, which meant that we could not report the pregnancy rate for the participants. Therefore, further large-scale studies are warranted to consolidate the findings of this study.

Both CoQ10 (200 mg/day) and Centrum (1 tablet/day) treatment for 3 months were effective in improving semen parameters, antioxidant capacity, and reducing SDF, but the improvement was greater with Centrum than with CoQ10. Therefore, Centrum combined antioxidants may provide more effective results than individual antioxidants such as CoQ10 in the treatment of infertile men with idiopathic OA due to the potential synergistic antioxidant action of the combined antioxidants. Furthermore, semen parameters, seminal antioxidant capacity, and SDF could be used as diagnostic biomarkers in men with IMI.

Conflict of interest

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

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ORCID

Ahmed T Alahmar <https://orcid.org/0000-0003-2100-5807>
 Rajender Singh <https://orcid.org/0000-0002-4592-6566>

Author contributions

Conceptualization: ATA. Data curation: ATA. Formal analysis: ATA. Methodology: ATA. Project administration: ATA. Visualization: ATA. Writing—original draft: ATA. Writing—review & editing: RS.

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Human chorionic gonadotropin therapy in hypogonadic severe-oligozoospermic men and its effect on semen parameters

Syed Waseem Andrabi¹, Gireesh Chandra Makker², Renu Makker², Geetanjali Mishra¹, Rajender Singh³

¹Department of Zoology, University of Lucknow, Lucknow; ²Makker IVF Centre, Lucknow; ³Division of Endocrinology, CSIR-Central Drug Research Institute, Lucknow, India

Objective: This study aimed to evaluate whether human chorionic gonadotropin (hCG) therapy is beneficial for improving semen parameters and clinical hypogonadism symptoms in hypogonadic oligozoospermic or severe oligozoospermic men with low or borderline testosterone levels.

Methods: A weekly dose of 250 µg (equivalent to approximately 6,500 IU) of hCG was administered subcutaneously for 3–6 months to 56 hypogonadic oligozoospermic or severe oligozoospermic men. Semen, biochemical, and genetic analyses were performed before the start of treatment followed by analyzing semen parameters every 3 months after the start of therapy. We grouped participants into responders and non-responders depending on positive changes in semen parameters.

Results: Out of 56 men, 47 (83.93%) responded, while 9 (16.07%) did not. Upon statistical analysis, it was found that age did not affect the overall outcomes ($p=0.292$); however, men with higher body mass index (BMI; 28.09 ± 3.48 kg/m²) showed better outcomes than those with low BMI (25.33 ± 3.06 kg/m²) ($p=0.042$). The duration of therapy (in months) was higher in non-responders than in responders ($p=0.020$). We found significant improvements in sperm concentration ($p=0.006$) and count ($p=0.005$) after 3 months of therapy. Sperm motility and progressive motility were also found to be higher in responders, but did not show statistically significant changes.

Conclusion: We conclude that hCG therapy can be beneficial in men with hypogonadic oligozoospermia or severe oligozoospermia.

Keywords: Human chorionic gonadotropin; Hypogonadism; Oligozoospermia

Introduction

Male and female factors contribute equally to about 15% of couples with fertility issues [1]. Men with hypogonadism have diminished levels of testosterone, and hypogonadism coupled with re-

duced gonadotropin levels (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]) is known as hypogonadic hypogonadism (HH). HH can either be congenital or acquired. The classical example of congenital hypogonadism is Kallmann syndrome, while acquired hypogonadism can occur due to pituitary lesions causing hyperprolactinemia or as a result of anabolic steroid usage. During spermatogenesis, which is regulated by hormones, the differentiation of stem cells starts in the testes. Gonadotropin-releasing hormone (GnRH) from the hypothalamus is released in a pulsatile manner to stimulate the production of FSH and LH in the anterior lobe of the pituitary gland [2]. FSH helps in spermatogenesis by directly stimulating Sertoli cells, while LH stimulates testosterone production from Leydig cells. Testicular testosterone regulates spermatogenesis by its action on Sertoli cells, while peripheral testosterone plays an important role in sexual function in men in the form of libido, erectile function, and ejaculation [3]. For successful spermatogenesis, the

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Corresponding author: **Gireesh Chandra Makker**

Makker IVF Centre, 3c, Tilak Marg, Hazratganj, Lucknow, Uttar Pradesh 226001, India
Tel: +91-522-4008289 E-mail: makkermed@gmail.com

Co-Corresponding author: **Rajender Singh**

Division of Endocrinology, CSIR-Central Drug Research Institute, Sector10, Jankipuram Extension, Sitapur Road, Lucknow, Uttar Pradesh 226031, India
Tel: +91-522-2612411-18 Fax : +91-522-22771941 E-mail: rajender_singh@cdri.res.in

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functioning of the hypothalamus-pituitary-gonadal axis is critical and any alterations in this axis can give rise to hypogonadism, which can result in spermatogenic failure and affect male fertility [4].

Generally, testosterone levels start declining after around 40 years, and by 80 years, they are reduced by almost half [5]. Hypogonadism is characterized by low serum testosterone levels coupled with low libido, erectile dysfunction, low energy, depression, anemia, infertility, and many more clinical symptoms [6-8]. Hypogonadism affects 38% of men over 45 years of age and 7% of men under 40 years of age [9,10]. Although the clinical symptoms of hypogonadism are primarily signs of aging in men [6], early-onset hypogonadism may also affect fertility in men of reproductive age. In the past few decades, its prevalence in younger generations has increased exponentially, probably due to the use of anabolic-androgenic steroids [9]. Human chorionic gonadotropin (hCG), an analogue of LH that has a longer half-life (36 hours), plays an important role in the treatment of endocrine testicular failures and stimulates testosterone production by acting on Leydig cells [9]. In this study, hCG was administered to 56 hypogonadic infertile men with oligozoospermia with the goal of improving their semen profile.

Methods

This study was approved by the Institutional Ethics Committee of the Central Drug Research Institute, Lucknow (CDRI/IEC/2015/A1). The experiments were carried in accordance with the guidelines approved for research on human samples. Written informed consent was obtained from patients for publication of the submitted article and any accompanying images. Signed copies of the consent form were provided.

In total, 104 infertile men belonging to ethnic groups speaking Indo-Aryan languages who had idiopathic severe oligozoospermia (sperm concentration > 5 million/mL) and hypogonadism symptoms were recruited at an infertility clinic between January 2014 to September 2019. All patients had completed puberty normally and had well-developed secondary sexual characteristics. A physical examination of testicular size was done by a well-trained urologist, and only patients with normal testicular sizes were included in this study. Men with a history of surgical intervention of the genital tract (e.g., for obstructive azoospermia or varicocele), drug abuse, radiation exposure, excessive alcohol consumption, abnormal karyotypes, and mental illness were excluded from the study. Out of 104 men, 25 were excluded due to a history of other treatments, while 18 other men were excluded due to incomplete hormonal therapy data. Five patients were excluded as they had Y-chromosome microdeletions, leaving 56 patients in this study. These patients had hypogonadism symptoms with initial total testosterone levels < 400 ng/dL, imply-

ing borderline testosterone levels. Informed written consent was obtained from all participants. Infertility in these men was defined as an inability to conceive after unprotected intercourse for more than 1 year. Female factor infertility was ruled out after a proper evaluation with a proper medical history of infertility treatment, as well as a general examination of the pelvic region and transvaginal ultrasonography showing no abnormality. All participants had primary infertility, and a detailed history including alcohol intake, smoking, family fertility history, lifestyle, libido, spontaneous erections, and anosmia was taken at their first visit.

Semen samples after 3–5 days of abstinence were collected by masturbation in a sterile container. Semen analysis was performed according to the 2010 World Health Organization guidelines [11]. The samples were allowed to liquefy and semen parameters were recorded. Oligozoospermia was confirmed by at least three semen analyses suggesting a below-normal sperm count.

1. Genetic analysis

To rule out genetic causes, karyotype and Y-chromosome microdeletion analyses were performed. Karyotype analysis confirmed a normal chromosomal complement (46,XY) in all men. Y-chromosome microdeletions were analyzed using six STS markers for the AZFa (sY86 and sY84), AZFb (sY127, and sY134) and AZFc (sY255 and sY254) regions according to the recommendations of the European Academy of Andrology and the European Quality Monitoring Network Group [12]. Five men with Y-chromosome microdeletions were excluded from the study.

