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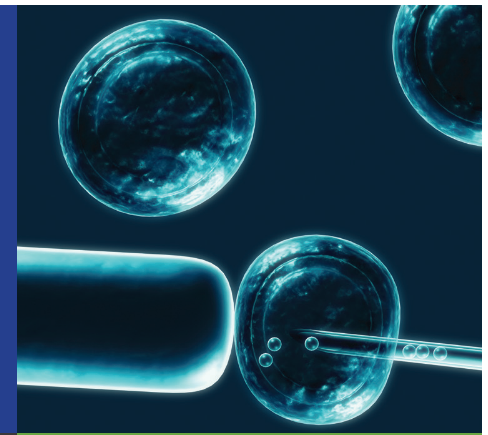

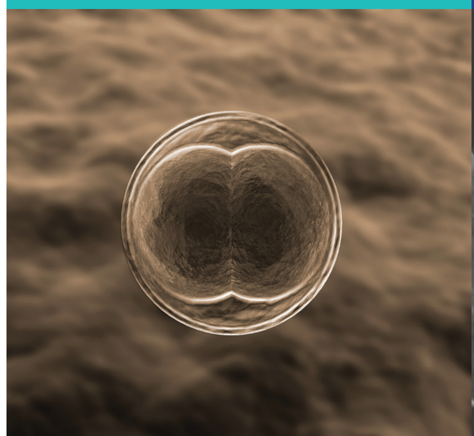

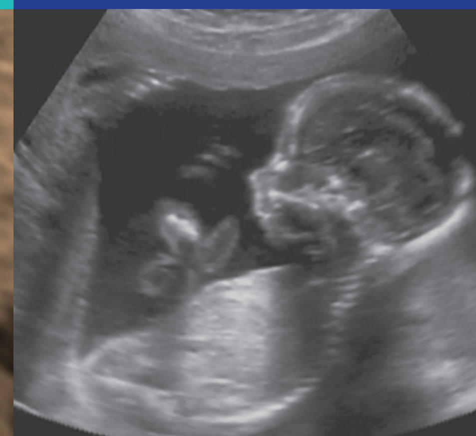

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Aims and Scope

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

Background

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

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Editorial Office
Department of Obstetrics and Gynecology CHA Bundang Medical Center, CHA University 59 Yatap-ro, Bundang-gu, Seongnam-si, Gyeonggi-do, Korea
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Controlled ovarian hyperstimulation for fertility preservation in women with breast cancer: Practical issues

So Yun Park, Kyungah Jeong, Eun Hye Cho, Hye Won Chung

Department of Obstetrics and Gynecology, Ewha Womans University College of Medicine, Seoul, Korea

In Korean women, a westernized lifestyle is associated with an increased risk of breast cancer. Fertility preservation has become an increasingly important issue for women with breast cancer, in accordance with substantial improvements in survival rate after cancer treatment. The methods of controlled ovarian hyperstimulation (COH) for fertility preservation in breast cancer patients have been modified to include aromatase inhibitors to reduce the potential harm associated with increased estradiol levels. Random-start COH and dual ovarian stimulation are feasible options to reduce the total duration of fertility preservation treatment and to efficiently collect oocytes or embryos. Using a gonadotropin-releasing hormone agonist as a trigger may improve cycle outcomes in breast cancer patients undergoing COH for fertility preservation. In young breast cancer patients with *BRCA* mutations, especially *BRCA1* mutations, the possibility of diminished ovarian reserve may be considered, although further studies are necessary. Herein, we review the current literature on the practical issues surrounding COH for fertility preservation in women with breast cancer.

Keywords: Breast neoplasms; Fertility preservation; Ovarian hyperstimulation

Introduction

Breast cancer is the most common cancer in women, and one in five patients are younger than 45 years old at diagnosis [1]. In light of substantial improvements in cancer treatment, fertility preservation has become an increasing priority for women with breast cancer [2,3]. In the United States, the overall 5-year survival rate in women with breast cancer has increased from 74.6% in 1975–1979 to 91.0% in 2007 [4].

A westernized diet, early menarche or late menopause, delayed marriage, and having fewer children later in life are all associated with the increasing risk of breast cancer that has been observed among Korean women. Since 1996, the proportion of early-stage breast cancers has significantly increased, and the incidence rate of female breast cancer was 67.2 cases per 100,000 women in 2010 [5]. Breast cancer is currently the second most common cancer in Korean women, after thyroid cancer. The current methods of controlled ovarian hyperstimulation (COH) for fertility preservation in breast cancer patients have been modified. To reduce the risks associated with increased estradiol levels, aromatase inhibitors (AIs) are co-administered, unlike in other cancers. In contrast to typical infertility treatment, random-start ovarian stimulation is used to minimize the time typically required for fertility preservation treatment prior to chemotherapy.

