CONNECTING YOUR EXPERTISE TO TAILORED TREATMENTS

GONAL-f®: The world’s most prescribed rFSH
고날-에프 팬으로 전 세계에서 330 만명 이상의 야기가 테어났습니다

- 김증된 약효2,3과 잘 확립된 안전성 프로파일4
- 침략의 유전자재조합기술5,6을 통해 제품의 구성요소가
  일관성이 있고 신뢰할 수 있습니다5,9
- Ready-to-use pre-filled pen 형태로 사용이 편리하고12
  세밀한 용량 조절이13,14 가능합니다

PERGOVERIS® PRE-FILLED PEN

THE WORLD’S FIRST AND ONLY COMBINATION OF RECOMBINANT hFSH•hLLH12,3
IN A CONVENIENT PRE-FILLED PEN4,5

DETAIL MAKES UP A FORMULA YOU CAN RELY ON

*See inside for indication, contraindications, precautions, warnings, and full prescribing information. Use only under medical supervision. • For marketing, contact Merck Sharp & Dohme. ©2018 Merck. All rights reserved. www.merck.com

Dedicated to the details that matter
Aims and Scope

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

Background

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

Distribution

CERM is not for sale, but is distributed to members of the Korean Society for Reproductive Medicine and relevant institutions. This journal is open access and full text PDF files are also available at the official website (http://www.eCERM.org). Circulation number of print copies is 400. For subscription of print copy, please contact the Korean Society for Reproductive Medicine (http://www.ksfs.or.kr/).

Open Access

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
PRESIDENTIAL MESSAGE

Korean Society for Reproductive Medicine 50th anniversary commemorative address
Seok Hyun Kim

REVIEW ARTICLE

Efficacy of ablation and sclerotherapy for the management of ovarian endometrioma: A narrative review
Byung Chul Jee

ORIGINAL ARTICLES

Melatonin and selenium supplementation in extenders improves the post-thaw quality parameters of rat sperm
Erfan Shahandeh, Mahboubeh Ghorbani, Tahereh Mokhlisabadi Farahani, Fatemeh Bardestani

Impact of imatinib administration on the mouse ovarian follicle count and levels of intra-ovarian proteins related to follicular quality
Se Jeong Kim, Tae Eun Kim, Byung Chul Jee

Prevalence of Y chromosome microdeletions among infertile Mongolian men
Erdenesuvd Damdinsuren, Purevjargal Naidansuren, Mensaikhan Gochoo, Bum-Chae Choi, Min-Youp Choi, Bolorchimeg Baldandorj

Effect of aqueous Nigella sativa extract on the functional parameters of post-thaw human spermatozoa during vitrification
Zohreh Nasiri, Fatemeh Ghorbani, Mohammad Seify, Aysan Sharbati

The relationship between reactive oxygen species, DNA fragmentation, and sperm parameters in human sperm using simplified sucrose vitrification with or without triple antioxidant supplementation
Theesit Juanpanich, Tayita Suttirojpattana, Rangsun Pampai, Teraporn Vutyavanich

The impact of hyperandrogenism on the outcomes of ovulation induction using gonadotropin and intrauterine insemination in women with polycystic ovary syndrome
Vu Ngoc Anh Ho, Toan Duong Pham, Nam Thanh Nguyen, Hieu Le Trung Hoang, Tuong Manh Ho, Lan Ngoc Vuong

Consecutive versus concomitant follicle-stimulating hormone and highly purified human menopausal gonadotropin: A milder response but better quality
Hassan Ali Maghraby, Abdel Fattah Mohamed Agameya, Manal Shafik Swelam, Nermeen Ahmed El Dabah, Ola Youssef Ahmed

Comparison of complication rates after transvaginal ultrasound-guided oocyte pick-up procedures with respect to ovarian response
Nur Dokuzyeylul Gungor, Tugba Gurbuz, Murat Onal

High efficiency of homemade culture medium supplemented with GDF9-β in human oocytes for rescue in vitro maturation
Mehdi Mohsenzadeh, Mohammad Ali Khalili, Fatemeh Anbari, Mahboubeh Vatanparast
Dear Members of the Korean Society for Reproductive Medicine,

This year marks the 50th anniversary of the Korean Society for Reproductive Medicine, which was established in 1972. It has already been half a century since we took our first steps toward the development of reproductive medicine in Korea. If you compare the history of our society to a person's life, we have passed through our adolescent years and are entering the prime of life, when we have the opportunity to take on the most important roles in our life. The growth and development of the Korean Society for Reproductive Medicine are the fruit borne by the passion of approximately 3,500 society members, steady research, and the efforts made by the preceding presidents and executives. We express our sincere gratitude for our members' hard work.

The Korean Society for Reproductive Medicine began with 17 researchers who participated in the Seventh International Fertility Society held in Japan in 1971. These researchers returned to Korea deeply impressed by global trends in reproductive medicine. Feeling the necessity for more organized and systematical clinical and basic research, they decided to form the Korean Society for Fertility (tentative name) to establish a further specialization within the field of obstetrics and gynecology and urology, with a particular focus on reproduction. On July 19, 1972, a general meeting of promoters was held at Seoul Mise En Grille with Professor Geon-Yeong Na of the Department of Obstetrics and Gynecology at Seoul National University as chairperson, and the inaugural meeting of the Korean Society for Fertility and Sterility was announced in the Seoul Shinmun on July 24, 1972.

To play a central role in promoting the development of both basic and clinical academic research and the medical technology for infertility, we have been issuing an official academic journal annually since 1974. After the first academic conference was held on January 31, 1974, an issue of the journal based on the articles presented at the conference was discussed, and the Korean Journal of Fertility and Sterility volume 1 issue 1 (the first issue) was published in December 1974. The journal was then published under the name Korean Journal of Reproductive Medicine from 2007 to 2010 and was re-named Clinical and Experimental Reproductive Medicine (CERM) in 2011. CERM was listed in the Emerging Sources Citation Index in February 2019 and is making progress day by day as a sophisticated academic journal in reproductive medicine.

The Korean Society for Reproductive Medicine covers the basic and clinical aspects of a wide range of topics such as infertility, assisted reproductive technology, preservation of fertility, reproductive endocrinology, reproductive immunity, reproductive genetics, and reproductive physiology and serves as a place for the exchange of academic research and information between basic medical researchers and clinicians to expand their vision of reproductive medicine. In addition to academic exchange, the Korean Society for Reproductive Medicine is expected to play a critical role in overcoming the low birthrate, a national problem faced by Korean society, which has experienced a severe drop in births.

This year, we celebrated the remarkable achievements made by the Korean Society for Reproductive Medicine over the last half-century and are ready to start the next wonderful half-century of the society. To further develop reproductive medicine, we will serve as a place for internal exchange among academics, encourage researchers who have achieved excellent accomplishments, and establish an environment where researchers in Korea and overseas can collaborate with each other by inviting renowned researchers overseas to give presentations. Moreover, we will make every effort to improve the rights and interests of our members by actively participating in policy-making related to reproductive medicine. We request your continued interest in the activities of the Korean Society for Reproductive Medicine.

Once again, we express our deepest gratitude to the members of the Korean Society for Reproductive Medicine for their passion and effort throughout the journey to the 50th anniversary of the society.

Thank you.

January 2022
Seok Hyun Kim,
25th President of the Korean Society for Reproductive Medicine
Introduction

Endometriosis typically presents as three types: superficial peritoneal lesions, deep infiltrating endometriosis, and ovarian endometrioma [1]. Of these types, ovarian endometrioma can be easily identified by ultrasonography; it is lined with endometrial tissue and contains a chocolate-colored fluid that arises from the accumulation of menstrual debris. Ovarian endometrioma accounts for 17% to 44% of all cases of endometriosis [2]. Lee et al. [3] analyzed 1,374 cases (1,350 women) confirmed as endometriosis by pathological reports during surgery performed at a single center for 9 years. The predominant location of endometriosis was in the ovaries (96.4%), followed by soft tissues (2.8%), the gastrointestinal tract (0.3%), and the urinary tract (0.2%). In ovarian endometrioma, unilateral lesions accounted for about two-thirds of cases, and bilateral lesions for about one-third.

In symptomatic women with ovarian endometriomas, a surgical approach is usually recommended. There are three main surgical techniques: cystectomy, ablation, and sclerotherapy. The degree of symptom relief and recurrence rate should be considered when assessing the therapeutic effects of various techniques. In addition, and more importantly, the preservation of ovarian reserve and the subsequent pregnancy rate should be considered, especially in women who desire pregnancy in the future.

The aim of this review is to summarize information regarding the efficacy of ablation and sclerotherapy compared to cystectomy in...
terms of preservation of ovarian reserve, the recurrence rate, and the pregnancy rate.

**Ovarian cystectomy**

Ovarian cystectomy is the preferred technique in terms of recurrence and the spontaneous pregnancy rate after surgery [4,5]. However, cystectomy often causes ovarian damage and diminished ovarian reserve. At 9 to 12 months after ovarian cystectomy, 39.5% and 57% reductions in serum anti-Müllerian hormone (AMH) levels were observed in patients with unilateral and bilateral endometriomas, respectively [6]. Since ovarian endometrioma consists of a pseudocapsule, cystectomy leads to the removal of the lining of endometrial tissues as well as the normal ovarian tissues [7]. Furthermore, the remaining normal ovarian tissues are usually coagulated for bleeding control, thereby further diminishing ovarian reserve. A greater decline in ovarian reserve could occur in older women and those with larger ovarian endometriomas, bilateral lesions, and advanced-stage disease [6,8-11]. Therefore, cystectomy has to be chosen very carefully in women who desire future pregnancy or who are infertile.

Cystectomy is a very difficult option to choose in women who already have a diminished ovarian reserve before surgery, and even in women with recurrent endometrioma after surgery. As a way to preserve ovarian reserve when cystectomy is performed, hemostasis by ovarian suturing or a hemostatic agent has been introduced.

Table 1 lists 11 comparative studies on serum AMH decrement (3 months or more postoperatively) after cystectomy of ovarian endometrioma with bipolar coagulation versus suturing (five studies), as well as bipolar coagulation versus a hemostatic agent (six studies). Although Baracat et al. [12] summarized comparative studies on serum AMH decrement after ovarian cystectomy, the meta-analysis included several studies that enrolled both endometrioma and non-endometrioma groups. Therefore, in this review, three studies that enrolled mixed groups were not included in Table 1 [13-15]. However, the study by Kang et al. [16] was included, because the serum AMH decrement in the endometrioma group could be extracted separately. The serum AMH decrement was calculated as follows: [(postoperative AMH level–preoperative AMH level)/preoperative AMH level] × 100 (%).

Among the five studies comparing bipolar coagulation versus suturing, the serum AMH decrements were similar in three studies [17-19]. However, two studies reported significantly smaller serum AMH decrements in the suturing group [20,21]. Among the six studies comparing bipolar coagulation versus hemostatic agent, the serum AMH decrements were similar in three studies [22,23,24]. However, three studies reported significantly smaller serum AMH decrements in the hemostatic agent group [16,25,26].

Interestingly, Araujo et al. [24] compared serum AMH decrements after all three methods (bipolar coagulation versus suturing versus a hemostatic agent), but the serum AMH decrements were similar in all groups. These 11 studies showed varying results for the results of the serum AMH decrement; therefore, no conclusions can be drawn.

### Table 1. Comparative studies on serum AMH decrement (3 months or more postoperative) after cystectomy for ovarian endometrioma with bipolar coagulation versus suturing and bipolar coagulation versus a hemostatic agent

<table>
<thead>
<tr>
<th>Study</th>
<th>Study type/No. of women in each arm</th>
<th>AMH measurement time after cystectomy (mo)</th>
<th>Bipolar coagulation (%)</th>
<th>Suturing (%)</th>
<th>Hemostatic (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrero et al. (2012) [17]</td>
<td>Randomized/50 vs. 50</td>
<td>3</td>
<td>–19</td>
<td>–13</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>–23</td>
<td>–18</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>–23</td>
<td>–18</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Takashima et al. (2013) [18]</td>
<td>Retrospective/21 vs. 23</td>
<td>3</td>
<td>–3</td>
<td>–17</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Tanprasertkul et al. (2014)</td>
<td>Randomized/25 vs. 25</td>
<td>3</td>
<td>–28</td>
<td>–21.6</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>–27</td>
<td>–31.2</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Asgari et al. (2016) [20]</td>
<td>Randomized/57 vs. 52</td>
<td>3</td>
<td>–53.4</td>
<td>–15.9</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Zhang et al. (2016) [21]</td>
<td>Randomized/69 vs. 69</td>
<td>3</td>
<td>–58</td>
<td>–28</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>–55</td>
<td>–28</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>–53</td>
<td>–26</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Sonmezzer et al. (2013) [22]</td>
<td>Randomized/15 vs. 15</td>
<td>3</td>
<td>–23</td>
<td>–19</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Song et al. (2015) [25]</td>
<td>Prospective/62 vs. 63</td>
<td>3</td>
<td>–42.2</td>
<td>–24.6</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Kang et al. (2015) [16]</td>
<td>Prospective/23 vs. 43</td>
<td>3</td>
<td>–41.1</td>
<td>–15.6</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Choi et al. (2018) [26]</td>
<td>Randomized 40 vs. 40</td>
<td>3</td>
<td>–41.9</td>
<td>–18.1</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>Chung et al. (2019) [23]</td>
<td>Randomized/47 vs. 47</td>
<td>3</td>
<td>–26.7</td>
<td>–12.7</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Araujo et al. (2021) [24]</td>
<td>Randomized/27 vs. 26 vs. 24</td>
<td>6</td>
<td>–6.7</td>
<td>–11</td>
<td>–13</td>
<td>NS</td>
</tr>
</tbody>
</table>

AMH, anti-Müllerian hormone; NS, not significant.
Thus, it remains unclear whether suturing or the use of hemostatic agents as a method of hemostasis results in smaller serum AMH decrements compared to bipolar coagulation.

In a systematic review and meta-analysis, 3-month postoperative AMH levels were significantly lower in patients who received bipolar coagulation group than in those for whom a non-thermal hemostasis method was used (mean difference, –0.79 ng/mL; 95% confidence interval [CI], –1.19 to –0.39) [27]. In that report, only three studies were included; in one study, 3-month postoperative AMH levels were compared between bipolar coagulation versus a hemostatic agent [22], while two studies compared 3-month postoperative AMH levels between bipolar coagulation and suturing [19,21].

Ablation versus cystectomy

Ablation is a method of incising an ovarian endometrioma to remove the internal fluid and ablate the lining of endometrial tissue. Ablation can be performed using bipolar coagulation, laser vaporization, or plasma energy [28]. Since the cyst wall is not removed, it is generally considered a better option than cystectomy in terms of ovarian reserve [11]. Table 2 lists 11 comparative studies on serum AMH decrement, recurrence of endometrioma, or the pregnancy rate after ablation versus cystectomy of ovarian endometrioma [8,10,29-37]. In most studies, bipolar coagulation was used as a method for ablation, but laser vaporization was used in four studies [10,29-31]. Plasma energy was used in only one study [32]. It is difficult to draw a definitive conclusion from these 11 studies on which ablation technique would be better. The reader should refer to each article for details on how to use a specific ablation technique.

1. Serum AMH decrement

A randomized study by Giampaolino et al. [8] indicated that both ablation and cystectomy had a negative impact on ovarian reserve. However, they found that endometrioma size was associated with the magnitude of AMH decrement after ablation or cystectomy. In 24 women with endometriomas measuring <5 cm, the degree of AMH decrement at 3 months was similar between ablation and cystectomy (~18.2% vs. ~17.6%), but in 22 women with endometriomas ≥5 cm in size, a smaller decline of serum AMH level was noted in the ablation group (~14.8% vs. ~24.1%, p < 0.05). Therefore, in cases with endometriomas ≥5 cm in size, ablation might be better than cystectomy for preserving serum AMH levels.

Another randomized study by Candiani et al. [30] indicated that ablation was better than cystectomy in terms of preserving serum AMH levels. In the ablation group, the mean preoperative and 3-month postoperative serum AMH levels were 2.3 and 1.9 ng/mL, respectively (p > 0.05), while in the cystectomy group, the corresponding levels were 2.6 and 1.8 ng/mL, respectively (p < 0.05). A prospective study by Saito et al. [10] showed that ablation was better than cystectomy in terms of ovarian reserve, especially in bilateral lesions. In women with bilateral lesions, the 1-, 6-, and 12-month postoperative AMH decrements were significantly smaller in the ablation group than in the cystectomy group. However, in women with unilateral lesions, the AMH decrements were similar between the ablation and cystectomy groups.

A randomized study by Shaltout et al. [33] demonstrated that the 6-month postoperative AMH decrement was significantly smaller in the ablation group than in the cystectomy group. Interestingly, they found that the insertion of oxidized regenerated cellulose (Surgicel; Ethicon, Somerville, NJ, USA) inside the cavity of the cyst significantly minimized the AMH decrement in the ablation group, but not in the cystectomy group. A retrospective study by Chen et al. [34] indicated that both ablation and cystectomy had a negative impact on ovarian reserve, but they found that ablation was better than cystectomy in terms of ovarian reserve. In the ablation group, the mean preoperative and 6-month postoperative serum AMH levels were 4.47 and 3.95 ng/mL, respectively (p < 0.05), while the corresponding levels in the cystectomy group were 4.25 and 3.40 ng/mL, respectively (p < 0.05). The mean change in AMH levels was significantly smaller in the ablation group (mean, ~0.52 ng/mL vs. ~0.85 ng/mL, p < 0.05).

In summary, five studies indicated that both ablation and cystectomy had negative impacts on ovarian reserve; however, smaller decrements in the serum AMH level after ablation were uniformly reported [8,10,30,33,34]. Ablation appears to be advantageous in terms of the preservation of ovarian reserve, especially in women with endometriomas ≥5 cm in size or bilateral lesions [8,10].

2. Recurrence rate

Seven studies compared the recurrence rate of endometrioma between ablation versus cystectomy [10,29,31,33-36]. Interestingly, five studies reported a higher recurrence rate in the ablation group than in the cystectomy group, but without a statistically significant difference [31,33-36]. In a randomized study, Carmona et al. [29] reported a significantly higher recurrence rate at the 1-year follow-up in the ablation group than the cystectomy group (31% vs. 11%, p < 0.05). However, the overall recurrence rate at the 5-year follow-up became similar between the two groups (37% vs. 22%, p > 0.05). In a prospective study, Saito et al. [10] reported no recurrence in any patients in the study population.

In a randomized study, Shaltout et al. [33] reported that the insertion of oxidized regenerated cellulose (Surgicel) inside the cavity of the cyst significantly lowered the recurrence rate in both the ablation group (27.1% vs. 10.9%) and the cystectomy group (24.4% to 9.1%). An earlier Cochrane review (published in 2008) included the afore-
### Table 2. Comparative studies on serum AMH decrement, recurrence of endometrioma, and the pregnancy rate after ablation versus cystectomy for ovarian endometrioma

<table>
<thead>
<tr>
<th>Study</th>
<th>Methods for ablation (No. of women in each arm)</th>
<th>AMH</th>
<th>Recurrence of endometrioma</th>
<th>Pregnancy rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beretta et al. (1998) [35]/randomized</td>
<td>Bipolar coagulation</td>
<td>NA</td>
<td>2 yr: 18.8% vs. 6.2% (NS)</td>
<td>2 yr: 23.5% vs. 66.7% (p &lt; 0.05)</td>
</tr>
<tr>
<td>Alborzi et al. (2004) [36]/randomized</td>
<td>Bipolar coagulation</td>
<td>NA</td>
<td>1 yr: 18.8% vs. 5.8% (NS)</td>
<td>2 yr: 31.3% vs. 17.3% (NS)</td>
</tr>
<tr>
<td>Alborzi et al. (2007) [37]/randomized</td>
<td>Bipolar coagulation</td>
<td>NA</td>
<td>1 yr: 18.8% vs. 5.8% (NS)</td>
<td>2 yr: 31.3% vs. 17.3% (NS)</td>
</tr>
<tr>
<td>Carmona et al. (2011) [29]/randomized</td>
<td>Laser vaporization</td>
<td>NA</td>
<td>1 yr: 31% vs. 11% (p &lt; 0.05)</td>
<td>5 yr: 37% vs. 22% (NS)</td>
</tr>
<tr>
<td>Giampaolino et al. (2015) [8]/randomized</td>
<td>Bipolar coagulation (endometrioma size &lt; 5 cm)</td>
<td>NA</td>
<td>3 mo: –18.2% vs. –17.6% (NS)</td>
<td>NA</td>
</tr>
<tr>
<td>Mircea et al. (2016) [32]/comparative</td>
<td>Plasma energy</td>
<td>NA</td>
<td>2 yr: 61.3% vs. 69.3% (NS)</td>
<td>3 yr: 84.4% vs. 78.3% (NS)</td>
</tr>
<tr>
<td>Candiani et al. (2018) [30]/randomized</td>
<td>Laser vaporization</td>
<td>Preoperative vs. 3 mo (ng/mL): 2.3 vs. 1.9 (NS)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Saito et al. (2018) [10]/prospective</td>
<td>Laser vaporization</td>
<td>1 mo/6 mo/12 mo: –69%–59%–53%</td>
<td>12 mo</td>
<td>NA</td>
</tr>
<tr>
<td>Shaltout et al. (2019) [33]/randomized</td>
<td>Bipolar coagulation (with Surgicel®)</td>
<td>6 mo: –33.5% vs. –54.1% (p &lt; 0.05)</td>
<td>2 yr: 27.1% vs. 24.4% (NS)</td>
<td></td>
</tr>
<tr>
<td>Candiani et al. (2020) [31]/retrospective</td>
<td>Laser vaporization</td>
<td>NA</td>
<td>29 mo: 10.9% vs. 9.1% (NS)</td>
<td>NA</td>
</tr>
<tr>
<td>Chen et al. (2021) [34]/retrospective</td>
<td>Bipolar coagulation</td>
<td>Preoperative/6 mo (ng/mL): 4.47/3.95 (difference, –0.52)</td>
<td>2 yr: 16.6%</td>
<td>73% during 32 mo</td>
</tr>
</tbody>
</table>

AMH, anti-Müllerian hormone; NA, not available; NS, not significant.

"p<0.05 when compared with the preoperative serum AMH level."
mentioned two studies [35,36] and concluded that cystectomy showed a significantly lower recurrence rate (odds ratio [OR], 0.41; 95% CI, 0.18–0.93) [4]. In that review, symptom recurrence was also significantly lower in the cystectomy group (dysmenorrhea: relative risk [RR], 0.15; 95% CI, 0.06–0.38; dyspareunia: RR, 0.08; 95% CI, 0.01–0.51; non-menstrual pelvic pain: RR, 0.10; 95% CI, 0.02–0.56). These seven studies clearly show that the recurrence rate tends to be higher after ablation than after cystectomy.

### 3. Pregnancy rate

Five studies reported the pregnancy rate after ablation versus cystectomy [32,34-37]. In a randomized study by Beretta et al. [35], the 2-year cumulative pregnancy rate was significantly lower in the ablation group than in the cystectomy group (23.5% vs. 66.7%, p < 0.05). In a randomized study by Alborzi et al. [36], the 1-year cumulative pregnancy rate was also significantly lower in the ablation group than in the cystectomy group (23.3% vs. 59.4%, p < 0.05).

However, in a subsequent randomized study by Alborzi et al. [37], the pregnancy rate after superovulation was similar between the ablation and cystectomy groups (30% vs. 35.7%). A multicenter case-control study by Mircea et al. [32] showed that the probability of spontaneous pregnancy at 24 and 36 months was similar between the ablation and cystectomy groups (61.3% and 84.4% vs. 69.3% and 78.3%, respectively). In a recent retrospective study, Chen et al. [34] also reported a similar spontaneous pregnancy rate between the ablation and cystectomy groups (73% during 32 months vs. 71% during 30 months).

An earlier Cochrane review (published in 2008) included the aforementioned three studies [35-37] and concluded that cystectomy showed a significantly higher pregnancy rate (OR, 5.21; 95% CI, 2.04–13.29) [4]. However, two subsequent studies reported a similar pregnancy rate between the ablation and cystectomy groups [32,34]. Therefore, more research is needed to demonstrate whether the pregnancy rate is different between ablation and cystectomy.

Some clinicians used a “combination technique” or a “three-stage procedure,” but there are very few comparative studies on these techniques. Therefore, they are briefly presented below. In the combination technique, a large part of the endometrioma wall is first removed by cystectomy, and the remaining 10%–20% of the endometrioma wall close to the hilum is ablated [38]. In a randomized study, the combination technique showed a similar recurrence rate to that achieved using cystectomy (2.0% vs. 5.9% at 6 months postoperatively) [39]. The three-stage procedure refers to drainage of the cyst during laparoscopy, followed by subsequent gonadotropin-releasing hormone agonist treatment, and then ablation of the remains during a second laparoscopy [40]. In a small randomized study, the three-stage procedure showed a smaller serum AMH decrement at 6 months postoperatively (mean, 4.5 to 3.99 ng/mL; p > 0.05) compared to cystectomy (mean, 3.9 to 2.9 ng/mL; p < 0.05) [41].

### Sclerotherapy versus cystectomy

Ovarian cystectomy and ablation are now usually performed via the laparoscopic approach. In contrast, sclerotherapy is a type of non-surgical management of ovarian endometrioma. Sclerotherapy involves performing direct percutaneous puncture of ovarian endometrioma to remove the internal fluid, inserting a sclerosing agent such as ethanol into the cyst cavity, and removing it after a certain period of time (“washing” method). Noma and Yoshida [42] reported a higher recurrence rate after ethanol washing for < 10 minutes than after ≥ 10 minutes (62.5% vs. 9.1%, p < 0.05).

Some groups used retention of ethanol, wherein the ethanol is left in situ. In a retrospective study of recurrent endometrioma cases, the washing method for 0–10 minutes showed a non-significantly higher recurrence rate (during 1 year) than the retention method (32.1% vs. 13.3%, p > 0.05) [43]. Another retrospective study of recurrent endometrioma cases showed that the washing method (for 10 minutes) led to a significantly lower cure rate (during 1 year) than the retention method (82% vs. 96%) [44].

However, a recent retrospective study of patients with recurrent or bilateral endometrioma found similar 1-year recurrence rates between the washing (for 10 minutes) and retention methods (48.1% versus vs. 37.5%) [45]. In that report, live birth rates (spontaneous or artificial conception) were also similar (40% vs. 46.2%). In another recent retrospective study, the washing method (for 1–3 minutes) showed a smaller AMH decrement at 6 months postoperatively than the retention method (~2.7% vs. ~23.6%, p < 0.05) [46]. In that report, the overall pregnancy rates (up to 9 years) were similar (47.2% vs. 54.5%). Thus, it remains unclear whether the washing method has a higher recurrence rate than the retention method in sclerotherapy of ovarian endometrioma. More research is needed to demonstrate whether the washing method results in a smaller serum AMH decrement than the retention method.

Direct puncture can be performed using a long aspiration needle (16–17 gauge) or a flexible catheter (i.e., catheter-directed sclerotherapy). In a prospective study (14 women with primary or recurrent endometrioma), catheter-directed sclerotherapy decreased endometrioma size (from 5.8 cm to 1.1 cm), and no recurrence of endometrioma was noted during a mean follow-up of 1 year [47]. The mean preoperative and 6-month postoperative serum AMH levels were similar (from 4.29 to 4.36 ng/mL, p > 0.05). Simple aspiration alone is usually not recommended because it has a very high recurrence rate (83%–91.5%) [48,49]. However, Zhu et al. [49] reported that repetitive aspiration tended to decrease the recurrence rate,
which was 5.4% after the sixth aspiration.

Table 3 lists eight studies [42,50-56] that compared sclerotherapy versus cystectomy for ovarian endometrioma or sclerotherapy versus no intervention in terms of the serum AMH decrement, recurrence of endometrioma, and the pregnancy rate. Five studies included women undergoing in vitro fertilization and embryo transfer (IVF-ET), and the primary endpoint of those studies was clinical pregnancy rate (or live birth rate) [50-54]. In a study by Alborzi et al. [54], sclerotherapy was performed at the time of ovum pickup, and thereafter patients were followed for clinical pregnancy by IVF-ET or recurrence.

1. Serum AMH decrement

Only two studies described the serum AMH decrement at 6 months postoperatively after sclerotherapy versus cystectomy [55,56]. In a study by Garcia-Tejedor et al. [55], preoperative serum AMH levels were similar between sclerotherapy versus cystectomy (2.20 vs. 1.09 ng/mL), and the 6-month postoperative serum AMH levels were also similar between the two groups (2.02 vs. 1.35 ng/mL). In a study by Koo et al. [56], a serum AMH decrement at 6 months postoperatively was not observed in the sclerotherapy group (2.3 to 2.6 ng/mL, p > 0.05), but a significant serum AMH decrement was found in the cystectomy group (3.0 to 1.6 ng/mL, p < 0.05). Thus, it remains inconclusive whether sclerotherapy is better than cystectomy in terms of ovarian reserve.

2. Recurrence rate

Four studies described the recurrence rate of endometrioma after sclerotherapy versus cystectomy [42,54-56]. Three studies reported a similar recurrence rate between sclerotherapy and cystectomy, but only one study by Alborzi et al. [54] reported a significantly higher recurrence rate in the sclerotherapy group than in the cystectomy group (34.1% vs. 14.0%, p < 0.05). The authors [54] explained that the unusually higher recurrence rate in the sclerotherapy group could be attributed to the longer follow-up period in their study. Nonetheless, the majority of currently available reports show a similar recurrence rate when comparing sclerotherapy versus cystectomy.

3. Pregnancy rate

Two studies described a similar spontaneous pregnancy rate between sclerotherapy and cystectomy [42,55]. Five studies described pregnancy rates via IVF-ET, but the participants in the two comparative arms were quite heterogeneous [50-54]. Yazbeck et al. [50] compared IVF-ET outcomes between sclerotherapy and cystectomy in a prospective study of patients with recurrent endometrioma. The ongoing pregnancy rates after one IVF cycle (48.3% vs. 19.2%, p = 0.04) and after three IVF cycles (55.2% vs. 26.9%, p = 0.03) were significantly higher in the sclerotherapy group.

Afatoonian et al. [51] compared IVF-ET outcomes between the sclerotherapy group for patients with recurrent endometrioma and currently recurring endometrioma (i.e., no intervention) in a randomized study, and the pregnancy rates after one IVF cycle were similar (27.8% vs. 15%, p > 0.05). In a retrospective study, Lee et al. [52] compared IVF-ET outcomes between patients who underwent sclerotherapy for recurrent endometrioma, currently recurring endometrioma group (after previous cystectomy), and current endometrioma groups. The pregnancy rates after one IVF cycle were similar (44.4% vs. 37.1% vs. 41.1%).

Miquel et al. [53] compared IVF-ET outcomes between a sclerotherapy group and a current endometrioma group in a retrospective study, and the live birth rate after multiple IVF cycles was significantly higher in the sclerotherapy group (31.3% vs. 14.5%, p < 0.05). In a prospective study, Alborzi et al. [54] compared IVF-ET outcomes between the sclerotherapy group and the cystectomy group, and the live birth rates after one IVF cycle were similar (29.5% vs. 38.6%). In that study, sclerotherapy was performed at the time of oocyte pickup; thus, the sclerotherapy group could be interpreted as currently having endometrioma, at least at the time of oocyte pickup.

The five studies regarding the pregnancy rate via IVF-ET in women with endometrioma can be summarized as follows. (1) For recurrent endometrioma, sclerotherapy may be more beneficial than cystectomy (based on one study) [50]. (2) For recurrent endometrioma, sclerotherapy may not be more beneficial than no sclerotherapy (based on two studies) [51,52]. (3) For endometrioma, sclerotherapy may be more beneficial than no sclerotherapy in terms of the cumulative live birth rate (based on one study) [53].

Thus, there is no concrete evidence that sclerotherapy helps to improve the IVF pregnancy rate (when compared to cystectomy or no sclerotherapy). However, the spontaneous pregnancy rate was similar between sclerotherapy and cystectomy. In women with recurrent endometrioma after surgery, cystectomy is a very difficult option to choose because of a diminished ovarian reserve. As an alternative, sclerotherapy can be a good option for recurrent endometrioma, but the sclerotherapy-related decrement of serum AMH and reproductive outcomes should be further evaluated.