2. Hormonal therapy

Based on the testosterone levels (low or borderline) and clinical symptoms, a diagnosis of adult-onset hypogonadism (AOH) was made. After diagnosing, the male partners were suggested to receive hCG therapy at a weekly dose of 250 µg. During the treatment, semen analysis was performed after every 4 weeks. No sperm was found in the ejaculate samples even after centrifugation for the first 2 months in any participants. However, after the third month, occasional sperm were found in the ejaculate in two men and at the end of 4 months, six other men responded by showing occasional sperm. All the responders agreed to continue the therapy until 6 months and three men showed normal semen parameters in the ejaculate, while three men showed oligozoospermic parameters. As advised, the couples had regular sexual intercourse.

Results

In this single-center study, the patients were treated by a single urologist with specific training in male infertility. A total of 56 men

met the inclusion criteria, all of whom received hCG therapy, followed by a classification of the patients into responders and non-responders depending upon the effect of hCG therapy on semen parameters. In responders, the sperm concentration was found to increase in the ejaculate, either during or after hCG therapy. In non-responders, no change in sperm concentration was found in the ejaculate post-treatment. In total, 47 men (83.93%) responded and showed an increase in sperm concentration, while nine men (16.07%) did not show any improvement in their semen parameters. The majority (57.1%) of the men were between 31 and 40 years of age.

Upon comparing the age between two groups, the mean \pm standard deviation age of responders (32.00 ± 4.31 years) and non-responders (30.40 ± 3.27 years) was not significantly different ($p=0.292$). Unexpectedly, the body mass index was significantly higher ($p=0.042$) in responders (28.09 ± 3.48 kg/m²) than in non-responders (25.33 ± 3.06 kg/m²). Upon comparing the duration of therapy, non-responders had a significantly longer ($p=0.020$) duration than responders (4.74 ± 1.03 vs. 3.77 ± 0.90 months). No significant difference in the length of marriage was found ($p=0.895$) between responders (6.98 ± 4.22 years) and non-responders (6.78 ± 3.70 years). Selected clinical characteristics of responders and non-responders are given in Table 1.

We also compared the results of pre-treatment hormonal analyses

Table 1. Comparison of clinical characteristics between responders and non-responders

Parameter	Outcome		p-value
	Responder	Non-responder	
Age (yr)	32.00 ± 4.31	30.40 ± 3.27	0.292
Duration of therapy (mo)	3.77 ± 0.90	4.74 ± 1.03	0.020
Body mass index (kg/m ²)	28.09 ± 3.48	25.33 ± 3.06	0.042
Length of marriage (yr)	6.98 ± 4.22	6.78 ± 3.70	0.895

Values are presented as mean \pm standard deviation.

Table 2. Pre-treatment hormonal comparison between responders and non-responders

Hormone	Outcome		p-value
	Responder	Non-responder	
LH (mIU/mL)	6.58 ± 3.63	3.88 ± 2.14	0.026
FSH (mIU/mL)	5.71 ± 2.53	7.38 ± 4.21	0.256
Total testosterone (ng/mL)	3.52 ± 0.68	3.38 ± 0.85	0.639
Free testosterone (pg/mL)	15.41 ± 7.90	8.47 ± 4.48	0.009
Prolactin (ng/mL)	14.42 ± 6.01	8.33 ± 3.96	0.005
Estradiol (pg/mL)	35.04 ± 15.41	34.76 ± 15.59	0.964

Values are presented as mean \pm standard deviation.

LH, luteinizing hormone; FSH, follicle-stimulating hormone.

between the responders and non-responders. LH, free testosterone, and prolactin levels were significantly higher in responders than in non-responders (Table 2). However, no significant difference was observed in FSH, total testosterone, and estradiol levels between responders and non-responders (Table 2).

The main target of hCG therapy was its effect on semen parameters. In the final observations of the two groups post-therapy, we found significant improvements in sperm concentration ($p=0.006$) and the total sperm count ($p=0.005$); however, motility and progressive motility improved, but the changes were not statistically significant (Table 3).

As a further analysis of the sperm concentration distribution pre- and post-therapy, we performed the paired-proportion test and found a significant increase ($p=0.012$) of 25% in sperm concentration and a significant increase ($p=0.019$) of 30.4% in the overall count (Table 4).

Discussion

In infertile men, it is important to diagnose the underlying cause of infertility in order to determine an appropriate fertility treatment. In infertile azoospermic and oligozoospermic men with AOH, sperm restoration can be performed using pulsatile GnRH therapy, which

Table 3. Effects of hCG therapy on semen parameters of pre- and post-therapy

Variable	Pre-therapy	Post-therapy	p-value
Concentration (million/mL)	3.03 ± 3.25	6.54 ± 9.40	0.006
Count (million)	6.57 ± 4.26	15.82 ± 24.97	0.005
Motility (%)	46.71 ± 23.37	52.59 ± 17.08	0.067
Progressive motility (%)	21.21 ± 17.07	23.95 ± 16.11	0.340

Values are presented as mean \pm standard deviation.

hCG, human chorionic gonadotropin.

Table 4. Sperm concentration and count distribution of pre- and post-hCG treatment

Variable	Pre-treatment	Post-treatment	% Difference
Concentration			
≤ 3 million/mL	43 (76.8)	29 (51.8)	-25.0
> 3 million/mL	13 (23.2)	27 (48.2)	0.25
Total	56 (100)	56 (100)	-
Count			
≤ 6 million	30 (53.6)	13 (23.2)	-30.4
> 6 million	26 (46.4)	43 (76.8)	0.304
Total	56 (100)	56 (100)	-

Values are presented as number (%).

hCG, human chorionic gonadotropin.

requires special equipment and is expensive compared to the simpler option of self-injectable gonadotropin therapy [13]. Gonadotropin therapy has also been reported to be more efficient than clomiphene citrate therapy [14].

The European Academy of Andrology suggested that when total testosterone levels are less than 350 ng/dL, the likelihood of hypogonadism is high. However, lower testosterone levels below the reference range indicate a higher likelihood of testosterone deficiency. In patients with borderline testosterone levels, free testosterone can be used to establish a clear diagnosis. There are limited options for treating men with hypogonadism, and the most successful method is testosterone replacement therapy (TRT) [6]. TRT is also effective for promoting muscle strength energy, bone mineral density, and improvements in sexual function and vitality [8]. From 2001 to 2011, TRT has become four times as common among men between 40 and 49 years of age and three times as common overall [15]. hCG can be beneficial for men requiring TRT, and over the past decade, the importance of hCG has grown exponentially due to its usage in hypogonadic men.

hCG can be a promising alternative in men with AOH. As an analogue of LH, hCG stimulates the production and release of intratesticular testosterone by acting on Leydig cells, with the added benefit of a better half-life and self-administration (subcutaneously) [8]. Various protocols have been used in men with hypogonadism to induce and maintain spermatogenesis. Monotherapy of hCG or in combination with human menopausal gonadotropin can be given to azoospermic males for 3–6 months to achieve spermatogenic restoration, as shown by the presence or increase of spermatozoa in the ejaculate [16]. In our case, monotherapy of 250 µg of hCG was successfully used to increase the sperm concentration in severely oligozoospermic men.

Furthermore, upon comparing pre-treatment hormonal profiles between responders and non-responders, we found that free testosterone was predictive of individuals' responses to hCG therapy, suggesting that along with testosterone, free testosterone levels should be also checked for diagnostic purposes in all patients before starting treatment. Testicular function improvements in the form of seminal parameters can be due to the beneficial effect of hCG therapy on Leydig and Sertoli cells. In our study, clinically modest response rates in sperm parameters (concentration and count) were observed, which could also be meaningful in further decision-making regarding various treatment options, including intrauterine insemination, *in vitro* fertilization, and intracytoplasmic sperm injection (ICSI).

Our results showed a positive response to therapy, indicating that the duration of treatment should be at least 3 months. Upon a further analysis of our results regarding the effect of hCG therapy on semen parameters, we found statistically significant improvements in

semen concentration from 3.03 ± 3.25 million/mL at baseline to 6.54 ± 9.40 million/mL on average after 3 months of therapy ($p=0.006$). Gonadotropic administration in infertile men with either azoospermia or oligozoospermia with high levels of FSH is usually useless. Hormonal therapy treatments in men with hypogonadic oligozoospermic have been overshadowed due to the use of ICSI, testicular sperm extraction (TESE), and micro-TESE. In men with hypogonadism, hCG therapy may be an option for spermatogenic restoration when fertility is desired. Our data suggest that hCG therapy is safe and effective as an alternative to exogenous testosterone therapy. In a direct evaluation of the effects of hCG on semen parameters, our results showed significant improvements in semen concentration and count. The only challenge patients face is financial, as hCG therapy is costly; however, most of the time, the desire to achieve parenthood overcomes this barrier. In men with low or borderline testosterone levels, hCG therapy should be discussed as an option to retain fertility.