The Korean Society for Fertility Preservation has suggested that an appropriate method of fertility preservation should be decided through individual patient counseling. Methods such as the cryopreservation of embryos, oocytes, or ovarian tissue and gonadotro-

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Corresponding author: **Kyungah Jeong**
Department of Obstetrics and Gynecology, Ewha Womans University Mokdong Hospital, Ewha Womans University College of Medicine, 1071 Anyangcheon-ro, Yangcheon-gu, Seoul 07985, Korea
Tel: +82-2-2650-2858 Fax: +82-2-2647-9860 E-mail: ogjeong@ewha.ac.kr

pin-releasing hormone (GnRH) agonist treatment during chemotherapy may be considered, and patients must consult with experts before chemotherapy or any other treatment [6]. Herein, practical concerns related to fertility preservation in women with breast cancer will be discussed. With this review, we aim to provide useful clinical information based on recent studies.

Letrozole administration during ovarian stimulation

Estrogen exposure is a well-known risk factor for estrogen receptor (ER)-positive breast cancer [7,8]. The role of estrogen in the carcinogenesis of ER-negative breast cancer has yet to be elucidated, and estrogens are thought to have little effect on breast carcinogenesis in ER-negative breast cancer [9]. However, several studies have suggested a possible association between estrogen-dependent reproductive characteristics such as high parity and the incidence of ER-negative breast cancer [10,11]. Therefore, treatment with letrozole may be useful even in patients with ER-negative breast cancer.

Letrozole is an AI that prevents estrogen production through competitive, reversible binding to the heme of the cytochrome P450 unit of aromatase [12]. Clinically, 5 mg/day letrozole (Femara; Novartis, East Hanover, NJ, USA) is administered on day 2 or 3 of the patient's cycle. The administration of letrozole is continued daily during COH and discontinued on the day of the trigger. After oocyte retrieval, letrozole is continued again for approximately 3 to 6 days until estradiol levels decrease to < 50 pg/mL [13].

Evidence suggests that the administration of letrozole during COH can significantly reduce peak estradiol concentrations. Peak estradiol concentrations (337–829 pg/mL) were found to be less than 1,000 pg/mL when letrozole was commenced on days 2–3 [14-18]. However, Azim et al. [19] reported that anastrozole did not suppress estradiol at its maximum tolerated oral dose of 10 mg/day compared to 5 mg/day of letrozole (2,515.07 ± 1,368.52 vs. 714 ± 440.83 pg/mL, respectively; $p = 0.01$), and letrozole was more potent than anastrozole for the suppression of estrogen.

While it potentially avoids the negative effects of estrogens on tumor growth, letrozole prevents the aromatization of androgens to estrogens, which may induce significant changes in the endocrine follicular environment and impact oocyte competence. Several studies have investigated whether the co-administration of letrozole has an impact on oocyte yield. Oktay et al. [16] and Checa Vizcaino et al. [20] both reported that oocyte yield was not significantly affected when letrozole was administered. In contrast, Domingo et al. [17] and Revelli et al. [18] demonstrated a small but significant decrease in oocyte yield. The administration of letrozole with gonadotropins significantly reduced the number of oocytes available for cryostorage in comparison with the use of gonadotropins alone in breast cancer patients [18]. The triggering of ovulation with a gonadotropin-releasing hormone agonist (GnRHa) has been suggested to help minimize its negative effect [21], and *in vitro* maturation is a useful strategy to improve the mature oocyte yield in breast cancer patients undergoing ovarian stimulation for fertility preservation [15].

A recent systematic review identified 1,002 records, of which 15 selected studies were included in the final analysis [22]. No evidence was found of a decline in relapse-free survival rates in women with breast cancer who received COH with the coadministration of letrozole compared with women who did not undergo fertility preservation procedures. Kim et al. [23] reported the largest long-term study, including over 5 years of safety data, regarding the use of COH with letrozole supplementation for fertility preservation via embryo or oocyte cryopreservation in women with breast cancer. The mean follow-up duration after diagnosis was 5.0 years in the fertility preservation group and 6.9 years among the control patients, who chose not to undergo fertility preservation. In the fertility preservation group, the hazard ratio for recurrence after ovarian stimulation was 0.77 (95% confidence interval, 0.28–2.13), and survival was not compromised compared with controls ($p = 0.61$). Neither *BRCA* gene mutation status ($p = 0.57$) nor whether fertility preservation was undergone before or after breast surgery ($p = 0.44$) significantly affected survival outcomes in the fertility preservation group. None of the tumor characteristics, including ER status, affected the survival rates after COH with letrozole supplementation [23]. COH with concurrent use of AIs appeared to be a safe fertility preservation option for young women with breast cancer at risk of fertility loss following chemotherapy.