Sclerotherapy can induce abdominal pain (due to ethanol leakage into the peritoneal cavity), intraperitoneal hemorrhage, peritonitis, ovarian abscess, and systemic absorption-related acute alcohol intoxication. Table 4 lists the aforementioned comparative or non-comparative studies and presents the complications of sclerotherapy in detail. The overall crude complication rate was 5.2% (36/693).
Table 3. Comparative studies of serum AMH decrement, recurrence of endometrioma, and the pregnancy rate after sclerotherapy versus cystectomy for ovarian endometrioma or versus no intervention

<table>
<thead>
<tr>
<th>Study</th>
<th>No. of women in each arm</th>
<th>Methods for sclerotherapy</th>
<th>AMH (ng/mL)</th>
<th>Recurrence of endometrioma</th>
<th>Pregnancy rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noma et al. (2001) [42] retrospective</td>
<td>Sclerotherapy (n = 74) 100% Ethanol washing (30 min)</td>
<td>NA</td>
<td>14.9% during 21 mo</td>
<td>52.1% (12/23) during 21 mo</td>
<td>38.4% (5/13) during 18.7 mo (NS)</td>
</tr>
<tr>
<td>Yazbeck et al. (2009) [50] prospective</td>
<td>Sclerotherapy for recurrent OMA (n = 31) IVF soon after 100% ethanol washing (10 min) Cystectomy for recurrent OMA (n = 26) IVF within unknown period after initial surgery</td>
<td>NA</td>
<td>12.9% during 26 mo</td>
<td>OPR after 1 IVF/3 IVFs: 48.3%/55.2%</td>
<td>19.2%/26.9% (p = 0.04/p = 0.03)</td>
</tr>
<tr>
<td>Aflatoonian et al. (2013) [51] randomized</td>
<td>Sclerotherapy for recurrent OMA (n = 20) IVF after 3 mo since 98% ethanol washing (10 min) Cystectomy for recurrent OMA (n = 20) IVF (no intervention)</td>
<td>NA</td>
<td>20% during 6 mo</td>
<td>CPR after 1 IVF: 0.278</td>
<td>15% (NS)</td>
</tr>
<tr>
<td>Lee et al. (2014) [52] retrospective</td>
<td>Sclerotherapy for recurrent OMA (n = 29) IVF after 3 mo since 98% ethanol washing (10 min)</td>
<td>Preoperative/6 mo: 2.20/2.02 (NS) During 20 mo 0.059</td>
<td>2.09/1.35 (NS)</td>
<td>28.6% (NS)</td>
<td>0% (NS)</td>
</tr>
<tr>
<td>Garcia-Tejedor et al. (2020) [55] prospective</td>
<td>Sclerotherapy (n = 17) Cystectomy (n = 14) 100% ethanol washing (15 min)</td>
<td>Preoperative/6 mo: During 20 mo 2.11/2.37 (NS) 0.059</td>
<td>During 20 mo 1.09/1.35 (NS)</td>
<td>28.6% (NS)</td>
<td>0% (NS)</td>
</tr>
<tr>
<td>Miquel et al. (2020) [53] retrospective</td>
<td>Sclerotherapy (n = 37) OMA (n = 37) IVF after 96% ethanol washing (10 min)</td>
<td>NA</td>
<td>2 yr–7 yr: 0.341</td>
<td>LBR after 1 IVF: 0.295</td>
<td>14.5% (67 cycles) (p &lt; 0.05)</td>
</tr>
<tr>
<td>Koo et al. (2021) [56] retrospective</td>
<td>Sclerotherapy (n = 20) Cystectomy (n = 51) 99% Ethanol washing (20 min) via a catheter-directed method</td>
<td>Preoperative/6 mo: During 20 mo 2.3/2.6 (NS) 7.8% during mean 21.7 mo (NS)</td>
<td>3.0/1.6 (p &lt; 0.05)</td>
<td>2 yr–7 yr: 0.341</td>
<td>NA</td>
</tr>
<tr>
<td>Alborzi et al. (2021) [54] prospective</td>
<td>Sclerotherapy (n = 44) Cystectomy (n = 57) At the time of OPU by 96% ethanol retention IVF after 1 yr since cystectomy</td>
<td>NA</td>
<td>14.0% (p &lt; 0.05)</td>
<td>LBR after 1 IVF: 0.295</td>
<td>38.6% (NS)</td>
</tr>
</tbody>
</table>

AMH, anti-Müllerian hormone; NA, not available; NS, not significant; OMA, endometrioma; IVF, in vitro fertilization; OPR, ongoing pregnancy rate; CPR, clinical pregnancy rate; LBR, live birth rate; OPU, ovum pickup.

*p<0.05 when compared with the preoperative AMH level.
Conclusions

The findings of this review can be summarized as follows. First, when cystectomy of ovarian endometrioma is performed, it remains unclear whether suturing or the use of hemostatic agents as a method of hemostasis results in a smaller serum AMH decrement than bipolar coagulation. Second, both ablation and cystectomy have a negative impact on ovarian reserve, but ablation results in a smaller serum AMH decrement than cystectomy. Thus, ablation can be recommended in terms of ovarian reserve. However, ablation tends to result in a higher recurrence rate than cystectomy. In the past, ablation has been reported to be disadvantageous in terms of the pregnancy rate in comparison to cystectomy; however, several recent reports have presented similar pregnancy rates between the two groups. Therefore, more studies are needed to demonstrate whether the pregnancy rate is different between ablation and cystectomy.

Third, when sclerotherapy of ovarian endometrioma is performed, it remains unclear whether the washing method has a higher recurrence rate than the retention method. In addition, more research is needed to show whether the washing method results in a smaller serum AMH decrement than the retention method. Last, it remains inconclusive whether sclerotherapy is better than cystectomy in terms of ovarian reserve. The recurrence rate appears to be similar after sclerotherapy and cystectomy. There is no concrete evidence that sclerotherapy helps to improve the IVF pregnancy rate when compared to cystectomy or no sclerotherapy. In the author's opinion, sclerotherapy should be applied carefully only to recurrent endometriomas when it would be difficult to perform cystectomy or ablation.

Conflict of interest

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

ORCID

Byung Chul Jee https://orcid.org/0000-0003-2289-6090

References

3. Lee HJ, Park YM, Jee BC, Kim YB, Suh CS. Various anatomic locations of surgically proven endometriosis: a single-center experi-


Melatonin and selenium supplementation in extenders improves the post-thaw quality parameters of rat sperm

Erfan Shahandeh¹, Mahboubeh Ghorbani², Tahereh Mokhlesabadi-farahani³, Fateme Bardestani⁴

1Department of Biology, Islamic Azad University of Hamedan, Hamedan; 2Department of Midwifery North Khorasan University of Medical Sciences, Bojnurd; 3Clinical Research and Department of Kamali Hospital Center, Alborz University of Medical Sciences, Alborz; 4Department of Anatomy, Faculty of Medicine, Yasuj University of Medical Sciences, Yasuj, Iran

Objective: The aim of this study was to determine the effects of melatonin and selenium in freezing extenders on frozen-thawed rat sperm.

Methods: Semen samples were collected from 20 adult male Wistar albino rats. Following dilution, the samples were divided into six groups: four cryopreserved groups with 1 mM and 0.5 mM melatonin and selenium supplements, and two fresh and cryopreserved control groups. The rapid freezing technique was used to freeze the samples. Flow cytometry was used to assess plasma membrane integrity, mitochondrial membrane potential, and DNA damage, while computer-assisted sperm analysis was used to assess motility.

Results: Total motility was higher in the 1 mM melatonin supplementation group than in the cryopreserved control group (mean±standard error of the mean, 69.89±3.05 vs. 59.21±1.31; p≤0.05). The group with 1 mM selenium had the highest plasma membrane integrity (42.35%±1.01%). The cryopreserved group with 0.5 mM selenium had the highest mitochondrial membrane potential, whereas the cryopreserved control group had the lowest (45.92%±4.53% and 39.45%±3.52%, respectively).

Conclusion: Cryopreservation of rat semen supplemented with 1 mM melatonin increased sperm motility after freeze-thawing, while supplementation with 0.5 mM selenium increased mitochondrial activity.

Keywords: Computer-assisted sperm analysis; Flow cytometry; Melatonin; Selenium

Introduction

Changes in sperm occur during the cryopreservation cycle, including membrane lipid peroxidation, membrane integrity changes, mitochondrial damage, acrosome damage, DNA denaturation, and chromatin damage [1]. The harmful changes in the semen samples of different species are caused by reactive oxygen species (ROS) and free radicals during the freezing process [2,3]. ROS, along with low antioxidant levels in seminal plasma, causes oxidative stress, which leads to a reduction in semen quality [4]. Enzymatic and antioxidant systems in sperm and seminal plasma are responsible for reducing or neutralizing free radicals, but these systems are not always sufficient for protection [5]. To reduce oxidative damage during the freezing process, an extender supplement with an appropriate antioxidant system is required [4]. Melatonin (N-acetyl-5-methoxytryptamine; MT) is an indoleamine hormone that is synthesized from tryptophan [6-8]. MT neutralizes the toxic effects of ROS by isolating reactive free radicals. This compound helps maintain cellular function by stimulating antioxidant enzymes and neutralizing toxic species such as nitric oxide, peroxynitrite anion, and hydrogen peroxide [9,10].

Evidence has shown that MT has the ability to improve sperm parameters, including total motility, plasma membrane integrity (PMI), survival rate, and DNA integrity, and decrease lipid peroxidation [4,11]. Selenium (Se) is a rare biological mineral that regulates mito-
chondrial function, thyroid hormone synthesis, and phagocytic function, among other metabolic functions [10]. Se acts as part of antioxidant enzymes such as glutathione peroxidase (GPx) [12]. By detoxifying hydrogen peroxide, this enzyme protects against peroxidative damage [13]. Several endogenous antioxidants protect epididymal sperm from oxidative stress [14]. Studies have shown that Se can improve the quality of sperm in impotent camels [15]. As a result of its antioxidant properties, this compound has been studied extensively in animal research [16]. The aim of the present study was to evaluate the antioxidant effect of MT and Se on the functional parameters of epididymal rat sperm, including motility, cell membrane and mitochondrial integrity, and DNA damage during freezing.

Methods

1. Animals

A total of 20 adult male Wistar albino rats weighing 150–200 g (10–12 weeks old) were used as sperm donors. The animals were obtained from the Animal Center at Yasuj University of Medical Sciences. All animal housing and surgical procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of Yasuj University of Medical Sciences, Yasuj, Iran. The rats were housed in a pathogen-free environment in animal cages with standard food and water at a temperature of 23°C ± 1°C with a relative humidity of 55% ± 10% under a 14-hour light/10-hour dark cycle.

2. Semen collection

The rats were killed using cervical dislocation. During surgery and under sterile conditions, the tails of the epididymis that contained sperm were collected and placed in a 35 mm culture dish containing 5 mL of HEPES buffered Tyrode’s lactate (TL-HEPES) solution with 3 mg/mL bovine serum albumin supplementation. Incisions were made at the tail of the epididymis using insulin injection needles to extract the sperm. The samples were then incubated at 37°C with 5% CO₂ for 5 minutes to remove the sperm from the epididymis. The sperm suspension was poured into a 5 mL conical tube and stored at 22°C for further experiments. The TL-HEPES extender solution was prepared as follows: 2.3 mM KCl, 114 mM NaCl, 0.4 mM NaH₂PO₄•H₂O, 0.2 mM CaCl•H₂O, 0.5 mM MgCl•6H₂O, 2 mM NaHCO₃, 10 mM lactic acid, 30 × 10⁻⁶ mol NaCl, and 1 mmol EDTA and were dissolved at a concentration of 5 × 10⁻⁴ sperm/mL. A total of 400 µL of acid detergent solution and 1,200 µL of AO staining solution were added to the mixture. After 15 seconds, the sperm were evaluated using flow cytometry [21].

3. Freezing and thawing procedure

Semen samples with a concentration of 20 × 10⁶ sperm/mL to 30 × 10⁶ sperm/mL were diluted in a TL-HEPES extender solution, and the semen of each rat was divided into six groups, with four groups containing MT and Se at concentrations of 0.5 mM and 1 mM and two groups containing no added material, which were used as fresh and cryopreservation control groups, respectively [18,19]. The samples were cooled to 4°C and equilibrated at this temperature for 2 hours. Next, 0.5 mL of each sample was placed on French straws and sealed using a sealing device. They were then cooled for 10 minutes in nitrogen vapor (suspended 3 cm above liquid nitrogen) before being poured into liquid nitrogen at –196°C. The frozen straws were thawed for 30 seconds in a 37°C water bath before evaluation [20].

4. Semen evaluation

1) Computer-Assisted Sperm Analysis

Sperm motility analysis was performed using computer-assisted sperm analysis (CASA; SCA, Microptic Co., Barcelona, Spain). In this study, a 10 µL thawed semen sample was placed on a Makler chamber slide that had already been preheated to 37°C. The evaluated parameters included motility (%), progressive motility (%), average path velocity (μm/sec), curvilinear velocity (μm/sec), linearity (%), straight-line velocity (μm/sec), straightness (%), wobble (%), and beat cross-frequency (Hz), as shown in Table 1.

2) Integrity of the DNA

The sperm DNA integrity was assessed using acridine orange (AO) staining. The heated samples were first put in a centrifuge for 5 minutes at 500 × g. The pellets were then mixed with a null ethylenediaminetetraacetic acid (EDTA) buffer solution containing 10 mmol Tris, 0.15 mol NaCl, and 1 mmol EDTA and were dissolved at a concentration of 5 × 10⁶ sperm/mL. A total of 400 µL of acid detergent solution and 1,200 µL of AO staining solution were added to the mixture. After 15 seconds, the sperm were evaluated using flow cytometry [21].

3) Plasma membrane integrity

The hypo-osmotic test was used to evaluate the sperm PMI. First, 50 µL of thawed semen was mixed with 50 µL of HOS solution (1.35 g fructose, 0.735 g sodium citrate with 100 mL distilled water at an osmotic pressure of 190 mOsmol). The resulting solution was then incubated in an incubator (37°C, 45 minutes). Finally, 10 µL of the sample was placed on a dry and preheated slide and covered with a slip cover. A contrast phase microscope (Olympus BX20) with ×400 magnification was used for microscopic evaluation. In each slide, 200 spermatozoa were studied, and sperm with swollen and twisted tails were considered to have integrated membranes [22].

5. Mitochondrial membrane integrity

Molecular probes of 5,5′,6,6′-tetramethylbenzimidazolyl-carbocyanine iodide (JC-1)-PI were used to evaluate the mitochondrial membranes of thawed sperms. For this purpose, thawed semen samples were put in a centrifuge for 5 minutes at 500 × g. The spermatozoa were then dissolved in 487 µL of phosphate-buffered
The addition of 1 mM and 0.5 mM MT and Se to the extender led to improved total motility compared to the frozen control group. In addition, 1 mM MT was associated with more total motility than the other groups. However, no statistically significant differences between the groups were observed for the other dynamic parameters (Table 1). A higher PMI was observed in the 1 mM Se group than in either MT group. In addition, the highest percentage of sperm with high MMP was observed in the 0.5 mM Se group, and the lowest was observed in the control group (45.92% ± 4.53% and 39.45% ± 3.52%, respectively). MT and Se reduced DNA damage at both concentrations (Table 2).

### Results

The addition of 1 mM and 0.5 mM MT and Se to the extender led to improved total motility compared to the frozen control group. In addition, 1 mM MT was associated with more total motility than the other groups. However, no statistically significant differences between the groups were observed for the other dynamic parameters (Table 1). A higher PMI was observed in the 1 mM Se group than in either MT group. In addition, the highest percentage of sperm with high MMP was observed in the 0.5 mM Se group, and the lowest was observed in the control group (45.92% ± 4.53% and 39.45% ± 3.52%, respectively). MT and Se reduced DNA damage at both concentrations (Table 2).

### Discussion

Sperm cytoplasm is prone to the overproduction of ROS and exposure to severe oxidative stress during the freezing process due to an insufficient immune response and concentration of antioxidants [21,22]. Balanced and optimal amounts of ROS are necessary for im-
 proved performance and enhance motility with increased cyclic adenosine monophosphate and protein phosphorylation; however, high amounts of ROS resulting from a lack of sufficient antioxidant capacity can have harmful and toxic effects [24]. Oxidative stress caused by free radicals produced in the sperm cytoplasm has a significant role in reducing sperm fertility [25]. During freezing, the antioxidant capacity of frozen sperm decreases. However, semen dilution processes can reduce or eliminate antioxidant compositions in semen and lead to increased oxidative stress [24]. Several main endogenous antioxidant systems such as GPx, glutathione reductase (GSH), and superoxide dismutase (SOD) have been introduced for sperm [26]. Studies have shown that MT has a positive effect on the activity of antioxidant enzymes and increased proteins of SOD, GPx, GSH and catalase by increasing the synthesis of intracellular antioxidants [27,28]. Se can also improve the antioxidant activity of GPx enzymes [10]. Sperm mitochondria have the ability to produce some amount of ROS products in response to oxidative phosphorylation functions [29]. Freezing increases the release of ROS, especially superoxide anion and hydrogen peroxide, by altering the structure of the mitochondrial membrane [30]. According to the results, MT and Se improved sperm MMP in the frozen groups by increasing the activity of antioxidant enzymes and decreasing intracellular ROS. The presence of MT in freezing solutions was associated with a higher frequency of samples with a high MMP and a lower rate of PMI (as an indicator of a healthy plasma membrane). This study observed a correlation between sperm MMP and PMI. These results are comparable to a study by Gungor et al. [23] that found that gallic acid gave sperm more energy, thereby improving MMP in comparison to the control group, but resulted in a significant reduction in PMI compared to the control group. The highest percentage of high MMP was observed in sperm from the 0.5 mM Se group. Mitochondrial activity was higher in the 0.5 mM Se group than in the frozen control group, but there were no significant differences between the MT and Se groups. It is notable that MT can protect the mitochondrial structure by reducing oxygen consumption and O2 production and, as a result, reduce lipid peroxidation and play an important role in mitochondrial activation [31]. In addition, supplementation with Se has the ability to improve the mitochondrial activity of sperm [32].

Proper motility is one of the essential characteristics of sperm related to fertility, and any disorder related to motility can prevent sperm from reaching the fertilization site [33]. Our study showed that the presence of 1 mM MT in the extender caused a significant increase in total motility. In addition, the highest degree of progressive motility corresponded to this same concentration of MT; however, no significant difference was observed in the frozen control group. The results of this part of the study are consistent with those of a study by Fadl et al. [18] that found that concentrations of 1 mM MT corresponded to the highest degree of progressive motility compared to other doses as well as the control group. In addition, the present study showed that adding Se to the extender did not significantly improve rat sperm motility after thawing. These results contradict those of a study by Khalil et al. [19] that found that Se at concentrations of 1 and 1.5 mM significantly increased sperm motility. Dorostkar et al. also evaluated different doses of Se (1 mg/mL, 2 mg/mL, 4 mg/mL, and 8 mg/mL) in buffalo and found that doses of 1 mg/mL and 2 mg/mL significantly improved sperm motility compared to the control group, while doses of 4 mg/mL and 8 mg/mL showed a decrease in sperm motility compared to the control group [34]. These contradictions might be explained by differences in sperm type, supplement concentrations, and sperm preparation and freezing processes.

The plasma membrane is involved in protecting the physiology of sperm cytosol. In freezing, osmotic changes influence membrane integrity and sperm homeostasis by changing salt concentrations [35]. In the current study, although the PMI in the group containing 1 mM Se did not differ from that of the frozen control group, it had the highest amount compared to the other groups. The motility scores for the Se groups were not higher than those of the MT group, but better and more acceptable results were observed for PMI parameters than in the MT group. In addition, PMI in the group with 0.5 mM Se concentration was not statistically significant compared to the frozen control group, while the concentration of 1 mM Se had the highest PMI. This finding can probably be attributed to the ability of Se (at a concentration of 0.5 mM) to reduce lipid peroxidation and regulate osmotic balance and pH.

The last part of the results of this study showed that adding MT and Se to the freezing medium significantly reduced DNA damage after thawing, which is consistent with the results of a study by Rezaian et al. [32] and Breininger et al. [36] that found that Se concentrations (5 µL and 1 mg and 2 mg, respectively) reduced DNA damage after freezing. Pool et al. also observed that all doses of supplementary MT (0.1 µM, 1 µM, 10 µM, and 100 µM) helped reduce DNA damage compared to the control group [31]. Adding these supplements to increase the antioxidant capacity of freezing solutions may increase the density of chromatin structure and protect sperm from freezing damage.

In this study, the presence of 1 mM MT in the extender corresponded to the highest degree of motility, and the addition of 0.5 mM Se was associated with the highest degree of mitochondrial function in post-thaw rat sperm. However, more extensive studies that examine a wider range of concentrations are required to further understand the impact of these factors on fertility.
Conflict of interest

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

ORCID

Erfan Shahandeh https://orcid.org/0000-0002-7004-4514
Mahboubeh Ghorbani https://orcid.org/0000-0002-5894-4464
Tahereh Mokhlesabadifarahani https://orcid.org/0000-0002-0921-4836
Fateme Bardestani https://orcid.org/0000-0002-6691-3211

Author contributions

Conceptualization: ES, FB. Data curation: TM, MG. Formal analysis: TM, MG. Methodology: MG. Project administration: ES. Visualization: TM. Writing–original draft: FB. Writing–review & editing: ES.

References


https://doi.org/10.5653/cerm.2022.05267
Impact of imatinib administration on the mouse ovarian follicle count and levels of intra-ovarian proteins related to follicular quality

Se Jeong Kim¹,², Tae Eun Kim³, Byung Chul Jee²,³

¹Department of Obstetrics and Gynecology, Fertility Center of CHA Ilsan Medical Center, CHA University School of Medicine, Seoul; ²Department of Obstetrics and Gynecology, Seoul National University College of Medicine, Seoul; ³Department of Obstetrics and Gynecology, Seoul National University Bundang Hospital, Seongnam, Republic of Korea

Objective: The impact of imatinib, a tyrosine kinase inhibitor, on ovarian follicles and several proteins related to follicular function and apoptosis was investigated in mice.

Methods: Saline, cyclophosphamide (Cp; 50 or 75 mg/kg), or imatinib (7.5 or 15 mg/kg) was injected once intraperitoneally into female B6D2F1 mice (18 mice in each group). In multiple ovarian sections, the number of various types of follicles and the proportion of good-quality (G1) follicles were counted. The levels of six proteins (anti-Müllerian hormone [AMH], BCL-xL, BAX, acid sphingomyelinase [A-SMase], caspase-3, and α-smooth muscle actin [α-SMA]) within the whole ovaries were quantified using Western blots.

Results: Compared to the saline group, a significant reduction of the primordial follicle count was observed in the group treated with imatinib 7.5 and 15 mg/kg, as well as in the group treated with Cp 75 mg/kg. Administration of Cp significantly decreased the proportion of G1 primordial follicles, but administration of imatinib did not. No differences in the AMH, anti-apoptotic BCLX-L, pro-apoptotic BAX, and A-SMase levels in the ovarian tissues were observed among the five groups. However, caspase-3 and α-SMA levels were significantly higher in the imatinib and Cp groups than in the saline group.

Conclusion: The administration of imatinib to mice significantly reduced the primordial follicle count and increased the protein levels of caspase-3 and α-SMA. Our findings suggest that imatinib potentially exerts ovarian toxicity via apoptotic processes, similarly to Cp.

Keywords: Caspase; Cyclophosphamide; Imatinib; Primordial follicle; Tyrosine kinase inhibitor

Introduction

Imatinib, a tyrosine kinase inhibitor, is widely used in patients with chronic myeloid leukemia (CML) or gastrointestinal stromal tumors [1,2]. Although the detrimental effects of chemotherapeutics on future fertility are a major concern for female cancer survivors, it is largely unknown whether imatinib causes ovarian damage [3]. The main target of imatinib is an oncogenic protein formed by the BCR-ABL fusion gene. Imatinib also inhibits other tyrosine kinases such as ABL, KIT, and platelet-derived growth factor receptor (PDGFR) [4-6]. Within the ovary, the KIT ligand and PDGF have been shown to independently promote primordial follicle activation, transition from the primordial to primary follicle, oocyte growth, granulosa cell proliferation, and follicle survival [7-9]. Because imatinib inhibits the c-KIT pathway, which is essential for ovarian follicle development, it may have a negative impact on ovarian follicle survival [10].

To date, studies on ovotoxicity of imatinib in humans are scarce. A case series reported that amenorrhea was usually not induced in women taking imatinib orally, and successful conception commonly occurred [11]. However, a few case reports have suggested that imatinib may induce the loss of ovarian reserve. One case report showed that long-term administration of imatinib (for 2 years) might lead to...
primary ovarian insufficiency [12]. In another case report, an imatinib user showed a severely impaired ovarian response to exogenous gonadotropin stimulation, but presented a normal ovarian response after stopping imatinib [13].

Several animal experiments have reported unclear results regarding the potential ototoxic effects of imatinib. In a zebrafish model, imatinib feeding once, twice, or three times per day caused frequency-dependent irreversible suppression of ovarian folliculogenesis [14]. In a mouse model, long-term injections of imatinib (for 4–6 weeks) induced diminished ovarian reserve [15]. In that report, mice treated with imatinib could yield in vivo fertilized zygotes through ovarian stimulation, but the development of zygotes in vitro and implantation of subsequent blastocysts were severely hampered [15].

Intraperitoneal injections of imatinib in human ovary-xenografted mice increased follicular atresia and induced bizarre-shaped follicles without oocytes [16]. Meanwhile, in a leukemic mouse model, administration of imatinib orally for two months did not affect the numbers of primordial, primary, and secondary follicles [17].

To our knowledge, the effect of imatinib on ovarian function is still unclear. Thus, we aimed to investigate the effect of one-time imatinib injections in mice on the quantity and quality of ovarian follicles and the levels of six proteins: anti-Müllerian hormone (AMH), BCL-xL, BAX, acid sphingomyelinase (A-SMase), caspase-3, and α-smooth muscle actin (α-SMA). Cyclophosphamide (Cp), an alkylating drug used in cancer treatment protocols, is a well-known ototoxic agent that induces follicle loss. We also tested the deleterious effects of Cp injections on ovarian follicles and compared the results to those of imatinib injections.

Methods

1. Mice

Six- to seven-week-old B6D2F1 female mice (Orient Bio, Seongnam, Korea) were used. They were raised in controlled sterile conditions at 22°C with a 12-hour light/dark cycle, and had free access to autoclaved pellet diet and water. The experimental protocols and animal handling procedures were ethically performed with the approval of the Institutional Animal Care and Use Committee (IACUC) of the Seoul National University Bundang Hospital (IACUC No. 53-2019-014).

2. Experimental design

After 1 week of adaptation, 90 mice were divided into five groups, and each agent was injected intraperitoneally once: 0.1 mL of normal saline (control group), Cp (Cp monohydrate; Sigma Aldrich, St Louis, MO, USA) 50 mg/kg, Cp 75 mg/kg, imatinib (Enzo Life Sciences, Farmingdale, NY, USA) 7.5 mg/kg, or imatinib 15 mg/kg. Cp was dissolved in phosphate-buffered saline (PBS) and prepared at different concentrations. The dose of imatinib was based on equivalence to the average human oral dose of 400–800 mg/day to treat CML (corresponding to 8–16 mg/kg in a human of 50 kg) [14,18]. A one-time injection of imatinib 7.5 mg/kg was also used in a previous mouse model study [19], whereas in this study, we used two doses of imatinib (7.5 mg/kg and 15 mg/kg). Imatinib powder was dissolved in PBS to obtain an imatinib stock solution. The final volume of intraperitoneal injection was all set at 0.1 mL. One week later, we sacrificed the mice by cervical dislocation and collected the bilateral ovaries.

3. Histological examinations and follicle counts

Nine mice in each group were used; thus, 18 ovaries in each group were obtained. All ovaries were fixed in 4% buffered paraformaldehyde, embedded in a paraffin block, and then cut into 4-µm sections serially, resulting in at least five sections per ovary. The ovarian sections were stained with Mayer’s hematoxylin-eosin solution (Merck-Serono, Darmstadt, Germany) for histologic examinations. Ovarian follicles were evaluated by two senior experts. Under a light microscope (Nikon, Tokyo, Japan) at ×400 magnification, ovarian follicles were classified into four types as defined in a previous study [20]: (1) Primordial follicle: a single layer of flattened pre-granulosa cells; (2) Primary follicle: a single layer of granulosa cells, including cuboidal forms; (3) Secondary follicle: at least two layers of cuboidal granulosa cells; (4) Antral follicle: multiple layers of cuboidal granulosa cells with an antrum.

Each follicle was evaluated for its integrity according to the following criteria as mentioned in a previous study [21]: (1) G1 (good quality) follicle: intact spherical follicle and oocyte; (2) G2 (fair quality) follicle: granulosa cells pulled away from the edge of follicles, but with an intact oocyte; (3) G3 (poor quality) follicle: disruption and/or loss of granulosa-theca cells, with pyknotic nuclei and/or a missing oocyte. Representative histological images of ovarian follicles are shown in Figure 1.

4. Western blotting

Nine other mice in each group were used for the Western blot analysis. The ovaries were suspended in a lysis buffer (20 mM Tris-HCl at a pH of 8.0, 137 mM NaCl, 1% Nonidet P-40, and 10% glycerol) supplemented with protease inhibitors (0.5 mM PMSF, 0.025 mM N-CBZ-L-phenylalanine chloromethyl ketone, 0.025 mM N-p-tosyl-lysine chloromethyl ketone, and 0.025 mM L-1-tosylamide-2-phenyl-ethylchloromethyl ketone). After centrifugation at 4°C for 10 minutes at 10,000 × g, the pellets were discarded and the supernatant was obtained. After boiling for 5 minutes, we loaded 50 µg of protein onto a 12% SDS-polyacrylamide gel and performed electrophoresis at 120 V for...
1.5 hours. The resolved proteins were transferred onto nitrocellulose membranes at 100 V for 2 hours. After incubation in a blocking buffer (5% non-fat milk, 0.05% Tween-20 in 20 mM TBS at a pH of 8.0) for 1 hour at room temperature, the blots were incubated overnight at 4°C with appropriate primary antibodies: AMH (1:400, sc-166752; Santa Cruz Biotechnology, Dallas, TX, USA), BCL-xL (1:100, sc-271121; Santa Cruz Biotechnology), BAX (1:300, sc-7480; Santa Cruz Biotechnology), A-SMase (1:200, ab83354; Abcam, Cambridge, UK), cleaved caspase-3 (1:500, 5a1e; Cell Signaling Technology, Danvers, MA, USA), and α-SMA (1:200, sc-53142; Santa Cruz Biotechnology). In Figure 2, representative bands from the Western blot analysis are presented. Specifically, the bands for BCL-xL appear to be double, and this phenomenon is common for phosphorylated BCL-xL [22]. AMH is a well-known marker of ovarian reserve. BCL-xL is an anti-apoptotic marker and BAX is a pro-apoptotic marker. A-SMase is an enzyme known to increase the levels of the pro-apoptotic sphingolipid ceramide; thus, it acts as a pro-apoptotic marker. Caspase-3 is a well-known marker of late apoptosis. α-SMA, which is detected in tissues with disrupted blood vessels, is a marker of vessel damage [23]. Next, the blot was incubated with anti-rabbit secondary antibodies conjugated with horseradish peroxidase (1:1000, catalog #A4914; Sigma-Aldrich). Scion Image for Windows (Scion Corp., Frederick, MD, USA) was used to analyze the chemiluminescence signal. For each protein, eight or nine replicates were used. In the experimental groups, each protein level was expressed relative to the protein level of the saline control group. Therefore, the protein level of the saline control group was always 1.0.

5. Statistical analysis

We used IBM SPSS ver. 22.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). Ovarian follicle counts were compared using one-way analysis of variance (ANOVA) followed by the Tukey multiple-comparison test. All samples were tested for the normality of the data distribution before ANOVA. A p-value < 0.05 was considered to indicate statistical significance. The
protein levels were compared using the Kruskal-Wallis test followed by the Mann-Whitney U-test. In this analysis, the threshold for statistical significance was a p-value < 0.01.

Results

The detailed ovarian follicle counts, including the proportion of G1 follicles in the five groups, are presented in Table 1. Notably, the number of primordial follicles was significantly lower in the group treated with imatinib 7.5 mg/kg (13.9 ± 7.0 vs. 23.3 ± 8.0, p = 0.001), as well as in the group treated with imatinib 15 mg/kg (15.5 ± 7.9 vs. 23.3 ± 8.0, p = 0.010) than in the control group. The Cp 75 mg/kg group also showed a lower number of primordial follicles than the control group (13.8 ± 10.7 vs. 23.3 ± 8.0, p = 0.002), but the Cp 50 mg/kg group did not.

The ovarian follicle counts in the five groups are also depicted in Figure 3. The primary and antral follicle counts were all similar among the five groups (Figure 3B and D). The secondary follicle count was significantly lower in the imatinib 15 mg/kg–treated group than in the other four groups (p = 0.001 for each) (Figure 3C).

Table 1. Ovarian subtype follicle counts and the proportions of G1 follicles in mice treated with Cp or imatinib

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Cp 50 mg/kg</th>
<th>Cp 75 mg/kg</th>
<th>Imatinib 7.5 mg/kg</th>
<th>Imatinib 15 mg/kg</th>
<th>p-value</th>
<th>a)</th>
<th>b)</th>
<th>c)</th>
<th>d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of primordial follicles</td>
<td>23.3 ± 8.0</td>
<td>19.6 ± 8.1</td>
<td>13.8 ± 10.7</td>
<td>13.9 ± 7.0</td>
<td>15.5 ± 7.9</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 primordial follicles (%)</td>
<td>64.5 ± 11.9</td>
<td>36.0 ± 14.5</td>
<td>40.0 ± 22.1</td>
<td>51.7 ± 23.2</td>
<td>58.7 ± 12.9</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of primary follicles</td>
<td>23.6 ± 7.9</td>
<td>22.7 ± 7.6</td>
<td>24.6 ± 11.7</td>
<td>23.6 ± 6.6</td>
<td>21.7 ± 7.2</td>
<td>0.506</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 primary follicles (%)</td>
<td>67.0 ± 12.5</td>
<td>55.0 ± 21.4</td>
<td>53.2 ± 18.8</td>
<td>60.5 ± 15.5</td>
<td>59.1 ± 11.6</td>
<td>0.174</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of secondary follicles</td>
<td>49.1 ± 10.4</td>
<td>49.2 ± 12.1</td>
<td>45.8 ± 24.6</td>
<td>50.8 ± 9.8</td>
<td>36.1 ± 10.2</td>
<td>0.004</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 secondary follicles (%)</td>
<td>51.7 ± 12.8</td>
<td>38.2 ± 11.2</td>
<td>37.6 ± 11.8</td>
<td>46.7 ± 9.2</td>
<td>38.4 ± 12.8</td>
<td>0.002</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of antral follicles</td>
<td>14.1 ± 6.1</td>
<td>16.0 ± 8.9</td>
<td>20.0 ± 10.3</td>
<td>18.1 ± 4.2</td>
<td>18.6 ± 6.4</td>
<td>0.107</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 antral follicles (%)</td>
<td>86.6 ± 12.0</td>
<td>75.0 ± 14.8</td>
<td>73.2 ± 14.5</td>
<td>79.6 ± 13.1</td>
<td>86.4 ± 6.9</td>
<td>0.015</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as mean±standard error. G1, good-quality; Cp, cyclophosphamide.

a) By one-way analysis of variance; b) p < 0.05, c) p < 0.01, d) p < 0.001 when compared with the control group (by the Tukey multiple-comparison test).