Conflict of interest

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

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ORCID

Syed Waseem Andrabi	https://orcid.org/0000-0001-8749-9016
Giresh Chandra Makker	https://orcid.org/0000-0002-9193-0558
Renu Makker	https://orcid.org/0000-0001-8720-8987
Geetanjali Mishra	https://orcid.org/0000-0001-8976-4602
Rajender Singh	

Author contributions

Conceptualization: RS. Data curation: SWA. Formal analysis: GCM, RM. Funding acquisition: RS. Methodology: SWA, GM. Project administration: RS. Visualization: RS. Writing—original draft: SWA. Writing—review & editing: RS, GM.

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Azoospermic men with isolated elevation of follicle-stimulating hormone represent a specific subpopulation of patients with poor reproductive outcomes

Safar Gamidov^{1,2}, Taras Shatylo¹, Alina Popova¹, Natig Gasanov¹, Gennadiy Sukhikh^{1,2}

¹Department of Andrology and Urology, V.I. Kulakov National Medical Research Center of Obstetrics, Gynecology, and Perinatology, Moscow;

²Department Obstetrics, Gynecology, and Perinatology, I.M. Sechenov First Moscow State Medical University (Sechenov University), Moscow, Russia

Objective: This study aimed to describe a distinct subpopulation of azoospermic patients with isolated elevation of follicle-stimulating hormone (iFSH) and poor outcomes of microdissection testicular sperm extraction (microTESE).

Methods: A retrospective analysis of microTESE outcomes was conducted among 565 patients with non-obstructive azoospermia (NOA). Testicular pathology was assessed by the dominant histological pattern and Bergmann-Kliesch score (BKS). Descriptive statistics were presented for the iFSH subgroup. Inhibin B levels, the sperm retrieval rate (SRR), and BKS were compared in iFSH patients and other NOA patients.

Results: The overall SRR was 33.3% per microTESE attempt. The median BKS was 0.6 (interquartile range, 0–2). Of all NOA patients, 132 had iFSH, and microTESE was successful only in 11 of those cases, with an SRR of 8.3%, while the total SRR in other NOA patients was 38.1% ($p < 0.001$). iFSH had a sensitivity of 32.1% (95% confidence interval [CI], 27.4%–36.8%) and specificity of 94.1% (95% CI, 90.8–97.5%) as a predictor of negative microTESE outcomes.

Conclusion: Patients with iFSH may harbor a distinct testicular phenotype with total loss of the germ cell population and poor outcomes of surgical sperm retrieval.

Keywords: Azoospermia; Follicle-stimulating hormone; Male infertility; Sperm retrieval

Introduction

Azoospermia is a highly adverse finding in the male partners of infertile couples. Although a minority of azoospermic men may benefit from surgical interventions and medical therapy, eventually leading to an emergence of sperm in the seminal fluid, most of them are naturally considered sterile and depend on sperm retrieval techniques

in order to achieve biological fatherhood. However, successful surgical sperm retrieval may be guaranteed only in cases of obstructive azoospermia. In contrast, in non-obstructive azoospermia (NOA), which is characterized by spermatogenic failure, the sperm retrieval rate (SRR) tends to be modest at best. The SRR in NOA essentially depends on the surgical technique and testicular phenotype.

Microdissection testicular sperm extraction (microTESE), originally proposed by Schlegel and Li [1], is the most appropriate technique for NOA. As for the testicular phenotype, there is no way of properly determining it before testicular biopsy per se, which substantially limits our understanding of the likelihood of sperm retrieval before attempting microTESE and makes patient counseling suboptimal. Even diagnostic testicular biopsy, as demonstrated by Berookhim et al. [2], has little predictive value due to the potential heterogeneity of seminiferous tubules and the random nature of this procedure.

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Corresponding author: Taras Shatylo

Department of Andrology and Urology, V.I. Kulakov National Medical Research Center of Obstetrics, Gynecology, and Perinatology, Oparina St, 4, Moscow 117997, Russia

Tel: +79276204925 E-mail: moscowandrology@gmail.com

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Endocrine parameters and testicular volume are currently viewed as possible markers of microTESE success. Follicle-stimulating hormone (FSH), despite being routinely used by clinicians in decision-making along with inhibin B, seems to be quite unreliable, with a poor area under the curve (AUC) value demonstrated in a meta-analysis based on five studies [3]. Even if we consider it to be a predictor for successful sperm retrieval, it is definitely not a linear one. Indeed, a weak U-shaped relationship between FSH level and the SRR was noted in at least 1 study [4].

It would be more logical to try to integrate these clinical and biochemical parameters together in order to identify basic patterns that correspond to distinct testicular phenotypes in NOA. In other words, the likelihood for successful sperm retrieval does not and should not depend on individual endocrine parameters. Rather, using endocrine parameters as a whole, we may deduce the current intrinsic state of testicular parenchyma, and the latter effectively defines the SRR. The evaluation of clinical data, hormone levels, and testicular pathology is currently disjointed. The widely used classification, which includes hypospermatogenesis, maturation arrest, and Sertoli cell-only (SCO) syndrome, does not reflect all the complexities that underlie these phenomena.

We aimed to perform a heuristic identification of basic testicular phenotypes by comparing observed clinical patterns with pathology results and reproductive outcomes. An often overlooked clinical pattern is isolated FSH elevation (iFSH) in the presence of normal or borderline luteinizing hormone (LH) and testosterone levels. This finding is quite frequent in NOA, but it is not as widely discussed as more obvious and well-known patterns, such as hypergonadotropic hypogonadism. Therefore, we decided to begin with an attempt to describe iFSH, which seems to be the most unfavorable endocrine pattern, as pointed out by Esteves et al. [5]. Our hypothesis was that iFSH may reflect a specific testicular phenotype characterized by a negligible SRR and adverse pathological findings.

Methods

1. Study population

A retrospective analysis of microTESE outcomes was conducted among 565 patients with primary infertility and NOA, who were treated at our center between October 2010 and December 2017. This retrospective study was approved by the Institutional Review Board of V.I. Kulakov National Medical Research Center. The World Health Organization (WHO) definition of infertility was used [6]. As all included patients were azoospermic, male factor infertility was obvious. Prior to microTESE, all patients were interviewed regarding their duration of infertility, previous fertility treatments, chronic

health conditions, family history, previous surgical treatments, medications, occupational hazards, and lifestyle risk factors. A physical examination, complete blood count, urinalysis, blood chemistry tests, endocrine profiling, ultrasonography, and electrocardiography were performed in all patients. Patients' endocrine profile was evaluated using a Cobas e411 analyzer for immunochemistry testing (Roche Diagnostics, Basel, Switzerland). The inhibin B level was measured using an Inhibin B Gen II assay (Beckman Coulter United Kingdom, Wycombe, UK). Salvage microTESE procedures, as well as cases when any type of hormonal therapy was administered, were not included in this analysis.

Semen analysis was performed according to the WHO laboratory manual [7]. Azoospermia was confirmed when at least two semen analyses revealed an absence of sperm in the ejaculate. Patients with azoospermia caused by spermatogenic failure (i.e., NOA) were selected by testicular pathology results showing characteristic alterations of tubular structure (hypospermatogenesis, maturation arrest, SCO syndrome, or tubular atrophy).

2. Interventions

All patients underwent microTESE, as originally described by Schlegel and Li [1]. Prior to the procedure all patients signed an informed consent form, which included a statement on the possible use of anonymized data for subsequent scientific analysis. The surgical approach involved an incision along the scrotal raphe and separation of the tunica dartos, with subsequent testis delivery. In several complex cases, when vasal or epididymal obstruction amenable to reconstruction was suspected (the precise nature of azoospermia was not yet known because there were no pathology results), lateral scrotal incisions were used instead, allowing the vas deferens to be inspected, but then, as it became obvious that the patient had NOA, regular sperm retrieval was commenced. When the seminiferous tubules were distinctly heterogeneous under optical magnification ($\times 15$ – 20), we performed dissection in order to find more or less intact tubules and attempted sperm retrieval, while taking one or two pieces of testicular parenchyma for a pathological examination. When the seminiferous tubules were visually judged to be of poor quality, the parenchyma appeared homogeneous, and a thorough dissection revealed no healthy tubules, we attempted random biopsies from different areas. During all steps of this procedure, an embryologist was present nearby the operating room to handle the specimens and to perform an initial search for viable sperm, guiding the ongoing surgical procedure. The tissue samples underwent standard mechanical processing, which included mincing and passing the suspension through a 24-G catheter multiple times, prior to microscopy.