Tamoxifen, sold under the brand name Nolvadex among others, is a selective ER modulator. It is a nonsteroidal triphenylethylene antiestrogen. In breast cancer patients, tamoxifen has been proven to have an excellent suppressive effect on cancer cell proliferation and tumorigenesis. Tamoxifen is often preferred over other treatments, as it does not antagonize endometrial development. Oktay et al. [14] compared tamoxifen and letrozole during COH for embryo cryopreservation in breast cancer patients. They used 60 mg/day of tamoxifen alone (Tam-*in vitro* fertilization [IVF]), 60 mg/day of tamoxifen in combination with low-dose follicle-stimulating hormone (FSH; TamFSH-IVF), or 5 mg of letrozole in combination with FSH (letrozole-IVF). The combination of low-dose FSH with tamoxifen (TamFSH-IVF) or letrozole (letrozole-IVF) resulted in higher embryo yields than Tam-IVF. The recurrence rates did not appear to be higher, but the letrozole protocol may be preferred due to lower peak estradiol levels. However, due to insufficient data, conclusions could not be drawn regarding the use of tamoxifen in women with breast cancer during COH [20,24]. Several relevant studies of AI or tamoxifen

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administration during COH in women with breast cancer are summarized in Table 1.

Random-start ovarian stimulation

Traditionally, ovarian stimulation is initiated in the early follicular phase within 3 days of the period before dominant follicle selection. The rationale for this timing includes the stimulation of a synchronous cohort of antral follicles recruited during the interfollicular transition and the induction of timely endometrial development to synchronize blastocyst development with the implantation window. However, the synchronization of endometrial development is not required if the aim of the cycle is oocyte or embryo cryopreservation for fertility preservation without fresh embryo transfer (ET) for immediate pregnancy. This is particularly pertinent in cases subject to time constraints, such as those of cancer patients awaiting gonadotoxic chemotherapy.

After an early referral to a fertility specialist, the prompt use of COH instead of the conventional protocol is very important in all reproductive-aged women undergoing cancer treatment who desire fertility preservation. To provide optimal cancer treatment with minimal delay, random-start ovarian stimulation can be performed. In patients with breast cancer who undergo surgical resection as the first step of cancer therapy, COH begins immediately for a 4- to 6-week window between surgery and adjuvant chemotherapy. Therefore, the conventional initiation of COH within 3 days of the period is impossible, and random-start ovarian stimulation can be used at the initial visit. COH can be initiated before and/or immediately after surgery. Oocyte or embryo cryopreservation can be accomplished with random-start COH within 2–3 weeks, and patients can proceed subsequently with surgery or additional cancer treatment such as chemotherapy, radiation, and/or hormonal therapy [25].

A GnRH antagonist can be administered to pause the patient's cycle, and this approach has been shown to have no negative impact on embryo quality and to improve synchronous follicular growth in poor responders [26]. The appropriate use of a GnRH antagonist can accomplish random-start COH regardless of the point in the menstrual cycle. In a prior study, ovarian stimulation without a GnRH antagonist was initiated if the follicle cohort following the lead follicle was smaller than 12 mm and remained smaller than 12 mm prior to a spontaneous luteinizing hormone (LH) surge. After an LH surge, administration of a GnRH antagonist was initiated when the secondary follicle cohort reached 12 mm to prevent a premature secondary LH surge. If the follicle cohort following the lead follicle reached 12 mm before the spontaneous LH surge, pituitary suppression with a GnRH antagonist was initiated and continued until the triggering of final oocyte maturation. Ovulation was induced with human chorionic gonadotropin (hCG) or a GnRH agonist when the dominant follicle reached 18 mm in diameter, and ovarian stimulation was initiated 2–3 days into the luteal phase [27]. To downregulate LH and initiate luteolysis, a GnRH antagonist was administered with recombinant FSH while simultaneously initiating follicular stimulation.

Martinez et al. [28] published the largest study reporting the outcomes of different random-start protocols with and without AIs. According to that study, random-start ovarian stimulation provided a significant advantage by decreasing the total time for an IVF cycle, and in emergent settings, ovarian stimulation can be initiated at a random cycle date for fertility preservation without compromising oocyte yield or maturity. Although random-start COH protocols are efficient in obtaining an appropriate number of mature oocytes/embryos, only a minority of the patients underwent thawing and ET, and not enough reports have been conducted to evaluate the rates of implantation and pregnancy. Nevertheless, comparable pregnancy rates after the transfer of embryos created from donor eggs obtained after luteal-phase start protocols are encouraging.

Danis et al. [29] published a systematic review of 19 studies published between January 2000 and June 2017 to evaluate the utility of random-start ovarian stimulation for fertility preservation. This recent review suggested that random-start stimulation was associated with a reduced time interval between the initiation of ovarian stimulation and oocyte or embryo cryopreservation. The yield of mature oocytes and their potential development into embryos was comparable between the conventional and random-start protocols, although the gonadotropin doses were higher in the random-start protocols.

Therefore, additional clinical studies are necessary to further assess the efficacy of this strategy, especially regarding the rates of clinical pregnancy and live-born infants originating from the use of cryopreserved embryos and oocytes obtained via random-start ovarian stimulation.

Dual ovarian stimulation

Dual ovarian stimulation consists of two successive instances of ovarian stimulation in the follicular and ensuing luteal phase, with oocyte retrieval at the end of each ovarian stimulation. Evidence is accumulating that follicular- and luteal-phase COH is feasible in patients with poor prognoses with a reduced ovarian reserve to increase their chances of conceiving in each menstrual cycle [30-32].