Figure 3. Ovarian subtype follicle counts in the five groups. Primordial (A), primary (B), secondary (C), antral (D) follicle counts. Values are presented as mean±standard error. Control, 0.1 mL of normal saline; Cp 50, cyclophosphamide 50 mg/kg; Cp 75, cyclophosphamide 75 mg/kg; Ima 7.5, imatinib 7.5 mg/kg; Ima 15, imatinib 15 mg/kg. a) p < 0.05 by one-way analysis of variance followed by the Tukey multiple-comparison test.
The proportions of G1 follicles in the five groups are depicted in Figure 4. Although administration of imatinib (at a dose of either 7.5 or 15 mg/kg) significantly decreased the number of primordial follicles, the proportion of G1 primordial follicles was well preserved, as shown in Figure 4A. Similarly, the proportion of G1 primary and antral follicles was also preserved in the two imatinib-treated groups, but the proportion of G1 secondary follicles was not (Figure 4C). Interestingly, the administration of Cp at either dose (50 or 75 mg/kg) significantly decreased the proportion of G1 follicles in almost all types compared to the control group (Figure 4).

To summarize the findings regarding primordial follicles, administration of imatinib (7.5 or 15 mg/kg) significantly reduced the number of primordial follicles, but with good preservation of the proportion of G1 primordial follicles. The administration of Cp 50 mg/kg did not reduce the number of primordial follicles, but reduced the proportion of G1 primordial follicles. The administration of Cp 75 mg/kg significantly reduced both the number of primordial follicles and the proportion of G1 primordial follicles.

The levels of the six ovarian proteins in the five groups are depicted in Figure 5. The ovarian AMH levels in the groups treated with Cp 50 mg/kg, Cp 75 mg/kg, and imatinib 15 mg/kg were similar to those in the control group, but the AMH level was rather high in the imatinib 7.5 mg/kg group ($p < 0.001$). The ovarian levels of anti-apoptotic BCL-xL and pro-apoptotic BAX and A-SMase were similar in all five groups. However, ovarian caspase-3 protein levels were significantly higher in the groups treated with Cp 50 mg/kg, Cp 75 mg/kg, imatinib 7.5 mg/kg, and imatinib 15 mg/kg than in the control group. In addition, α-SMA levels were significantly higher in the groups treated with imatinib or Cp than in the saline control group.

**Discussion**

In the present study, we examined the ovotoxicity of imatinib as well as Cp in a mouse model. Administration of imatinib 7.5 and 15 mg/kg significantly reduced the number of primordial follicles, an indicator of ovarian reserve. Increased ovarian caspase-3 and α-SMA levels were observed in imatinib-treated mice. These findings suggest that imatinib induces primordial follicle loss, possibly via an apoptotic mechanism involving caspase-3 and vascular damage.

A decreased number of primordial follicles was also observed in the Cp 75 mg/kg group, while increased ovarian caspase-3 levels were observed in both the Cp 50 and 75 mg/kg groups. Caspase-3 is a major downstream effector enzyme in the late apoptosis process, and it has also been reported that Cp induces increased levels of caspase-3 [24].

Studies have reported that imatinib induces the loss of primordial...
Follicles in animal models, which is consistent with our findings [14-16]. Furthermore, we here found, for the first time, that imatinib exerts an ovotoxic effect through an apoptotic mechanism involving caspase-3, similarly to Cp. Given that there was no difference in ovarian expression of BCL-xL, BAX, and A-SMase between the imatinib- or Cp-treated groups and the control, it can be inferred that BCL-xL, BAX, and A-SMase do not seem to take part in the apoptotic mechanism that causes primordial follicle reduction.

Imatinib has been proposed as an agent to prevent primordial follicle loss caused by cisplatin [19]. This ovoprotective effect was presumed to derive from the fact that imatinib could inhibit the c-Abl-TAp63 pathway, which is one of the main mechanisms of cisplatin-induced follicular apoptosis. However, subsequent studies have challenged these results, and the potential ovoprotective effect of imatinib remains a topic of debate. Therefore, since our study demonstrates imatinib's ovotoxicity, caution is needed regarding the use of imatinib to protect against ovarian damage induced by anti-cancer drugs.

The so-called “burnout” hypothesis has been suggested as a mechanism for ovarian damage induced by Cp [25,26]. According to this hypothesis, Cp inhibits the dormancy of primordial follicle pool by destroying growing follicles. This induces premature activation of primordial follicles, thereby reducing the number of primordial follicles. We found in this study that Cp reduced the primordial follicle count without affecting the primary, secondary, or antral follicle count. Therefore, Cp does not seem to cause “burnout” phenomenon, at least at the doses we studied. Luan et al. [24] also demonstrated that Cp specifically depletes primordial follicles by directly inducing apoptotic cell death, rather than depleting the primordial follicle pool by activating or destroying growing follicles.

In the present study, we also found that imatinib reduced the primordial follicle count, but did not affect other growing follicle counts. Analogously, imatinib does not seem to induce the “burnout” phenomenon, at least at the doses we studied. We found that imatinib reduced the primordial follicle count, similarly to Cp. However, the extent of primordial follicle damage by imatinib might be modest in comparison with the damage caused by Cp, because the proportion of G1 follicles was maintained in the imatinib-treated groups. This observation could be clinically important, because the ovarian reserve may recover after discontinuing imatinib if G1 follicles are maintained during imatinib administration. Further studies are needed to determine how long the decrement of primordial follicles lasts after imatinib administration and when the number of primordial follicles recovers after imatinib discontinuation.

In the present work, ovarian AMH levels were well preserved after 1 week of exposure to imatinib or Cp. This could be explained by the good preservation of primary and secondary follicles after imatinib or Cp administration, since they are the main sources of AMH pro-
duction. Meanwhile, it has been reported that serum AMH levels dropped within 3 days after Cp administration, and then rebounded to equivalent levels 7 days after Cp treatment [24]. Those researchers found that healthy granulosa cells in follicles replaced damaged granulosa cells and secreted AMH. Further research is needed to clarify how ovarian AMH levels change over time after imatinib administration.

Our study has several limitations. Although we demonstrated the ovotoxicity of imatinib, the underlying mechanism of imatinib-induced ovotoxicity should be further elucidated. We exposed mice for 1 week via a single dose of imatinib. Further studies are needed to evaluate the effects of various exposure durations and doses of imatinib. In conclusion, imatinib administration to mice negatively affects the primordial follicle count. This detrimental effect might be induced via caspase-3-dependent apoptosis and vascular damage, similarly to Cp. The potential ovotoxicity induced by imatinib treatment should be further studied both biologically and clinically.

Conflict of interest

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

ORCID

Se Jeong Kim https://orcid.org/0000-0003-1879-3451
Tae Eun Kim https://orcid.org/0000-0001-7570-4481
Byung Chul Jee https://orcid.org/0000-0003-2289-6090

Author contributions

Conceptualization: SJK, BCJ. Data curation: SJK, BCJ. Formal analysis: all authors. Methodology: SJK, BCJ. Project administration: SJK, BCJ. Visualization: SJK. Writing-original draft: all authors. Writing-review & editing: all authors.

References


Prevalence of Y chromosome microdeletions among infertile Mongolian men

Erdenesuvd Damdinsuren1,2,*, Purevjargal Naidansuren2,*, Mendsaikhan Gochoo1, Bum-Chae Choi2,3, Min-Youp Choi3, Bolorchimeg Baldandorj1

1Department of Obstetrics and Gynecology, Mongolian National University of Medical Sciences School of Medicine, Ulaanbaatar; 2Mon-CL Fertility Center, Ulaanbaatar, Mongolia; 3Center for Recurrent Miscarriage and Infertility, Creation and Love Women’s Hospital, Kwangju, Republic of Korea

Objective: Y chromosome microdeletions are the second most common genetic cause of male infertility after Klinefelter syndrome. The aim of this study was to determine the patterns of Y chromosome microdeletions among infertile Mongolian men.

Methods: A descriptive study was performed on 75 infertile men from February 2017 to December 2018. Y chromosome microdeletions were identified by polymerase chain reaction. Semen parameters, hormonal levels, and testis biopsy samples were examined.

Results: Among 75 infertile men, two cases of Y chromosome microdeletions were identified. The first case had an AZFa complete deletion and the other had an AZFc partial deletion. This study found that the proportion of Y chromosome microdeletions among infertile Mongolian men was 2.66%.

Conclusion: The findings can be applied to in vitro fertilization and assisted reproductive technology, and our results will help clinicians improve treatment management for infertile Mongolian couples.

Keywords: Azoospermia; Infertility; Semen analysis; Y chromosome

Introduction

Infertility occurs in 10%–15% of all couples worldwide, and male infertility is responsible for 40%–50% of infertility cases [1,2]. The prevalence of infertility in Mongolia was 8.7% in 2003 and 11.6% in 2013 [3]. According to findings from the Child and Maternity Hospital, male factor infertility constitutes 25.6% of all infertility cases [4]. Y chromosome microdeletions are the second most common genetic cause of male infertility. In the general population, Y chromosome microdeletions occur in 1 in 4,000 men, but the frequency is significantly higher among infertile men. The association between Y chromosome microdeletions and defective spermatogenesis has been previously studied. The incidence of Y chromosome microdeletions is 2%–10% or even higher among azoospermic patients with no sperm count, or oligospermia patients with a sperm count of less than 5 million/mL [5]. The distal end of the long arm of the Y chromosome includes the azoospermia factor (AZF) locus, which contains the genes necessary for spermatogenesis. The AZF locus has been mapped to a region in band q11.23 of the Y chromosome. Microdeletions occur in the AZF region on the long arm of the Y chromosome, which includes AZFa, AZFb, and AZFc [6]. The diagnosis of Y chromosome microdeletions can establish the cause of the patient’s azoospermia and oligozoospermia and enable a prognosis to be formulated.

The purpose of this study was to investigate the frequency of Y chromosome microdeletions among infertile men who visited the Mon-CL Fertility Center, Ulaanbaatar, Mongolia, for evaluation, and to introduce a modern infertility diagnosis, contributing to further treatment. The standard method applied by the European Academy of Andrology/European Molecular Genetics Quality Network (EAA/
EMQN) was used to evaluate patients. The data from this study demonstrate a low frequency (2.66%) of Y chromosome microdeletions in azoospermic and severe oligozoospermic infertile men in the Mongolian population.

Methods

1. Study population

Having obtained approval from the local institutional review board, this prospective descriptive study was carried out from February 2017 to December 2018. The inclusion criterion was as follows: screening for microdeletions was carried out only in men affected by azoospermia or severe oligozoospermia (sperm concentration of less than 5 million/mL). Patients were excluded if they had (1) semen analysis only performed once; (2) treatment with chemotherapeutic agents, radiotherapy, or testicular tumors; (3) karyotype abnormalities; or (4) retrograde ejaculation. According to the National Statistics Center of Mongolia, there were approximately 853,018 men (50.6% of the total male population) from the age of 15 to 49 in 2019. The confidence interval for the results using the given formula is 95%, with percentiles ranging from 10th to 95th in this study. Seventy-five azoospermic and severe oligozoospermic infertile patients were included in this study.

2. Semen analysis

In all participants, semen analysis was performed at least twice, at 1-month intervals, following 3–7 days of sexual abstinence. These samples were collected and examined after 30 minutes of liquefaction. The mean values from different semen analyses were reported and the average results were used. The reference values set by the World Health Organization in 2010 were used: a sperm count over 15 million/mL was considered normal, a sperm count of ≤ 5 million/mL was defined as severe oligozoospermia, and the absence of sperm was defined as azoospermia.

3. DNA analysis by polymerase chain reaction

Blood was taken from all participants for DNA analysis. DNA was extracted using DNA extraction kits (Chorosh Onosh, Ulaanbaatar, Mongolia). Next, DNA amplification by multiplex polymerase chain reaction (PCR) was performed using sequence-tagged sites primers for the AZFa sub-region (sY84 and sY86), the AZFb sub-region (sY127 and sY134), the AZFc sub-region (sY254, sY255), and the sex-determining region of the Y gene (sY14). Samples showing microdeletions on the first screening were verified by subsequent multiplex PCR amplification two more times. A complete description of the primers used for detecting Y-chromosome microdeletions and the amplification sets is shown in Table 1. The multiplex PCR amplification conditions were optimized as follows: initial denaturation at 95°C for 10 minutes, followed by 32 cycles at 95°C for 30 seconds, 60°C for 90 seconds, and 72°C for 60 seconds; with a final extension at 72°C for 1 minute. The PCR products were separated by electrophoresis on a 1.6% agarose gel stained with ethidium bromide. They were then viewed under ultraviolet trans-illumination. Negative controls with a DNA template were included with each reaction.

4. Hormone assay

Serum samples were obtained by venipuncture from all participants to measure levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and total testosterone (TT). The serum samples were allowed to clot for 30 minutes and the samples were separated by centrifugation for 10 minutes. All hormone assays were estimated using the Elecsys FSH, Elecsys LH, and Elecsys TT on a Cobas e 411 analyzer (Roche Diagnostics, Indianapolis, IN, USA).

5. Testicular biopsy

Multiple testicular sperm extraction (TESE) procedures were performed in azoospermic patients for diagnosis and treatment. If sperm was not retrieved by TESE, these testicular samples were fixed in 10% formalin solution, processed, embedded with paraffin, sectioned, stained with hematoxylin and eosin, and then examined for histological anomalies. Based on the most predominant and favorable histopathological pattern, testicular histology was classified as normal spermatogenesis, hypospermatogenesis, maturation arrest, Sertoli cell-only syndrome, or tubular hyalinization.

Table 1. The STS primer set used to detect Y chromosome microdeletions

<table>
<thead>
<tr>
<th>STS</th>
<th>Region</th>
<th>Sequence 5'→3'</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>s14</td>
<td>Yp</td>
<td>F: GAATATCCGGCTCTCCGG 470</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GCTGGTGCTCATTTGAG</td>
<td></td>
</tr>
<tr>
<td>AZFa</td>
<td>sY84</td>
<td>F: AGAGGGTTGTTAAGACGCT 326</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GCCTACCTGGGAGCTTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sY86</td>
<td>F: GTGACACAAGACTATGGCT 318</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ACACAGAGGGACACACCT</td>
<td></td>
</tr>
<tr>
<td>AZFb</td>
<td>sY127</td>
<td>F: GGTACCACAAGCAGGGAG 281</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTACGGCAAGTATAGGGGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sY134</td>
<td>F: GTCCGCTTCCATATAAAGC 300</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CCACTGGCAAAAATCTCAA</td>
<td></td>
</tr>
<tr>
<td>AZFc</td>
<td>sY254</td>
<td>F: GGTTGTACGAGGGGACAC 380</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GAAGGTTATCTACCAAAGGCA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sY255</td>
<td>F: GTTAGGATCCGGCTGGGAT 123</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTCGTCATGTCGACCCAC</td>
<td></td>
</tr>
</tbody>
</table>

STS, sequence tagged site; F, forward; R, reverse.
6. Data analysis

All collected data were evaluated with the Statistical Package for GraphPad Prism ver. 9.0.0 (GraphPad, San Diego, CA, USA). The data were presented as median and range, or number and percentage. The unpaired t-test was used for comparisons, and p-values of < 0.05 were considered to indicate statistical significance.

7. Ethical approval and consent to participate

This was a descriptive study conducted at the Mon-CL Fertility Center, Ulaanbaatar, Mongolia. The study was approved by the Institutional Review Board of the Mongolian National University of Medical Sciences (IRB No. 2017/3-05). Each patient who agreed to participate in the study provided written informed consent before enrollment.

Figure 1. Flowchart of study participants. A total of 1,007 men underwent semen analyses. Of these, 75 patients who were diagnosed with infertility were analyzed for Y chromosomal microdeletions. Six patients (8.0%) had severe oligozoospermia and 69 patients (92.0%) had azoospermia. Thirty-nine underwent testicular sperm extraction (TESE). Sperm was retrieved from 25 patients (64.1%), but not from 14 patients (35.9%). The tissue samples of these 14 patients were sent for biopsies. PCR, polymerase chain reaction.
Results

1. Patient characteristics

Patient characteristics are presented in Figure 1. The patients’ age ranged from 24 to 46 years (34.51 ± 5.42 on average). The average infertility period was 7.4 ± 5.1 years. The mean body weight was 87.92 ± 14.41 kg, and the average body mass index was 27.85 ± 5.25 kg/m². A total of 1,007 men underwent semen analyses from February 2017 to December 2018. From the total sample, 75 patients who were diagnosed with infertility were analyzed for Y chromosome microdeletions. Six patients (8.0%) had severe oligozoospermia and 69 patients (92.0%) had azoospermia. From this group, 39 patients underwent TESE. The sperm retrieval rate was 64.1% (25 of 39 patients). Testicular samples from the 14 patients whose sperm was not retrieved were sent to the histopathology laboratory for analysis.

2. Semen analysis

The mean age of azoospermic patients was 34.6 ± 5.89 years old, and severe oligozoospermic patients averaged 33.7 years old. The average pH of the samples from all participants was 7.7. The mean semen volume of all patients was 3.05 mL (range, 0.2–7 mL). The mean semen volume was 2.94 ± 1.79 mL in patients with azoospermia and 3.96 ± 1.62 mL in those with severe oligozoospermia. The average sperm count or concentration in 1 mL of semen in patients with severe oligozoospermia was 1.60 ± 1.64 million/mL (Table 2).

3. Y chromosome microdeletions

Seventy-five patients were analyzed for Y chromosome microdeletions. A microdeletion in the Y chromosome was detected in the AZFa region (sY84 and sY86) in 1 patient and partially detected in the AZFc region (sY254) in 1 other patient. Deletions in the AZFb re-

Table 2. Results of semen analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Azoospermia (n = 69)</th>
<th>Severe oligozoospermia (n = 6)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>34.61 ± 5.39</td>
<td>33.67 ± 5.89</td>
<td>0.684</td>
</tr>
<tr>
<td>pH</td>
<td>7.70 ± 0.41</td>
<td>7.65 ± 0.36</td>
<td>0.752</td>
</tr>
<tr>
<td>Semen volume (mL)</td>
<td>2.94 ± 1.79</td>
<td>3.96 ± 1.62</td>
<td>0.189</td>
</tr>
<tr>
<td>Sperm count (million/mL)</td>
<td>0</td>
<td>1.60 ± 1.64</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation. Unpaired t-test calculated using GraphPad Prism. Differences were considered to be statistically significant when \( p < 0.05 \).

Figure 2. Polymerase chain reaction (PCR) results of Y chromosome microdeletions. (A) A representative picture of the Y chromosome non-deletion group. Lane 1: size marker; lane 2: negative control; lane 3: negative control (female patient blood); lane 4: positive control (sY127); lane 5: positive control (sY14); lane 6: AZFa- sY84; lane 7: AZFa- sY86; lane 8: AZFb-sY127; lane 9: AZFb-sY134; lane 10: AZFc-sY254; lane 11: AZFc-sY255. (B) A representative picture of an AZFc (sY-254) microdeletion. Lane 1: size marker; lane 2: negative control (distilled water); lane 3: negative control (female patient blood); lane 4: positive control (sY127); lane 5: positive control (sY14); lane 6: AZFa- sY84; lane 7: AZFa- sY86; lane 8: AZFb-sY127; lane 9: AZFb-sY134; lane 10: AZFc-sY254; lane 11: AZFc-sY255. (C) A representative picture of an AZFa (sY84 and sY86) microdeletion. Lane 1: size marker; lane 2: negative control (distilled water); lane 3: negative control (female patient blood); lane 4: positive control (sY127); lane 5: positive control (sY14); lane 6: AZFa- sY84; lane 7: AZFa- sY86; lane 8: AZFb-sY127; lane 9: AZFb-sY134; lane 10: AZFc-sY254; lane 11: AZFc-sY255. SM, size marker; NC, negative control; PC, positive control.
gion were not detected (Figure 2). Thus, Y chromosome microdeletions were detected in 2 patients (2.66%) (Table 3). PCR results of the 75 patients are shown in Supplementary Figure 1. The age of the patient with a partial deletion of AZFc was 40 years; he was diagnosed with azoospermia, did not undergo TESE, his FSH level was 23.85 mIU/mL, his LH level was 12.0 mIU/mL, and his TT level was 5.0 ng/mL (Table 4). The hormone levels of the Y chromosome microdeletion group and the non-deletion group were compared. The average FSH level of the two patients (2.66%) with a microdeletion was 40.93 ± 17.07 mIU/mL; the average LH level was 12.5 ± 0.71 mIU/mL, and the average TT level was 4.53 ± 0.66 ng/mL. In the 73 patients (97.4%) with no microdeletions, the average FSH level was 14.86 ± 14.58 mIU/mL, the average LH level was 8.17 ± 5.41 mIU/mL, and the average TT level was 3.09 ± 2.16 ng/mL (Table 5). The mean level of FSH in the Y-chromosome microdeletion group was significantly higher than in the non-deletion group (p = 0.016). The LH and TT levels showed no significant differences.

### Table 3. Prevalence of Y chromosome microdeletions

<table>
<thead>
<tr>
<th>Deleted loci</th>
<th>Case</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZFa</td>
<td>1</td>
<td>1.33</td>
</tr>
<tr>
<td>AZFb</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AZFc</td>
<td>1</td>
<td>1.33</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>2.66</td>
</tr>
</tbody>
</table>

### Table 4. Clinical features of the Y chromosome microdeletions

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sperm concentration</th>
<th>Testicular histology</th>
<th>Hormone</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZFa deleted</td>
<td>31</td>
<td>Azoospermia</td>
<td>Sertoli cell-only syndrome</td>
<td>FSH: 58.0, LH: 12.0, TT: 5.0</td>
</tr>
<tr>
<td>AZFc partially deleted</td>
<td>40</td>
<td>Azoospermia</td>
<td>Refused biopsy</td>
<td>FSH: 23.85, LH: 13.01, TT: 4.06</td>
</tr>
</tbody>
</table>

FSH, follicle-stimulating hormone; LH, luteinizing hormone; TT, total testosterone.

### Table 5. Hormone levels of the Y chromosome microdeletion and non-deletion groups

<table>
<thead>
<tr>
<th>Hormone level</th>
<th>Y chromosome microdeletion group (n = 2)</th>
<th>Y chromosome non-deletion group (n = 73)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (mIU/mL)</td>
<td>40.93 ± 17.07</td>
<td>14.86 ± 14.58</td>
<td>0.016**</td>
</tr>
<tr>
<td>LH (mIU/mL)</td>
<td>12.5 ± 0.71</td>
<td>8.17 ± 5.41</td>
<td>0.268</td>
</tr>
<tr>
<td>TT (ng/mL)</td>
<td>4.53 ± 0.66</td>
<td>3.09 ± 2.16</td>
<td>0.357</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviation. Unpaired  t-test calculated using GraphPad Prism.

**Differences were considered to be statistically significant when p<0.05.

### Discussion

Y chromosome microdeletions are one of the most common caus-
Figure 3. Patterns of Sertoli cell-only syndrome. (A) Testicular atrophic tubules with hyalinization (H&E, 4×10, 10×40). (B, C) Tubules with thickened basal membranes are lined by Sertoli cells, some of which are altered in shape and detached from the basal membrane, and devoid of germ cells (H&E, 4×10, 10×40). (D) Expansion of interstitial space along with increased connective tissue. Atrophic appearance in the seminiferous tubules. Basement membrane thickening (H&E, 4×10, 10×10).
es of male infertility [5]. The Y chromosome AZF region contains many genes that are important for spermatogenesis. The region known as the AZF includes AZFa, AZFb, and AZFc [7]. The AZFa region contains USP9Y and DBY. The AZFb region contains CDY2, EI-F1AY, HSFY, PRY, RBMYL1, RP54Y5, SMCY, and XKRY. The AZFc region contains BPY2, CDY1, CSPG4LY, DAZ, and GOLGA2LY [8]. A study by Tiepolo and Zuffardi [9] demonstrated that Y chromosome microdeletions are involved in testicle differentiation and testicle maturation. Y chromosome microdeletions play an important role in predicting sperm extraction from testes. Some studies have shown that Y chromosome microdeletions are associated with testicular cancer and recurrent pregnancy loss [10,11].

Our Y chromosome microdeletion study is the first-ever study in Mongolia carried out to investigate this issue among infertile patients. Y chromosome microdeletions were found in two (2.66%) of 75 patients with azoospermia and severe oligozoospermia. The reported incidence of Y chromosome microdeletions in infertile men varies between studies from 1% to 55% [12,13].

According to a 2008 report, the frequency of AZF microdeletions among infertile men was less than 2.5% in Sweden, Germany, and Austria, whereas it was 10% (the highest) in Australia, China, and Brazil [5]. A comparative study carried out across Asia among patients with idiopathic azoospermia or severe oligozoospermia showed frequencies of 19.4% in China, 10.6%–11.7% in Taiwan, 15.8% in Japan, 9.6%–12.0% in India, 3.2% in Saudi Arabia, 3.3% in Turkey, and 2.6% in Kuwait [14–16]. We used six different markers—for the AZFa region, sY84 and sY86; for the AZFb region, sY127 and sY134; and for the AZFc region, sY254 and sY255—according to the guidelines published by the EAA/EMQN. In a 2012 study of Y chromosome microdeletions in 115 patients in Iran, 1.7% showed deletions in the AZFa and AZFb regions. Those researchers used the same six markers that we used in our study [17]. In a 2011 study, Akin et al. reported Y chromosome microdeletions in 7 patients (3.93%) among 178 infertile men. They were detected in the AZFc and AZFa regions [18]. In a study of 1,738 infertile men by Zhang et al. [19] in China, the frequency of Y chromosome microdeletions was 8.57%. Of note, the frequency of microdeletions in AZFa was 2.2%. In a study of 3,654 men by Totoni et al. [20], the frequency of Y chromosome deletions was 5.06%. The proportion of deletions in AZFa was 2.16%, which is similar to our results [19]. Most patients with AZFa deletions were diagnosed with Sertoli cell-only syndrome [21]. In a case with the complete deletion of AZFa, no sperm were retrieved. However, in cases of partial deletion, it has been reported that sperm can be retrieved by TESE [21,22]. In our study, the patient with a Y chromosome AZFa microdeletion was 31 years old, with azoospermia; no sperm was retrieved from his testis, and a histologic examination showed Sertoli cell-only syndrome. These results are similar to those reported by Kamp et al. [21].

Both patients with Y chromosome microdeletions had azoospermia, and FSH and LH hormone levels were higher than normal. The FSH level was 40.93 ± 17.07 mIU/mL and the LH level was 12.5 ± 0.71 mIU/mL, but there was a significant difference in the FSH hormone level compared to the non-Y chromosome microdeletion group. These results are similar to the results reported by Wang et al. [23] and Kumar et al. [24].

The mean age of patients in the sperm retrieval group was 38.3 ± 5.1 years, while the mean age of the sperm non-retrieval group was 31.5 ± 3.63 years. This difference was statistically significant. A study by Tsai et al. [25] showed that in vitro fertilization (IVF) results, pregnancy rates, and miscarriage rates were correlated with male age. According to the results of a micro-TESE study by Enatsu et al. [26], the average age of the patients in whom sperm were retrieved from the testis was 35.0 ± 5.6 years, while the average age of the sperm non-retrieval group was 33.2 ± 4.9 years (p < 0.05).

The 14 patients with no sperm retrieved by TESE were diagnosed with testicular histopathology. Histopathological examination showed Sertoli-cell-only syndrome in 12 patients (85.71%) and seminiferous tubule hyalinization in 2 patients (14.29%). Of the 25 patients who had sperm retrieved from TESE, 18 patients (72%) had IVF treatment. Two of these patients (11.1%) conducted embryo banking, and 16 (88.9%) had an embryo transfer. The partners of six patients (37.5%) had successful clinical pregnancies. One partner gave birth to twins, three delivered single babies, and two patients had missed abortions during the first trimester. The current data show that there is a low frequency of Y chromosome microdeletions in azoospermic and severe oligozoospermic infertile men in the Mongolian population. In patients with non-obstructive azoospermia, AZFa, AZFb, and AZFb/c microdeletions occur in 1%–2% of cases, but sperm is not retrieved by TESE, so there is no need for unnecessary TESE procedures. In patients with AZFc microdeletions, sperm formation functions normally. IVF can be performed with the sperm of a patient with microdeletions in the AZFc region, potentially resulting in a successful pregnancy. However, microdeletions within the AZFc region will likely be inherited by male children. We recommend further studies with a larger group of patients and control subjects screened for this microdeletion in order to confirm our results.

Conflict of interest

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

The authors would like to thank Dr. Sang-Jin Song for providing a Y chromosome microdeletion setting for this research. We would also like to thank Dr. Enkhee O, the Head of the Department of Adult Pathology, National Center for Pathology of Mongolia for analyzing the biopsy results and Dr. Gantumur Battogtokh, R&D Center, Up-ex-Med Co. Ltd, South Korea, who helped with the writing and submission of the manuscript. The authors would also like to express their gratitude to all individuals who participated as patients in this study.

ORCID

Erdenesuvd Damdinsuren https://orcid.org/0000-0001-8535-3101
Purevjargal Naidansuren https://orcid.org/0000-0001-7996-4061
Mendsaikhan Gochoo https://orcid.org/0000-0002-9909-9244
Bum-Chae Choi https://orcid.org/0000-0002-6626-2359
Min-Youp Choi https://orcid.org/0000-0002-3107-7807
Bolorchimeg Baldandorj https://orcid.org/0000-0003-1525-9958

Author contributions

Conceptualization: ED, PN, BB. Data curation: ED, PN, MG. Formal analysis: ED, PN, MG. Methodology: BCC, MYC. Writing–original draft: ED, PN. Writing–review & editing: BB, BCC, MYC, MG.

Supplementary material

Supplementary material can be found via https://doi.org/10.5653/cerm.2021.05099.

References

4. Bayasgalan G. The major clinical forms and risk factors of male infertility in Mongolia. Ulaanbaatar: Mongolian National University of Medical Sciences; 2005.
18. Akin H, Onay H, Turker E, Ozkinay F. Primary male infertility in...


Effect of aqueous Nigella sativa extract on the functional parameters of post-thaw human spermatozoa during vitrification

Zohreh Nasiri¹, Fatemeh Ghorbani², Mohammad Seify³,⁴, Aysan Sharbati⁵

¹Department of Biochemistry, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran; ²Department of Biochemistry, Faculty of Medicine, Tehran University of Medical Sciences, Tehran; ³Department of Reproductive Biology, Shahid Sadoughi University of Medical Sciences, Yazd; ⁴Research and Clinical Center for Infertility, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd; ⁵Department of Medical Surgical Nursing, Faculty of Nursing and Midwifery, Iran University of Medical Sciences, Tehran, Iran

Objective: Sperm vitrification leads to the production of reactive oxygen species (ROS) that can damage the functional parameters of sperm. The present study aimed to investigate the antioxidant effect of Nigella sativa extract on motility, plasma membrane function, mitochondrial membrane potential (MMP), DNA damage, and intracellular ROS production.

Methods: A total of 20 sperm samples were used. Samples were divided into six experimental groups, including groups with aqueous extract from N. sativa seeds at concentrations of 1% to 6%, a cryopreserved control group, and a fresh control group.

Results: Statistical analysis showed significantly higher total sperm motility at concentrations of 3% to 6% than in the vitrified semen control group. Additionally, progressive motility and all motion characteristics at all concentrations were significantly higher than in the vitrified semen control group. The presence of N. sativa seed extract also improved the quality of the sperm parameters assayed in all experimental groups (1%–6%; intracellular ROS production, DNA damage, MMP, and sperm membrane function) compared to the control group.

Conclusion: Higher concentrations of N. sativa led to improvements in all sperm parameters and sperm quality. These findings indicate that N. sativa seed extract is effective for improving the quality of sperm after vitrification.