3. Studied variables

In this retrospective study, we analyzed the following variables for each sperm retrieval attempt: patient's age, serum levels of inhibin B, testosterone, LH, FSH, estradiol, sonographically measured total testicular volume (using a built-in formula: length \times width \times height \times 0.52), presence of varicocele, presence of genetic causes of testicular failure (e.g., azoospermia factor deletions, Klinefelter syndrome, and other karyotype abnormalities), history of cytotoxic chemotherapy, and previous scrotal/inguinal surgery. The definition of iFSH was based on local laboratory reference values; it was defined as a serum FSH level $>$ 12.4 mIU/mL and an LH level \leq 8.6 mIU/mL. Characteristics of the study population are shown in Table 1. If any individual case lacked some of the aforementioned data, it was excluded from final analysis.

4. Reproductive outcomes

We evaluated sperm retrieval as a direct outcome of the microTESE procedure. It should be emphasized that individual sperm retrieval attempt was considered to be successful when sperm of appropriate quality were found in sufficient quantity for cryopreservation and/or immediate intracytoplasmic injection (ICSI). Cases where solitary non-viable or non-usable sperm were found during microTESE were dismissed as failed attempts. A receiver operating characteristic (ROC) curve analysis was performed for potential predictors of successful sperm retrieval.

We also evaluated the live birth rate (LBR) in the wives of patients who were included in this study. The precise embryological outcomes of ICSI such as the fertilization rate, embryo quality, biochemical pregnancy rate, and clinical pregnancy rate, were not studied, as

Table 1. Characteristics of the study population

Variable	Value
Age (yr)	33 (30–38)
Inhibin B (pg/mL)	53 (20–103)
Testosterone (nmol/L)	12 (8–18)
LH (mIU/mL)	6.7 (4.2–12)
FSH (mIU/mL)	14 (6–25)
Estradiol (pg/mL)	93 (59–138)
Testicular volume (cm ³)	15 (11–22)
Klinefelter syndrome	27 (4.5)
Robertsonian translocation	22 (3.6)
AZFc deletion	36 (5.9)
Varicocele	175 (28.9)
Cryptorchidism	33 (5.4)
Chemotherapy or radiation therapy	12 (2.0)

Values are presented as median (interquartile range) or number (%). LH, luteinizing hormone; FSH, follicle-stimulating hormone; AZFc, azoospermia factor c.

this information was not available for a significant proportion of patients; however, this gap in the data is not critically important considering the nature of this study and the availability of final LBR data.

5. Pathology outcomes

The testicular pathology results were stratified into hypospermatogenesis, maturation arrest, SCO, and complete tubular atrophy according to the predominant pattern as identified by a pathologist. Considering possible inconsistencies between this “dominant pattern” and the true likelihood of successful sperm retrieval, we also evaluated the percentage of tubules with different stages of spermatogenesis (no spermatogenesis, maturation arrest with presence of round spermatids, and complete spermatogenesis). In order to facilitate a statistical analysis, we also presented this data as a numerical variable based on the Bergmann-Kliesch score (BKS) [8]. SCO was defined as a complete absence of germ cells, with only Sertoli cells being present in a sample. Maturation arrest was reported if seminiferous tubules contained germ cells, but none of them had reached beyond a specific stage of spermatogenesis (such as round spermatids, spermatocytes, or spermatogonia). If the sample contained elongated spermatids, but in fewer than 75% of seminiferous tubules (BKS $<$ 8), the term “hypospermatogenesis” was applied. Finally, samples with a BKS of 8–10 were considered to demonstrate preserved normal spermatogenesis.

6. Statistical analysis

Descriptive statistics were presented for the iFSH subgroup. The normality of the distribution of numerical variables was checked with the Kolmogorov-Smirnov test. We compared the SRR as a primary endpoint in azoospermic iFSH patients and in other NOA patients using the chi-square test. The BKS and levels of inhibin B (thought to be more or less independent of gonadotropin signaling) were compared using the Mann-Whitney *U*-test. The difference in the SRR between iFSH patients and the rest of the sample was used as the primary outcome measure, and differences in BKS and inhibin B levels were secondary outcome measures. The *p*-values $<$ 0.05 were considered to indicate statistical significance. IBM SPSS ver. 23 (IBM Corp., Armonk, NY, USA) was used for the statistical analysis.

Results

1. Reproductive outcomes of microTESE

Sperm were successfully retrieved in 188 cases, and the overall SRR was 33.3% per microTESE attempt. ICSI with surgically retrieved spermatozoa led to 78 live births overall. The LBR was 13.8% per couple. The ROC analysis for potential predictors of microTESE success revealed that the AUC for inhibin B was 0.763 (95% confidence interval

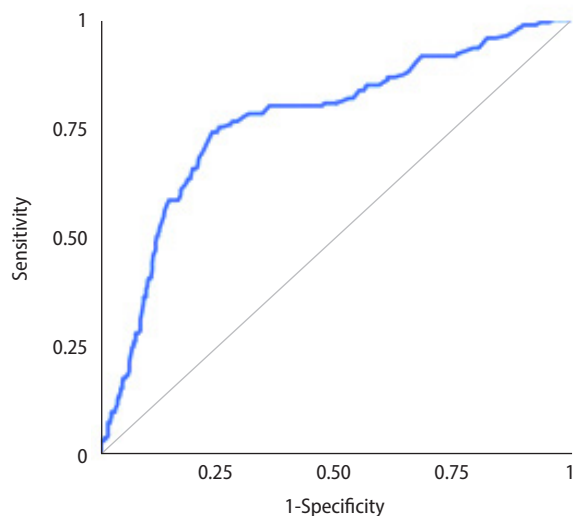


Figure 1. Receiver operating characteristic curve for inhibin B as a potential predictor of successful microdissection testicular sperm extraction.

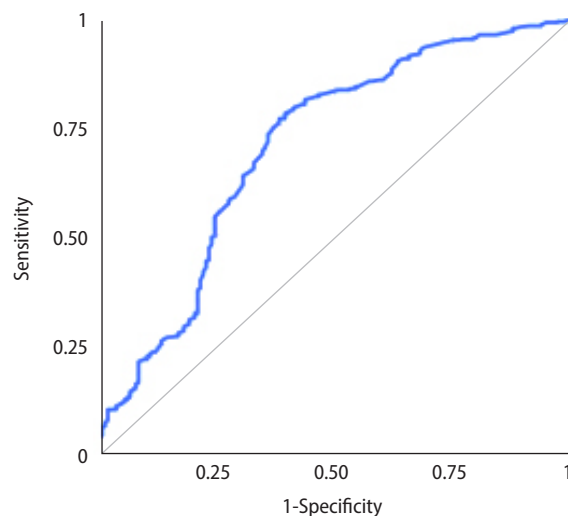


Figure 2. Receiver operating characteristic curve for follicle-stimulating hormone as a potential predictor of successful microdissection testicular sperm extraction.

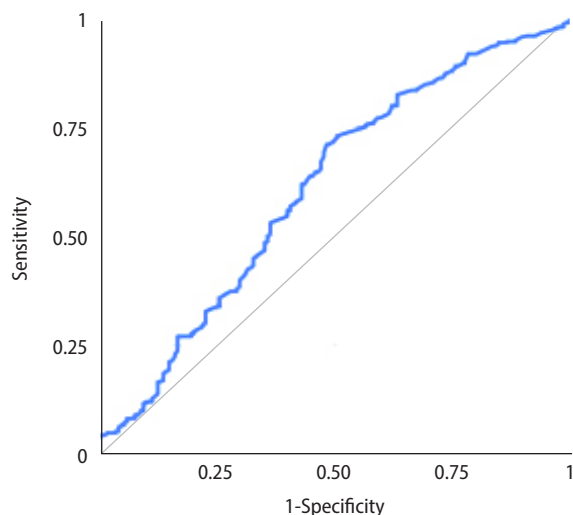


Figure 3. Receiver operating characteristic curve for luteinizing hormone as a potential predictor of successful microdissection testicular sperm extraction.

[CI], 0.718–0.808) (Figure 1). The AUC for testosterone was 0.543 (95% CI, 0.492–0.594), and the AUC for testicular volume was 0.762 (95% CI, 0.718–0.807). Inverted ROC curves for gonadotropin levels are shown in Figures 2 and 3. The AUC for FSH was 0.704 (95% CI, 0.654–0.754), while LH performed poorly, with an AUC of 0.608 (95% CI, 0.555–0.66).