Kuang et al. [30] investigated the efficiency of dual ovarian stimulation with a combination of gonadotropins, clomiphene citrate, and AI in the follicular and luteal phases in patients with poor ovarian response. In this pilot study, the first stimulation was conducted using a combination of 25 mg per day of clomiphene citrate starting on

Table 1. Summary of studies involving aromatase inhibitor/tamoxifen administration during ovarian stimulation in women with breast cancer

Study	Study design	Protocol	Peak estradiol (pg/mL)	Oocyte outcome
Kim et al. (2016) [23]	Prospective, non-randomized, controlled study -120 Women: COH prior to surgery, 14 patients; postoperative COH, 106 patients -217 Women: no COH	Letrozole (5 mg)+150–300 U of FSH daily	564.5 ± 436.3	13.3 ± 8.4
Meirow et al. (2014) [24]	Prospective consecutive enrollment -27 Women with ER– breast cancer: COH -43 Women with ER+ breast cancer: COH+tamoxifen	ER– group: COH with variable dose of FSH ER+ group: COH with variable dose of FSH+tamoxifen (20 mg) daily	COH vs. COH+tamoxifen: 5,093 ± 4,364 vs. 6,924 ± 4,146 (p = 0.095)	COH vs. COH+tamoxifen: 10.2 ± 6.1 vs. 12.7 ± 8.0 (p = 0.183)
Revelli et al. (2013) [18]	Retrospective cohort study -50 Women with ER+breast cancer: FSH+letrozole - 25 Women with ER–breast cancer: FSH only	Letrozole (5 mg)+FSH daily	FSH+letrozole vs. FSH only: 446 ± 357 vs. 1,553 ± 908 (p = 0.001)	FSH+letrozole vs. FSH only: 6.6 ± 3.5 vs. 8.0 ± 5.0 (p = 0.038)
Domingo et al. (2012) [17]	Retrospective multi-center study -142 Women with breast cancer: FSH+letrozole -66 Women with non-breast cancer: FSH only -97 Women without cancer (male factor infertility): FSH only	Women with breast cancer: FSH+letrozole (5 mg)+150–225 U of FSH daily Women with non-breast cancer: 150–225 U/day of FSH only Women without cancer: 150–225 U/day of FSH only	FSH+letrozole vs. FSH only (non-breast cancer) vs. FSH only (male factor): 381 ± 191 vs. 1,744 ± 1,242 vs. 2,109 ± 1,260 (p < 0.001)	FSH+letrozole vs. FSH only (non-breast cancer) vs. FSH only (male factor): 9.8 ± 7.1 vs. 12.2 ± 6.5 vs. 12.4 ± 5.4 (p = 0.003)
Checa Vizcaino et al. (2012) [20]	Prospective data collection, retrospective analysis -9 Women with breast cancer, FSH+letrozole -10 Women with non-breast cancer, FSH only	Women with breast cancer: letrozole (5 mg)+150–225 U of FSH daily Women with non-breast cancer: 150–225 U/day of FSH only	FSH+letrozole vs. FSH only: 829 ± 551.11 vs. 1,666.4 ± 739.42 (p = 0.006)	FSH+letrozole vs. FSH only: 16.3 ± 7.21 vs. 15.4 ± 8.19 (no significant difference)
Azim et al. (2007) [19]	Prospective sequential cohort study -47 Women, FSH+letrozole -7 Women FSH+anastrozole	FSH+letrozole: letrozole (5 mg)+FSH daily FSH+anastrozole: maximum tolerated dose of 10 mg of anastrozole+FSH daily	FSH+letrozole vs. FSH+anastrozole: 714.38 ± 440.83 vs. 2,515.07 ± 1,368.52 (p = 0.01)	FSH+letrozole vs. FSH+ anastrozole: 11.57 ± 7.14 vs. 9.71 ± 8.5 (no significant difference)

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Table 1. Continued

Study	Study design	Protocol	Peak estradiol (pg/mL)	Oocyte outcome
Oktay et al. (2006) [16]	Prospectively recruited women with breast cancer compared with retrospectively identified age-matched controls who underwent IVF to treat tubal disease -47 Women with breast cancer, FSH+letrozole -56 Women with tubal disease, FSH only	FSH+letrozole: letrozole (5 mg)+150–300 U of FSH daily FSH only: 300 U of FSH daily	FSH+letrozole vs. FSH only: 483.4 ± 278.9 vs. 1,464 ± 664.9 ($p < 0.001$)	FSH+letrozole vs. FSH only: 8.7 ± 4.8 vs. 9.7 ± 5.1 ($p = 0.43$)
Oktay et al. (2005) [14]	Prospective cohort study -12 Women, tamoxifen only IVF (TamOnly) -7 Women, FSH+tamoxifen IVF (TamFSH) -11 Women, FSH+letrozole IVF (LetFSH)	TamOnly: tamoxifen (60 mg) daily TamFSH: FSH (150 U)+tamoxifen (60 mg) daily LetFSH: letrozole (5 mg)+FSH (150 U) daily	TamOnly vs.TamFSH: 419 ± 39 vs. 1,182 ± 271 ($p < 0.05$) TamOnly vs. LetFSH: 419 ± 39 vs. 380 ± 57 ($p > 0.05$) TamFSH vs. LetFSH: 1,182 ± 271 vs. 380 ± 57 ($p < 0.05$)	TamOnly vs.TamFSH: 1.5 ± 0.3 vs. 5.1 ± 1.1 ($p < 0.05$) TamOnly vs. LetFSH: 1.5 ± 0.3 vs. 8.5 ± 1.6 ($p < 0.001$) TamFSH vs. LetFSH: 5.1 ± 1.1 vs. 8.5 ± 1.6 ($p > 0.5$)