Keywords: Cryopreservation; Human sperm; Nigella sativa; Oxidative stress

Introduction

The process of sperm cryopreservation plays an important role in artificial insemination. Although artificial insemination using fresh sperm tends to be more successful, the fertility rate decreases when cryopreserved sperm is used [1]. During cryopreservation, large amounts of reactive oxygen species (ROS) are produced, resulting in increased lipid peroxidation. The sperm plasma membrane contains high amounts of unsaturated fatty acids that are oxidized by ROS [2]. The cryopreservation process can cause intracellular ice crystallization, osmotic stress, and cold shock, which could ultimately negatively affect sperm structure and function [3]. Oxidative stress products can affect sperm characteristics such as motility, cell membrane potential, mitochondrial membrane potential (MMP), and DNA activity, disrupting sperm function and fertility [4]. Cryopreservation can be undertaken using two methods: slow freezing and vitrification. While slow freezing increases damage to sperm and tissue, it has been the most conventional and common cryopreservation method for many years. Vitrification tends to be used as an alternative method to slow freezing. Vitrification is a relatively new method for sperm cryopreservation that has advantages over conventional methods, such as reducing intracellular ice crystals. Moreover, compared to slow freezing, vitrification is cheaper and freezes sperm in...
Sperm naturally contains many antioxidant systems capable of reducing intracellular damage [6]. An antioxidant system in the seminal plasma consists of the enzymatic antioxidants, catalase, and superoxide dismutase (SOD), with its two isozymes playing a critical role in eliminating ROS production. SOD preserves the quality of spermatozoa by reducing lipid peroxidation and oxygen toxicity [7]. The presence of SOD and catalase reduces lipid peroxidation by removing the superoxide anion produced by reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in neutrophils [8]. Glutathione peroxidase, which is another important enzymatic antioxidant, removes peroxyl from hydrogen peroxide. Moreover, natural antioxidants such as vitamin E, pyruvate, urate, ascorbate, glutathione, vitamin A, albumin, ubiquinol, taurine, hypotaurine, vitamin C, beta-carotenes, and carotenoids are non-enzymatic antioxidants present in seminal plasma [9]. The vitrification process reduces the antioxidant protection of the sperm membrane [10]. However, an imbalance between oxidative stress products and the antioxidant system of sperm during the vitrification process is the principal cause of cryopreservation damage [11,12].

Many recent studies have been conducted on the use of antioxidants to reduce the damaging effects of the cryopreservation process. *Nigella sativa* is a medicinal plant known as black seed, black cumin, or *shouneez* that has several therapeutic effects on different diseases. For instance, a study showed that *N. sativa* oil improved the sperm count, motility, morphology, semen volume, and pH of raw semen in fertile men [13]. Another study showed that *N. sativa* improves the raw sperm parameters and acrosomal function of cyclophosphamide-induced testis toxicity in mice [14]. It was reported that *N. sativa* extract (NSE) at all concentrations (1%–6%) improved the quality of buffalo sperm that had been damaged in the vitrification process by an antioxidant mechanism [15]. *N. sativa* seeds contain carbohydrates, eight of the nine essential amino acids, vitamins, minerals, and antioxidants [16]. Various sources have reported the nutritional composition of *N. sativa* (20%–85% of protein, 38.20% of fat, 7%–94% of fiber, and 31.94% of total carbohydrates). Glutamate, arginine, aspartate, cysteine, and methionine are the major and minor amino acids. Black cumin seeds also contain iron, copper, zinc, phosphorus, calcium, thiamine, niacin, pyridoxine, and folic acid [17]. *N. sativa* has been proven to have antifungal and antibacterial effects, as well as antioxidant properties [18]. *N. sativa* is believed to contain essential elements such as thymoquinone, dithymoquinone, 4-terpineol, carvacrol, anethole, thymol, and alpha-pinene [15]. Evaluation of *N. sativa* oil using thin-layer chromatography showed that thymoquinone, carvacrol, and 4-terpineol have a very strong antioxidant effect on the sperm parameters of vitrified buffalo sperm [15]. Although studies conducted on rabbits, mice, and humans have shown that NSE improves the quality of raw sperm parameters [19-21], no study has yet been conducted on the antioxidant effect of aqueous NSE on the functional parameters of human sperm during the vitrification process. The main hypothesis of this study was that NSE, which reduces ROS production in sperm due to its antioxidant mechanism, improves the quality of vitrified semen. Therefore, this study aimed to evaluate the antioxidant properties of aqueous NSE on motility, plasma membrane integrity, MMP, DNA damage, and intracellular ROS production in human sperm.

**Method**

1. **Subjects and semen collection**

This study was conducted at the Research Center and Laboratory of Tehran University of Medical Sciences using 20 normal sperm samples obtained from men referred to the Aban Infertility Center from February 2020 to April 2020. Samples were obtained from patients via masturbation after 3 to 7 days of abstinence from sex. This study was approved by the ethics committee of Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1398.887). Written informed consent was obtained from participants. The sperm parameters were assessed according to World Health Organization standards (2010), and sperm motility and concentration were assessed using the SCA CASA system (Sperm Class Analyzer ver. 5.1; Microptic S.L., Barcelona, Spain). Only sperm samples with a concentration of more than 1 × 10⁹ sperm/mL, progressive motility of 70%, and a volume of 2 mL to 6 mL were used for this study.

2. **NSE preparation**

*N. sativa* seeds were prepared by a plant classification expert from a local medicinal plant market. The aqueous extract was prepared based on the protocols used in previous studies with similar backgrounds [15]. *N. sativa* seeds were crushed after being washed with distilled water and dried at 50°C. Next, 10 g of seed powder was mixed with 50 mL of distilled water for 15 to 20 minutes. The mixture then rested for 30 minutes. After centrifugation, 1,340 × g, 15 minutes), the supernatant was separated using Whatman filter paper. The filtration was performed twice to increase accuracy. The extract sterilization was carried out using Acrodisc (Merck, Kenilworth, NJ, USA), and the extract was stored in special sterile containers at 4°C for several days until use. Finally, the intended concentrations of the extract were prepared based on previous studies [15]. Concentrations of 1% containing 30 µL of extract and 170 µL of distilled water, 2% containing 60 µL of extract and 140 µL of distilled water, 3% containing 90 µL of extract and 110 µL of distilled water, 4% containing 120 µL of extract and 80 µL of distilled water, 5% containing 150 µL of extract and 50 µL of distilled water, and 6% containing 180 µL of extract and 20 µL of distilled water were prepared [15].
3. Vitrification and warming

The micro-droplet technique was used for the vitrification method [22]. In this process, samples were first mixed with human tubal fluid (HTF; Sigma-Aldrich, St. Louis, MO, USA) solution and then added to a solution containing 5% human serum albumin (Sigma-Aldrich) and 0.5 mol/L sucrose. Next, the sperm solution was divided equally into six experimental groups, and concentrations of 1% to 6% extract were added to each of the groups, respectively. No NSE was added to the solution for the raw semen control group. Finally, 30 µL of the prepared suspension was incubated in a tank containing liquid nitrogen for 1 week. At the thawing stage, the samples and 5 mL of the preheated HTF solution at 37°C were first mixed with 1% HAS, and, after incubation, the suspension was kept at 37°C near 5% CO₂ for 5 minutes. Finally, the samples were centrifuged at 400 × g for 5 minutes and suspended in 50 µL of HTF [4].

4. Assessment of sperm motion characteristics

In this assessment, a 10-µL sperm sample was first placed on a pre-heated Makler slide at 37°C and examined using the CASA system. The aforementioned parameters, including total motility (%), progressive motility (%), average path velocity (VAP; µm/sec), curvilinear velocity (VCL; µm/sec), linearity (LIN; %), and straight-line velocity (VSL; µm/sec), were examined. Five microscopic fields and 400 total spermatozoa were examined.

5. Assessment of functional sperm membrane

To investigate the sperm plasma membrane function, the hypo-osmotic solution test was used. To prepare the solution, 0.73 g of sodium citrate and 1.35 g of fructose (Merck) were dissolved in 100 mL of distilled water (osmolality ~190 mOsmol/kg). Finally, 50-µL sperm samples were dissolved in 500 µL of HOS solution (at 37°C for 45 minutes). Next, 10 µL of this solution was transferred to a slide, and the samples were evaluated using phase-contrast microscopy (Olympus BX20, Olympus, Tokyo, Japan) [23].

6. Assessment of mitochondrial membrane plasma

Lipophilic cationic dye, JC-1 (T4069, Sigma-Aldrich), was used to investigate MMP. After being centrifuged (500 × g, 5 minutes), the samples were dissolved in phosphate-buffered saline (PBS) until a solution containing 5% human serum albumin (Sigma-Aldrich) and 0.5 mol/L sucrose. Next, the sperm solution was divided equally into six experimental groups, and concentrations of 1% to 6% extract were added to each of the groups, respectively. No NSE was added to the solution for the raw semen control group. Finally, 30 µL of the prepared suspension was incubated in a tank containing liquid nitrogen for 1 week. At the thawing stage, the samples and 5 mL of the preheated HTF solution at 37°C were first mixed with 1% HAS, and, after incubation, the suspension was kept at 37°C near 5% CO₂ for 5 minutes. Finally, the samples were centrifuged at 400 × g for 5 minutes and suspended in 50 µL of HTF [4].

7. Assessment of DNA damage

Acridine orange fluorescence was used to determine DNA damage. The centrifuged samples (500 × g, 5 minutes) were added to a Tris-null EDTA (ethylenediaminetetraacetic acid) buffer solution containing 1 mmol EDTA, 10 mmol Tris, and 0.15 mol NaCl. Using this technique, green fluorescence, indicating normal chromatin, and red fluorescence, indicating abnormal chromatin, were detected using FL1 (500–530 nm) and FL2 (620 nm) detectors, respectively [25].

8. Assessment of intracellular ROS production

Dihydroethidium (DHE) was used to measure intracellular ROS production. DHE is oxidized by the superoxide anion and binds to DNA, and it produces red fluorescence. The samples were suspended in PBS solution after being thawed at a concentration of 1 × 10⁶ sperm/mL. Next, 10 µL of DHE solution (Sigma-Aldrich) was added to the previous solution and kept at 25°C for 20 minutes. Finally, the flow cytometry technique was used. With this technique, red fluorescence was detected using FL2 (525–625 nm) [23].

9. Flow cytometry analysis

Flowcytometric analysis of the sperm parameters was conducted using FACSCaliber (BD Biosciences, San Jose, CA, USA). The samples were excited using an argon laser at 488 nm. SYBR-14 and propidium iodide (Pi; Molecular Probes, Eugene, OR, USA) were dissolved in anhydrous DMSO (4 µM) and distilled water (1 mM), respectively. Aliquots of the two working solutions were mixed at a 1:1 ratio, and 10 µM was added per counting tube, resulting in a concentration of 50 nM SYBR-14 and 12 µM PI. Three events were assessed using this technique, which was replicated three times. Gating settings for the sperm population (gated events) used to exclude non-sperm events were based on Hoechst 33342 staining. A total of 10,000 spermatozoa were assessed using flow cytometry and analyzed using Cyflogic (ver. 1.2.1; CyFlo Ltd., Turku, Finland).

10. Statistical analysis

All data were analyzed using the Kolmogorov Smirnov test to confirm a normal distribution of values. One-way analysis of variance and the Tukey test were used to determine differences between the various groups. Statistical analysis was performed using SPSS ver. 16.0 (SPSS Inc., Chicago, IL, USA). The results are shown as means with standard errors. Differences were considered to be statistically significant at p < 0.05.

Results

1. Effects of NSE on sperm parameters (total motility, progressive motility, and motion characteristics)

Vitrification caused a significant decrease in the percentage of total motility, progressive motility, and motion characteristics (VSL,
VCL, LIN, and VAP) compared to the raw semen control group \((p < 0.05)\). No significant difference was observed in total motility in the groups containing 1% and 2% concentrations compared to the vitrified semen control group, while the groups with 3% to 6% concentrations preserved the quality of sperm better compared to the control group \((p \leq 0.05)\). The addition of the NSE at concentrations of 1% to 6% also improved the percentage of progressive motility and motion characteristics (VSL, VCL, LIN, and VAP) compared to the control group \((p \leq 0.05)\) (Table 1).

### 2. Effects of NSE on sperm membrane function, MMP, DNA damage, and intracellular ROS production

Vitrification resulted in a noticeable decrease in sperm membrane function and MMP and an increase in intracellular ROS production and DNA damage compared to the raw semen control group \((p < 0.05)\). The presence of NSE at all 6 concentration levels caused a significant increase in sperm membrane function and MMP compared to the vitrified semen control group \((p \leq 0.05)\), while no significant increase was observed compared to the raw semen control group. Moreover, all concentrations of NSE led to a significant reduction in intracellular ROS production and DNA damage compared to that of the vitrified semen control group \((p \leq 0.05)\) (Table 2).

### Discussion

The present study investigated the effect of different concentrations of aqueous NSE on the parameters of human sperm during vitrification. This study’s findings revealed that the vitrification process significantly damaged sperm quality, which was assessed according to their percentage of total motility and motion parameters. NSE, as a tool for sperm cryoprotection, preserved the normal quality of sperm parameters. Although NSE improved progressive motility and motion parameters at all concentrations, the percentage of total motility improved the most at concentrations of 3% to 6%. Moreover, while other variables evaluated in this study were also affected by the vitrification technique, the presence of NSE reversed the effects of vitrification. The quality of the functional sperm membrane and MMP decreased and intracellular ROS production increased in the vitrified semen control group compared to the raw semen control group. NSE improved the above parameters at all concentrations.

The ROS produced during the cryopreservation process have an adverse effect on sperm structure and function. Therefore, the production of ROS should be minimized to improve sperm function during cryopreservation. However, multiple studies have shown that the cryopreservation process reduces sperm antioxidants \([26,27]\).

### Table 1. Motility parameters of raw, control, and post-thaw human sperm samples supplemented with different concentrations of aqueous NSE

<table>
<thead>
<tr>
<th>Group</th>
<th>Raw semen control</th>
<th>Vitrified semen control</th>
<th>1%</th>
<th>2%</th>
<th>3%</th>
<th>4%</th>
<th>5%</th>
<th>6%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total motility (%)</td>
<td>64.1 ± 14.7</td>
<td>25.5 ± 11.4(^a)</td>
<td>29.3 ± 8.4</td>
<td>29.5 ± 8.2</td>
<td>35.7 ± 8.1(^b)</td>
<td>35.8 ± 8.9(^b)</td>
<td>32.2 ± 8.1(^b)</td>
<td>32.2 ± 8.5(^b)</td>
</tr>
<tr>
<td>Progressive motility (%)</td>
<td>45.3 ± 5.6</td>
<td>12.7 ± 5.1(^a)</td>
<td>41.6 ± 5.6(^b)</td>
<td>42.1 ± 5.6(^b)</td>
<td>45.8 ± 5.2(^b)</td>
<td>46.9 ± 5.7(^b)</td>
<td>44.1 ± 5.3(^b)</td>
<td>43.9 ± 5.1(^b)</td>
</tr>
<tr>
<td>VCL (µm/sec)</td>
<td>45.3 ± 4.1</td>
<td>22.5 ± 4.7(^a)</td>
<td>47.6 ± 4.4(^b)</td>
<td>47.9 ± 4.6(^b)</td>
<td>47.8 ± 4.5(^b)</td>
<td>48.7 ± 4.1(^b)</td>
<td>47.1 ± 4.3(^b)</td>
<td>47.5 ± 4.5(^b)</td>
</tr>
<tr>
<td>VSL (µm/sec)</td>
<td>27.4 ± 7.6</td>
<td>14.1 ± 7.4(^a)</td>
<td>25.1 ± 7.5(^b)</td>
<td>25.7 ± 7.6(^b)</td>
<td>27.5 ± 7.4(^b)</td>
<td>28.7 ± 8.1(^b)</td>
<td>28.2 ± 7.6(^b)</td>
<td>28.1 ± 7.5(^b)</td>
</tr>
<tr>
<td>VAP (µm/sec)</td>
<td>35.5 ± 7.1</td>
<td>22.6 ± 7.5(^a)</td>
<td>34.7 ± 7.4(^b)</td>
<td>37.5 ± 7.1(^b)</td>
<td>39.6 ± 7.5(^b)</td>
<td>39.9 ± 7.1(^b)</td>
<td>38.1 ± 7.7(^b)</td>
<td>38.8 ± 7.3(^b)</td>
</tr>
<tr>
<td>LIN (%)</td>
<td>60.24 ± 6.43</td>
<td>42.36 ± 6.75(^a)</td>
<td>57.14 ± 6.12(^b)</td>
<td>58.02 ± 6.19(^b)</td>
<td>61.59 ± 6.17(^b)</td>
<td>61.62 ± 6.34(^b)</td>
<td>60.59 ± 6.15(^b)</td>
<td>60.70 ± 6.62(^b)</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard error. NSE, Nigella sativa extract; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity.

\(^a\)\(p<0.05\), significant differences compared to the raw semen control group; \(^b\)\(p<0.05\), significant differences compared to the vitrified semen control group.

### Table 2. Sperm membrane function, DNA damage, intracellular ROS, and MMP of raw, control, and post-thaw human sperm samples supplemented with different concentrations of aqueous NSE

<table>
<thead>
<tr>
<th>Group</th>
<th>Raw semen control</th>
<th>Vitrified semen control</th>
<th>NSE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>65.2 ± 2.4</td>
<td>33.6 ± 2.8(^a)</td>
<td>67.1 ± 2.5(^b)</td>
</tr>
<tr>
<td>Sperm membrane function</td>
<td>59.5 ± 5.4</td>
<td>29.2 ± 5.7(^a)</td>
<td>37.7 ± 5.4(^b)</td>
</tr>
<tr>
<td>MMP</td>
<td>7.4 ± 4.3</td>
<td>10.5 ± 4.1(^a)</td>
<td>3.6 ± 1.3(^b)</td>
</tr>
<tr>
<td>DNA damage</td>
<td>32.3 ± 7.2</td>
<td>53.1 ± 7.5(^a)</td>
<td>43.9 ± 7.5(^b)</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard error. ROS, reactive oxygen species; MMP, mitochondrial membrane potential; NSE, Nigella sativa extract.

\(^a\)\(p<0.05\), significant differences compared to the raw semen control group; \(^b\)\(p<0.05\), significant differences compared to the vitrified semen control group.
addition, studies have proven that reduced antioxidant activity leads to oxidative stress reactions that disrupt the sperm parameters, such as sperm motility, plasma membrane integrity, and DNA [4]. As a medicinal plant, N. sativa has strong antioxidant properties. This property is due to the presence of effective compounds such as thymoquinone, thymol, and dithymoquinone. Vitamin E, a potent antioxidant, is also one of the components present in the seeds of N. sativa [28]. N. sativa contains elements such as iron, sodium, potassium, and copper that increase sperm maturity and can also be cofactors for several enzymes that protect semen [29]. The antioxidant properties of thymoquinone include the ability to eliminate hydroxyl and superoxide radicals [30]. The antioxidant properties of thymoquinone may depend on the redox property of the quinine structure of the thymoquinone molecule that causes it to pass through morphological barriers and penetrate intracellular spaces, reducing ROS production. The thymoquinone present in NSE can increase non-enzymatic antioxidant activity such as glutathione and, in addition to spontaneous reaction with glutathione, NADH, and NADPH, produce species such as glutathione and dihydrothymoquinone that protect sperm from oxidative stress damage [31]. The results of this study confirm the protective properties of N. sativa against oxidative stress. In the groups containing NSE at all concentrations (1%–6%), a significant decrease in intracellular ROS production was observed. The extent of the reduction increased according to the concentration of NSE, from 1% to 6%, compared to the vitrified semen control group [15]. A previous study found that NSE was capable of controlling free radicals, such that the antioxidant activity of NSE increased from 1% to 6%, and this result is in agreement with that of our study. Motility is one of the main characteristics of sperm related to successful fertility. Sperm with normal motility can reach the location of the egg and result in normal fertility. Therefore, an understanding of sperm motility is very important in research studies.

Our results showed an upward trend in sperm motility at concentrations of 1% to 4% compared to the cryopreserved control group. While this increase was observed at low concentrations of 1% to 4%, at higher concentrations of 5% and 6%, motility began to decrease. Kolahdooz et al. [13] showed that N. sativa oil improved sperm parameters (count, morphology, and motility) in infertile men. Supplementation with N. sativa oil in the vitrified semen of goats showed that N. sativa could preserve the quality of sperm parameters and the sperm membrane [32]. N. sativa oil has been reported to improve sperm parameters, testosterone level, and MMP in rats exposed to an obesogenic diet [33]. A study conducted in 2018 showed that aqueous NSE in cryopreserved buffalo spermatozoa improved sperm parameters, DNA integrity, and plasma membrane integrity due to its antioxidant activity, and these results are similar to those of this study [15]. Increased sperm motility at concentrations of 1% to 4% and decreased sperm motility at concentrations above 4% are likely due to thymoquinone. Thymoquinone has antioxidant properties at low concentrations but causes an increase in ROS production at concentrations higher than 4%, which leads to a reduction in motility [34,35]. The results related to sperm motility also hold true for the results of motion characteristics (VSL, VCL, LIN, VAP). ROS are produced in mitochondria during the oxidative phosphorylation process. In the experimental groups, NSE at concentrations of 1% to 6% showed a significant increase in MMP compared to the vitrified semen control group and this increase exhibited an upward trend up to a concentration of 4% according to previous results. This increase in MMP was probably caused by a reduction in intracellular ROS production due to the antioxidant properties of NSE. Previous studies have observed improvements in the sperm quality of humans and different animal species due to the presence of NSE [20,21]. Our assessment of sperm DNA damage showed that aqueous NSE reduced DNA damage in the groups with concentrations of 1% to 6% compared to that in the vitrified semen control group and the raw semen control group. This decrease can be attributed to the effects of NSE on DNA structure and density. These results are in agreement with the findings of previous studies showing that NSE supplementation in extenders improved DNA integrity [36].

The presence of aqueous NSE during the sperm vitrification process protects sperm against oxidative stress and reduces ROS production. This reduction can improve sperm parameters, such as motility, plasma membrane function, MMP, and DNA damage. This property can be attributed to the robust antioxidant activity of the constituent elements of NSE. Although further research is needed on the effects of plant extracts on sperm function, given the available evidence, NSE can be suggested as a powerful and inexpensive antioxidant to improve vitrification conditions.

Conflict of interest

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

Author contributions

Conceptualization: AS. Data curation: ZN, FG. Formal analysis: MS. Acquisition: MS. Methodology: MS, AS. Project administration: FG. Visualization: MS. Writing—original draft: FG, AS. Writing—review & editing: ZN.

References

1. AbdelHafez F, Bedaiwy M, El-Nashar SA, Sabanegh E, Desai N.


26. Bilo deau JF, Chatterjee S, Sirard MA, Gagnon C. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle
The relationship between reactive oxygen species, DNA fragmentation, and sperm parameters in human sperm using simplified sucrose vitrification with or without triple antioxidant supplementation

Theesit Juanpanich 1, Tayita Suttirojpattana 1, Rangsun Pampai 2, Teraporn Vutyavanich 3

1 Chiang Mai IVF Center, Chiang Mai; 2 Embryo Technology and Stem Cell Research Center, School of Biotechnology, Suranaree University of Technology, Nakhon Ratchasima; 3 Department of Obstetrics and Gynecology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

Objective: This study examined whether the addition of triple antioxidants (3A)—10 µM acetyl-L-carnitine, 10 µM N-acetyl-L-cysteine, and 5 µM α-lipoic acid—in freezing-thawing medium during human sperm cryopreservation using the sucrose vitrification (SuV) and liquid nitrogen vapor (Vapor) techniques could improve post-thaw survival of spermatozoa.

Methods: We analyzed 30 samples from healthy human sperm donors. Each sample was allocated into one of five groups: fresh control, SuV, SuV+3A, Vapor, and Vapor+3A. The sperm motility, morphology, viability, intracellular and extracellular reactive oxygen species (ROS) levels, and sperm DNA fragmentation (SDF) were evaluated.

Results: The cryopreserved spermatozoa had significantly reduced percentages of motility ($p<0.05$) and viability ($p<0.05$). Antioxidant supplementation non-significantly improved these parameters ($p>0.05$). No significant differences were found in sperm morphology between the fresh and frozen-thawed groups ($p>0.05$). After freezing, the extracellular ROS levels in the frozen-thawed groups were significantly higher ($p<0.05$) than in the fresh group. However, we did not find any differences in intracellular ROS parameters among these groups ($p>0.05$). The SDF was higher in the SuV and Vapor groups than in the fresh group, but without statistical significance ($p=0.075$ and $p=0.077$, respectively).

Conclusion: Cryopreservation had detrimental effects on sperm motility, viability, and extracellular ROS levels, without changing the morphology or intracellular ROS levels. Antioxidant supplementation was slightly effective in preventing SDF in frozen-thawed spermatozoa.

Keywords: Antioxidants; DNA fragmentation; Human; Spermatozoa; Sucrose; Vitrification

Introduction

Cryopreservation of human spermatozoa has been widely used in assisted reproductive technology (ART) for more than 60 years [1]. Since the introduction of cryopreservation, the development of new protocols aiming to optimize the quality of thawed sperm samples has been of major interest to researchers in the field of andrology. It is well known that sperm vitrification using a high concentration of a permeable cryoprotective agent (CPA) can damage sperm cells, inducing osmotic injury and physiological alterations [2]. Other approaches consider the utilization of protocols that avoid the use of CPA as an alternative way to preserve sperm function and viability. For example sucrose, a non-permeating agent, has been successfully used for human sperm cryopreservation [3,4]; however, studies on the relationship between ROS formation and sperm DNA fragmentation (SDF) are limited.

Reactive oxygen species (ROS) are formed as natural byproducts of cellular aerobic metabolism and function as signal molecules that...
regulate cell-to-cell communication [5]. Normally, a small amount of ROS molecules can be destroyed by the scavenging system itself. However, at the pathological level, ROS can negatively impact cellular function, resulting in sperm damage [6]. Excessive levels of ROS molecules can not only impair sperm motility, but also induce SDF by endonuclease activity via the apoptotic cascade pathway [7].

In previous studies, antioxidants such as acetyl-L-carnitine (ALC), N-acetyl-L-cysteine (NAC), and α-lipoic acid (ALA) have been shown to exert protective effects individually on several tissues and might be beneficial in mammalian gametes [8–10]. L-carnitine and ALC are found naturally in epididymal fluid. They play a critical role in sperm metabolism, which directly affects sperm motility and fertilization [8,11]. Moreover, ALC improves the in vitro blastocyst development rate in mouse embryos by preventing oxidative stress-induced DNA damage [12]. NAC, a precursor of glutathione (GSH), is widely used as a thiol-containing antioxidant and modulator of the intracellular redox state [13]. The addition of GSH to the culture medium increased the percentage of fertilization and enhanced embryo development [9]. GSH has an antioxidant defensive capacity during the sperm freezing-thawing process [14]. Sperm quality may improve as a result of cysteine’s effect on GSH levels. ALa is a universal antioxidant that acts as a cofactor for mitochondrial enzyme activity [15]. It helps with ATP generation, converts pyruvate to acetyl-CoA by oxidative decarboxylation, and is involved in the citric acid cycle via mitochondrial alpha-ketoglutarate dehydrogenase activity [16]. ALA supplementation in sperm freezing medium improved sperm motility and acrosome integrity, and protected sperm from freezing-thawing-induced DNA damage [10].

In nature, various antioxidant systems act together in concert to provide protection against oxidative stress and promote repair. Previous studies that concentrated on the use of individual antioxidants did not replicate natural conditions. The supplementation of combinations of antioxidants should exert a synergistic effect and provide better protection against oxidative-induced injuries than single antioxidant supplements [17]. Limited research has been conducted on the effects of antioxidant combinations in sperm freezing-thawing medium. A study by Truong and Gardner [18] supplemented ALC, NAC, and ALC in sperm and oocyte washing medium, as well as in embryo culture medium. They showed that a combination of these three antioxidants provided better protection against ROS than their individual counterparts [19]. However, it is not known whether this combination of antioxidants might affect sperm quality after cryopreservation.

The rationale for combining the three antioxidants is as follows: ALC serves as a universal scavenger of free radicals and reduces DNA damage, while NAC is an important substrate for the synthesis of GSH, and ALA is capable of regenerating other antioxidants, including GSH, which plays a critical role in protecting cells from oxidative damage. We developed a simplified sucrose freezing medium for vitrification of human spermatozoa. In this study, we investigated the effect of triple antioxidant supplementation in freezing-thawing medium on both intracellular and extracellular ROS production, and we also evaluated SDF by imaging flow cytometry.

### Methods

#### 1. Chemicals

All chemicals were purchased from Sigma-Aldrich Chemicals (Sigma Chemical, St. Louis, MO, USA), unless otherwise stated.

**2. Semen collection and preparation**

Thirty normozoospermic semen samples from patients who had been referred to the in vitro fertilization clinic of Korat Health Center were included in this study. This study was approved by the Ethics Committee for Research Involving Human Subjects of Suranaree University of Technology, Thailand (EC-63-80). All participants signed an informed consent form before participating in this study. Alcohol drinkers and smokers were excluded from this study, as well as those with a chronic illness or serious systemic disease, genital infection, or varicocele. The semen samples were collected by masturbation after abstinence of 2–7 days and allowed to liquefy for 30 minutes at 37°C. Semen analysis was performed according to the guidelines of the World Health Organization [20], and only semen samples exhibiting parameters within the normal ranges were used in the study.

Liquefied semen samples were placed on the top of two layers (40% and 80% fractions) of Sil-Select Stock solution (FertiPro NV, Beemem, Belgium) and centrifuged at 350 × g for 10 minutes to separate immotile and motile sperm from seminal plasma. The pellets were then re-suspended in washing medium consisting of 3 mL of Earle's Balanced Salt Solution (EBSS; Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 0.3% human serum albumin (Life Global, Guilford, CT, USA), 0.03 M sodium pyruvate, and 10 mM HEPES, and centrifuged at 200 × g for 5 minutes. This washing step was repeated twice. After discarding the supernatant, the final pellet was re-suspended in 500 µL of the washing medium and allocated into five aliquots: (1) fresh control, (2) sucrose vitrification (SuV), (3) sucrose vitrification supplemented with triple antioxidants (SuV+3A), (4) the vapor method (Vapor), and (5) the vapor method supplemented with triple antioxidants (Vapor+3A).

**3. Sperm cryopreservation and thawing**

The sperm samples were cryopreserved by two different protocols (the SuV and liquid nitrogen vapor methods). In this study, the sucrose vitrification medium was phosphate buffered saline (PBS) solu-
tion containing 10% (w/v) bovine serum albumin and 0.5 M sucrose. For the sucrose vitrification method, each sperm sample (100 µL/aliquot) was diluted 1:1, with sucrose freezing medium supplemented with 10 µM ALC; Abcam, Cambridge, UK), 10 µM NAC, and 5 µM ALA (SuV+3A) or without triple antioxidants (SuV). The concentrations of the three antioxidants in this study were based on a previous study by Truong and Gardner [18]. Then, the samples were loaded into 0.25-mL straws and incubated at 4°C for 10 minutes. After 10 minutes, the straws were inserted into the holes of a pre-cooled homemade aluminum block, which was previously immersed in liquid nitrogen [21]. The vitrified straws were left on liquid nitrogen for at least 1 week before subsequent experiments.

For the liquid nitrogen vapor method (Vapor), each sperm sample (100 µL/aliquot) was diluted with an equal volume of Spermfreeze medium (Fertipro, Beernem, Belgium) supplemented with (Vapor+3A) or without triple antioxidants (Vapor), as described in the previous paragraph. The mixtures were loaded into 0.25-mL straws and incubated at room temperature for 10 minutes. The straws were placed in a horizontal position at a distance of 5–7 cm above the level of liquid nitrogen for 15 minutes, and they were directly plunged into liquid nitrogen. The vitrified straws were left on liquid nitrogen for at least 1 week before subsequent experiments.

In the warming steps, the straws were thawed in 25°C water, washed in EBSS medium supplemented with or without triple antioxidants, and centrifuged at 200 × g for 3 minutes. After centrifugation, the supernatant was discarded, the final pellet was re-suspended in 100 µL of the washing medium, and the sperm parameters were immediately assessed.

### 4. Measurements of sperm motility, morphology, and viability

The post-thaw samples were immediately assessed for sperm motility and kinematic parameters using a computer-assisted semen analyzer (CASA; HTM IVOS II, Hamilton Thorne Biosciences, Beverly, MA, USA). Progressive motility (%), total motility (%), average path velocity (VAP, µm/sec), straight line velocity (VSL, µm/sec), curvilinear velocity (VCL, µm/sec), amplitude of lateral head displacement (ALH, µm), beat-cross frequency (Hz), straightness (STR, [VSL/VAP] × 100), and linearity (LIN, [VSL/VCL] × 100) were evaluated. The morphology of sperm was assessed by staining with Diff-Quick (Arnamparn, Nonthaburi, Thailand) and analyzed by an HTM IVOS II CASA equipped with a Dimensions II Strict Morphology software system using Kruger’s strict criteria. A total of 200 spermatozoa were analyzed in each slide at ×400 magnification.

Sperm viability was assessed using 0.5% (w/v) eosin-Y dissolved in 0.9% NaCl. A 10-µL sperm suspension was mixed with 10 µL of 0.5% eosin-Y, then, the mixture was placed on a glass slide and covered with a coverslip. The samples were immediately assessed for sperm viability using a compound microscope (Olympus, Tokyo, Japan). A total of 200 spermatozoa were analyzed in each slide [22]. The spermatozoa were classified as live (unstained heads) or dead sperm (stained red or dark pink heads) and reported as the percentage of live sperm.