2. Pathology outcomes of microTESE

SCO or severe atrophy affecting 100% of the seminiferous tubules

was found in 183 cases (32.4%). Complete maturation arrest was relatively rare, and was found in nine cases (1.6%). In 63 cases (11.1%), SCO histology and maturation arrest overlapped. In the remaining 310 cases (54.9%), at least 1% of the seminiferous tubules contained elongated spermatids, which may be described as hypospermatogenesis.

Only a minority of cases ($n = 27$, 4.8%) had a BKS of 8–9, which presumably corresponds to normal spermatogenesis at the site where the biopsy was taken (not reflecting spermatogenesis in the entire testes). No patients had a BKS of 10. In 201 cases (35.6%), the BKS was within the range of 1 to 7, which is interpreted as mixed testicular atrophy. In 123 cases (21.8%), the BKS was below 1, meaning that only 1%–9% of tubules contained elongated spermatids. Thus, the median BKS was 0.6 (interquartile range, 0–2).

Among 27 biopsy attempts with a BKS of 8–9 on pathology, 24 were successful (88.9%). The three failures are explained by the fact that not enough viable sperm usable for ICSI were found despite the presence of elongated spermatids. Since we do not perform elongated spermatid injection, these attempts were deemed unsuccessful. Eighty-five biopsy attempts (42.3%) were successful in patients with mixed testicular atrophy, while, surprisingly, 79 biopsy attempts (64.2%) were successful when the BKS was below 1. As expected, the SRR was 0% when all seminiferous tubules had SCO or complete atrophy.

3. Outcomes in the iFSH group

Overall, 132 of all NOA patients had iFSH prior to the sperm retrieval attempt. MicroTESE was successful in only 11 cases. The SRR in this

category was 8.3%. In comparison, the total SRR of other NOA patients taken together was 38.1%. The difference was statistically significant ($p < 0.001$). The odds ratio for successful sperm retrieval in the iFSH group was 0.160 (95% CI, 0.084–0.305).

Descriptive statistics for the iFSH patients are presented in Table 2. Interestingly enough, only 24 patients in the iFSH group had serum testosterone concentrations below 9 nmol/L, which reflects mostly normal Leydig cell function in this subpopulation. The median inhibin B level was 31 pg/mL and 65 pg/mL in the iFSH group and the rest of the patients, respectively ($p < 0.001$). During microdissection, the testicular parenchyma was markedly homogeneous, with the appearance of yellow-brown “barren” tubules without any sites visually resembling areas of focal spermatogenesis (Figure 4).

Indeed, on testicular pathology, 82 samples (63.6%) had complete SCO histology or tubular atrophy, and in one case (0.8%) there was complete maturation arrest. In 36 other cases (27.3%), maturation arrest co-existed with SCO histology without any evidence of advanced stages of spermiogenesis. However, in the remaining 11 cases

(17.8%), at least 1% of seminiferous tubules contained elongated spermatids and microTESE resulted in positive sperm retrieval. Among them, unexpectedly, there was one case with a BKS of 8, three cases of mixed testicular atrophy (BKS of 4, 4, and 1, respectively), and the other cases had BKS of 0.1–0.6. The median BKS was 0 and 0.6 in the iFSH group and the rest of the patients, respectively ($p < 0.001$).

We evaluated the predictive parameters of iFSH status as a potential stand-alone predictor for negative microTESE (Table 3). It demonstrated poor sensitivity (32.1%; 95% CI, 27.4%–36.8%) coupled with excellent specificity (94.1%; 95% CI, 90.8%–97.5%). The positive predictive value for microTESE failure was 91.7% (95% CI, 86.9%–96.4%), and the negative predictive value for microTESE failure was 40.9% (95% CI, 36.2%–45.5%).

Discussion

FSH is one of the main hormonal factors that regulate spermatogenesis. Its biological actions are mediated through receptors located on the surface of Sertoli cells [9]. It is well known that mutations affecting FSH or FSH receptor structure may have deleterious impacts on male fertility, although such cases are rarely observed in clinical practice [10,11]. Although the idea that iFSH-associated azoospermia has very poor SRR outcomes is not essentially new, we hypothesize that it represents the most adverse phenotype in the azoospermia landscape [5].

In research and clinical practice, the FSH level is treated as a serum marker for sperm retrieval failure, but this premise is biased. There is a weak, if any, linear association between the numerical value of serum FSH concentration and the likelihood of preserved spermatogenesis. The pooled analysis by Yang et al. [12] revealed an AUC of

Table 2. Characteristics of patients with isolated elevation of serum FSH

Variable	Value
Age (yr)	32 (29–36)
Inhibin B (pg/mL)	31 (13–60)
Testosterone (nmol/L)	13 (9.6–17.8)
LH (mIU/mL)	5.85 (4.58–7.10)
FSH (mIU/mL)	17.5 (14.9–22.7)
Estradiol (pg/mL)	87 (47–127)
Testicular volume (cm ³)	14.6 (11–17)
Klinefelter syndrome	4 (3.0)
Robertsonian translocations	5 (3.8)
AZFc deletion	11 (8.3)
Varicocele	33 (25.0)
Cryptorchidism	6 (4.5)
Chemotherapy or radiation therapy	7 (5.3)

Values are presented as median (interquartile range) or number (%). FSH, follicle-stimulating hormone; LH, luteinizing hormone; AZFc, azoospermia factor c.

Table 3. Sensitivity, specificity, and predictive values of isolated elevation of FSH for microTESE failure

Variable	Sperm retrieval failure	Successful sperm retrieval	Measure
iFSH	121	11	PPV: 91.7%
non-iFSH	256	177	NPV: 40.9%
Measure	Sensitivity: 32.1%	Specificity: 94.1%	Accuracy: 52.7%

FSH, follicle-stimulating hormone; microTESE, microdissection testicular sperm extraction; iFSH, isolated elevation of serum FSH; PPV, positive predictive value; NPV, negative predictive value.



Figure 4. Homogenous seminiferous tubules in a patient with isolated elevation of follicle-stimulating hormone.

0.72 ± 0.04 in a ROC curve analysis of FSH as a sperm retrieval predictor; this AUC is moderate at best and reflects the inadequacy of FSH as a standalone marker. In a recent study by Zeadna et al. [13], FSH was likewise not found to be a valuable predictor of the SSR.

In a study by Kelsey et al. [14], the FSH level had a fair AUC of 0.89 in simply distinguishing azoospermic and non-azoospermic childhood cancer survivors; however, with specificity and sensitivity of 81% and 83%, respectively, in this setting it is doubtful that the FSH level would be able to solve the significantly more delicate task of distinguishing azoospermic patients with focal spermatogenesis. Controversially, some authors have even described a positive association between FSH and the SRR [15]. We assume that this discrepancy can be resolved if we do not interpret the FSH level as a continuous variable, but rather as a flag of certain testicular phenotypes, such as the one we attempted to describe in this paper.

Interestingly, FSH levels in patients with iFSH-associated azoospermia tend to be only mildly or moderately elevated. This pattern can be explained by the involvement of a large number of factors in hypothalamic-pituitary-gonadal (HPG) regulation. Severe disruption of spermatogenesis is characterized by low levels of inhibin B, which means that this negative feedback loop is inactive, leading to uninhibited LH and FSH production. However, when Leydig cells are functioning normally, testosterone and estradiol exert proper negative feedback, which limits gonadotropin release so that LH remains borderline normal and FSH remains mildly elevated. This difference is due to the different effects of gonadotropin-releasing hormone (GnRH) on their production; in short, FSH seems to be less dependent on GnRH pulse frequency and seems to have a basal constitutive secretion [16]. Furthermore, inhibin B directly blocks activin-induced FSH secretion by pituitary gonadotropes, while its influence on LH secretion is limited [17]. This scenario is possible in cases of germ cell aplasia or, possibly, selective death of germ cells due to viral infection or another testicular insult that leaves interstitial testosterone-producing cells intact. In contrast with iFSH-associated azoospermia, in complete testicular failure with impaired spermatogenesis and sex steroid synthesis, both negative feedback loops are broken, which results in full-blown hypergonadotropic hypogonadism.