COH, controlled ovarian hyperstimulation; FSH, follicle-stimulating hormone; ER, estrogen receptor; IVF, *in vitro* fertilization.

day 3 of the cycle until ovulation was triggered, 2.5 mg of letrozole per day starting on day 3 for a total of 4 days, and 150 IU of human menopausal gonadotropin (hMG) every other day starting on day 6 until the triggering of ovulation. The second stimulation was initiated after the first oocyte retrieval, provided that two or more antral follicles were identified, and it consisted of 2.5 mg of letrozole and 225 IU/day of hMG, which were both administered from the day of retrieval until the second trigger of ovulation. A relatively large dose of hMG was used to perform ovarian stimulation after the first oocyte retrieval due to the ovarian insensitivity to hMG stimulation during the luteal phase relative to the follicular phase. For both the first and second stimulations, final oocyte maturation was induced with a GnRH agonist (triptorelin, 100 μg) when follicular maturation was reached. The authors reported similar developmental potential for the antral follicles from the luteal phase compared with those from the follicular phase in terms of mature oocytes, fertilization rate, cleavage rate, and the number of top-quality embryos obtained [30].

In a more recent prospective paired non-inferiority observational study, Ubaldi et al. [31] reported a similar number of euploid blastocysts per metaphase II oocyte injected after follicular- and luteal-phase COH in the same menstrual cycle. In follicular-phase COH versus luteal-phase COH, no differences were observed in the number of retrieved cumulus-oocyte complexes (5.1 ± 3.4 vs. 5.7 ± 3.3, respectively), metaphase II oocytes (3.4 ± 1.9 vs. 4.1 ± 2.5, respectively), biopsied blastocysts (1.2 ± 1.2 vs. 1.4 ± 1.7, respectively), or euploid blastocysts (0.6 ± 0.8 vs. 0.7 ± 0.8, respectively). The types of chromosomal abnormalities observed in aneuploid embryos from the follicular-phase COH and luteal-phase COH were also similar, suggesting no impact on the meiotic maturation of oocytes.

Two studies were recently published and reported promising results of double ovarian stimulation [32,33]. Cardoso et al. [33] conducted a retrospective and comparative study of 13 patients who underwent unsuccessful IVF cycles with a conventional antagonist ovarian stimulation protocol and repeated the attempt with a double stimulation protocol. They used the conventional ovarian stimulation antagonist protocol involving a high dose of gonadotropins (225 IU of FSH and 75 IU of hMG), and the triggering was carried out with a single subcutaneous injection of hCG (250 μg). The ovarian double stimulation protocol was similar to the conventional protocol, except triggering was initiated using a GnRH agonist (triptorelin, 0.2 mg). The patients subjected to dual ovarian stimulation exhibited a statistically significant increase in the mean number of oocytes collected, from 5.3 to 9.3 mature oocytes [33].

Liu et al. [34] conducted a retrospective case-control study involving a total of 116 women aged ≥ 38 years who were treated with double ovarian stimulation. The women were divided into 4 groups according to the follicular-phase ovarian stimulation protocol: a

GnRH agonist short protocol, a GnRH antagonist protocol, a mild stimulation protocol, and a progestin pituitary downregulation protocol. The starting dose of gonadotropin was 150–300 IU of FSH, and luteal phase stimulation was performed with 225 IU of hMG daily within 1–3 days of oocyte retrieval. Both instances of triggering were performed using 250 IU of hCG. The numbers of oocytes retrieved and metaphase II oocytes ($p=0.002$ and $p=0.003$, respectively), fertilized oocytes ($p=0.003$), cleaved embryos ($p=0.002$), and top-quality embryos ($p=0.031$) with luteal-phase stimulation were higher than those retrieved with follicular-phase stimulation [34].

The first study that investigated stimulation with an identical protocol in the follicular and luteal phases of the same menstrual cycle found a similar number of euploid blastocysts in patients with reduced ovarian response. The evidence of multiple follicular waves during a single menstrual cycle in women with diminished ovarian reserve raised important implications. In particular, it indicated that random-start COH offers an efficient strategy for fertility preservation in young cancer survivors to save time before anticancer therapies. Dual stimulation can maximize the number of oocytes obtained per menstrual cycle and in turn increases the chance of obtaining reproductively competent embryos in the shortest possible time. Randomized controlled trials comparing pregnancy outcomes after dual stimulation are eagerly awaited [35].