### 5. Determination of extracellular ROS levels

The extracellular ROS level was assessed by a chemiluminescence technique, using a Glomax 20/20 luminometer (Turner Biosoys, Sunnyvale, CA, USA). The result was presented as relative light units (RLU) of counted photons per minute or mV/s. Briefly, 10 µL of sperm samples from each aliquot were diluted with 400 µL of PBS and mixed with 10 µL of luminol reagent (5-amino-2,3 dihydro-1,4 phthalazinedione). Then, each sample was measured twice, the average value of RLU/sec was corrected by dividing with the sperm concentration, and the final value of extracellular ROS was expressed in units of RLU/sec/10^6. A final extracellular ROS value lower than 20 RLU/sec/10^6 was classified as normal [23].

### 6. Determination of intracellular ROS levels

The intracellular sperm ROS level was evaluated using cell-permeable 2’7’-dichlorofluorescein diacetate (DCFH-DA), which was oxidized by the free intracellular H2O2 molecules into green fluorescence dichlorofluorescein (DCF). A total amount of 100 µM DCFH-DA and 2.5 µM propidium iodide (PI) was separately added to a concentration of 5 × 10^6 sperm/mL from each sample, followed by incubating at 37°C in 5% CO2 for 10 and 2 minutes, respectively. After incubation, the samples were washed with PBS and analyzed using an imaging flow cytometer (Amnis-Merck, Seattle, WA, USA) equipped with a charge-coupled device (CCD) camera, and a laser operated at 20 mW as a light source. At least 5,000 events were collected for each sample and analyzed by FlowSight (Amnis-Merck). Sperm populations were identified by plotting the forward scatter and side scatter, excluding other debris. Green fluorescence (DCF) was evaluated between 500 and 530 nm, while red fluorescence (PI) was evaluated between 580 and 630 nm (excitation 488 nm; emission, 530 nm in the FL-2 channel and 632 nm in the FL-5 channel). The percentage of viable DCF-positive cells (DCF+, PI–) and the mean fluorescence were calculated using image analysis software (IDEAS, Amnis-Merck).

### 7. Detection of SDF by sperm chromatin structure assay

The sperm chromatin structure assay (SCSA) is a flow cytometric assay that relies on the fact that abnormal sperm chromatin is highly susceptible to physical induction of partial DNA denaturation in situ [24]. It measures the intensity of acridine orange (AO) fluorescence using flow cytometry. The SCSA was performed according to the procedure described by Evenson et al. [25], with some modifications.
In brief, 100 µL of sample from each aliquot was diluted in TNE buffer (0.5 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 7.4) to a final concentration of $5 \times 10^6$ sperm/mL. Then, 200 µL of low-pH denaturing solution (0.15 M NaCl, 0.08 N HCl, 0.01% Triton X-100, pH 1.2) was directly added to the diluted sample and incubated for 30 seconds. Then, the sample was stained with 30 µL of staining solution (0.2 M NaH$_2$PO$_4$, 1 mM disodium EDTA, 0.15 M NaCl, 0.1 M citric acid monohydrate, pH 6.0) containing 6 µg/mL AO and loaded into an imaging flow cytometer (Amnis-Merck) equipped with a CCD camera and a laser operated at 20 mW. The sample was exposed to 488-nm laser light. At least 5,000 events from each sample were collected by FlowSight and analyzed using image analysis software (IDEAS). AO fluoresces green when it binds to native DNA (530 ± 30 nm) and red when it binds to fragmented DNA (> 630 nm), as shown in Figure 1.

### 8. Statistical analysis

All data, the mean numbers of sperm motility and kinematics, sperm viability, normal morphology, ROS levels, and percentage of DNA fragmentation were presented as mean ± standard error of the mean and compared by one-way analysis of variance (ANOVA). The percentage data were arc sine-transformed to obtain a normal distribution before analysis with one-way ANOVA using SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA). The differences were compared by the post-hoc Fisher’s protected least significant difference test. Significant differences were defined as a *p*-value less than 0.05.

### Results

#### 1. Characteristics of participants

The average age of the 30 donors was 34.5 ± 0.8 years. The semen parameters, including semen volume, concentration, and motility before sperm preparation were $2.8 \pm 0.2$ mL, $57.5 \pm 5.6 \times 10^6$ sperm/mL, and 71.7% ± 2.5%, respectively.

#### 2. Effect of triple antioxidant supplementation on sperm motility, viability, and morphology

The cryoprotective effects on motility parameters of vitrified spermatozoa are illustrated in Table 1. The total motility after warming was significantly lower in all vitrified sperm groups than in the fresh control group (*p* < 0.05), and a similar phenomenon was also observed for progressive motility (*p* < 0.05). Statistically significant differences were found in some parameters associated with the cryopreservation process, including VAP, VCL, ALH, and STR (*p* < 0.05), while VSL was not significantly different (*p* > 0.05) from the fresh control group. All vitrified sperm groups showed significantly lower viability than the control group (*p* < 0.05). However, no significant differences in terms of post warming sperm morphology were observed among the fresh control and vitrified groups (*p* > 0.05).

---

**Figure 1.** Determination of human sperm DNA fragmentation by imaging flow cytometry. On the left panel, yellow to red-stained cells indicate DNA fragmentation and green-stained cells indicate intact DNA in sperm, respectively. On the right panel, the sperm DNA fragmentation was evaluated individually by the intensity of acridine orange using imaging flow cytometry. High to moderate DNA fragmentation is shown in red and yellow colors, respectively. Normal to low DNA fragmentation is shown in green. SCSA, sperm chromatin structure assay; HDS, high DNA stainability; DFI, DNA fragmentation index; BF, bright field; AO, acridine orange; Mod, moderate.

https://doi.org/10.5653/cerm.2021.05120
Table 1. CASA motility, kinetic parameters, viability, and morphology of fresh and frozen-thawed human spermatozoa supplemented with or without the use of triple antioxidants (n=30)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fresh control</th>
<th>SuV</th>
<th>SuV+3A</th>
<th>Vapor</th>
<th>Vapor+3A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility (%)</td>
<td>95.3 ± 0.5</td>
<td>73.3 ± 2.0</td>
<td>76.9 ± 1.7</td>
<td>71.7 ± 2.3</td>
<td>74.7 ± 2.1</td>
</tr>
<tr>
<td>Progressive fraction (%)</td>
<td>91.1 ± 0.9</td>
<td>65.8 ± 2.1</td>
<td>69.4 ± 1.8</td>
<td>64.6 ± 2.5</td>
<td>66.8 ± 2.1</td>
</tr>
<tr>
<td>VAP (µm/sec)</td>
<td>61.0 ± 2.1</td>
<td>46.0 ± 1.4</td>
<td>50.0 ± 1.2</td>
<td>50.7 ± 1.2</td>
<td>50.2 ± 1.4</td>
</tr>
<tr>
<td>VSL (µm/sec)</td>
<td>39.1 ± 1.9</td>
<td>35.5 ± 1.4</td>
<td>39.3 ± 1.2</td>
<td>36.2 ± 1.2</td>
<td>37.1 ± 1.1</td>
</tr>
<tr>
<td>VCL (µm/sec)</td>
<td>127.5 ± 4.3</td>
<td>94.5 ± 3.0</td>
<td>101.0 ± 2.9</td>
<td>111.4 ± 3.1</td>
<td>109.1 ± 3.2</td>
</tr>
<tr>
<td>ALH (µm)</td>
<td>7.2 ± 0.2</td>
<td>5.2 ± 0.2</td>
<td>5.5 ± 0.2</td>
<td>6.1 ± 0.2</td>
<td>7.5 ± 1.6</td>
</tr>
<tr>
<td>BCF (Hz)</td>
<td>26.1 ± 0.7</td>
<td>26.7 ± 0.5</td>
<td>27.0 ± 0.4</td>
<td>27.9 ± 0.6</td>
<td>28.2 ± 0.5</td>
</tr>
<tr>
<td>STR (%)</td>
<td>65.0 ± 1.6</td>
<td>73.6 ± 1.2</td>
<td>74.8 ± 1.0</td>
<td>69.6 ± 0.9</td>
<td>71.7 ± 0.8</td>
</tr>
<tr>
<td>LIN (%)</td>
<td>33.2 ± 1.2</td>
<td>38.5 ± 1.1</td>
<td>39.5 ± 1.0</td>
<td>34.0 ± 0.8</td>
<td>35.6 ± 0.8</td>
</tr>
<tr>
<td>Eosin viability (%)</td>
<td>91.8 ± 2.0</td>
<td>69.4 ± 2.9</td>
<td>74.0 ± 2.5</td>
<td>69.8 ± 2.2</td>
<td>71.3 ± 2.2</td>
</tr>
<tr>
<td>Normality (%)</td>
<td>16.4 ± 1.5</td>
<td>17.2 ± 1.7</td>
<td>18.7 ± 1.7</td>
<td>20.0 ± 1.6</td>
<td>19.7 ± 1.6</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard error of the mean.

CASA, computer-assisted sperm analysis; SuV, sucrose vitrification; 3A, triple antioxidants; Vapor, liquid nitrogen vapor; VAP, average path velocity; VSL, straight line velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; STR, straightness; LIN, linearity.

Different superscript letters within a row indicate significant differences, p<0.05. a) STR: (VSL/VAP)×100; b) LIN: (VSL/VCL)×100.

3. Effect of triple antioxidant supplementation on sperm ROS level

The levels of ROS in the sperm suspension were measured by a chemiluminescence assay. The extracellular ROS levels in sperm suspensions were significantly higher after the freeze-thawed process (SuV, 0.74 ± 0.09; SuV+3A, 0.82 ± 0.16; Vapor, 1.05 ± 0.21; Vapor+3A, 1.07 ± 0.20 RLU/sec/10⁶) than in fresh control group (0.22 ± 0.03 RLU/sec/10⁶, p < 0.05) (Figure 2). The mean intensity of DCF fluorescence (intracellular ROS levels) did not significantly differ between the fresh control and vitrified groups (p > 0.05) (Figure 3).

4. Effect of triple antioxidant supplementation on SDF

To evaluate the protective effect of antioxidants on sperm DNA integrity, the DNA fragmentation rate of spermatozoa was assessed by flow-based SCSA after the freezing-thawing process. As shown in Table 2, the DNA fragmentation rate of spermatozoa did not differ significantly between the fresh control and vitrified groups. However, the DNA fragmentation rate showed a tendency to be higher following the freezing-thawing process in the SuV and Vapor groups than in the fresh control group (p = 0.075, p = 0.077, respectively). The rate of high DNA stainability (HDS) was significantly higher in the Vapor and Vapor+3A groups than in the fresh control and SuV groups. Supplementation with antioxidants in the freezing and thawing medium had a positive effect on reducing the DNA fragmentation rate, but it was not significant.

Discussion

Sperm cryopreservation, which is routinely utilized in human ART programs, is closely associated with the use of permeable CPA or a combination of permeable and non-permeable CPAs [26]. However, permeable CPAs are inseparably linked with the problem of toxicity, which damages cell membranes and results in reduced sperm motility and loss of sperm function [27]. Non-permeable CPAs play a supporting role that substantially enhances the effectiveness of permeable CPA. They do not directly penetrate the membrane, resulting in...
decreased toxicity compared to permeable CPAs [3]. Sperm viability and motility after cryopreservation are important parameters that predict the likelihood of in vitro fertilization [28]. In this study, we demonstrated the feasibility of human sperm vitrification using only sucrose as a non-permeable CPA. The method yielded high recovery rates of viable and motile sperm cells. Our results revealed significantly lower sperm viability and motility in the vitrified groups than in the fresh control group, which is in agreement with earlier studies [29-31]. Cryopreservation has deleterious effects on sperm motility by damaging the plasma membrane and mitochondrial function [29,32]. However, we did not find statistically significant differences in the viability and motility parameters of post-thawed samples between sucrose vitrification and the commercial sperm freezing medium. Previous studies have focused on other motility parameters as cru-

Table 2. Comparison of DNA fragmentation test between fresh and freeze-thawed spermatozoa with or without triple antioxidant supplementation (n = 30)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fresh control</th>
<th>SuV</th>
<th>SuV+3A</th>
<th>Vapor</th>
<th>Vapor+3A</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA fragmentation (%)</td>
<td>7.3 ± 1.2</td>
<td>15.3 ± 4.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.4 ± 1.6</td>
<td>14.0 ± 3.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.5 ± 1.8</td>
</tr>
<tr>
<td>High DNA stainability (%)</td>
<td>1.2 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.7 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.2 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.3 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard error of the mean.<br>SuV, sucrose vitrification; 3A, triple antioxidants; Vapor, liquid nitrogen vapor.<br><sup>a,b</sup>Different superscript letters within a row indicate significant differences, p<0.05; <sup>c</sup>SuV and Vapor showed a non-significant tendency to be higher than the fresh control (p=0.075 and p=0.077, respectively).

Figure 3. Quantitative intracellular H₂O₂ generation was evaluated by the measurement of dichlorofluorescein (DCF) fluorescence intensity using imaging-flow cytometry. Flow-cytometric histograms show the amount of intracellular H₂O₂ generation H₂O₂ in sperm. (A) Fresh nonfrozen group, (B) SuV group, (C) SuV+3A group, (D) Vapor group, and (E) Vapor+3A group. Values are presented as mean±standard error of the mean. Significant differences were defined as p-values less than 0.05. SuV, sucrose vitrification; 3A, triple antioxidants; Vapor, liquid nitrogen vapor freezing.

https://doi.org/10.5653/cerm.2021.05120
cial for the prediction of male fertility [33]. Some reports have found a correlation between certain CASA motility parameters (such as ALH, VSL, VCL, and LIN) and human fertility [34]. In addition, ALH values were shown to be a reliable predictor for achieving clinical pregnancy [35]. Researchers also found that STR and LIN values had a significant positive correlation with fertility [34]. In our study, we found a deleterious effect of the freezing-thawing procedure on sperm kinetics, such as VAP, VCL, and ALH. Our motility values are comparable with those of previous reports [36,37]. Furthermore, cryopreservation has a detrimental effect not only on the percentage of total motility, but also on progressive motility. As a result, a decrease in viability and motility values in frozen sperm may affect fertility compared with fresh sperm.

There is evidence that cryopreservation can cause cellular damage by different pathways. The excessive production of ROS is known to play an important role in this regard [29,38]. A large amount of ROS production may cause the accumulation of high levels of peroxides and free radical molecules, which can negatively impact normal sperm function, resulting in loss of sperm motility and viability [27]. In our study, the extracellular ROS levels were significantly higher in all frozen-thawed aliquots. This result is consistent with other research on human sperm cryoinjury [39], which indicated that the extracellular ROS levels increased during cryopreservation by increasing mitochondrial and membrane NADPH oxidase-5 (NOX5) activity. The increased ROS production following cryopreservation may be a result of mechanical damage to the sperm plasma membrane [32,40]. An alternative explanation is that the balance between ROS production and antioxidant scavenging systems is disrupted during the freezing-thawing process.

Recent studies have suggested that the use of antioxidants such as cysteine [41], alpha-tocopherol [42], and GSH [43] may exert beneficial effects on reducing the harmful effects of ROS. A trend now exists for the use of natural antioxidants due to toxicological concerns related to synthetic antioxidants [6,44]. In this study, we used a combination of natural antioxidants (ALC, NAC, and ALA) in the cryopreservation and post-thaw media. Supplementation of these antioxidants could enhance post-thaw motility and viability. However, they had no effect on extracellular ROS after the freezing-thawing process. The relationship between antioxidant supplementation and their beneficial effects remains a controversial topic in the management of cellular oxidative stress. Several studies have reported that supplementation with a combination of antioxidants had no effect on the antioxidants level [45,46], whereas other reports have described positive therapeutic effects [47,48]. It may obscure rather than clarify the discussion of these situations to view the principle of these effects as a clear mechanism.

In our study, the use of triple antioxidants did not decrease the levels of extracellular ROS. Supplementation of NAC (a substrate for the synthesis of GSH), and ALA (stimulator of GSH synthetase) was probably not a good choice because sperm cells, unlike other cells, shed most of their cytoplasm during maturation. As a result, intracytoplasmic enzymatic antioxidant defense mechanisms could be lost or markedly decreased. The findings that SDF had a tendency to decrease without a concomitant reduction in ROS levels could imply that other mechanisms were involved. Although ROS are among the most studied reactive molecules, there are at least three other groups of such species, designated by their reactive heteroatom as reactive nitrogen species, reactive sulfur species, and reactive halogen (chlorine and bromine) species [49]. These endogenous molecular species might not have been detectable as ROS in our detection system. ALC could function as a universal scavenger of reactive species, and thus confer partial protection against DNA damage.

Flow cytometry is a useful tool to identify sperm populations with dysfunctional ability due to intracellular ROS generation [50]. Cryodamage to spermatozoa is likely to be multifactorial mechanisms. In our study, we found that intracellular ROS levels did not differ between the fresh control and frozen-thawed groups. Measuring intracellular ROS using DCF dye is an indirect, non-specific method to determine all the real ROS generated inside sperm cells [51]. This finding was contrary to the levels of extracellular ROS, which significantly increased after cryopreservation by vitrification and vapor freezing method. The low level of intracellular ROS levels detected in this study could perhaps be explained by the principle of the DCFH-DA assay and the unique compartmentalization of sperm cells. To measure intracellular ROS, DCFH-DA must diffuse into viable sperm cells and be deacetylated by cellular esterase in human sperm, forming DCFH. DCFH is a polar molecule that is membrane-nonpermeable and is later oxidized by ROS inside the cells into fluorescent DCF, which can be detected by flow cytometry. Unlike other cells, the sperm nucleus in the head is physically separated from the mitochondria in the midpiece. ROS generated inside the mitochondria react with DCFH and produce DCF that is retained inside the midpiece [52]. The dye could not show fluorescent signals because there was a very scanty amount of mitochondria. Only a small amount of ROS, not neutralized by the sperm antioxidant system, produces weak fluorescent signals. In addition, cellular stress during freezing-thawing procedures has been found to cause impairment of the sperm plasma membrane. ROS molecules (especially H2O2), which were not detected as intracellular ROS, may have passed through the aquaporin pores in the midpiece [53], and were detected as extracellular ROS by the luminol chemiluminescence technique.

Flow-based SCSA is currently the gold standard for DNA fragmentation screening in infertile men to predict fertility outcomes [54]. The Sperm DNA Fragmentation Study Group also recommends that
the SCSA, sperm chromatin dispersion, terminal deoxynucleotidyl transferase dUTP nick end labeling, and comet assays are reliable as stand-alone SDF tests, although they may explore slightly different aspects of DNA fragmentation. The results of SCSA are based on the DNA fragmentation index (DFI) or the percent of cells outside the main population (COMPaT), which correspond to sperm cells containing DNA damage. HDS is considered as indicating immature spermatozoa [24]. Earlier research showed that DFI ≥ 30% and HDS ≥ 15% were associated with low fertilization and pregnancy rates [24]. There are many causes of SDF, which may impact male fertility, such as lifestyle factors, infection, varicocele, defective protamination during spermatogenesis, and errors in cryopreservation [55-57].

The present study indicated that vitrification had an adverse effect on SDF during cryopreservation. This result is consistent with previous studies showing that the number of sperm with fragmented DNA was associated with a freezing-thawing procedure [50]. We did not find any relationship between ROS and DNA fragmentation. Besides ROS, other pathways or factors could contribute to SDF. Therefore, the exact mechanisms influencing SDF remain unclear. Further study of these pathways will enhance our understanding of SDF and could provide an effective basis for prevention through antioxidant supplementation. In our study, supplementation with triple antioxidants did not significantly decrease SDF compared to the non-supplemented group. Furthermore, the main limitation of this study was the variation in sperm quality among participants used for these experiments.

In summary, a simplified vitrification medium, consisting of sucrose, compared favorably with the conventional liquid nitrogen vapor freezing protocol. Triple antioxidants in this study, aimed at increasing the activity of the enzymatic antioxidant pathways inside the sperm cytoplasm, did not have significant effects on improving sperm motility, viability, and DNA fragmentation. In future studies, extracellular antioxidants should be considered instead of those that rely on the endogenous enzymatic pathway, as mature sperm contain a scant amount of cytoplasm. The commonly used method of flow cytometric measurements of ROS production based on DCFH-DA is probably inappropriate for sperm because of their unique structure. ROS might not be the only reactive radicals involved in sperm damage after cryopreservation. Clinical outcomes, such as sperm motility, viability, DNA fragmentation, fertilization, and live birth, might be better indicators than ROS production.

**Conflict of interest**

This work was funded by Chiang Mai IVF Center. Tayita Suttirojpattana and Theesit Juanpanich are employees of Chiang Mai IVF Center. No other potential conflicts of interest relevant to this article were reported.

**Acknowledgments**

The author would like to thank the Korat Health Center for their kind assistance with this study.

**ORCID**

Theesit Juanpanich https://orcid.org/0000-0002-0286-9362

Tayita Suttirojpattana https://orcid.org/0000-0001-5901-3277

Rangsun Parnpai https://orcid.org/0000-0002-4764-9101

Teraporn Vutyavanich https://orcid.org/0000-0001-6750-3137

**Author contributions**

Conceptualization: TV, TS, TJ. Data curation: TJ, TS. Formal analysis: TS. Methodology: TJ, TV. Project administration: TV, RP. Visualization: TS, TJ. Writing--original draft: TS, TJ. Writing--review & editing: all authors.

**References**

8. Banihani S, Agarwal A, Sharma R, Bayachou M. Cryoprotective effect of L-carnitine on motility, vitality and DNA oxidation of hu-

https://doi.org/10.5653/erm.2021.05120
36. Donnelly ET, McClure N, Lewis SE. Cryopreservation of human semen and prepared sperm: effects on motility parameters and


The impact of hyperandrogenism on the outcomes of ovulation induction using gonadotropin and intrauterine insemination in women with polycystic ovary syndrome

Vu Ngoc Anh Ho¹², Toan Duong Pham¹², Nam Thanh Nguyen¹², Hieu Le Trung Hoang¹², Tuong Manh Ho¹², Lan Ngoc Vuong¹²³

¹IVFMD, My Duc Hospital, Ho Chi Minh City; ²HOPE Research Center, My Duc Hospital, Ho Chi Minh City; ³University of Medicine and Pharmacy at Ho Chi Minh City, Ho Chi Minh City, Viet Nam

Objective: This study aimed to investigate the impact of hyperandrogenism (HA) on the outcomes of ovulation induction (OI) using gonadotropin and intrauterine insemination (IUI) in patients with polycystic ovary syndrome (PCOS).

Methods: This was a retrospective cohort study including 415 patients undergoing OI using gonadotropin and IUI treatment between January 2018 and December 2020 at a single infertility center. Baseline characteristics, clinical and laboratory parameters, and pregnancy outcomes were investigated.

Results: Among the study population, there were 105 hyperandrogenic (25.3%) and 310 non-hyperandrogenic patients (74.7%). The live birth rate was lower in the HA group than in the non-HA group, but this difference did not reach statistical significance due to the limited sample size (14.3% vs. 21.0%, relative risk=0.68; 95% CI, 0.41–1.14, p=0.153). No predictive factors for live birth were identified through logistic regression analysis.

Conclusion: HA did not negatively affect the outcomes of OI using gonadotropin and IUI cycles in Vietnamese women with PCOS. The result may not be applicable elsewhere due to the large variation in the characteristics of women with PCOS across races and populations.

Keywords: Hyperandrogenism; Intrauterine insemination; Live birth; Ovulation induction; Polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) is a common neuroendocrine disorder, affecting 6%–9% of women of reproductive age [1]. According to the Rotterdam consensus (2003), the diagnosis of PCOS is based on the presence of at least two out of three groups of symptoms: ovulatory dysfunction, hyperandrogenism (HA), and polycystic ovary morphology (PCOM) on ultrasonography [2]. The health problems associated with this syndrome are diverse and have significant negative impacts on quality of life and fertility [3]. For sub-fertile women with PCOS, lifestyle modifications, such as regular physical activity, healthy eating habits, and diet balancing, are the first-line treatment options for infertility [4-6]. When lifestyle modifications fail, ovulation induction (OI) is a simple, non-invasive, low-cost approach that can be considered an alternative [6]. The most common drug of choice is clomiphene citrate and gonadotropin, while letrozole and metformin may also be used off-label [6]. OI with intrauterine insemination (IUI; OI+IUI) is the option of choice when there is coexisting suboptimal semen quality [7]. Although the effectiveness is unclear, performing IUI in ovulation-induced cycles is widely used for women with PCOS without male-related factors [8]. In women with PCOS, it is postulated that HA plays a critical role in...
the origins of PCOS [9]. Studies have found that HA remodels follicular development competence [10] and increases the risk of miscarriage and other adverse maternal-fetal outcomes, especially in Asian women [11,12]. In addition, HA has been demonstrated to increase hypertension in pregnancy, leading to preterm birth [13]. HA has been found to negatively affect the live birth rate (LBR) in women with PCOS after assisted reproductive techniques [14]. However, there have not been many studies on the effects of HA on OI and the outcomes of IUI. Thus, we decided to perform this study to evaluate the impact of HA on the treatment outcomes of OI using gonadotropin and IUI.

Methods

1. Study setting and population

This was a retrospective study at IVFMD, My Duc Hospital, Ho Chi Minh City, Viet Nam analyzing women with PCOS between January 2018 to December 2020. The study was approved by the Institutional Review Board of My Duc Hospital (08/21/DD-BVMD), on August 3, 2021. Patients’ information was kept confidential. All treatment data were agreed to be used for scientific research purposes.

Infertile women with PCOS aged 18 to 38 years who underwent the first cycle of OI with gonadotropin followed by IUI were eligible for the study. Patients were diagnosed with PCOS based on the Rotterdam criteria and must have had at least one patent Fallopian tube, as shown on hysterosalpingography. In addition, the male partner had normal sperm or mild male factor infertility (total sperm count ≥ 10 million). Women with uterine abnormalities (submucosal fibroids, intra-uterine cavity polyps, bicornuate uterus, and synechiae of the uterine cavity), tubal damage, male factor infertility, severe male factor infertility, or using frozen semen were excluded.

Based on the Rotterdam criteria, patients were diagnosed with PCOS when they met at least two of the following criteria: HA (modified Ferriman–Gallway score ≥ 3 [6,15]), a total testosterone level ≥ 1.8 nmol/L [16], or a free androgen index > 6 [17]; ovulation dysfunction (cycle length < 21 or > 35 days or < 8 cycles/year or amenorrhea (> 90 days); PCOM (≥ 20 follicles per ovary or ovarian volume of > 10 mL on transvaginal ultrasonography using transducers with a frequency bandwidth of 8 MHz, ensuring no corpora lutea, cysts, or dominant follicles were present). There were two groups of patients in this study: hyperandrogenic (HA) and non-hyperandrogenic (non-HA) women.

2. IUI procedure

From day 2 to day 4 of the menstrual cycle, OI was performed using human menopausal gonadotropin (hMG; IVF-M 75 IU, LG Chem, Seoul, Korea). The administered daily dose of hMG was 75 IU/day. Doses were individually adjusted based on the ovarian response, with a maximum daily dose of 150 IU. Monitoring was performed according to the clinic’s procedures. Transvaginal ultrasonography was performed using transducers with a frequency bandwidth of 8 MHz (Samsung HS30, Seoul, Korea) to measure follicles’ diameters. Patients were scheduled for a check-up on day 7 of stimulation. After that, follicular monitoring was performed every 2–3 days, depending on the number and size of follicles. Ovulation was triggered when the leading follicle’s diameter reached 18 mm, using human chorionic gonadotropin (IVF-C 5000 IU, LG Chem) at a dose of 5,000 IU. The IUI cycles were canceled or converted to in vitro fertilization (IVF) or in vitro maturation (IVM) for patients who had (1) more than three follicles with a diameter of ≥ 14 mm observed or (2) ovarian unresponsiveness to the hMG maximum daily dosage of 150 IU after 21 days of stimulation. In patients who had three or more follicles with a diameter of 14 mm but refused to cancel IUI cycle, a bolus of gonadotropin-releasing hormone (GnRH) agonist (Diphereline 0.1 mg; Lősen Pharma Biotech, Signes, France) at a dose of 0.1 mg was indicated to induce ovulation.

IUI was performed around 36 to 40 hours after ovulation triggering. The couples were instructed to have regular intercourse during stimulation, with the last intercourse to be no more than 2 days prior to insemination. Semen was collected and washed within 1 hour using both the swim-up technique and sperm density gradient centrifugation. The volume of the prepared semen sample used for insemination was 0.4 mL. Insemination was subsequently performed by physicians using a soft catheter (Gynétics, Lommel, Belgium). Bed rest after IUI was optional, depending on patients’ preferences.

Micronized progesterone (Cyclogest 200 mg; 400 mg/day, vaginal; Actavis, Parsippany-Troy Hills, NJ, USA) was used for luteal phase support for 14 days after insemination. A pregnancy test was performed by measuring the serum beta human chorionic gonadotropin (β-hCG) level 2 weeks after IUI. A level of β-hCG of 5 mIU/mL or above was considered pregnancy. Transvaginal ultrasonography was performed 3 weeks later.

3. Outcome measures

The primary outcome was the LBR. Live birth was defined as an infant born after 24 weeks with vital signs, heart rate, and muscle tone [18]. The secondary outcomes were the positive β-hCG, clinical pregnancy, ongoing pregnancy, ectopic pregnancy, miscarriage rates; the multiple pregnancy rate; the rates of ovarian hyperstimulation syndrome (OHSS); hypertensive disorders of pregnancy (HDP), and gestational diabetes mellitus (GDM); the rate of cycles with mono-/multi-follicular growth; and the rates of cycle cancellation and cycles converted to IVF or IVM.

https://doi.org/10.5653/cerm.2022.05204
4. Statistical analysis

Data were analyzed using descriptive statistics (mean and standard deviation for normally distributed variables, or median and interquartile range for skewed variables). Differences between groups were analyzed using one-way analysis of variance with the post hoc Tukey honest significant difference test or the Kruskal Wallis test for normally distributed or skewed variables, respectively, and the chi-square test for categorical variables. Univariable and multivariable logistic regression analyses were performed to identify factors associated with live birth. All variables with a p-value < 0.25 in the univariate analysis were included in the multivariable analysis. All analyses were performed using the R statistical package (R version 3.3.3; R Foundation, Vienna, Austria). Statistical significance was defined as p < 0.05.

Results

1. Baseline characteristics

In total, 415 women with PCOS were enrolled in this study from January 2018 to December 2020. Of these patients, 105 (25.3%) were diagnosed with HA, and 310 did not have HA (74.7%). The women in this study were relatively young, with a mean age of 28.3 years. Both the HA and non-HA women were non-obese, with a mean body mass index (BMI) of 23.4 and 21.8 kg/m², respectively. The anti-Müllerian hormone level was significantly higher in the HA women than in the non-HA women (9.05 vs. 7.77 ng/mL, respectively). Most non-HA women had PCOM, while the prevalence was 93.3% in HA women.

In HA group, there were seven cases without PCOM and six cases without ovulation dysfunction. The types and duration of infertility and the total motile sperm count were comparable between the two groups defined according to the PCOS phenotype. The total gonadotropin consumption and the duration of ovarian stimulation did not differ between the two groups. In most cycles, there was only one dominant follicle, and the most common method for ovulation triggering was hCG. There was also no difference in the cancellation rate. Similarly, the IVM and IVF conversion rates were comparable. Patients’ demographic and clinical characteristics are shown in Table 1.

2. Treatment outcomes

Overall, the LBR in HA women was lower than in non-HA women (14.3% and 21.0%, respectively). However, statistical significance was not reached (p = 0.153). The majority of pregnancies resulted in singletons. The prevalence of pregnancies with twins in HA and non-HA women was 20% and 12.3%, respectively. The birth weights of babies born were also comparable between the two groups. There were no significant differences between the two groups regarding the rates of positive pregnancy tests, ectopic pregnancies, miscarriages, and preterm births. The percentages of pregnancies with HDP and GDM were comparable between the two groups. There were no cases of OHSS. The details related to treatment outcomes are shown in Table 2.

No predictive factors for live birth were identified after logistic regression analysis (Table 3). There was no correlation between obesity and the treatment outcomes in women with HA (Supplementary Table 1).

Discussion

Our study evaluated the impact of HA on OI+IUI outcomes and reported long-term treatment outcomes. The results from our study demonstrated that the LBR was lower, although not significantly, in the HA group than in the non-HA group of women undergoing OI+IUI treatment due to the limited sample size. Our study has certain limitations. The first limitation is the retrospective nature of the study. Secondly, this is a single-center study that may not fully represent the overall population of women with PCOS. Thirdly, the study was performed among Vietnamese women, which may limit the generalizability of the findings due to the differences in characteristics of women with PCOS across races and populations.