As for the difference in the SRR, germ cell aplasia or precise depopulation due to currently unrecognized factors would lead to a virtually zero chance of sperm retrieval (iFSH scenario). However, gross damage to the testicular parenchyma may actually leave some tubules relatively intact, with evidence of their continuing function being masked by the overwhelming HPG response when two major loops of negative feedback are severed (the hypergonadotropic hypogonadism scenario). In fact, it is the latter scenario in which microTESE technique works best. A study by Yu et al. [18] paradoxically demonstrated that patients with narrower seminiferous tubules and

higher FSH levels had better chances of successful microTESE. Their findings may actually reflect the same phenomenon and even provide some indirect morphological proof. The relatively good predictive value of FSH for sperm retrieval in conventional TESE (cTESE), as observed by Gnessi et al. [19], could be a result of bias. Patients with hypergonadotropic hypogonadism and focal spermatogenesis benefit less from cTESE than they would benefit from microTESE, which negates the principal difference between them and patients with iFSH. This results in a more uniform SRR and similarly poor outcomes in all FSH groups. Some successful cTESE attempts in mildly hypergonadotropic patients with hypospermatogenesis would be possible, which would give the appearance of a “classic” inverse relationship between FSH and the SRR. Indeed, the aforementioned meta-analysis by Yang et al. [12] showed a reasonable AUC of 0.72 for FSH, but it included studies on both cTESE and microTESE. The same logic would apply to testicular sperm aspiration (TESA), which is essentially random, like cTESE, while being less invasive. A recent study by Liu et al. [20] revealed a negative correlation between the FSH level and the SRR for TESA, as expected; however, there was a positive correlation between the FSH level and microTESE success. In a study by Zhu et al. [21], FSH had an AUC of 0.87 for successful surgical sperm retrieval, but the majority of patients underwent simple cTESE. Nonetheless, the meta-analysis by Li et al. [3], based strictly on microTESE studies, demonstrated an abysmal AUC of 0.612. The AUC in our series (0.704) is well within the range of values observed by other researchers.

In microTESE, a U-shaped association between FSH and the SRR was described. The concept of iFSH-associated azoospermia fits this concept perfectly. In these adverse cases, FSH tends to be moderately elevated, which forms a “low SRR valley” in the U-shaped FSH-SRR curve. Ramasamy et al. [4] observed this “valley” with an SRR of 51% at FSH levels less than 15 IU/mL, while the SRR in other groups was over 60%. In another study, the worst prognosis for sperm retrieval was observed for FSH levels within the 10–15 IU/mL range, though it described a specific subpopulation of patients with SCO histology on diagnostic testicular biopsy [2]. Zhang et al. [22] also demonstrated poor microTESE outcomes with a “low SRR valley” in patients with moderate elevation of FSH, although only when the testicular volume was low. The authors [22] analyzed FSH and LH levels separately, so it is unknown whether their results could be influenced by the iFSH pattern. If the “low SRR valley” hypothesis is true, it invalidates the concept of an “FSH cut-off point” to select patients in whom microTESE is allegedly pointless. However, Chen et al. [23] reportedly found that an FSH cut-off point of 19.4 IU/L predicted the absence of testicular sperm, although their study was based only on a direct comparison of average FSH levels in patients with positive and negative testicular biopsies. The mean reported FSH level in patients with

successful sperm retrieval was only 7.94 ± 4.95 IU/L, which hints at the possible contamination of this group by obstructive and transitory azoospermia cases.

Most iFSH patients had idiopathic azoospermia, which is likely due to unrecognized mutations that are deleterious for germ cells. A recent study by Das et al. [24] is of interest in this context, as idiopathic NOA patients had significantly lower (though still out of reference range) levels of FSH than patients with a known etiology of NOA. The authors [24] did not report LH levels in their cohort, so it is not possible to speculate whether their patients had iFSH. However, considering that testosterone levels were higher in idiopathic NOA patients, this might be the case. We also had seven patients who previously underwent cytotoxic cancer treatment, which, as a side effect, possibly eradicated the germ cell epithelium. Norwegian researchers specifically described this as a possible adverse event in patients treated for malignant lymphomas, naming it “exocrine hypogonadism” [25]. The FSH elevation observed in human males with age may also be related to a gradual depletion of germ cells [26]. In theory, any man who would live indefinitely long would eventually develop iFSH-elevated azoospermia.

Our study is limited by its retrospective nature, lack of an adequate control group, and the fact that iFSH definition was based on local laboratory reference values. FSH production depends on many factors beyond feedback, including the general responsiveness of the HPG axis, gonadotrope population, GnRH pulse frequency, and basal FSH production. Laboratory reference values are not universal in a biological sense, and even while cases with a moderately elevated FSH level and a borderline elevated LH level could potentially bear the same iFSH-associated phenotype, they were not considered to have iFSH due to the reliance on reference values. Therefore, results obtained in this study should not be generalized, but could be used to inform further research.

In this paper, we made an attempt to describe isolated FSH elevation as a distinct clinical pattern in patients with azoospermia, and also frequently mentioned hypergonadotropic hypogonadism as another typical pattern in azoospermic patients, mainly as a reference point for comparisons. Other specific phenotypes of NOA may exist, and they need to be properly described. In our opinion, numerical variables that reflect endocrine parameters are easy to analyze and serve as convenient candidate markers of reproductive outcomes and nomogram material, but we need to move on to recognize non-linear associations and patterns in NOA.

Conflict of interest

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

ORCID

Safar Gamidov	https://orcid.org/0000-0002-9128-2714
Taras Shatylko	https://orcid.org/0000-0002-3902-9236
Alina Popova	https://orcid.org/0000-0003-1163-5602
Natig Gasanov	https://orcid.org/0000-0003-4695-9789
Gennadiy Sukhikh	https://orcid.org/0000-0002-7712-1260

Author contributions

Conceptualization: SG, TS, AP, GS. Investigation: SG, TS, AP, NG. Data curation: SG, TS, AP. Formal analysis: TS. Methodology: TS. Writing—original draft: TS. Writing—review & editing: SG, AP, NG, GS.

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Efficacy of subcutaneous granulocyte colony-stimulating factor infusion for treating thin endometrium

Kaberi Banerjee, Bhavana Singla, Priyanka Verma

Advance Fertility and Gynaecology Centre, New Delhi, India

Objective: This study was conducted to assess the efficacy of subcutaneous granulocyte colony-stimulating factor (G-CSF) for treating thin endometrium.

Methods: Data from 88 infertile women with thin endometrium (<7 mm) aged 23 to 40 years were evaluated retrospectively over a period of 1 year. In group 1, subcutaneous infusion of G-CSF (300 µg/mL) was administered to 44 women along with other supplemental treatments. If the lining did not exceed 7 mm within 72 hours, a second infusion was administered. In group 2, which also had 44 women, only estradiol valerate and sildenafil were administered, while subcutaneous G-CSF infusion was not. Embryo transfers were performed once the lining exceeded 7.5 mm. The efficacy of G-CSF was evaluated by assessing the thickness of the endometrium before embryo transfer, pregnancy rates, and clinical pregnancy rates.

Results: There were no differences between the groups regarding demographic variables, egg reserves, sperm parameters, the number of embryos transferred, and embryo quality. The pregnancy rate was significantly higher in group 1 (60%, 24 of 40 cases) than in group 2 (31%, 9 of 29 cases) ($p < 0.001$). The clinical pregnancy rate was also significantly higher in group 1 (55%) than in group 2 (24%) ($p < 0.001$).

Conclusion: Subcutaneous G-CSF infusion improved the thickness of the endometrium when it was thin. To the best of our knowledge, this is the first documented study to clearly demonstrate the benefits of subcutaneous G-CSF infusion for treating thin endometrium.

Keywords: Granulocyte colony-stimulating factor; Infusion; Subcutaneous; Thin endometrium

Introduction

Despite recent technological developments, *in vitro* fertilization (IVF) is still not a surefire solution to infertility, and implantation failure remains a major problem that leads to unsuccessful IVF. Successful implantation depends on many factors such as egg quality, sperm quality, embryo quality, the receptivity of the endometrium, and the quality of the embryo transfer technique [1]. The endometrium is

very important for embryo implantation, and endometrial thickness is a marker of endometrial receptivity. It is used as a prognostic factor in embryo transfers [2,3]. Adequate endometrial thickness is essential for a successful pregnancy. A thin endometrium adversely affects the rate of successful reproduction using IVF. The definition of a thin endometrium varies across studies, but it is generally defined as an endometrium of <7 mm on the day of human chorionic gonadotropin (hCG) injection during fresh embryo transfer cycles and <7–8 mm on the day that progesterone supplementation is added for frozen-thawed cycles [4–6]. The reported prevalence of thin endometrium in assisted reproductive technology patients is between 1.5% and 9.1% [6,7].