In a retrospective observational study that involved 50 cycles in 34 patients who underwent fertility preservation due to breast cancer, follicular-phase or luteal-phase ovarian stimulation with an AI was performed. The numbers of oocytes retrieved at the first and second oocyte pickup did not differ significantly between conventional stimulation and dual stimulation. The researchers demonstrated the efficacy of luteal-phase stimulation and dual stimulation with the AI protocol among breast cancer patients in regard to increasing the number of oocytes harvested within the limited time available before the initiation of cancer treatment [36].

Triggering with GnRHa versus hCG

Reddy et al. [37] compared the cycle outcomes and the incidence of ovarian hyperstimulation syndrome (OHSS) when oocyte maturation was triggered by a GnRHa versus hCG in breast cancer patients undergoing fertility preservation. A total of 129 women aged ≤ 45 years, diagnosed with stage ≤ 3 breast cancer, and with normal ovarian reserve who desired fertility preservation were compared in the retrospective cohort study. COH was achieved utilizing letrozole and gonadotropins. Serum anti-Müllerian hormone (AMH) levels were similar between the GnRHa and hCG groups (2.7 ± 1.9 vs. 2.1 ± 1.8 ng/mL, respectively; $p=0.327$). One case of mild or moderate OHSS was reported in the GnRHa group compared to 12 in the hCG group (2.1% vs. 14.4%, respectively; $p=0.032$). The maturation and fertilization rates and the number of cryopreserved embryos were significantly higher in the GnRHa group. Therefore, the researchers suggested that the GnRHa trigger improved cycle outcomes as evidenced by the number of mature oocytes and cryopreserved embryos, while significantly reducing the risk of OHSS in breast cancer patients undergoing fertility preservation [37].

A GnRHa trigger can be effective in the induction of oocyte maturation and prevention of OHSS on IVF cycles using an antagonist protocol. Yilmaz et al. [38] reported the comparison of different triggers in different cycles of the same patients. The authors reasoned that the GnRHa trigger is clinically more successful than hCG with regard to OHSS prevention and reproductive outcomes on fresh IVF/intracytoplasmic sperm injection cycles, although more extensive studies are needed to draw firm conclusions.

In a recent retrospective study by Lin et al. [39], a total of 427 completed GnRHa-downregulated IVF cycles with fresh ET were enrolled. The results of that study suggested that dual triggering of final oocyte maturation with GnRHa and a standard dose of hCG can significantly improve live birth rates, clinical pregnancy rates, and the fertilization rate in women with diminished ovarian reserve undergoing GnRHa-downregulated IVF-intracytoplasmic sperm injection cycles. Clinical results of letrozole-COH efficiency in terms of oocyte quality and pregnancy outcomes in breast cancer patients are still limited, and large data sets will probably be available in several years. The first evaluation of the impact of letrozole-COH on the oocyte microenvironment in patients with breast cancer has suggested that a GnRHa trigger may improve oocyte quality [40].

However, Asada et al. [41] reported that the usual dose of GnRHa could fail to trigger oocyte maturation. They suggested that this finding may be attributable to a suboptimal endogenous LH surge due to the severe downregulation of the hypothalamus-pituitary axis. The researchers recommended that a rescue protocol entailing re-triggering oocyte maturation using hCG could produce a successful outcome. Chen et al. [42] published a systemic review and meta-analysis that indicated comparable or significantly improved outcomes with the use of GnRH agonists in addition to hCG compared with hCG alone for the triggering of final oocyte maturation.

BRCA gene mutations and ovarian reserve

AMH is a sensitive serum marker that approximates the primordial follicle reserve. Titus et al. [43] compared serum AMH concentrations in young breast cancer patients (n = 84) with (n = 24 per group; mean age, 34.8 ± 4.8 years) and without (n = 60 per group; mean age, 36.3 ± 3.5 years) *BRCA* mutations. The patients with mutations displayed significantly lower serum concentrations of AMH than the

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patients without them (1.22 ± 0.92 ng/mL vs. 2.23 ± 1.56 ng/mL, respectively; $p < 0.001$). When the researchers analyzed the impact of *BRCA1* versus *BRCA2* mutations on ovarian reserve compared to those who tested negative for the same mutations (n = 60 per group; mean age, 36.3 ± 3.5 years), the significance remained for *BRCA1* mutations (n = 15 per group; two patients had both *BRCA1* and *BRCA2* mutations; mean AMH concentration, 1.12 ± 0.73 ng/mL; $p < 0.001$) but not for *BRCA2*-only mutations (n = 9 per group; mean AMH concentration, 1.39 ± 1.20; $p = 0.127$). The researchers used transgenic mouse models to confirm that women with *BRCA1* mutations had diminished ovarian reserve. The data are also consistent with the decreased response to ovarian stimulation and earlier menopausal age shown previously in human *BRCA1* mutation carriers [43].