The results from our study showed an LBR consistent with those reported by previous studies investigating IUI outcomes in women with PCOS. Huang et al. [19] conducted a study on 1068 IUI cycles and reported an overall LBR of 13.2%. In cycles with multi-follicular growth, the LBR was slightly higher, at 15.8%. It is also worth noting that 49.9% of cycles in our study achieved monofollicular growth. The LBR in our study was also comparable to the LBR in cycles included in a systematic review [20]. The percentages of cancellation or conversion to IVF and IVM treatment were comparable between HA and non-HA groups. Previous studies on IUI considered factors such as age [21], obesity [22], ovulation dysfunction [23,24], ovarian reserve [21,25-27], and the presence of HA [28] as predictors for pregnancy. This study could not demonstrate the hypothesis that HA has a negative impact on pregnancy outcomes after OI+IUI. This contrasts with results from the latest systematic review by Ma et al. [11], which stated that the rates of clinical pregnancy, miscarriage, and adverse pregnancy outcomes were higher in patients with HA. Moreover, De Vos et al. [29] found that the cumulative live birth rate (CLBR) after fresh or frozen embryo transfer in patients with hyperandrogenic PCOS phenotypes was significantly lower than in normoandrogenic patients. In particular, the CLBR of the hyperandrogenic phenotypes A and C were 25.8% and 27.8%, compared with the rates of 48% in patients with the normoandrogenic phenotype D (p = 0.002 and p = 0.01, respectively) and 53.3% in controls with polycystic ovarian morphology (p < 0.001 and p = 0.001, respective-
The median free testosterone index of patients in our study was low, at a level of 6.71 (Q1 = 4.80, Q3 = 9.26). This is similar to the findings of another study in Vietnamese women with PCOS by Cao et al. [30]. Given the fact that the free testosterone index in our study was impressively lower than that of other ethnicities [11,28], it was hypothesized that the severity of HA in our patients was less than that of different populations. Moreover, the presence of HA can likewise potentially affect treatment outcomes differently in Vietnamese individuals. There is an essential role of obesity in treatment outcomes in women with PCOS in the interaction with HA. For patients undergoing IVF or intracytoplasmic sperm injection, Romanski et al. [31] showed a significant trend for a decreased LBR and increased miscarriage rate as BMI increased. Furthermore, patients with a BMI > 40 kg/m² had worse IVF treatment outcomes than normal-weight patients. High BMI could also affect OH-IUI treatment outcomes negatively. A recent retrospective study by Guan et al. [22] investigating 831 IUI cycles showed that obese women might require more gonadotropin doses and more days of stimulation. Furthermore, patients with a BMI > 40 kg/m² had worse IVF treatment outcomes than normal-weight patients. High BMI could also affect OH-IUI treatment outcomes negatively. A recent retrospective study by Guan et al. [22] investigating 831 IUI cycles showed that obese women might require more gonadotropin doses and more days of stimulation. Moreover, obesity is recognized in the literature as an aggravating factor of endocrine-metabolic disorders, insulin resistance, response to ovarian stimulation, and adverse events in pregnancy and the neonatal period [32-38]. As mentioned previously, the women in our study were non-obese. This is similar to other studies showing a lower prevalence of obesity in East Asian women with PCOS than in other populations such as Hispanic, Caucasian, and African descent [39-42]. Therefore, the low BMI could explain the consistency in treatment outcomes between both groups of patients in our study. In other words, in our less severely hyperandrogenic and non-obese patients,

Table 1. Demographic and clinical characteristics of the patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Non-HA (n = 310)</th>
<th>HA (n = 105)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of female partner (yr)</td>
<td>28.3 ± 3.1</td>
<td>28.3 ± 3.6</td>
<td>0.893a</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>21.8 ± 2.8</td>
<td>23.4 ± 3.4</td>
<td>&lt; 0.001a</td>
</tr>
<tr>
<td>Anti-Müllerian hormone (ng/mL)</td>
<td>7.77 (5.77–10.51)</td>
<td>9.05 (6.57–12.22)</td>
<td>0.007b</td>
</tr>
<tr>
<td>Testosterone (ng/dL)</td>
<td>1.21 (0.87–1.50)</td>
<td>2.00 (1.72–2.22)</td>
<td>&lt; 0.001b</td>
</tr>
<tr>
<td>Free testosterone index</td>
<td>2.69 (1.68–3.97)</td>
<td>6.71 (4.80–9.26)</td>
<td>&lt; 0.001b</td>
</tr>
<tr>
<td>Polycystic ovary morphology</td>
<td></td>
<td></td>
<td>&lt; 0.001c</td>
</tr>
<tr>
<td>Yes</td>
<td>310 (100.0)</td>
<td>98 (93.3)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0</td>
<td>7 (6.7)</td>
<td></td>
</tr>
<tr>
<td>Ovulation disorder</td>
<td></td>
<td></td>
<td>&lt; 0.001c</td>
</tr>
<tr>
<td>Yes</td>
<td>310 (100.0)</td>
<td>99 (94.3)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0</td>
<td>6 (5.7)</td>
<td></td>
</tr>
<tr>
<td>Duration of infertility (yr)</td>
<td>2.0 (1.0–3.0)</td>
<td>2.0 (1.0–3.0)</td>
<td>0.426b</td>
</tr>
<tr>
<td>Type of infertility</td>
<td></td>
<td></td>
<td>0.403c</td>
</tr>
<tr>
<td>Primary</td>
<td>219 (71.3)</td>
<td>80 (76.2)</td>
<td></td>
</tr>
<tr>
<td>Secondary</td>
<td>88 (28.7)</td>
<td>25 (23.8)</td>
<td></td>
</tr>
<tr>
<td>Duration of stimulation (day)</td>
<td>12.0 (10.0–15.0)</td>
<td>13.0 (10.8–16.0)</td>
<td>0.085b</td>
</tr>
<tr>
<td>Total dose of follicle-stimulating hormone (IU)</td>
<td>1,050.0 (750.0–1425.0)</td>
<td>902.5 (693.8–1556.2)</td>
<td>0.551b</td>
</tr>
<tr>
<td>Type of trigger</td>
<td></td>
<td></td>
<td>&lt; 0.001c</td>
</tr>
<tr>
<td>GnRH agonist</td>
<td>84 (32.2)</td>
<td>10 (11.6)</td>
<td></td>
</tr>
<tr>
<td>hCG</td>
<td>177 (67.8)</td>
<td>76 (88.4)</td>
<td></td>
</tr>
<tr>
<td>Follicle size with diameter ≥ 14 mm on the day of trigger</td>
<td>0.036c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>142 (46.1)</td>
<td>65 (62.5)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>57 (18.5)</td>
<td>14 (13.5)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>35 (11.4)</td>
<td>7 (6.7)</td>
<td></td>
</tr>
<tr>
<td>≥ 4</td>
<td>74 (24.0)</td>
<td>18 (17.3)</td>
<td></td>
</tr>
<tr>
<td>Cycle with cancellation</td>
<td>76 (24.5)</td>
<td>19 (18.1)</td>
<td>0.188c</td>
</tr>
<tr>
<td>Cycle converted to IVF/IVM</td>
<td></td>
<td></td>
<td>0.210c</td>
</tr>
<tr>
<td>IVM</td>
<td>1 (1.32)</td>
<td>1 (5.26)</td>
<td></td>
</tr>
<tr>
<td>IVF</td>
<td>6 (7.89)</td>
<td>6 (31.6)</td>
<td></td>
</tr>
<tr>
<td>Total motile sperm count (millions)</td>
<td>7.6 (2.4–11.5)</td>
<td>8.7 (3.2–12.2)</td>
<td>0.441b</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviation, median (interquartile range), or number (%).
HA, hyperandrogenism; GnRH, gonadotropin-releasing hormone; hCG, human chorionic gonadotropin; IVF, in vitro fertilization; IVM, in vitro maturation.

a) Student t-test; b) Mann-Whitney U-test; c) Chi-square test.
the effects of the PCOS phenotype on treatment outcomes may not differ. Additionally, a subgroup analysis was performed in order to further investigate the impact of obesity on treatment outcomes in HA women. Similarly, there was no significant difference between non-obese and overweight or obese HA women.

There are still many concerns about gonadotropin administration

Table 2. Pregnancy outcomes of the first IUI cycle

<table>
<thead>
<tr>
<th>Variable</th>
<th>Non-HA (n = 310)</th>
<th>HA (n = 105)</th>
<th>RR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive β-hCG test</td>
<td>87 (28.1)</td>
<td>25 (23.8)</td>
<td>0.85 (0.58–1.25)</td>
<td>0.446a</td>
</tr>
<tr>
<td>Clinical pregnancy</td>
<td>80 (25.8)</td>
<td>22 (21.0)</td>
<td>0.81 (0.54–1.23)</td>
<td>0.360a</td>
</tr>
<tr>
<td>Ongoing pregnancy</td>
<td>68 (21.9)</td>
<td>18 (17.1)</td>
<td>0.78 (0.49–1.25)</td>
<td>0.331a</td>
</tr>
<tr>
<td>Live birth</td>
<td>65 (21.0)</td>
<td>15 (14.3)</td>
<td>0.68 (0.41–1.14)</td>
<td>0.153a</td>
</tr>
<tr>
<td>Singleton</td>
<td>57 (87.7)</td>
<td>12 (80.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Twins</td>
<td>8 (12.3)</td>
<td>3 (20.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Singleton</td>
<td>3,148.2 ± 441.8</td>
<td>3,240 ± 416.9</td>
<td></td>
<td>0.539b</td>
</tr>
<tr>
<td>Twin</td>
<td>2,271.4 ± 185.8</td>
<td>1,800 ± 905.5</td>
<td></td>
<td>0.260b</td>
</tr>
<tr>
<td>Ovarian hyperstimulation syndrome</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ectopic pregnancy</td>
<td>3 (1.0)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miscarriage &lt; 12 wk</td>
<td>9 (2.9)</td>
<td>4 (3.8)</td>
<td>1.31 (0.41–4.17)</td>
<td>0.746b</td>
</tr>
<tr>
<td>Multiple pregnancy</td>
<td>13 (4.19)</td>
<td>3 (2.86)</td>
<td>0.68 (0.2–2.34)</td>
<td>0.770b</td>
</tr>
<tr>
<td>Preterm delivery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 24 wk</td>
<td>3 (1.0)</td>
<td>3 (2.9)</td>
<td>2.95 (0.61–14.4)</td>
<td>0.173b</td>
</tr>
<tr>
<td>&lt; 28 wk</td>
<td>3 (1.0)</td>
<td>4 (3.8)</td>
<td>3.94 (0.9–17.3)</td>
<td>0.072b</td>
</tr>
<tr>
<td>&lt; 34 wk</td>
<td>5 (1.6)</td>
<td>4 (3.8)</td>
<td>2.36 (0.65–8.63)</td>
<td>0.239b</td>
</tr>
<tr>
<td>&lt; 37 wk</td>
<td>14 (4.5)</td>
<td>6 (5.7)</td>
<td>1.27 (0.5–3.21)</td>
<td>0.604b</td>
</tr>
<tr>
<td>Hypertension</td>
<td>4 (1.3)</td>
<td>3 (2.9)</td>
<td>2.21 (0.5–9.73)</td>
<td>0.376b</td>
</tr>
<tr>
<td>Gestational diabetes</td>
<td>14 (4.5)</td>
<td>6 (5.7)</td>
<td>1.27 (0.5–3.21)</td>
<td>0.604b</td>
</tr>
</tbody>
</table>

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

IUI, intrauterine insemination; HA, hyperandrogenism; RR, relative risk; CI, confidence interval; β-hCG, beta human chorionic gonadotropin.

a) Chi-square test; b) Student t-test.

Table 3. Logistic regression analysis of factors associated with live birth

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No live birth (n = 335)</th>
<th>Live birth (n = 80)</th>
<th>OR (95% CI); p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate</td>
<td></td>
</tr>
<tr>
<td>Hyperandrogenism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>245 (73.1)</td>
<td>65 (81.2)</td>
<td>Ref.</td>
</tr>
<tr>
<td>Yes</td>
<td>90 (26.9)</td>
<td>15 (18.8)</td>
<td>0.63 (0.33–1.14); 0.132</td>
</tr>
<tr>
<td>Age of female partner (yr)</td>
<td>28.3 (3.3)</td>
<td>28.3 (2.9)</td>
<td>1.00 (0.93–1.08); 0.923</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>22.2 (3.1)</td>
<td>22.3 (3.0)</td>
<td>1.02 (0.94–1.10); 0.678</td>
</tr>
<tr>
<td>Anti-Müllerian hormone (ng/mL)</td>
<td>8.4 (6.1–10.8)</td>
<td>7.0 (5.4–10.5)</td>
<td>0.96 (0.90–1.02); 0.192</td>
</tr>
<tr>
<td>Testosterone (ng/dL)</td>
<td>1.4 (1.1–1.8)</td>
<td>1.3 (1.0–1.7)</td>
<td>0.74 (0.47–1.15); 0.178</td>
</tr>
<tr>
<td>Free testosterone index</td>
<td>3.6 (1.9–5.6)</td>
<td>3.6 (2.3–5.1)</td>
<td>0.99 (0.91–1.09); 0.910</td>
</tr>
<tr>
<td>Duration of infertility (yr)</td>
<td>2.0 (1.0–3.0)</td>
<td>2.0 (1.5–3.0)</td>
<td>1.01 (0.88–1.15); 0.920</td>
</tr>
<tr>
<td>Type of infertility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>242 (72.7)</td>
<td>57 (72.2)</td>
<td>Ref.</td>
</tr>
<tr>
<td>Secondary</td>
<td>91 (27.3)</td>
<td>22 (27.8)</td>
<td>1.03 (0.58–1.76); 0.916</td>
</tr>
<tr>
<td>Duration of stimulation (day)</td>
<td>12.0 (10.0–15.0)</td>
<td>12.0 (10.0–15.0)</td>
<td>0.99 (0.94–1.05); 0.812</td>
</tr>
<tr>
<td>Total dose of follicle stimulating hormone (units of 150 IU)</td>
<td>6.5 (4.5–9.5)</td>
<td>8.0 (5.0–10.8)</td>
<td>1.05 (0.98–1.13); 0.146</td>
</tr>
<tr>
<td>Total motile sperm count (millions)</td>
<td>7.8 (2.6–12.0)</td>
<td>7.9 (2.3–11.8)</td>
<td>1.01 (0.98–1.04); 0.436</td>
</tr>
</tbody>
</table>

Values are presented as number (%) or median (interquartile range).

OR, odds ratio; CI, confidence interval.
in OI because of the high occurrence of OHSS and multiple pregnancies associated with its use. There were no cases of OHSS in our study. A possible reason could be the strict implementation of an OHSS prevention strategy at our center, including a GnRH agonist trigger. A GnRH agonist trigger was indicated when there were more than three follicles at a diameter of ≥ 14 mm on the day of trigger. The percentage of cycles with a GnRH agonist trigger was significantly lower in the HA group than in the non-HA group (11.6% vs. 32.2%, p = 0.01). However, there was no significant difference in the multiple pregnancy rate between the two groups (2.86% vs. 4.19%, p = 0.77). The percentage of twins was also comparable, and no higher-order multiple pregnancies were recorded. This incidence was similar to that of the aforementioned study [15].

In conclusion, HA in Vietnamese women with PCOS did not have a negative effect on OI+IUI outcomes, unlike the findings of previous studies in other races. The result may not be applicable elsewhere due to the large variation in the characteristics of women with PCOS across races and populations.

Conflict of interest

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

Author contributions

Conceptualization: VNAH, TDP, NTN, TMH, LNV. Data curation: VNAH, TDP, LNV. Formal analysis: VNAH, TDP, NTN, LNV. Methodology: VNAH, TDP, TMH, LNV. Visualization: VNAH, TDP, NTN, HLTH, LNV. Writing—original draft: VNAH, TDP, NTN, HLTH, LNV. Writing—review & editing: all authors.

Supplementary material

Supplementary material can be found via https://doi.org/10.5653/cerm.2022.05204.

References


Objective: This study investigated the impact of two stimulation protocols using highly purified human menopausal gonadotropin (HP-hMG) on the endocrine profile, follicular fluid soluble Fas levels, and outcomes of intracytoplasmic sperm injection (ICSI) cycles.

Methods: This prospective clinical trial included 100 normal-responder women undergoing ovarian stimulation for ICSI; 55 patients received concomitant follicle-stimulating hormone (FSH) plus HP-hMG from the start of stimulation, while 45 patients received FSH followed by HP-hMG during mid/late follicular stimulation. The primary outcome was the number of top-quality embryos. The secondary outcomes were the number and percentage of metaphase II (MII) oocytes and the clinical pregnancy rate.

Results: The number of MII oocytes was significantly higher in the concomitant protocol (median, 13.0; interquartile range [IQR], 8.5–18.0 vs. 9.0 [8.0–13.0] in the consecutive protocol; \(p=0.009\)); however, the percentage of MII oocytes and the fertilization rate were significantly higher in the consecutive protocol (median, 90.91; IQR, 80.0–100.0 vs. 83.33 [75.0–93.8]; \(p=0.034\) and median, 86.67; IQR, 76.9–100.0 vs. 77.78 [66.7–89.9]; \(p=0.028\), respectively). No significant between-group differences were found in top-quality embryos (\(p=0.693\)) or the clinical pregnancy rate (65.9% vs. 61.8% in the consecutive vs. concomitant protocol, respectively). The median follicular fluid soluble Fas antigen level was significantly higher in the concomitant protocol (9,731.0 pg/mL; IQR, 6,004.5–10,807.6 vs. 6,350.2 pg/mL; IQR, 4,382.4–9,418.4; \(p=0.021\)).

Conclusion: Personalized controlled ovarian stimulation using HP-hMG during the late follicular phase led to a significantly lower response, but did not affect the quality of ICSI.

Keywords: Follicle-stimulating hormone; Human chorionic gonadotropin; Intracytoplasmic sperm injection outcomes; Purified human menopausal gonadotropin; Soluble Fas

Introduction

Although the role of follicle-stimulating hormone (FSH) in in vitro fertilization (IVF) cycles has been well established, and FSH alone can lead to successful follicular development, the role of luteinizing hor-
therefore, other sources of LH activity became important. Highly purified hMG preparations use human chorionic gonadotropin (hCG) of either pituitary or trophoblastic origin as a source of LH bioactivity, and hCG provides a more potent effect than LH [6,7]. LH activity driven by hCG improves the ovarian response to FSH stimulation and enhances the implantation potential of embryos [8].

FF provides an important microenvironment for the proper development of oocytes [9]. Its components reflect the metabolic state of ovarian granulosa and theca cells [10]. Soluble Fas (Apo-1, CD95) is a cell-surface molecule that is a member of the tumor necrosis factor family and mediates apoptosis [11]. Apoptosis is involved in the physiology of reproduction, including follicular atresia, endometrial proliferation implantation, blastocyst attachment, and placent al proliferation [12,13]. It was found that lower levels of FF soluble Fas antigen (sFas) were associated with higher pregnancy rates, as sFas may support embryo implantation, prevent damage of the embryo, and affect blastocyst attachment and the proliferation of placenta cells [14,15]. However, other studies stated that lower concentrations of FF sFas were associated with more apoptosis in stimulated cycles and resulted in poor quality of oocytes and embryos [9]. No studies have evaluated the effect of different gonadotropin protocols on follicular apoptosis. The hypothesis of the present study was that the LH activity of highly purified human menopausal gonadotropin (HP-hMG) from the early follicular phase might be harmful to oocyte development, and sFas was used as a marker of follicular apoptosis that may occur as a result of adding LH from the early follicular phase.

Methods

1. Study design and subjects

One hundred patients were recruited from private IVF/intracytoplasmic sperm injection (ICSI) centers in Alexandria, Egypt, between April 2020 and June 2021. The subjects were recruited by convenience sampling. Fifty-five consecutive patients at IVF centers who matched the inclusion criteria received conventional concomitant protocol. Forty-five patients with cross-matched criteria started the new consecutive protocol after agreement of their physicians. Before the couples were enrolled into our study, they underwent a standard protocol of investigations, including a semen analysis, ovarian reserve testing, and transvaginal ultrasonography for uterine assessment and an antral follicular count.

The inclusion criteria were women aged ≤ 37 years with regular ovulatory cycles and an expected normal response if undergoing ICSI with pituitary downregulation. The exclusion criteria were women with polycystic ovarian syndrome and poor responders according to the Bologna criteria [16]. The power was 96.8%, with an effect size of 0.8 calculated based on the number of oocytes in group 1 versus group 2 (results of the current study) using the G*Power program [17]. This study protocols were approved by the local Ethics Committee of Alexandria University. Every patient was extensively counseled and provided written informed consent prior to participating in the study.

2. Ovarian stimulation protocol

In group 1 (concomitant protocol), 55 patients received concomitant FSH and HP-hMG from the start of stimulation. In group 2 (consecutive protocol), 45 patients received FSH only at the beginning of ovarian stimulation, followed by the addition of HP-hMG when the follicles reached 12 mm or more. HP-hMG was continued until the day of triggering of ovulation. FSH was administered as recombinant FSH (rFSH), follitropin alfa (Gonal-F; Merck Serono, Geneva, Switzerland), Gonapure (Minapharm Pharmaceuticals, 10th of Ramadan City, Egypt) or highly purifiedurofollitropin (Postimom; IBSA Institut Biochimique SA, Lugano, Switzerland). HP-hMG was administered as either Meriofert (IBSA Institut Biochimique SA) or Menopur (Ferring Pharmaceuticals, Saint-Prex, Switzerland). The doses of gonadotropins were individualized according to the patient’s age, body mass index, and previous response to ovulation stimulation, and ranged from 225 IU to 300 IU daily.

Pituitary down-regulation was done by either a gonadotropin-releasing hormone (GnRH) long agonist protocol using triptorelin acetate 0.1 mg (Decapeptyl; Ferring Pharmaceuticals) or by a GnRH antagonist protocol, with a daily dose of subcutaneous cetrorelix (Cetro 0.25 mg, Merck Serono) on days 5–6 of stimulation. The ovarian response was monitored by the serum estradiol (E2) concentration using COBAS e411 (Roche Diagnostics, Mannheim, Germany), and the diameter of follicles measured by transvaginal ultrasonography. Once the leading follicle reached 18 mm in diameter, ovulation was triggered using 1,000 IU of hCG.

Oocyte retrieval was done using transvaginal ultrasonography 36 hours after triggering ovulation, followed by ICSI for mature oocytes 2–4 hours later. Fertilization and cleavage were assessed and the embryos were classified according to their morphological appearance. Embryos were transferred on day 4 or 5. The luteal phase was supported with a daily 100-mg dose of progesterone in oil intramuscularly and vaginal suppositories (400 mg twice daily) starting on the day of oocyte retrieval. Pregnancy was assessed 14 days after embryo transfer by analyzing the serum hCG level. Clinical pregnancy was defined by the presence of a gestational sac on transvaginal ultrasonography 5–7 weeks after embryo transfer.

3. Measurement of sFas in FF

FF samples were aspirated from large follicles (≥ 16 mm) at the
time of oocyte retrieval, centrifuged immediately at 1,200 rpm for 10 minutes at room temperature to remove cellular contents and debris, and then the supernatant was collected and stored at −80° until assayed. FF was assessed for quantitative determination of sFas as a marker of oocyte apoptosis using a sandwich enzyme-linked immunosorbent assay (Human sFas/TNFRSF6, Quantikine; R&D Systems, Minneapolis, MN, USA) [18]. A monoclonal antibody specific for sFas was pre-coated on each well. Diluent RD 1-8 was added to each well. The FF samples were thawed at room temperature, and centrifuged at 3,000 rpm for 10 minutes. The supernatant was diluted 10-fold prior to the assay using 25 μL of sample + 225 μL of Calibrator Diluent RD5L (diluted 1:2). Samples (100 μL) were added to each well and incubated for 2 hours at room temperature. Then wells were washed by buffer (400 μL) three times for a total of four washes and aspirated. 200 μL of human Fas conjugate were added to each well, followed by incubation for another 2 hours at room temperature. The aspiration/wash was repeated. Substrate solution (200 μL) was added to each well, followed by incubated for 30 minutes at room temperature under protection from light. Stop solution (50 μL) was added to each well. The color in the wells changed from blue to yellow. The optical density (OD) of each well was determined within 30 minutes, using a micro-plate reader set to 450 nm and 540 nm, and then the readings at 540 nm were subtracted from those at 450 nm to correct for optical imperfections in the plate. The corrected OD was plotted on a curve and the resultant concentration was multiplied by 10 to obtain the sFas level in picograms per milliliter (Figure 1).

4. Outcomes

The primary outcome was the number of top-quality embryos. The secondary outcomes were the number and percentage of metaphase II (MII) oocytes and the clinical pregnancy rate.

5. Statistical analysis

Data were analyzed using IBM SPSS ver. 20.0. (IBM Corp., Armonk, NY, USA). Qualitative data were described as numbers and percentages. The Kolmogorov-Smirnov test was used to verify the normality of distribution. Quantitative data were described using the range (minimum and maximum), mean, standard deviation, or median and interquartile range (IQR), as appropriate. A p-value < 0.05 was considered to indicate statistical significance. The statistical analyses were carried out using the Mann-Whitney test, the Fisher exact test, the chi-square test, and the Student t-test. The Pearson coefficient was calculated to evaluate correlations between two normally distributed quantitative variables.

Results

No significant differences were found in the baseline characteristics between the two groups, as shown in Table 1. Basal hormone levels (day 2 LH and E2) and hormone levels on the day of triggering (LH, E2, progesterone, and hCG) were not significantly different between the two groups (Table 2). The median serum progesterone level on the day of triggering was 0.63 ng/mL in the concomitant protocol and 0.7 ng/mL in the consecutive protocol (p = 0.667). The median value of serum hCG was 0.48 mIU/mL in the concomitant protocol and 0.60 mIU/mL in the consecutive protocol (p = 0.098). When comparing the two groups regarding the parameters of ICSI outcomes (Table 2), the duration of treatment was significantly shorter with the concomitant protocol, which may have been because higher doses of gonadotropins were used. With the consecutive protocol, significantly fewer oocytes were retrieved, but there was a higher percentage of MII oocytes and a higher fertilization rate, which may be explained by apoptosis of small and medium-sized follicles while larger follicles remained unaffected. However, there were no statistically significant differences between the two groups in the number of top-quality embryos or the clinical pregnancy rate. A subgroup analysis according to the GnRH analogue used in the concomitant and consecutive protocols is illustrated in Tables 3 and 4, respectively. Univariate analysis was done between serum hCG on the day of triggering and the total dose of HP-hMG, and a significant positive correlation was observed in group 1 (r = 0.63, p < 0.001) (Figure 2); this was attributed to the fact that the LH source in HP-hMG is mainly of hCG origin. There were no cases of moderate or severe ovarian hyperstimulation syndrome in both groups. The FF level of sFas (an apoptotic marker) was significantly higher in group 1 (median, 9,731.0 pg/mL; IQR, 6,004.5–10,807.6 pg/mL) than in group 2 (median, 6,350.2 pg/mL; IQR, 4,382.4–9,418.4 pg/mL).
Table 1. Demographic characteristics of women receiving early or late HP-hMG

<table>
<thead>
<tr>
<th>Variable</th>
<th>Concomitant FSH+HP-hMG (n = 55)</th>
<th>Consecutive FSH then HP-hMG (n = 45)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>29.07 ± 4.08</td>
<td>27.89 ± 4.55</td>
<td>0.174</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.80 (22.1–26.0)</td>
<td>24.0 (22.1–26.6)</td>
<td>0.685</td>
</tr>
<tr>
<td>Duration of infertility (yr)</td>
<td>4.50 (3.0–6.0)</td>
<td>3.50 (2.5–5.0)</td>
<td>0.205</td>
</tr>
<tr>
<td>Cause of infertility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubal</td>
<td>5 (9.1)</td>
<td>4 (8.9)</td>
<td>1.000</td>
</tr>
<tr>
<td>Uterine</td>
<td>0</td>
<td>1 (2.2)</td>
<td>0.450</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>3 (5.5)</td>
<td>2 (4.4)</td>
<td>1.000</td>
</tr>
<tr>
<td>Peritoneal</td>
<td>1 (1.8)</td>
<td>2 (4.4)</td>
<td>0.587</td>
</tr>
<tr>
<td>Male</td>
<td>26 (47.3)</td>
<td>22 (48.9)</td>
<td>0.872</td>
</tr>
<tr>
<td>Unexplained</td>
<td>21 (38.2)</td>
<td>13 (28.9)</td>
<td>0.320</td>
</tr>
<tr>
<td>AMH (ng/dL)</td>
<td>2.73 ± 0.73</td>
<td>2.67 ± 0.83</td>
<td>0.706</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation, median (interquartile range), or number (%).

HP-hMG, highly purified human menopausal gonadotropin; FSH, follicle-stimulating hormone; BMI, body mass index; AMH, anti-Müllerian hormone.

Independent t-test; Mann-Whitney U-test; Fisher exact test.

Table 2. Endocrine profile and cycle characteristics and outcomes in both groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Concomitant FSH and HP-hMG (n = 55)</th>
<th>Consecutive FSH then HP-hMG (n = 45)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2 LH (IU/L)</td>
<td>4.80 (3.0–7.2)</td>
<td>5.10 (3.8–6.2)</td>
<td>0.620</td>
</tr>
<tr>
<td>Day 2 E2 (pg/mL)</td>
<td>30.90 (16.3–45.0)</td>
<td>29.50 (20.0–43.0)</td>
<td>0.506</td>
</tr>
<tr>
<td>Hormone on the day of triggering</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH (IU/L)</td>
<td>2.25 (1.4–3.7)</td>
<td>2.37 (1.8–3.0)</td>
<td>0.887</td>
</tr>
<tr>
<td>E2 (pg/mL)</td>
<td>3,000 (2,409.5–4,100.5)</td>
<td>2,982.0 (2,155.0–4,115.5)</td>
<td>0.631</td>
</tr>
<tr>
<td>P4 (ng/mL)</td>
<td>0.63 (0.5–0.9)</td>
<td>0.70 (0.5–0.9)</td>
<td>0.667</td>
</tr>
<tr>
<td>hCG (mIU/mL)</td>
<td>0.48 (0.3–0.9)</td>
<td>0.60 (0.5–0.8)</td>
<td>0.089</td>
</tr>
<tr>
<td>Duration of treatment (day)</td>
<td>10.35 ± 1.42</td>
<td>11.04 ± 1.48</td>
<td>0.018</td>
</tr>
<tr>
<td>Total dose of gonadotropins</td>
<td>2,400 (1,650–4,500)</td>
<td>2,175 (1,500–4,050)</td>
<td>0.002</td>
</tr>
<tr>
<td>No. of oocytes retrieved</td>
<td>16.0 (12.0–20.0)</td>
<td>12.0 (10.0–15.0)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>No. of metaphase II oocytes</td>
<td>13.0 (8.5–18.0)</td>
<td>9.0 (8.0–13.0)</td>
<td>0.009</td>
</tr>
<tr>
<td>Percentage of metaphase II oocytes</td>
<td>83.33 (75.0–93.8)</td>
<td>90.91 (80.0–100.0)</td>
<td>0.034</td>
</tr>
<tr>
<td>OHSS</td>
<td>2 (3.6)</td>
<td>0</td>
<td>0.500</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>77.78 (66.7–89.9)</td>
<td>86.67 (76.9–100.0)</td>
<td>0.028</td>
</tr>
<tr>
<td>Implantation rate (%)</td>
<td>50 (0–100)</td>
<td>50 (0–100)</td>
<td>0.511</td>
</tr>
<tr>
<td>No. of top-quality embryos</td>
<td>7.0 (3.5–8.0)</td>
<td>6.0 (5.0–8.0)</td>
<td>0.693</td>
</tr>
<tr>
<td>Clinical pregnancy</td>
<td>34 (61.8)</td>
<td>29 (65.9)</td>
<td>0.064</td>
</tr>
<tr>
<td>Multiple pregnancy</td>
<td>8 (15.1)</td>
<td>14 (31.8)</td>
<td>0.049</td>
</tr>
</tbody>
</table>

Values are median (interquartile range), mean ± standard deviation, or number (%).

FSH, follicle-stimulating hormone; HP-hMG, highly purified human menopausal gonadotropin; LH, luteinizing hormone; E2, estradiol; P4, progesterone; hCG, human chorionic gonadotropin; OHSS, ovarian hyperstimulation syndrome.

Mann-Whitney U-test; Independent t-test; Fisher exact test; Chi-square test; Significant p-value < 0.05.

pg/mL; p = 0.021); an explanation for this may be that more oocytes were rescued from apoptosis in the concomitant protocol. In The subgroup analysis, there was no statistically significant difference between sFas levels in patients with different causes of infertility. We did not find a correlation between the FF sFas and serum hCG levels on the day of triggering. There was no significant correlation between FF sFas and the fertilization rate (r = 0.088, p = 0.44).

Discussion

LH plays a critical role in the follicular phase during ovulation induction. When a growing follicle reaches 10 mm, LH can stimulate granulosa cell function [19]. Most studies have compared rFSH versus HP-hMG throughout the protocol. Several meta-analyses have compared the effects of rFSH and HP-hMG, and the results were in-
conclusive [20–23]. In this study, we implemented a more personalized protocol for HP-hMG either from the beginning of ovarian stimulation or the late follicular phase to follow the physiology of the follicular phase. Kan et al. [8] stated that the addition of HP-hMG to rFSH on the day of GnRH antagonist administration might improve the cumulative pregnancy rate in patients aged ≤ 30 years. However, in our results, we did not find significant difference in the cumulative pregnancy rate between the two groups.