There are various treatment regimens that aim to improve endometrial thickness, but only some have been found to yield actual improvements. Granulocyte colony-stimulating factor (G-CSF) has recently been suggested as a possible treatment for thin endometrium

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Corresponding author: **Bhavana Singla**

Advance Fertility and Gynaecology Centre, 6, Ring Rd, Lajpat Nagar 4, New Delhi 110049, India

Tel: +91-98-7109-6615 Fax: +91-93-5000-5568 E-mail: drbhavanasingla@gmail.com

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[8,9]. G-CSF is a glycoprotein that combines growth factor and cytokine activities. It is secreted in various tissues, including reproductive tissues such as the endothelium and ovarian follicles, as well as immunocytes (e.g., macrophages) [10]. It stimulates neutrophilic granulocyte proliferation and differentiation and acts on decidual macrophages, affecting implantation. It also recruits dendritic cells, promotes Th-2 cytokine secretion, activates T regulatory cells, and stimulates various proangiogenic effects that also affect implantation [11]. In most studies, intrauterine infusions of G-CSF were administered to improve the uterine lining [8,9]. However, G-CSF can also be administered subcutaneously to improve the thickness of the endometrium [12]. In this study, we examined the efficacy of subcutaneous G-CSF infusion for treating thin endometrium.

Methods

The study was conducted at the Advance Fertility and Gynaecology Centre in New Delhi, India, from January 2019 to December 2019. Patients with primary and secondary infertility between the ages of 23 to 40 years were included in the study. In total, 88 infertile women were examined, and the inclusion criteria were as follows. (1) A history of at least one previous IVF failure and thin endometrium (< 7 mm) 15 to 18 days into a regular 28-to-30-day cycle. (2) A history of canceled embryo transfer due to thin endometrium (< 7 mm) on the day of hCG injection. (3) A history of thin endometrium (< 7 mm) 12 or 13 days after beginning estrogen supplementation (6 to 10 mg/day).

In group 1, 44 women undergoing either fresh or frozen cycles were examined. Those undergoing fresh cycles received subcutaneous injections of G-CSF (300 µg/mL) starting on day 7 of hormonal injection along with oral estradiol valerate (4 to 6 mg/day) to increase endometrial thickness and vaginal sildenafil (50 mg/day) to increase uterine blood flow. Those undergoing frozen cycles received oral estradiol valerate (6 mg/day) starting on day 2 of the menstrual cycle and subcutaneous injections of G-CSF (300 µg/mL) starting on the 7th day after beginning medication, along with an increased dose of oral estradiol valerate (10 mg/day) and vaginal sildenafil (50 mg/day). If the endometrial thickness did not exceed 7.5 mm within 72 hours, a second injection was given, and the other supplements were continued. A final G-CSF injection was administered on the day of oocyte retrieval for those undergoing fresh cycles and on the day that progesterone was added for those undergoing frozen cycles.

In group 2, which also included 44 women, estradiol valerate and sildenafil were given to those undergoing fresh and frozen cycles like in group 1; however, subcutaneous G-CSF infusion was not administered (Figure 1). Embryo transfer was performed only when the endometrial thickness exceeded 7.5 mm and there was subjective improvement in the echotexture of the lining based on two-dimen-

sional ultrasonography. The efficacy of G-CSF was evaluated by assessing endometrium thickness before embryo transfer, the pregnancy rate, and the clinical pregnancy rate.

Results

There were no significant differences between the groups regarding demographic variables, ovarian reserve (donor eggs were used in instances of low ovarian reserve), sperm parameters, number of embryos transferred, and embryo quality (Table 1).

In group 1, the embryo transfer was cancelled in four cases, and in group 2, there were 15 cancellations due to thin endometrium even after treatment, showing statistical significance ($p = 0.008$). The pregnancy rate was significantly higher in group 1 (60%, 24 out of 40 cases) than in group 2 (31%, 9 out of 29 cases) ($p < 0.001$). The clinical pregnancy rate was also significantly higher in group 1 (55%) than in group 2 (24%) ($p < 0.001$) (Table 2).

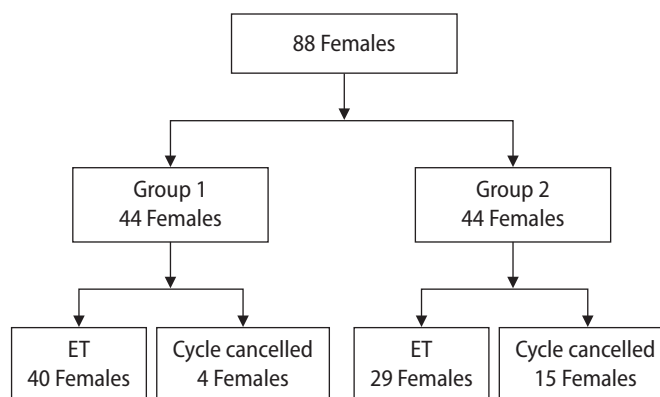


Figure 1. Flow diagram of study design. ET, embryo transfer.

Table 1. Demographic profile

Parameter	Group 1 (with G-CSF)	Group 2 (without G-CSF)	<i>p</i> -value
Number of patients	44	44	
Age (yr)	31.6	31.8	0.12
Length of infertility (yr)	4.3	5	0.47
Primary	25	23	1.12
Secondary	19	21	1.12
BMI (kg/m ²)	20.8	21	0.22
Antral follicle count	8.4	8.1	0.47
AMH level (ng/mL)	2.7	2.8	0.17

G-CSF, granulocyte colony-stimulating factor; BMI, body mass index; AMH, anti-Müllerian hormone.

Table 2. IVF outcome

Parameter	Group 1 (with G-CSF, n = 44)	Group 2 (without G-CSF, n = 44)	p-value
ET > 7.5 mm	40	29	< 0.001
Cycle cancelled	4	15	< 0.001
Pregnancy rate	24/40 (60)	9/29 (31)	< 0.001
Clinical pregnancy rate	22/40 (55)	7/29 (24)	< 0.001

Values are presented as number or number (%).

IVF, *in vitro* fertilization; G-CSF, granulocyte colony-stimulating factor; ET, endometrial thickness.

Discussion

Endometrial thickness is a marker of endometrial receptivity and is important for embryo implantation. Various treatments have been suggested in studies for improving endometrial thickness, but some remain unproven due to the limited number of subjects. In cases of thin endometrium, embryos from that particular cycle are frozen, and the endometrium is prepared again in order to perform an optimal embryo transfer.

G-CSF is an emerging treatment method for thin endometrium that has shown promising results. In our study, we assessed the effect of subcutaneous G-CSF infusion for improving the thickness of the endometrium. Zhang et al. [13] conducted a meta-analysis of 10 randomized control studies involving 1,016 IVF embryo transfer cycles, found that treatment with G-CSF infusion improved clinical outcomes after embryo transfer when performed using both local and systematic infusion, especially in cases of repeated implantation failure, and concluded that further randomized control trials are needed to investigate the efficacy of G-CSF infusion for patients with thin endometrium [13]. Most previous studies assessed the intrauterine effects of G-CSF infusion on thin endometrium. Gleicher et al. [8] and Kunicki et al. [9] found that intrauterine G-CSF infusion was effective for treating chronically thin endometrium and that the thickness of the endometrium significantly increased after G-CSF infusion, though it did not vary between conception and non-conception cycles. Both studies measured an ongoing clinical pregnancy rate of approximately 19%. Barad et al. [14] reported that G-CSF did not affect endometrial thickness, implantation rates, or clinical pregnancy rates among healthy IVF patients with normal endometrium or older IVF patients. Davari-Tanha et al. [15] conducted a double-blind placebo randomized control trial with 100 subjects in whom 300- μ g intrauterine infusions of G-CSF were performed, and it was found that the infusions may have increased the chemical pregnancy and implantation rates of patients with recurring implantation failure; however, the clinical pregnancy rate and miscarriage rate were not affected. Very few studies have assessed the efficacy of subcutaneous G-CSF infusion for treating repeated implantation failure. Aleyasin et al. [12]

found that single-dose systemic subcutaneous G-CSF infusion before implantation significantly increased the rates of successful IVF, implantation, and pregnancy (44.6%) in infertile women with repeated IVF failure. Scarpellini and Sbracia [16,17] found that G-CSF infusion might be effective for treating unexplained recurrent miscarriage and repeated implantation failure. Kamath et al. [18], in a Cochrane review, expressed uncertainty about the role of G-CSF for treating thin endometrium, and stated that the quality of the evidence suggesting that G-CSF infusion may improve the clinical pregnancy rate in women who have experienced two or more IVF failures was low. Zhao et al. [19] found that subcutaneous G-CSF infusion resulted in significantly higher pregnancy and implantation rates compared to the control group, whereas G-CSF administered via local uterine infusion had no beneficial effects on pregnancy and implantation rates in cases of recurrent IVF failure. Another recent study found that G-CSF infusion improved endometrial thickness regardless of whether the intrauterine or subcutaneous method was used. Although the intrauterine method showed slightly better results than the subcutaneous method, the degree of improvement was not statistically significant. Hence, the subcutaneous method can still be offered to patients, making it a viable option for performing G-CSF infusions to improve endometrial thickness and flow in patients with thin endometrium undergoing an embryo transfer [20].