Recently, Peccatori et al. [44] suggested that individuals with *BRCA1* mutations have significantly lower mature oocyte yields. This independence of the role of *BRCA1* mutations was confirmed by the comparison of this study group with a group of patients without *BRCA* mutations who were being treated for breast cancer. Oocyte cryopreservation is a feasible option that should be suggested to these patients, especially considering the frequent recommendation of prophylactic bilateral salpingo-oophorectomy at the age of 40 for both women with *BRCA1* mutations and those with *BRCA2* mutations.

In contrast, a retrospective cohort study demonstrated that *BRCA* carriers with and without malignancy had comparable ovarian reserve and response to ovarian stimulation to noncarriers undergoing fertility preservation [45]. However, data from Korean patients indicated that breast cancer patients with *BRCA* mutations had significantly lower serum AMH levels, although no significant differences were found between those with *BRCA1* and those with *BRCA2* mutations. The researchers recommended that fertility preservation be considered more aggressively in young breast cancer patients with *BRCA* mutations [46].

Winship et al. [47] published the protocol for a prospective observational study that will be the first large-scale study aimed at exploring the measurement of ovarian reserve in both *BRCA1* and *BRCA2* mutation carriers compared with age-matched patients without these mutations. The results of this study have the potential to clarify the association between circulating AMH and follicle density and thus help elucidate the mechanism of oocyte loss during reproductive life, particularly for women who are thought to have reduced ovarian reserve, such as *BRCA* mutation carriers.

Women with *BRCA* mutations (in consideration of the differences between *BRCA1* and *BRCA2* mutations) may be informed about the possible advantages of oocyte cryopreservation, along with the general risks, cost, and effectiveness of the procedure. Putting aside the unresolved debate on the impact of *BRCA* mutations on ovarian reserve, a personalized assessment of serum AMH or antral follicle count at the time of counseling can also be useful. Based on current knowledge, standardized clinical management cannot be recommended. However, even if a policy of fertility preservation in young, healthy *BRCA* carriers cannot be advocated across the board, it deserves consideration and should be carefully discussed on an individual basis [48].

Further research on the impact of *BRCA* mutations on fertility is necessary to confirm these findings and provide a better understanding of the underlying pathophysiology. Given that conflicting evidence exists regarding diminished fertility in this specific population, larger prospective trials are required to clarify the impact of *BRCA* mutations on ovarian aging.

Conclusion

In Korea, requests for fertility preservation treatment in breast cancer patients are becoming increasingly common. Random-start COH and dual ovarian stimulation with the co-administration of letrozole

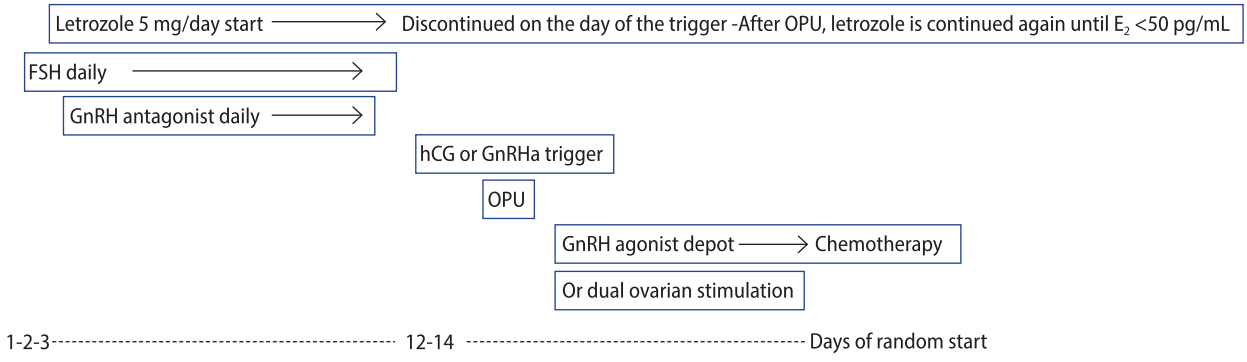

Figure 1. Controlled ovarian hyperstimulation protocol for fertility preservation in women with breast cancer. OPU, ovum pick-up; E_2, estradiol; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; hCG, human chorionic gonadotropin; GnRHa, gonadotropin-releasing hormone agonist.

are feasible for the efficient harvest of oocytes or embryos and the reduction of the total duration of fertility preservation treatment, and these techniques work without raising peak estradiol levels and correspondingly increasing breast cancer risk. The use of a GnRHa trigger may improve cycle outcomes in breast cancer patients undergoing fertility preservation. In young breast cancer patients with *BRCA* mutations, particularly *BRCA1* mutations, more effective fertility preservation treatment should be considered. The COH protocol for fertility preservation in women with breast cancer is outlined in Figure 1. Additional clinical studies with larger samples are required to confirm the optimal protocol for fertility preservation in young women with breast cancer.