Our results were consistent with the study of Filicori et al. [24], who added increasing doses of hCG as a source of LH activity from day 8 of stimulation (ranging from 0 IU to 50 IU, 100 IU, and 200 IU) with declining FSH and found that preovulatory E2 levels did not differ in all treatment groups. The number of small (≤ 10 mm) preovulatory follicles was significantly reduced in all patients who received exogenous hCG. In this study, we found a lower number of oocytes with the sequential protocol; this selective regression of smaller follicles seems to be an advantageous process. This is the first study to show that LH activity from late follicular stimulation increases FF sFas levels. LH may lead to atresia of small follicles; this modulates the response to involve a lower number of oocytes but a higher oocyte

### Table 3. Comparison according to the GnRH analogue used in the concomitant protocol

<table>
<thead>
<tr>
<th>Variable</th>
<th>Long agonist (n = 25)</th>
<th>Antagonist (n = 30)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormone at day of triggering</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2 (pg/mL)</td>
<td>3,000</td>
<td>3,000</td>
<td>0.780</td>
</tr>
<tr>
<td>P4 (ng/mL)</td>
<td>0.63</td>
<td>0.69</td>
<td>0.866</td>
</tr>
<tr>
<td>hCG (mIU/mL)</td>
<td>0.72</td>
<td>0.33</td>
<td>0.007</td>
</tr>
<tr>
<td>No. of oocytes retrieved</td>
<td>14</td>
<td>20</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>No. of metaphase II oocytes</td>
<td>10</td>
<td>15</td>
<td>0.051</td>
</tr>
<tr>
<td>No. of top quality embryos</td>
<td>7.0</td>
<td>6.0</td>
<td>0.093</td>
</tr>
<tr>
<td>Clinical pregnancy</td>
<td>18 (72)</td>
<td>16 (53.3)</td>
<td>0.156</td>
</tr>
</tbody>
</table>

Values are median or number (%).

GnRH, gonadotropin-releasing hormone; E2, estradiol; P4, progesterone; hCG, human chorionic gonadotropin.

Mann-Whitney U-test; Chi-square test; Significant p-value <0.05.

### Table 4. Comparison according GnRH analogue in consecutive protocol

<table>
<thead>
<tr>
<th>Variable</th>
<th>Long agonist (n = 36)</th>
<th>Antagonist (n = 9)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormone at day of triggering</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2 (pg/mL)</td>
<td>3,045.5</td>
<td>2,241</td>
<td>0.420</td>
</tr>
<tr>
<td>P4 (ng/mL)</td>
<td>0.7</td>
<td>0.7</td>
<td>0.854</td>
</tr>
<tr>
<td>hCG (mIU/mL)</td>
<td>0.6</td>
<td>0.49</td>
<td>0.410</td>
</tr>
<tr>
<td>No. of oocytes retrieved</td>
<td>12</td>
<td>12</td>
<td>0.627</td>
</tr>
<tr>
<td>No. of metaphase II oocytes</td>
<td>10</td>
<td>9</td>
<td>0.932</td>
</tr>
<tr>
<td>No. of top-quality embryos</td>
<td>6.0</td>
<td>6.0</td>
<td>0.336</td>
</tr>
<tr>
<td>Clinical pregnancy</td>
<td>22 (62.9)</td>
<td>7 (77.8)</td>
<td>0.709</td>
</tr>
</tbody>
</table>

Values are median or number (%).

GnRH, gonadotropin-releasing hormone; E2, estradiol; P4, progesterone; hCG, human chorionic gonadotropin.

Mann-Whitney U-test; Chi-square test.

---

**Figure 2.** Scatter plot showing correlation between total dose highly purified human menopausal gonadotropin (HP-hMG) and serum human chorionic gonadotropin (HCG) measured at the day of trigger.
quality. The role of sFas as an apoptotic marker that predicts oocyte quality was debatable. Sarandakou et al. [18] concluded that sFas plays a critical role in controlling oocyte maturation, as the concentration of FF sFas was higher in follicles with MII oocytes. In agreement with their results, we found that FF sFas was significantly higher in group 1, in which there was higher number of follicles at oocyte retrieval. In contradiction to work that was done by Abdelmeged et al. [15], we found that FF sFas was not related to the etiology of infertility or pregnancy. They reported that the levels of sFas in serum and FF varied according to patients' diagnosis, with significantly higher levels in patients with uterine factor infertility and low concentrations in the serum and FF of patients with male factor and unexplained infertility. However, in this study, there was only 1 patient with uterine factor infertility. Abdelmeged et al. [15] found a significant correlation between the levels of sFas in the serum of pregnant and non-pregnant women. However, in accordance with their findings, we observed no relationship between FF sFas and the fertilization rate, which is also consistent with the results of Onalan et al. [25] and Jose de los Santos et al. [26], who did not find correlations between the FF sFas and the fertilization rate or the cumulative pregnancy rate.

There are very few articles that have investigated the consecutive protocol or studied follicular apoptosis with different gonadotropin protocols. All the available articles studied sFas in relation to different causes of infertility and differences between serum and FF levels, whereas no previous studies have analyzed its level in different gonadotropin protocols even though the sFas-Fas ligand system is regulated by gonadotropins. Non-randomization might have led to selection bias, but we tried to minimize this risk by matching the cases in both groups according to age, BMI, and ovarian reserve.

We concluded that personalized controlled ovarian stimulation using consecutive FSH and HP-hMG resulted in lower apoptotic activity and a lower number of oocytes, with a higher fertilization rate. Neither the concomitant nor consecutive protocol resulted in accumulation of serum hCG or a rise in serum progesterone levels. Neither protocol caused moderate or severe ovarian hyperstimulation syndrome.

**Conflict of interest**

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

**Acknowledgments**

The authors would like to thank the staff and embryologists at the ICSI centers where the study was done, for their help with data collection and follicular fluid sample collection.

**ORCID**

Ola Youssef Ahmed https://orcid.org/0000-0001-7690-2594

**Author contributions**

Conseptualization: HAM. Data curation; OY, NAED. Formal analysis; OYA. Methodology; HAM. Project administration; HAM. Visualization; OYA. Writing–original draft: OYA. Writing–review & editing: HAM, AFMA, MSS, NAED.

**References**


Comparison of complication rates after transvaginal ultrasound-guided oocyte pick-up procedures with respect to ovarian response

Nur Dokuzeylul Gungor¹, Tugba Gurbuz², Murat Onal³

¹Department of Reproductive Endocrinology and IVF Center, BAU Goztepe Medical Park Hospital, Istanbul; ²Department of Obstetrics and Gynecology, Medistate Hospital, Istanbul, Turkey; ³Department of Reproductive Endocrinology and IVF Center, Gynolife Hospital, Lefkosa, Cyprus

Objective: The aim of this study was to compare the complication rates of oocyte pick-up (OPU) procedures via transvaginal ultrasonography in patients with different levels of ovarian reserve.

Methods: In total, 789 patients who underwent OPU procedures for in vitro fertilization (IVF) were included in the study.

Results: Individuals with normal ovarian reserve had a 2.947-fold higher risk of complications in OPU procedures than individuals with low ovarian reserve, and individuals with high ovarian reserve had a 7.448-fold higher risk of complications than individuals with low ovarian reserve. In addition, a higher number of IVF trials was associated with an increased risk of complications.

Conclusion: The results of this study show that OPU has a higher risk of complications, particularly severe pain, in patients with high ovarian reserve. It is thought that complications can be reduced by preferring mild stimulation in patients with high ovarian reserve. Collecting fewer oocytes is also associated with a lower risk of complications from OPU. Even if a patient’s reserve is very good, fewer and higher-quality oocytes should be targeted with the use of the lowest possible dose of drugs.

Keywords: Assisted reproductive techniques; Complications; Oocyte retrieval; Ovarian reserve

Introduction

According to data from the World Health Organization, 48.5 million couples are affected by infertility worldwide [1]. The aim of modern assisted reproductive technologies is to shorten the time until pregnancy, reduce the costs related to infertility, and ultimately, promote the live birth of a healthy baby, which can be achieved with appropriate treatment and measures that strengthen patient safety [2]. In vitro fertilization (IVF), in which fertilization takes place after mature eggs are collected from the ovaries, is a widely used method in the treatment of infertility [3].

Oocyte pick-up (OPU) via transvaginal ultrasonography is a commonly used oocyte collection method that is less invasive than other methods [4]. As this process is easy and effective for collecting eggs, it is frequently preferred as the gold standard approach [5]. Despite these advantages, sometimes damage to the surrounding tissues or complications due to various problems are encountered during or after the procedure. These complications can occur in the early or late period after OPU, and the majority are non-life-threatening conditions, such as bleeding (vaginal or intraabdominal), infection, post-procedural adhesion, and pain [6-8]. More serious complications, such as abscesses, sepsis, fistulas, urethral or bladder injuries, and ovarian torsion are rarely encountered [9]. In addition, the risk of complications of OPU is affected by variables such as the experience of the surgeon [4] and the features of the equipment being used [10,11]. In addition to complications arising from the procedure itself, undesirable effects due to anesthesia and sedation can also occur.

It has been reported that 9%–24% of patients who undergo ovari-
were included in the study. The exclusion criteria were a lack of com-
possible etiological factors for poor ovarian response include a de-
crease in the number of follicles due to age, endometriosis, chromo-
somal and genetic changes, a history of ovarian surgery, pelvic adhe-
sions, and metabolic, autoimmune, and infectious diseases [14-18].
High rates of cycle cancellation and implantation failure have been
reported in women with poor ovarian response, despite the imple-
mentation of strategies designed to optimize stimulation [19]. Al-
though previous studies have examined the complications that oc-
cur after OPU and various factors affecting complications, the num-
ber of studies focusing on the relationship between OPU complica-
tions and ovarian response level is quite limited. In this context, the
current study investigated the relationship between ovarian re-
response and OPU complications in patients who underwent IVF.

Methods

1. Study group
A total of 789 patients who underwent oocyte retrieval for IVF
were included in the study. The exclusion criteria were a lack of com-
plete clinical records, a history of ovarian surgery, a genetic disease
or chromosomal abnormality, a body mass index (BMI) ≥ 30 kg/m²,
age over 38 years, and the presence of uterine malformations.

2. Ethical considerations
This retrospective cohort study was conducted on patients who
underwent OPU procedures between January 2018 and December
2019 at the In Vitro Fertilization Center of BAU Goztepe Medical Park
Hospital, Istanbul, Turkey. The Ethics Committee of Beykoz University,
Turkey approved the conduct of this study (July 17, 2020; No.
2020/4). During the study period, all procedures and steps of the
study were carried out in accordance with the Helsinki Declaration
and Good Clinical Practice guidelines. Informed consent was ob-
tained from all individual participants included in the study.

3. Intervention
A gonadotropin-releasing hormone (GnRH) antagonist protocol
was utilized for ovarian stimulation in all patients. The gonadotropin
dose was individualized according to each patient’s ovarian reserve
and anti-Müllerian hormone (AMH) level. Gonadotropin stimulation
was started by applying 150–450 IU of recombinant follicle-stimulat-
ing hormone (FSH; Gonal-F, Merck, Istanbul, Turkey), and/or human
menopausal gonadotropin (Menoral; IBSA Pharmaceutical, Lugano,
Switzerland) as the initial dose on the second or third day of the
menstrual cycle in all women. Serial vaginal ultrasonography was
used to monitor ovarian response. In order to prevent premature lu-
teinization, 0.25 µg of a GnRH antagonist (Cetrotide 250 µg, Merck
was added daily when the leading follicle reached a diameter of 14
mm. When the mean diameter of two or three leading follicles
reached 17 mm or more, recombinant human chorionic gonadotro-
pin (Ovitrele 250 µg, Merck) was used to trigger ovulation. The OPU
procedure was carried out after trigger success, between 34 and 36
hours after administration. The equipment used constituted a dou-
ble-lumen needle (17 gauge) and a pump set at 125 mmHg vacuum
(Cook Medical, Bloomington, IN, USA). All follicles sized 14 to 20 mm
were obtained without flushing. A glass pipette was used to separate
the cumulus-oocyte complexes from the sample, and they were
transferred to the laboratory under necessary precautions after
washing with G-IVF media (G-IVF Plus, Vitrolife, Kungsbacka, Swe-
den). A single dose of cefazolin (1 g) was administered to OPU pa-
tients during the procedure. The conscious sedation and analgesia
technique was used for pain control during OPU. For sedation, 2 µg/
kg of fentanyl and 0.05 mg/kg of midazolam were administered. Se-
dation was generally preferred in cases with low follicle develop-
ment, in cases where a short period of time was predicted. For this
purpose, pethidine (0.5–1 mg/kg), midazolam (0.01–0.03 mg/kg),
fentanyl (1–2 µg/kg), lignocaine (10 mL of 1%) and diclofenac (50
mg) were administered. All OPU procedures were performed by a
single physician with 15 years of experience (MDG).

4. Measurements
The following parameters were recorded: age, BMI, smoking, caus-
es of infertility, AMH levels, day-3 FSH, peak estradiol (E2) level in the
cycle, number of previous IVF trials, anesthesia type, duration of OPU,
the number of collected oocytes, and complications. The study

789 patients who underwent oocyte retrieval for IVF
were included in the study. The exclusion criteria were a lack of com-
plete clinical records, a history of ovarian surgery, a genetic disease
or chromosomal abnormality, a body mass index (BMI) ≥ 30 kg/m²,
age over 38 years, and the presence of uterine malformations.

This retrospective cohort study was conducted on patients who
underwent OPU procedures between January 2018 and December
2019 at the In Vitro Fertilization Center of BAU Goztepe Medical Park
Hospital, Istanbul, Turkey. The Ethics Committee of Beykoz University,
Turkey approved the conduct of this study (July 17, 2020; No.
2020/4). During the study period, all procedures and steps of the
study were carried out in accordance with the Helsinki Declaration
and Good Clinical Practice guidelines. Informed consent was ob-
tained from all individual participants included in the study.

A gonadotropin-releasing hormone (GnRH) antagonist protocol
was utilized for ovarian stimulation in all patients. The gonadotropin
dose was individualized according to each patient’s ovarian reserve
and anti-Müllerian hormone (AMH) level. Gonadotropin stimulation
was started by applying 150–450 IU of recombinant follicle-stimulat-
ing hormone (FSH; Gonal-F, Merck, Istanbul, Turkey), and/or human
menopausal gonadotropin (Merional; IBSA Pharmaceutical, Lugano,
Switzerland) as the initial dose on the second or third day of the
menstrual cycle in all women. Serial vaginal ultrasonography was
used to monitor ovarian response. In order to prevent premature lu-
teinization, 0.25 µg of a GnRH antagonist (Cetrotide 250 µg, Merck
was added daily when the leading follicle reached a diameter of 14
mm. When the mean diameter of two or three leading follicles
reached 17 mm or more, recombinant human chorionic gonadotro-
pin (Ovitrele 250 µg, Merck) was used to trigger ovulation. The OPU
procedure was carried out after trigger success, between 34 and 36
hours after administration. The equipment used constituted a dou-
ble-lumen needle (17 gauge) and a pump set at 125 mmHg vacuum
(Cook Medical, Bloomington, IN, USA). All follicles sized 14 to 20 mm
were obtained without flushing. A glass pipette was used to separate
the cumulus-oocyte complexes from the sample, and they were
transferred to the laboratory under necessary precautions after
washing with G-IVF media (G-IVF Plus, Vitrolife, Kungsbacka, Swe-
den). A single dose of cefazolin (1 g) was administered to OPU pa-
tients during the procedure. The conscious sedation and analgesia
technique was used for pain control during OPU. For sedation, 2 µg/
kg of fentanyl and 0.05 mg/kg of midazolam were administered. Se-
dation was generally preferred in cases with low follicle develop-
ment, in cases where a short period of time was predicted. For this
purpose, pethidine (0.5–1 mg/kg), midazolam (0.01–0.03 mg/kg),
fentanyl (1–2 µg/kg), lignocaine (10 mL of 1%) and diclofenac (50
mg) were administered. All OPU procedures were performed by a
single physician with 15 years of experience (MDG).

The following complications were recorded: severe pain, bleeding,
urinary tract infection, ruptured endometrioma, and pelvic abscess.
Severe pain was defined as the presence of pain even after the use of
non-opioid analgesics, with a score of 6 or more when patients were
asked to score their pain between 1 and 10 on a visual analog scale
(VAS). The VAS is a valid, subjective measure for acute and chron-
ic pain. Scores were recorded by a handwritten mark on a 10-cm line
that represents a continuum between “no pain” and “worst pain ever
suffered” [20]. Patients with a diagnosis of urinary tract infection and
complaints after the procedure (burning while urinating and dysuria)
were diagnosed by performing a complete urine test and urine cul-
ture, and were given appropriate treatment. During the procedure,
we sometimes had to pass through endometriomas while trying to
obtain access to the follicle and retrieve oocytes. If we aspirated the
endometrioma content, we considered it as a case of endometrioma

www.eCERM.org
rupture. The only complication requiring hospitalization was pelvic abscess in one patient, in whom laparoscopic bilateral salpingectomy and abscess evacuation were performed.

5. Statistical analysis

All analyses were performed using IBM SPSS ver. 21 (IBM Corp., Armonk, NY, USA). Q-Q and histogram plots were used to determine whether variables were normally distributed. Data are given as mean ± standard deviation or median (range) for continuous variables according to the normality of distribution, and as frequency (percentage) for categorical variables. Normally distributed variables were analyzed with one-way analysis of variance. Pairwise comparisons of these variables were performed with the Tamhane test. Non-normally-distributed variables were analyzed with the Kruskal Wallis test. Pairwise comparisons of these variables were performed with the Bonferroni correction. Categorical variables were analyzed with the chi-square test. Multiple logistic regression analysis (backward conditional method) was performed to identify significant risk factors for complications. Two-tailed p-values of less than 0.05 were considered to indicate statistical significance.

Results

The mean age of the study group was 29.91 ± 5.20 years (range, 21–38 years). Patients with low, normal, and high ovarian response were found to be similar in terms of age, BMI and smoking. The frequency of polycystic ovarian syndrome was higher in the HR than in other groups (p < 0.001). AMH levels were higher (p < 0.001) and day-3 FSH levels were lower (p < 0.001) in patients with HR than in patients with PR. In patients with HR, the number of previous trials (p < 0.001) was lower, while the operation duration (p < 0.001) and the number of retrieved oocytes (p < 0.001) were significantly higher than in other patients (Table 1). The frequency of complications (p = 0.003) and severe pain (p = 0.002) were higher in patients with HR than in patients with PR. The most frequent complication was severe pain (Table 2, Figure 1).

Table 1. Summary of patient characteristics with regard to ovarian reserve

<table>
<thead>
<tr>
<th>Variable</th>
<th>Ovarian reserve</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PR (n = 209)</td>
<td>NR (n = 266)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>32 (21–38)</td>
<td>32 (21–38)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24.46 ± 2.48</td>
<td>24.45 ± 2.44</td>
</tr>
<tr>
<td>Smoking</td>
<td>55 (26.32)</td>
<td>82 (30.83)</td>
</tr>
<tr>
<td>Cause of infertility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polycystic ovarian syndrome</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tubal factor</td>
<td>15 (7.18)</td>
<td>30 (11.28)</td>
</tr>
<tr>
<td>Unexplained infertility</td>
<td>82 (39.23)</td>
<td>97 (36.47)</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>28 (13.40)</td>
<td>57 (21.43)</td>
</tr>
<tr>
<td>Male factor</td>
<td>64 (30.62)</td>
<td>69 (25.94)</td>
</tr>
<tr>
<td>Preimplantation genetic diagnosis</td>
<td>0</td>
<td>9 (3.38)</td>
</tr>
<tr>
<td>Fertility preservation</td>
<td>20 (9.57)</td>
<td>4 (1.50)</td>
</tr>
<tr>
<td>Anti-Müllerian hormone (IU/mL)</td>
<td>0.5 (0.2–0.9)</td>
<td>2 (1.1–4.22)</td>
</tr>
<tr>
<td>Day-3 FSH (IU/mL)</td>
<td>12.20 ± 1.17</td>
<td>7.20 ± 1.59</td>
</tr>
<tr>
<td>Peak E₂ level (pg/mL)</td>
<td>562 (256–1,200)</td>
<td>1,652 (869–2,625)</td>
</tr>
<tr>
<td>Previous IVF trial (n)</td>
<td>3 (1–6)</td>
<td>2 (1–6)</td>
</tr>
<tr>
<td>1</td>
<td>4 (1.91)</td>
<td>54 (20.3)</td>
</tr>
<tr>
<td>2</td>
<td>66 (31.58)</td>
<td>105 (39.47)</td>
</tr>
<tr>
<td>3</td>
<td>100 (47.85)</td>
<td>76 (28.57)</td>
</tr>
<tr>
<td>≥4</td>
<td>39 (18.66)</td>
<td>31 (11.65)</td>
</tr>
<tr>
<td>Anesthesia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local</td>
<td>5 (2.39)</td>
<td>2 (0.75)</td>
</tr>
<tr>
<td>General</td>
<td>204 (97.61)</td>
<td>264 (99.25)</td>
</tr>
<tr>
<td>Duration of OPU (min)</td>
<td>12.04 ± 2.66</td>
<td>14.34 ± 2.27</td>
</tr>
<tr>
<td>Collected oocytes</td>
<td>4 (1–5)</td>
<td>10 (7–12)</td>
</tr>
</tbody>
</table>

Values are presented as median (range), mean ± standard deviation, or number (%). PR, poor response; NR, normal response; HR, high response; FSH, follicle-stimulating hormone; E₂, estradiol; IVF, in vitro fertilization; OPU, oocyte pick-up. A, B, C The same letters denote a non-statistically significant difference between groups.

https://doi.org/10.5653/cerm.2021.04875
We performed multiple logistic regression analysis to identify significant risk factors for complications. We found that individuals with NR had a 2.947-fold higher risk of complications than individuals with PR (odds ratio [OR], 2.947; 95% confidence interval [CI], 1.123–7.736; \( p = 0.028 \)), and individuals with HR had a 7.448-fold higher risk of complications than individuals with PR (OR, 7.448; 95% CI, 2.787–19.908; \( p < 0.001 \)). In addition, we found that a higher number of previous IVF trials was associated with an increased risk of complications (\( p = 0.001 \)) (Table 3). Other variables included in the model were found to be non-significant, as follows: age (\( p = 0.646 \)), BMI (\( p = 0.129 \)), smoking status (\( p = 0.134 \)), cause of infertility (\( p = 0.163 \)), AMH levels (\( p = 0.426 \)), day-3 FSH values (\( p = 0.488 \)), peak E2 levels (\( p = 0.805 \)), type of anesthesia (\( p = 0.629 \)), and procedure duration (\( p = 0.587 \)).

Discussion

The OPU procedure is the most important stage of complication development in IVF. The majority of complications are minor, but severe complications may occur rarely and can cause life-threatening problems. In this study, the relationship between ovarian response and complications during OPU was investigated. It was found that, as the ovarian reserve increased, AMH and peak cycle E2 levels increased and day 3 FSH levels decreased. The number of oocytes collected and the time spent during the procedure were higher in patients with HR. The most common complication was severe pain, and it was also determined that a higher number of collected oocytes was associated with an increased frequency of complications, especially pain.

Various biochemical markers can be used to estimate ovarian reserve, including FSH, the ratio of FSH to luteinizing hormone, E2, inhibin B, and AMH [21]. Among these, AMH (a hormone secreted from granulosa cells) has been shown to be correlated with the antral follicle count in numerous studies [22-25]. Kim et al. [26] compared AMH and FSH levels with regard to ovarian reserve and reported that the AMH level was high and FSH level was low in patients with diminished ovarian reserve. In our study, in accordance with the literature, it was found that patients with HR had higher AMH and peak cycle E2 levels, but lower day-3 FSH levels.

Although OPU is performed with transvaginal ultrasonography, various complications may occur during and after the procedure. In prior studies, the complications frequently encountered after OPU were bleeding, infection, and severe pain. After OPU, it was reported that 2%–3% of patients experienced severe pain that could last up to the second day [27,28]. In our study, the most common complication (detected in 5.7% of cases) was severe pain, which is a higher rate

Table 2. Complications after transvaginal ultrasound-guided oocyte pick-up procedures

<table>
<thead>
<tr>
<th>Complication</th>
<th>Ovarian reserve</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PR (n = 209)</td>
</tr>
<tr>
<td>Total</td>
<td>6 (2.87)</td>
</tr>
<tr>
<td>Severe pain</td>
<td>4 (1.91)</td>
</tr>
<tr>
<td>Bleeding</td>
<td>0</td>
</tr>
<tr>
<td>Urinary tract infection</td>
<td>2 (0.96)</td>
</tr>
<tr>
<td>Ruptured endometrioma</td>
<td>0</td>
</tr>
<tr>
<td>Pelvic abscess</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are presented as number (%). PR, poor response; NR, normal response; HR, high response; NA, not applicable.

Figure 1. Severe pain rates with regard to ovarian reserve. NS, not significant.

Table 3. Significant risk factors for complications in multiple logistic regression analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>( \beta ) coefficient</th>
<th>Standard error</th>
<th>Wald</th>
<th>( p )-value</th>
<th>Exp (( \beta ))</th>
<th>95% CI for Exp (( \beta ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian response (poor)</td>
<td>1.081</td>
<td>0.492</td>
<td>17.754</td>
<td>&lt; 0.001</td>
<td>2.947</td>
<td>1.123–7.736</td>
</tr>
<tr>
<td>Ovarian response (normal)</td>
<td>4.817</td>
<td>0.282</td>
<td>&lt; 0.001</td>
<td>7.448</td>
<td>2.787–19.908</td>
<td></td>
</tr>
<tr>
<td>Ovarian response (high)</td>
<td>16.024</td>
<td>0.001</td>
<td>10.896</td>
<td>&lt; 0.001</td>
<td>1.605</td>
<td>1.212–2.126</td>
</tr>
<tr>
<td>Number of procedures</td>
<td>0.473</td>
<td>0.143</td>
<td>61.470</td>
<td>&lt; 0.001</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>Constant</td>
<td>-5.006</td>
<td>0.638</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dependent variable: presence of complications; Nagelkerke \( R^2 = 0.070 \). CI, confidence interval.
than reported in previous studies. This may be due to the fact that, in our study, patients who expressed their pain as 6 or higher on the VAS scale were defined as having severe pain. Instead, other studies defined severe pain as pain requiring hospitalization. The use of anesthesia during the operation and analgesics after OPU at different doses and at different times may also have affected the level of pain. In most studies, the most frequently reported complication after OPU was vaginal bleeding [9]. The majority of vaginal bleeding events reported after OPU are mild, and severe bleeding is very rare. Furthermore, case reports describing intrabdominal bleeding after OPU indicate that this complication is exceedingly rare [28,29]. In our study, bleeding developed in 0.3% of patients—a similar result to those reported in much of the literature [4]. The second most frequently reported complication of OPU is infection [9]. Infections have been reported at different frequencies (range, 0.03%–0.6%); however, serious consequences have been demonstrated [30,31]. In our study, occurred in 0.6% of patients. We applied a single dose of cefazolin (1 g) to our patients during the procedure, which may have reduced the frequency of infections. The frequency of complications detected in our study is consistent with the literature, indicating that they are usually predictable.

As the number of retrieved oocytes increases, the time spent during the operation and the risk of complication development due to OPU could also increase. As expected, a higher number of retrieved oocytes translated to an increased frequency of complications, especially severe pain. This may be a direct result of the increased duration of the procedure. Liberty et al. [32] showed that the frequency of complications related to OPU was higher in polycystic ovarian syndrome patients, possibly because more oocytes are retrieved in these patients. Levi-Setti et al. [4] showed that an increased number of oocytes collected and a longer time spent collecting oocytes is associated with the risk of complications. Ludwig et al. [33] reported that after oocyte retrieval, severe to very severe pain occurred in 3.1% of patients, and the pain increased in parallel with the number of oocytes collected. In another study, Singhal et al. [34] showed that the pain scores of patients with an OPU procedure duration exceeding 12 minutes were significantly higher than those with a shorter duration. However, they reported that the pain score was not correlated with the number of oocytes collected. It is therefore important to note that each oocyte retrieval procedure (and its length) could increase the probability of post-procedural adverse effects.

Recent studies reported additional concerns regarding OPU for severely obese women (BMI > 40 kg/m²), including anesthetic-related risks and technical difficulties such as an inability to access the ovaries [4,35]. In this article, BMI was not associated with an increased frequency of complications. This may have been due to the exclusion of patients with obesity from our study. BMI may not be associated with OPU complications up to a certain threshold. This issue could be explored in greater detail in studies that include patients with a BMI of 30 kg/m² and above.

Finally, we found that higher number of previous IVF trials was associated with an increased risk of complications. In patients with less ovarian reserve, more IVF attempts may be required to achieve pregnancy. In other words, patients with more trials may have less ovarian reserve. Indirectly, patients with an increased number of IVF attempts may have an increased risk of complications because they have poor ovarian reserve.

This study has several limitations, such as those associated with the nature of retrospective studies. It has been shown that the experience of the surgeon performing OPU is related to the risk of complications [4]. In our study, OPU was performed by a single doctor with 15 years of experience; thus, the frequency of complications should be assessed in accordance with this fact. In previous studies, the aspiration needle gauge has been shown to affect pain levels after OPU [10,11,36]. In our study, the fact that these conditions facilitate or reduce pain levels may cause misinterpretations in comparison with other studies. Lastly, patients who may have sought treatment at different health institutions due to complications after OPU could not be evaluated in our study. This may have caused the frequency of complications to be underestimated.

In conclusion, it was found that the most common complication in patients who underwent OPU was pain. The risk of complications was higher in patients with normal and high ovarian response than in those with poor ovarian response. We also found that an increased number of previous IVF cycles was associated with a higher risk of complications related to OPU. As pain is common in these patients, it is concluded that pain management may be crucial. Patients with normal or high ovarian response during OPU should be monitored more closely for the development of complications. Comprehensive and prospective studies on OPU complications would be useful.

Conflict of interest

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

ORCID

Nur Dokuzeylul Gungor  https://orcid.org/0000-0002-7234-3876
Tugba Gurbuz  https://orcid.org/0000-0003-3555-3767
Murat Onal  https://orcid.org/0000-0001-5881-6561

https://doi.org/10.5653/cerm.2021.04875
Author contributions

Conceptualization: all authors. Data curation: all authors. Formal analysis: all authors. Writing—original draft: all authors. Writing—review & editing: all authors.

References


27. Ozaltın S, Kumbasar S, Savan K. Evaluation of complications devel-


High efficiency of homemade culture medium supplemented with GDF9-β in human oocytes for rescue in vitro maturation

Mehdi Mohsenzadeh, Mohammad Ali Khalili, Fatemeh Anbari, Mahboubeh Vatanparast

1 Gerash Al-Zahra Fertility Center, Gerash University of Medical Sciences, Gerash; 2 Research and Clinical Center for Infertility, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd; 3 Molecular Medicine Research Center, Research Institute of Basic Medical Sciences, Rafsanjan University of Medical Sciences, Rafsanjan, Iran

Objective: Optimizing culture media for the incubation of immature oocytes is a vital strategy to increase the oocyte maturation rate during in vitro maturation (IVM) programs. This study evaluated the IVM and fertilization rates of human germinal vesicle (GV) and metaphase I (MI) oocytes using two different maturation media (commercial and homemade) with or without growth differentiation factor 9-β (GDF9-β) supplementation.

Methods: Immature oocytes from intracytoplasmic sperm injection (ICSI) cycles were collected and assigned to one of two IVM culture media (commercial or homemade; cleavage-stage base). After maturation, MII oocytes were examined under an inverted microscope for the presence of the polar body, zona pellucida (ZP) birefringence, and meiotic spindle (MS) visualization after maturation in four conditions (commercial or homemade medium, with or without GDF9-β). ICSI was done for matured oocytes, and fertilization was confirmed by the visualization of two distinct pronuclei and two polar bodies.

Results: No significant differences were found between the two culture media in terms of the time and rate of oocyte maturation or the rate of fertilization (p>0.05). Growth factor supplementation increased the 24-hour maturation rate for both GV and MI oocytes only in homemade medium. The maturation rate after 24 hours was higher for MI oocytes (p<0.05). Similar results were observed for MS visualization and ZP structure in both types of media (p>0.05).

Conclusion: Higher rates of oocyte maturation and fertilization were observed after application of homemade medium supplemented with GDF9-β. Therefore, this combination may be recommended as an alternative for clinical IVM programs.

Keywords: Fertilization; Growth differentiation factor 9; In vitro oocyte maturation techniques; Spindle apparatus

Introduction

In recent years, in vitro fertilization (IVF) cycles have achieved considerable success rates. However, IVF is accompanied by the risk of major complications of multiple pregnancies and ovarian hyperstimulation syndrome, a potentially life-threatening condition. The only way to prevent this syndrome is to eliminate ovarian triggering by hormonal administration [1]. One strategy is to perform in vitro maturation (IVM) of immature oocytes as an alternative to exogenous hormonal protocols [2]. Since IVM does not require ovarian stimulation, it has received more attention in recent years [1]. Patients with polycystic ovary syndrome (PCOS), unexplained infertility, poor quality embryos, normal ovulation, a poor response, and a need for fertility preservation are suitable for IVM. The rescue of oocytes that do not successfully mature during stimulated cycles is another important benefit of this technique [3].

IVM is not a new procedure, and the first IVF procedure was done using an immature rabbit oocyte [4]. Some types of IVM are rescue
IVM [5], conventional or standard IVM, and biphasic IVM [4]. Rescue maturation is motivated by the fact that approximately 15% of oocytes from conventional IVF cycles are immature [6,7]. Rescue IVM has shown very limited success; hence, this technique is not routinely used [8]. However, improving the quality of IVM oocytes would be of great clinical value for maximizing the number of mature oocytes and embryos, especially in poor responders [9,10].