Our study demonstrated the beneficial effects of G-CSF infusion using the subcutaneous method for treating thin endometrium, which is easier to administer than the intrauterine infusion method and does not require any extra steps. Subcutaneous G-CSF infusion was found to increase endometrial thickness and pregnancy rates in the study subjects. Other studies have examined the effects of subcutaneous G-CSF infusion in cases of recurrent IVF failure and unexplained recurrent miscarriages, but its effects on thin endometrium have not yet been established. Larger cohort studies are required in the future to further examine the effects of subcutaneous G-CSF infusion on thin endometrium. To the best of our knowledge, this is the first documented study to clearly demonstrate the beneficial effects of subcutaneous G-CSF infusion on thin endometrium.

Conflict of interest

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

ORCID

Kaberi Banerjee
Bhavana Singla
Priyanka Verma

<https://orcid.org/0000-0002-3744-7004>
<https://orcid.org/0000-0003-3151-9334>
<https://orcid.org/0000-0003-0011-3593>

Author contributions

Conceptualization: KB. Data curation: PV. Formal analysis: BS. Methodology: BS. Project administration: KB. Visualization: BS. Writing—original draft: BS. Writing—review & editing: BS.

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- Original articles are papers reporting the results of basic and clinical investigations that are sufficiently well documented to be acceptable to critical readers.
- Case reports deal with clinical cases of medical interest or innovation.
- Brief communications are short original research articles on issues important to medical and biological researchers.
- Letter to editor includes a reader's comment on an article published in CERM and a reply from the authors.

VI. PREPARATION OF MANUSCRIPTS

1. General guideline

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

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2. Original article

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

1) Title page

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

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

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

4) **Methods:** Describe the research plan, the materials (or subjects), and the methods used, in that order. Explain in detail how the disease was confirmed and how subjectivity in observations was controlled. When

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

- 5) **Results:** The results should be presented in logical sequence in the text, tables, and illustrations. Do not repeat in the text all data in the tables or figures, but describe important points and trends.
- 6) **Discussion:** Observations pertaining to the results of research and other related materials should be interpreted for your readers. Emphasize new and important observations; do not merely repeat the contents in the Introduction or Results. Explain the meaning of the observed opinion along with its limits, and within the limits of the research results connect the conclusion to the purpose of the research.
- 7) **Acknowledgments:** Persons who have contributed intellectually to the paper but whose contributions do not justify authorship may be named and their function or contribution described, e.g., “scientific adviser,” “data collections,” or “participation in clinical trial.” Such persons must have given their permission to be named. Authors are responsible for obtaining written permission from the persons acknowledged by name, because readers may infer their endorsement of the data and conclusions.
- 8) **ORCID (Open Researcher and Contributor ID):** Authors are recommended to provide an ORCID. To obtain an ORCID, authors should register in the ORCID website: <https://orcid.org>. Registration is free to every researcher in the world.
- 9) **Author contributions:** What authors have done for the study should be described in this section. To qualify for authorship, all contributors must meet at least one of the seven core contributions by CRediT (conceptualization, methodology, software, validation, formal analysis, investigation, data curation), as well as at least one of the writing contributions (original draft preparation, review and editing). Authors

may also satisfy the other remaining contributions; however, these alone will not qualify them for authorship. Contributions will be published with the final article, and they should accurately reflect contributions to the work. The submitting author is responsible for completing this information at submission, and it is expected that all authors will have reviewed, discussed, and agreed to their individual contributions ahead of this time. The information concerning sources of author contributions should be included in this section at submitting the final version of manuscript (at the first submission, this information should be included in title page).

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.

- 10) **References:** Number references consecutively in the order in which they are first mentioned in the text. References are identified by Arabic numerals in square bracket []. Unpublished observations, and personal communications should not be used as references, although references to written, not oral communications may be inserted (in parentheses) in the text. Abstracts published in a citable journal may be cited. To cite a paper accepted but not yet published, state the paper’s DOI number. References must be verified by the author(s) against the original documents. The titles of journals should be abbreviated according to the style used in Index Medicus (United States National Library of Medicine). List all authors in an article, but if the number exceeds six, give six followed by et al. Other types of references not described below should follow Citing Medicine: The NLM Style Guide for Authors, Editors, and Publishers (<http://www.ncbi.nlm.nih.gov/books/NBK7256/>).

Examples of references

(1) Journal article

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

(2) Website

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

(3) Book

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

(4) In press

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

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

12) **Figures:** Each figure should be submitted in a separate file, at a resolution of 600 dpi for photos and 1,200 dpi for line art. Lettering and identifying marks should be clear, and type size should be consistent on each figure. Capital letters should be used for specific areas of identification in a figure. Symbols, lettering, and numbering should be distinctly recognizable so that when the figure is reduced for publication each item will still be legible. Titles and detailed explanations belong in the figure legends, not on the illustrations themselves. Do not include figure legends in the same file as the figure.

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

3. Review article

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

4. Case report

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

5. Brief communication

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

6. Letter to the editor

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

VII. AUTHOR'S MANUSCRIPT CHECKLIST

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

VIII. PEER REVIEW PROCESS

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

responding authors. Revised manuscripts must be submitted online by the corresponding author. The corresponding author must indicate the alterations that have been made in response to the referees' comments item by item in response note. Failure to resubmit the revised manuscript within 8 weeks of the editorial decision is regarded as a withdrawal. Please notify the editorial office if additional time is needed or if you choose not to submit a revision. Authors can track the progress of a manuscript on the journal's web-site. Articles that are accepted for publication are listed in the "Articles in Press" section of the journal's website. The manuscript, when published, will become the property of the journal. All published papers become the permanent property of the Korean Society for Reproductive Medicine, and must not be published elsewhere without written permission.

Any appeal against the editorial decision to publish a text must be made within 2 weeks of the date of the decision letter. Authors who wish to appeal a decision should contact the Editor-in-Chief, explaining in detail their reasons for the appeal. All appeals will be discussed with at least one other associate editor. If the associate editor(s) does not agree, the appeal will be discussed at a full editorial meeting. CERM does not consider any second appeals and will reject any that are submitted regarding a manuscript.

IX. MANUSCRIPT ACCEPTED FOR PUBLICATION

1. Final version

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

2. Manuscript corrections

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

3. Galley proof

CERM provides the corresponding author with galley proofs for their correction. Corrections should be kept to minimum on these proofs to avoid a complete rewriting of the manuscript at that time. The Editor retains the prerogative to question minor stylistic alterations and major alterations that have been made by Editors that might affect the scientific content of the paper. Fault found after the publication is a responsibility of the authors. We urge our contributors to proofread and their accepted manuscript very carefully before acknowledging the manuscript as completed and ready for publishing. The corresponding author may be contacted by the Editorial Office, depending on the nature of correction in proof. If the proof is not returned to the Editorial Office within 48 hours, it may be necessary to reschedule the paper for a subsequent issue.

X. ARTICLE PROCESSING CHARGES

There is no page charge except for color printing. For color printing, a fee of KRW 150,000 (USD 150) will be charged per page. A minimum of 10 offprints will be provided on request, at the author's expense. An Offprint Order Form outlining the cost will be sent to the corresponding author with the page proofs.

XI. FEEDBACK AFTER PUBLICATION

1. Errors

If the authors or readers find any errors present in the manuscript as written, or any contents information that should be revised, these changes can be requested from the Editorial Board. The Editorial Board may consider erratum, corrigendum, or a retraction. If there are any revisions to the article, there will be a CrossMark description to announce the final draft. If there is a reader's opinion on the published article with the form of Letter to the Editor, it will be forwarded to the authors for subsequent review. The authors are able to reply to the reader's letter. The letter to the editor and the author's reply may be also published.

2. Complaints and appeals

The policy of *CERM* is primarily aimed at protecting the authors, reviewers, editors, and the publisher of the journal. The process of handling complaints and appeals follows the guidelines of the COPE as noted as available from: <https://publicationethics.org/appeals>.

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Clinical and Experimental Reproductive Medicine
Obstetrics and Gynecology CHA Bundang Medical Center,
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Address: 59 Yatap-ro, Bundang-gu, Seongnam-si,
Gyeonggi-do, Korea
Tel: +82-31-727-8701
E-mail: hwas0605@cha.ac.kr