Two major types of IVM media exist: commercial and homemade. Homemade medium is prepared from various base media, which are routinely used in IVF laboratories, such as human tubal fluid (HTF) [1], culture medium 199 [11,12], or cleavage-stage [13] and embryo-stage medium [14]. Since cleavage and embryo media are used for the aim of IVM, in addition to their defined use as embryo culture, they are also considered homemade. Previous studies have compared the efficiency of different culture media to optimize human oocytes in IVM conditions [1,10,15]. In a study by Fesahat et al. [10], the efficacy of four different culture media to promote human metaphase I (MI) oocyte maturation was compared. The rates of fertilization and embryo development were also evaluated in oocytes undergoing IVM in stimulated cycles. They concluded that various types of commercial media did not lead to more success in maturation, fertilization, and embryo development for MI oocytes than homemade media. In another study, tissue culture medium 199 and HTF media were compared concerning the human maturation, fertilization, and embryo quality rates, and it was found that, despite the widespread use of HTF in IVF laboratories, it was unsuitable for the immature oocytes retrieved from PCOS patients [1]. In this regard, the effects of supplementation with different growth factors have been studied. In a recent study, Chatroudi et al. [16] investigated the effect of human IVM medium supplementation with growth differentiation factor 9 (GDF9) and cumulus cells (CCs). Their results showed that supplemented medium enhanced fertilization and embryo formation rates, as well as the viability of blastocysts from vitrified cleavage embryos. Other studies also reported promising effects of GDF9 supplementation in enhancing the IVM results in mice [2] and pigs [17]. GDF9, which is secreted by oocytes, is capable of stimulating CC expansion in vivo and, during CC expansion, it promotes the hyaluronic acid-rich matrix [18]. It was reported that the morphology of both the oocyte and the surrounding CCs, especially the size of CCs and the amount of expansion, is the most important criterion for IVM [16]. In the study by Fesahat et al. [19], homemade medium was supplemented with human follicular fluid, but it might be easier to make and handle homemade media with GDF9.

Successful meiotic maturation of oocytes plays a crucial role in normal fertilization and subsequent embryo development [20,21]. Therefore, the maturation process and the choice of medium are critical steps in IVM technology [1]. Applying formulated commercial media has been reported to have several disadvantages, such as a short shelf life and high cost when compared with the standard medium that is used at fertility centers [10]. The aim of the current study was to compare the outcomes of IVM in terms of maturation, duration of maturation, oocyte quality, and fertilization rates between two IVM culture media: cleavage-based medium and commercial medium. Since there is insufficient evidence regarding the effects of GDF9-β IVM medium supplementation on the development and quality of IVM oocytes, we supplemented both types of culture media with this factor.

**Methods**

This study was approved by the Research Ethics Committee of Rafsanjan University of Medical Sciences, Rafsanjan, Iran (IR. RUMS.1399.241), and followed the Helsinki Declaration of 1975. All procedures involving human participants were done in accordance with the standards and ethical rules of the Yazd Fertility Center. All patients provided written informed consent for participation.

Sibling immature oocytes were collected from 82 intracytoplasmic sperm injection (ICSI) cycles between December 2017 and July 2019. The inclusion criteria were male factor infertility and women aged < 38 years, with at least four immature oocytes. Four immature sibling oocytes were assigned to four main groups: commercial and homemade media with and without GDF9-β. The exclusion criteria were endometriosis, PCOS, and severe male factor infertility.

**1. Ovarian stimulation protocol**

All patients underwent the multiple-dose gonadotropin-releasing hormone (GnRH)-antagonist controlled ovarian hyperstimulation protocol with recombinant follicle-stimulating hormone (rFSH; Cinnal-f, Cinnagen, Tehran, Iran) or Gonal-f (Merck Serono, Geneva, Switzerland) started on the second day of the menstrual cycle. Once the dominant follicle (13–14 mm) was detected by sonography, a GnRH antagonist (Cetrotide; Serono international, Geneva, Switzerland) was initiated, and rFSH was continued up to the day of ovulation triggering. Recombinant human chorionic gonadotropin (hCG; PD preg: Pooyesh Darou, Tehran, Iran) was administered for final maturation, when at least one follicle reached a diameter of 18 mm, 36 hours prior to oocyte retrieval.

**2. IVM medium**

A commercial medium (MediCult IVM System, Origio, Denmark) and a homemade medium were used for IVM. The base for the homemade medium was G2 (Vitrolife, Gothenburg, Sweden), which is used for the culture of human embryos from the 8-cell stage to the blastocyst stage [10]. The homemade medium was supplemented with human follicular fluid, but it might be easier to make and handle homemade media with GDF9.
with 75 mIU/mL FSH and 75 mIU/mL luteinizing hormone (LH; Ferring Pharmaceuticals, Suffern, NY, USA). The commercial medium had two parts: LAG medium (vial 1) and IVM medium (vial 2). Vial 2 was supplemented with an hCG solution (100 mIU/mL), an FSH solution (Ferring Pharmaceuticals, Suffern, NY, USA; 75 mIU/mL), and human serum albumin. According to the use of growth factor supplementation (GDF9-β, recombinant human factor 9, Sigma-Aldrich, St. Louis, MO, USA), there were four groups of IVM culture media: cleavage medium (G2) and commercial medium, with and without GDF9-β.

3. Oocyte collection and IVM culture
Approximately 2–3 hours after ovarian puncture, the cumulus-oocyte complexes were denuded using both enzymatic exposure (80 IU/mL hyaluronidase; Irvine Scientific, Santa Ana, CA, USA) and mechanical pipetting. The denuded oocytes were then checked for nuclear maturity. However, between the germinal vesicle (GV) and MI stages, GV breakdown (GVBD) and the circular bivalent stage (absence of a polar body [PB]) occur. Since these two stages cannot be recognized using polarizing optics or birefringence from the GVBD phase, an oocyte without GV or a PB is considered as MI-arrested. In the literature, GVBD is mentioned as the first visible event of oocyte meiotic maturation, followed by chromosome condensation and alignment at the MI plate. These oocytes are MI oocytes [22,23]. Both GV and MI oocytes were collected and assigned into four main culture groups; homemade medium without GDF9-β, homemade medium with GDF9-β (200 ng/mL) [2,16], commercial medium without GDF9-β, and commercial medium with GDF9-β. Thus, there were a total of eight subgroups, as shown in Figure 1.

For IVM with G2 medium, four immature oocytes were cultured in 25-µL droplets of the medium in a triple gas incubator with 5% CO2, 5% O2, and 90% air, under mineral oil (MediCult, Lyon, France). For the commercial medium, according to the manufacturer’s instructions, four-well plates were used: the two superior wells for vial I (LAG medium) and the two inferior wells for vial II (IVM medium). After oocyte pick-up, the immature oocytes were incubated in 0.5 mL of LAG medium in the superior wells, in a CO2 environment at 37°C for 2–3 hours prior to transfer to the final maturation medium. Each group of oocytes was then transferred into 0.5 mL of IVM medium in the two inferior wells of the four-well culture dish and incubated for 24 or 48 hours.

Oocyte maturity was confirmed under an inverted microscope (Nikon Co, Tokyo, Japan), with the presence of the first PB in the perivitelline space (Figure 2A). If the oocyte was mature, PolScope imaging was applied to analyze the zona pellucida (ZP) birefringence and meiotic spindle (MS). However, immature oocytes were left in the incubator for another 24 hours. At the end of 48 hours, the oocytes were checked for maturity. Mature oocytes were injected according to the standard ICSI protocol, and then 3–4 injected oocytes were cultured in a single drop of culture medium (20 µL droplets of G-1 Medium; Vitrolife), under mineral oil.

4. PolScope imaging and ICSI
An inverted microscope (TE300, Nikon) equipped with a polarizing optical system (OCTAX polarAIDE, Octax) was used for PolScope imaging. A droplet of buffered medium (G-Mops-V1; Vitrolife, 5 mL) was placed in a glass-bottomed dish (WillCo-dish; Bellco Glass, Vineland, NJ, USA) covered with mineral oil (Irvine Scientific). After maturation, the MI oocytes were loaded in the mentioned droplets and scored for ZP birefringence and MS visualization. For ZP birefringence, green was considered as high quality, yellow as moderate quality, and red as low quality (Figure 2B and C). Following oocyte morphology assessment, the ICSI procedure took place [24]. Sixteen hours after ICSI, normal fertilization was confirmed by the visualization of two distinct pronuclei and two PBs under an inverted microscope (Nikon). Since the institutional policy was not to transfer the embryos generated from IVM, all zygotes were discarded due to ethical issues.

Figure 1. Flowchart of the immature oocytes assigned groups. MI, metaphase I; GV, germinal vesicle; GDF9-β, growth differentiation factor 9-β.
5. Statistical analysis

Since most of the findings were categorical data, the chi-square test was performed for statistical analyses. When the data frequency was less than 5%, the Fisher exact test was used. The fertilization rates between groups were compared using the chi-square test. Multinomial logistic regression was used for dependent variables with more than two categories. IBM SPSS ver. 20 (IBM Corp., Armonk, NY, USA) was applied for data analysis, and a $p$-value < 0.05 was considered as indicating statistical significance. In each table, $p$-values from the chi-square test are reported for analyses of the relationships between two variables, whereas for analyses with three or more variables, $p$-values were calculated using multinomial logistic regression, whenever the result from the chi-square test was significant.

Results

The descriptive statistical analysis is presented in Table 1. Data from 124 ICSI cycles were gathered, of which 82 met our criteria and had at least 4 immature oocytes. In these cases, a total of 885 oocytes were retrieved, including 338 immature (214 GV and 124 MI) and 547 mature oocytes. A total of 82 ICSI cycles underwent IVM. The chi-square test did not show a significant difference in the distribution of oocytes between homemade and commercial media (Table 1). After IVM, 267 oocytes were matured and 71 oocytes remained immature or degenerated. After 48 hours of IVM, the total maturation rate was 81.5% for the homemade medium and 76.4% for the commercial IVM medium. However, there was no significant difference between the total conversion rate (after 48 hours) between the commercial and homemade medium ($p = 0.27$) (Table 2). Multinomial logistic regression showed a higher 24-hour maturation rate for MI than for GV oocytes ($p = 0.04$) (Table 2). Multinomial logistic regression also demonstrated a higher maturation rate after 24 hours for both GV and MI oocytes when homemade IVM medium was supplemented with GDF9-β. However, no significant difference was observed for commercial medium (Tables 3 and 4).

The chi-square test showed no significant differences in ZP birefringence between the two types of IVM media ($p = 0.24$) or between MI and GV oocytes ($p = 0.11$). However, high-quality results for ZP birefringence were found more frequently when GDF9-β was added to the culture medium ($p = 0.01$) (Figure 3). The spindle was visible in 55.8% of the matured oocytes. The chi-square test showed that the spindle was more frequently visible ($p = 0.01$) when MI oocytes were derived from MI oocytes (65%) than from GV oocytes (50.0%). However, there were no significant differences between the two types of culture media regarding MS visualization when GDF9-β was added to the culture medium ($p = 0.17$). The fertilization rate was higher in GV oocytes than in GV oocytes, as well as in 24-hour matured oocytes in comparison to 48-hour matured oocytes ($p < 0.05$). The fertilization rate showed no significant difference according to the type of culture medium or GDF9-β supplementation. However, the rate was higher when the spindle was visible in the matured oocyte ($p < 0.05$) (Table 5). There was no correlation between the fertilization rate and ZP quality.

---

Table 1. Descriptive statistics of the rescued immature oocytes

<table>
<thead>
<tr>
<th>Variable</th>
<th>GV</th>
<th>MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female age (yr)</td>
<td>30.5 ± 3.9</td>
<td>30.5 ± 3.9</td>
</tr>
<tr>
<td>Total number of retrieved oocytes$^a$</td>
<td>214</td>
<td>124</td>
</tr>
<tr>
<td>Total number of MII injected oocytes</td>
<td>164</td>
<td>103</td>
</tr>
<tr>
<td>Total fertilization rate</td>
<td>93 (56.7)</td>
<td>73 (70.9)</td>
</tr>
<tr>
<td>Spindle visualization</td>
<td>77 (36)</td>
<td>72 (58.1)</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation or number (%). GV, germinal vesicle; MI, metaphase I. $^a$Chi-square test.
Discussion

After 24 hours of incubation, the total maturity rate was 79.9% for the homemade IVM medium and 76.4% for the commercial IVM medium. The IVM success rate has been reported from 16.4% to 88.3%, depending on the time of incubation [25]. Possible reasons for this wide range may include the use of different types of IVM, with full or mild stimulation and with or without hCG priming, as well as differences in the cause of infertility, the medium used, and medium supplementation. No significant differences were found between the two types of media regarding the maturation rates. This finding is in accordance with previous studies that reported no significant advantage of commercial IVM media over standard media [10,26]. Furthermore, some good outcomes have been reported for non-commercial media; for instance, in this study, we used G2 medium, a routinely used medium, as the base medium for IVM. The limited expiration date of commercial IVM media is one of their disadvantages; moreover, they are costly and not routinely used in contrast to standard media.

Table 2. Comparison of the maturation rate between commercial and homemade medium and between GV and MI oocytes

<table>
<thead>
<tr>
<th>Maturation</th>
<th>Homemade medium</th>
<th>Commercial medium</th>
<th>GV</th>
<th>MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hr</td>
<td>80 (46.2)</td>
<td>63 (38.2)</td>
<td>80 (37.4)</td>
<td>63 (50.8)</td>
</tr>
<tr>
<td>48 hr</td>
<td>61 (35.3)</td>
<td>63 (38.2)</td>
<td>84 (39.3)</td>
<td>40 (32.3)</td>
</tr>
<tr>
<td>Immature</td>
<td>24 (13.9)</td>
<td>16 (9.7)</td>
<td>35 (16.4)</td>
<td>5 (4.0)</td>
</tr>
<tr>
<td>Degeneration</td>
<td>8 (4.6)</td>
<td>23 (13.9)</td>
<td>15 (7.0)</td>
<td>16 (12.9)</td>
</tr>
<tr>
<td>Total number</td>
<td>173</td>
<td>165</td>
<td>214</td>
<td>124</td>
</tr>
</tbody>
</table>

*p*-value 0.27<sup>a</sup> 0.04<sup>a</sup>

Values are presented as number (%).

GV, germinal vesicle; MI, metaphase I.

<sup>a</sup>Multinomial logistic regression showed that there was no significant difference in the 24-hour maturation rate in comparison to the 48-hour rate in both culture media, but the MI oocytes matured more often within 24 hours than the GV oocytes.

Table 3. Comparison of the maturation rate between homemade and commercial media, with and without growth factor supplementation, for GV oocytes

<table>
<thead>
<tr>
<th>Maturation</th>
<th>Homemade medium</th>
<th>Commercial medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GV with GDF9</td>
<td>GV without GDF9</td>
</tr>
<tr>
<td>24 hr</td>
<td>29 (46.0)</td>
<td>16 (31.4)</td>
</tr>
<tr>
<td>48 hr</td>
<td>16 (25.4)</td>
<td>26 (51.0)</td>
</tr>
<tr>
<td>Immature</td>
<td>14 (22.2)</td>
<td>7 (13.7)</td>
</tr>
<tr>
<td>Degeneration</td>
<td>4 (6.3)</td>
<td>2 (3.9)</td>
</tr>
<tr>
<td>Total number</td>
<td>63</td>
<td>51</td>
</tr>
</tbody>
</table>

*p*-value 0.01<sup>a</sup> 0.70<sup>a</sup>

Values are presented as number (%).

GV, germinal vesicle; GDF9, growth differentiation factor 9; IVM, in vitro maturation; MI, metaphase I.

<sup>a</sup>Multinomial logistic regression. The results showed that when homemade IVM medium was supplemented with GDF9-β, higher maturation rates were achieved after 24 hours, for both GV and MI oocytes.

Table 4. Comparison of the maturation rate between homemade and commercial IVM media, with and without growth factor, for MI oocytes

<table>
<thead>
<tr>
<th>Maturation</th>
<th>Homemade medium</th>
<th>Commercial medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MI with GDF9</td>
<td>MI without GDF9</td>
</tr>
<tr>
<td>24 hr</td>
<td>22 (78.6)</td>
<td>13 (41.9)</td>
</tr>
<tr>
<td>48 hr</td>
<td>5 (17.9)</td>
<td>14 (45.2)</td>
</tr>
<tr>
<td>Immature</td>
<td>1 (3.6)</td>
<td>2 (6.5)</td>
</tr>
<tr>
<td>Degeneration</td>
<td>0</td>
<td>2 (6.5)</td>
</tr>
</tbody>
</table>

*p*-value 0.01<sup>a</sup> 0.24<sup>a</sup>

Values are presented as number (%).

IVM, in vitro maturation; MI, metaphase I; GDF9, growth differentiation factor 9; GV, germinal vesicle.

<sup>a</sup>Multinomial logistic regression. The results showed that there were no higher maturation rates after 24 hours, for both GV and MI oocyte when the commercial medium was supplemented with GDF9-β.
Our results showed no significant differences between the GV and MI maturation rates after 48 hours, but the maturation rate was higher in MI oocytes after 24 hours of culture. This may be due to the fact that MI oocytes are more developmentally advanced than GV oocytes. Other studies also showed that an extended duration of IVM is accompanied by better clinical outcomes [25,27]. It is well established that immature oocytes from different developmental stages need different timing to reach maturation [25]. However, oocytes that do not reach maturation may need more time to mature or may be subject to certain inherent errors in meiotic maturation [28,29]. Therefore, caution is needed when using later-matured oocytes in the assisted reproductive technology cycles, as studies have shown that converting faster to MII is accompanied with better embryonic development in an IVM program [30].

The addition of oocyte-specific factors during IVM is a relatively new concept. Our results showed a higher maturation rate after 24 hours for both GV and MI oocytes when homemade IVM medium was supplemented with GDF9-β, whereas GDF9-β supplementation did not have a significant impact in commercial medium. This finding is in line with the study of Yeo et al. [2], according to which oocytes' developmental competence decreased in IVM. This may be
due to oocyte–CC communication disruption and inappropriate levels of oocyte factors such as GDF9-β. They also reported that IVM supplementation with exogenous GDF9-β was accompanied by better embryo development and fetal viability [2]. In this regard, Chatroudi et al. [16] showed that IVM medium supplementation with GDF9-β increased fertilization and embryo formation, as well as enhancing blastocysts’ viability after embryo cryopreservation. In 2006, Hussein et al. [31] showed that during bovine IVM, GDF9-β or bone morphogenetic proteins 15 enhanced oocyte developmental potential and consequently blastocyst formation. The results of this study confirm these effects in human IVM programs. A point that must be addressed is that the positive effect of GDF9-β was found only in homemade medium, but not in commercial medium. This may have been because of interactions between this growth factor and those found in the commercial medium. Unfortunately, the biological role of GDF9-β and other oocyte-specific factors in IVM is still unclear. Clarifying this issue would be of great importance for understanding the fundamental process of oocyte maturation and development to improve IVM media and thereby increase IVM-ICSI success rates. Another reason for this positive effect of GDF9 in homemade medium, but not commercial medium, may be the fact that commercial media are more complex than homemade media, which are made in-house. Commercial media, which are prepared with high quality control and standardized formulations, create an optimal environment for the immature oocytes, with no need for supplementation [32].

Regarding the ZP structure, no significant differences were found according to the type of culture medium or maturation timing (24 or 48 hours). The only difference was related to GDF9-β supplementation, which led to a higher frequency of high-quality (green color) findings. The MI and GV oocytes had the same ZP quality score. No previous study has compared ZP birefringence between GV and MI oocytes in IVM programs. Omid et al. [9] reported that the ZP structure was not influenced by IVM process, but according to other studies that evaluated the effect of GDF9 supplementation, the division and expansion of CCs are thought to constitute a mechanism explaining growth factor efficacy [16]. It has also been reported that ZP is synthesized by both oocytes and granulosa cells [33], and GDF9 may affect the ZP score through the regulation of granulosa cells.

Although the results from PolScope showed no signs of ZP structure disruption during IVM, scanning electron microscopy of caprine oocytes showed a higher frequency of the mature type II ZP surface in the ovulated oocytes than in IVM oocytes. The authors concluded that in a caprine model, the ZP structure is related to oocyte maturity [34]. Our results also showed more ZP marked in green (indicating high quality) when the culture medium was supplemented with GDF9-β. This is the first time that the ZP structure has been evaluated in conditions of IVM medium supplemented with GDF9-β. However, previous studies reported better human IVM outcomes with exogenous GDF9-β and concluded that it might be a promising approach for IVM [2,16].

The total MS visualization rate was 55.8%, while other studies reported a range from 51.9% to 73.8% [35,36]. The MS is composed of microtubules that can be temporarily depolymerized by a decrease in temperature, a prolonged process of visualization, or a pH change [37]. Our results showed no significant differences regarding spindle visualization between the two types of culture media, or when the maturation medium was supplemented with GDF9-β, but there was a higher likelihood of spindle detection in the MI-matured oocytes. This finding is in line with the study by Braga et al. [36], which showed that the spindle was more visible when MI oocytes were derived from MI rather than GV oocytes. More spindle detection was reported in in vivo matured than in IVM oocytes [38]. It has also been suggested that the IVM process may have detrimental effects on MS organization [39]. A prior study reported that spindle properties may be influenced by variables such as oocyte manipulation [40]. The results showed that GV-matured in vitro oocytes had impaired maturation and lower spindle detection in comparison to MI-stage oocytes. The nuclear maturation process consists of GVBD, meiosis resumption, and extrusion of the first PB. It is thought that during the IVM process, the in vitro environment may have an important effect on the human oocyte’s maturation potential. MI oocytes are developmentally more advanced than GV oocytes, as they reach a higher level of maturity during the first 24 hours.

Higher fertilization rates were recorded from MI-matured than from GV-matured oocytes. Another study likewise reported fertilization rates of 69% and 53% after maturation of MI and GV oocytes, respectively. The reason for the higher fertilization rate for MI oocytes may be the fact that MI oocytes are more developed and need a shorter time for maturation. Immature oocytes that matured during the first 24 hours had a higher fertilization rate than those that required 48 hours of maturation. Another study also showed that a prolonged incubation time (up to 48 hours) did not improve the fertilization rate of IVM oocytes [10]. In the literature, 24–30 hours of incubation is described as the ideal time for IVM outcomes [41,42].

It was shown that GV oocytes that matured during 30 hours of incubation were more competent for later development than those needing a longer time. Faster maturation was accompanied by better embryonic development [30]. Thus, although a longer time may allow a more mature nucleus to be achieved, this does not necessarily indicate oocyte cytoplasmic competence. Furthermore, compromised reproductive outcomes in IVM transfer cycles [43] may be a consequence of low cytoplasmic competency in the matured oocytes during IVM. Much progress has recently been made regarding
IVM efficacy, including biphasic IVM with pre-IVM culture, in which the aim is to achieve more cytoplasmic competency. In this new method, with a pre-IVM step, surrounding CCs are kept for paracrine signaling, and this method also tries to maintain the oocyte (GV) in a meiotically arrested stage. Meanwhile, by creating conditions similar to the post-LH surge follicular environment for the initiation and progression of meiosis, the achievement of developmental competence of the oocyte during 24 hours is facilitated. This method has potential for increasing the rates of synchronous maturation and MII formation [44,45].

Higher fertilization rates were seen when the spindle was visible before ICSI. This finding is in line with other studies that showed a higher fertilization rate when the MS was observed [36,39]. It is believed that the spindle and chromosome alignment of immature oocytes can be affected by the IVM process. S disturbance and the subsequent abnormal chromosomal organization result in embryo aneuploidy [37]. There was no significant difference in the fertilization rate between the two types of culture media, and there was also no significant correlation between the fertilization rate and ZP score. Ashourzadeh et al. [24] also showed no correlation between the ZP score and the fertilization rate in an IVM program.

In conclusion, a commercial medium designed especially for IVM oocytes with a standardized formulation was associated with good outcomes. Homemade culture medium supplemented with GDF9-β can also be used as a substitute IVM medium. MI oocytes are advanced in terms of both nuclear and cytoplasmic development compared to GV oocytes. In addition, IVM oocytes with a PB extrusion may not be parallel with cytoplasmic maturation, so caution must be used when performing rescue IVM of GV oocytes in clinical practice. Among various approaches for morphologic characterization, MS visualization is considered a powerful criterion for the prediction of normal oocytes. A limitation of this study is the lack of data regarding embryo development. It must be mentioned that our policy was not to transfer IVM-derived embryos in ICSI cycles, so the zygotes were discarded after checking for two pronuclei for ethical reasons.

Conflict of interest

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

ORCID

Mehdi Mohsenzadeh https://orcid.org/0000-0002-9540-3472
Mohammad Ali Khalili https://orcid.org/0000-0002-8932-0224
Fatemeh Anbari https://orcid.org/0000-0001-6967-2305
Mahboubeh Vatanparast https://orcid.org/0000-0002-1133-5004

Author contributions

Conceptualization: MM, MV. Data curation: FA, MV. Formal analysis: MV. Methodology: MV, MAK. Project administration: MV. Visualization: MM, FA. Writing–original draft: MV, MAK. Writing–review & editing: all authors.

References

827–32.
I. ABOUT THE JOURNAL

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

The areas of interest or the scopes of CERM are as follows;
- Infertility practice
- Assisted reproduction
- Reproductive medicine
- Fertility preservation
- Reproductive endocrinology
- Reproductive physiology
- Reproductive surgery
- Reproductive immunology
- Reproductive genetics
- Reproductive urology/andrology
- Basic science for reproduction
- Developmental biology
- Human and animal reproduction

CERM is covered by the PubMed, SCOPE, and emerging sources of citation index (eSCI).

It is published quarterly on the 1st day of March, June, September and December.

II. ETHICS IN PUBLISHING

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

1. Statement of human and animal right

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

2. Statement of IRB/IACUC approval

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

3. Registration of clinical trial research

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

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

- Correction of authorship: CERM does not correct authorship after publication unless a mistake has been made by the editorial staff. Authorship may be changed before publication but after submission when an authorship correction is requested by all of the authors involved with the manuscript.
- Non-author contributor: Any researcher, who does not meet all four ICMJE criteria for authorship discussed above but contribute substantively to study in terms of idea development, manuscript writing, conducting research, data analysis, and financial support should have their contributions listed in the ‘Acknowledgments’ section of the article. We encourage authors to fully acknowledge the contribution of patients and the public to their research where appropriate.

5. Process for scientific misconduct

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

6. Conflict-of-interest statement

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

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

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

8. Editorial responsibilities

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

III. COPYRIGHTS, OPEN ACCESS, AND CLINICAL DATA SHARING POLICY

1. Copyrights

A submitted manuscript, when published will become the property of the journal. The copyrights of all published materials are owned by the Korean Society for Reproductive Medicine, the Korean Society for Assisted Reproduction, the Pacific Society for Reproductive Medicine and Ko-
Upon acceptance of an article, authors will be asked to transfer the copyright for their content to the Korean Society for Reproductive Medicine, the Korean Society for Assisted Reproduction, the Pacific Society for Reproductive Medicine and Korean Society for Fertility Preservation. This transfer will ensure the widest possible dissemination of information to the readers. A letter will be sent to the corresponding author confirming receipt of the manuscript. A form facilitating transfer of copyright will be provided to the author of the manuscript at that time. If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article.

2. Open access

Articles published in CERM are open-access, distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0), which permits unrestricted non-commercial use, distribution, and the reproduction in any medium, provided that the original work is properly cited.

3. Archiving policy

Full text of CERM has been archived in PubMed Central (PMC)/Europe PMC (https://www.ncbi.nlm.nih.gov/pmc/journals/1702/) and National Library of Korea (https://www.nl.go.kr/) from the 46th volume, 2019. According to the deposit policy (self-archiving policy) of Sherpa/Romeo (http://www.sherpa.ac.uk/), authors cannot archive pre-print (i.e., pre-refereeing), but they can archive post-print (i.e., final draft post-refereeing). Authors can archive publisher’s version/PDF. CERM provides the electronic backup and preservation of access to the journal content in the event the journal is no longer published by archiving in PubMed Central and National Library of Korea.

4. Open data policy

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

5. Clinical data sharing policy

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

IV. MANUSCRIPT SUBMISSION

Manuscripts for submission to CERM should be prepared according to the following instructions. CERM follows ICMJE Recommendations, if not otherwise described below. Any physicians or researchers throughout the world can submit a manuscript if the scope of the manuscript is appropriate. Manuscripts can be submitted either in English.

Only those manuscripts which are original, have not been published elsewhere, and are not currently being considered for inclusion in another publication will be considered for publication in CERM. All manuscripts should be submitted online via the journal’s website (http://submit.ecerm.org/) by the corresponding author. Submission instructions are available at the website. All articles submitted to the journal must comply with these instructions. Failure to do so will result in return of the manuscript and possible delay in publication. Send all correspondence regarding submitted manuscripts to:

Byung Chul Jee, M.D.
Editor-in-Chief, Clinical and Experimental Reproductive Medicine
Address: Department of Obstetrics and Gynecology, Seoul National University Bundang Hospital, 82 Gumi-ro 173 beon-gil, Bundang-gu, Seongnam 13620, Korea
Tel: +82-31-787-7254, Fax: +82-31-787-4054
E-mail: blasto@snubh.org

V. CATEGORIES OF PUBLICATIONS

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

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

VI. PREPARATION OF MANUSCRIPTS

1. General guideline

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

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 pos-
sible. 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 in-
appropriate, 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 popula-
tion, 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, in-
clude 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. Empha-
size new and important observations; do not merely repeat the con-
tents in the Introduction or Results. Explain the meaning of the ob-
served opinion along with its limits, and within the limits of the re-
search 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 ad-
diser,” “data collections,” or “participation in clinical trial.” Such persons
must have given their permission to be named. Authors are respon-
se 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 recom-
ended to provide an ORCID. To obtain an ORCID, authors should reg-
ister in the ORCID website: https://orcid.org. Registration is free to ev-
ery 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 contribut-
ors 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 con-
tributions (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 pub-
lished with the final article, and they should accurately reflect contri-
butions to the work. The submitting author is responsible for complet-
ing this information at submission, and it is expected that all authors
will have reviewed, discussed, and agreed to their individual contribu-
tions 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 & edit-
ing: BCJ.

10) References: Number references consecutively in the order in which
they are first mentioned in the text. References are identified by Ara-
bic numerals in square bracket [ ]. Unpublished observations, and
personal communications should not be used as references, al-
though references to written, not oral communications may be in-
serted (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 au-
thor(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
cbi.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 re-
moval on in vitro fertilization day 2 significantly improves the subse-
quent development and clinical outcomes of fragmented human em-

(2) Website
American Society for Reproductive Medicine. Headlines in reproductive
medicine [Internet]. Birmingham: American Society for Reproductive
org/headlines/.
3. 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 website. 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 (do) 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.
The material contained in the manuscript has not been published, has not been submitted, or is not being submitted elsewhere for publication. Each author must indicate below that either (a) no financial conflict of interest exists with any commercial entity whose products are described, reviewed, evaluated or compared in the manuscript, except for that disclosed under “Acknowledgments” or (b) a potential conflict of interest exists with one or more commercial entities whose products are described, reviewed, evaluated or compared in the manuscript through the existence of one or more of the following relationships: the author is a full or part-time employee of a company; has an existing or optional equity interest in a company; owns or partly owns patents licensed to a company; has an ongoing retainer relationship with a company for which he/she receives financial remuneration; or has received financial compensation for this publication or for the work involved in this publication.

We agree with the preceding conditions and provide the appropriate signatures and information below accordingly: All authors should provide the appropriate signatures and any potential conflicts of interest should be disclosed.

Author's Name: ___________________________ Date: ___________________________ Signature: ___________________________

Disclosure statements concerning conflict of interest:

Author's Name: ___________________________ Date: ___________________________ Signature: ___________________________

Disclosure statements concerning conflict of interest:

Author's Name: ___________________________ Date: ___________________________ Signature: ___________________________

Disclosure statements concerning conflict of interest:

Author's Name: ___________________________ Date: ___________________________ Signature: ___________________________

Disclosure statements concerning conflict of interest:

Author's Name: ___________________________ Date: ___________________________ Signature: ___________________________

Disclosure statements concerning conflict of interest:
Copyright Transfer Form

The copyright to this article is transferred to the Korean Society for Reproductive Medicine, effective if and when the article is accepted for publication by Clinical and Experimental Reproductive Medicine.

The copyright covers the exclusive right to reproduce and distribute the article, including reprints, translations, photographic reproductions, micro-form, electronic form (offline and online) or any other reproductions of similar nature. The corresponding author declares that all co-authors of this article have been informed of the submission.

The corresponding author warrants that this contribution is original, that he/she has full power to make this grant, that he/she has not granted or assigned any rights in the article to any other person or entity, that the article is copyrightable, and that it does not infringe upon any copyright, trademark, or patent.

The corresponding author signs for and accepts responsibility for releasing this material on behalf of all authors.

Journal: Clinical and Experimental Reproductive Medicine

Article Title: ____________________________________________

Corresponding Author: __________________________________

E-mail: ________________________________________________

Place and Date: _________________________________________

Signature: _____________________________________________

Please send the completed form via e-mail to:

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