Conflict of interest

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

ORCID

So Yun Park	https://orcid.org/0000-0002-3572-9673
Kyungah Jeong	https://orcid.org/0000-0002-9673-1152
Eun Hye Cho	https://orcid.org/0000-0002-1370-0992
Hye Won Chung	https://orcid.org/0000-0002-6162-9158

Author contributions

Conceptualization: KJ. Writing–original draft: KJ, SYP, EHC. Writing–review & editing: KJ, HWC.

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The role of gonadotropin-releasing hormone agonists in female fertility preservation

Jae Hoon Lee[1,2], **Young Sik Choi**[2,3]

[1]Department of Obstetrics and Gynecology, Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul; [2]Institute of Women's Life Medical Science, Yonsei University College of Medicine, Seoul; [3]Department of Obstetrics and Gynecology, Severance Hospital, Yonsei University College of Medicine, Seoul, Korea

Advances in anticancer treatments have resulted in increasing survival rates among cancer patients. Accordingly, the quality of life after treatment, particularly the preservation of fertility, has gradually emerged as an essential consideration. Cryopreservation of embryos or unfertilized oocytes has been considered as the standard method of fertility preservation among young women facing gonadotoxic chemotherapy. Other methods, including ovarian suppression and ovarian tissue cryopreservation, have been considered experimental. Recent large-scale randomized controlled trials have demonstrated that temporary ovarian suppression using gonadotropin-releasing hormone agonists (GnRHa) during chemotherapy is beneficial for preventing chemotherapy-induced premature ovarian insufficiency in breast cancer patients. It should also be emphasized that GnRHa use during chemotherapy does not replace established fertility preservation methods. All young women facing gonadotoxic chemotherapy should be counseled about and offered various options for fertility preservation, including both GnRHa use and cryopreservation of embryos, oocytes, and/or ovarian tissue.

Keywords: Chemotherapy; Fertility preservation; Gonadotropin-releasing hormone agonist; Premature menopause

Introduction

Advances in anticancer treatments have resulted in increasing survival rates among cancer patients. In South Korea, the overall cancer mortality rate decreased by 2.7% annually between 2002 and 2016. A significant improvement was also evident in the 5-year survival rate, which increased to 70.6% for patients diagnosed with cancer between 2012 and 2016, compared with 41.2% for patients diagnosed between 1993 and 1995 [1]. Accordingly, the idea that quality of life after cancer should be actively considered during cancer treatment has become widely accepted. In particular, the preservation of fertility in younger patients receiving cancer treatment has gradually become an essential factor for consideration [2-4]. The main problems associated with chemotherapy in female cancer survivors include early menopause and an increased subfertility rate [5]. For fertility preservation in women, embryo and oocyte cryopreservation are considered to be the standard practice and are widely available [2-4]. However, because embryo and oocyte cryopreservation requires approximately 2 weeks, it is difficult to perform cryopreservation in patients with cancers for which treatment is more urgent due to rapid progression. In addition, cryopreservation may not be an option for all women for economic reasons.

There has been tremendous interest in medical agents that can potentially preserve fertility from the ovarian toxicity of chemotherapy, and gonadotropin-releasing hormone agonists (GnRHa) have been considered to be the most likely category of drugs for this purpose. In 1981, an animal study demonstrated that GnRHa administration protected male mice from gonadal damage caused by cyclophosphamide [6]. An observational long-term follow-up study of children treated with chemotherapy for Hodgkin disease showed that prepubertal administration of chemotherapy agents resulted in

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Corresponding author: **Young Sik Choi**
Department of Obstetrics and Gynecology, Severance Hospital, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea
Tel: +82-2-2228-2230 Fax: +82-2-313-8357 E-mail: YSCHOI08@yuhs.ac
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less ovarian damage than in similarly treated adult patients, and it was suggested that GnRHa could exert a protective role by creating a temporary "prepubertal" state in women of reproductive age [7]. The first randomized controlled trial (RCT) of GnRHa for preserving fertility during chemotherapy was conducted by Waxman et al. [8] in 1987. The authors reported that buserelin was not effective in preserving fertility. Since this first RCT was reported, several other RCTs and observational studies have been conducted in patients with various diseases, including breast cancer, hematological malignancies, and autoimmune diseases. However, these studies have reported conflicting results [9].

Although the mechanisms of protection have yet to be fully understood, as the results of large-scale RCTs were recently released, researchers' understanding of the conditions under which GnRHa can preserve fertility has been expanded and clarified. This trend was reflected in the 2018 American Society of Clinical Oncology (ASCO) guidelines for fertility preservation [4]. The 2018 ASCO guidelines stated for the first time that GnRHa may partially help in preserving fertility in patients undergoing chemotherapy. These changes in the ASCO guidelines have also influenced other guidelines released after 2018 [10,11]. Nevertheless, evidence regarding the efficacy of GnRHa for fertility preservation is still regarded as insufficient, and GnRHa use during chemotherapy does not replace established fertility preservation methods. In this review, we describe the scope of utility and limitations of GnRHa in preserving fertility, as well as the mechanisms by which GnRHa protects the ovaries from chemotherapy.

How do chemotherapeutic agents damage the ovary?

Although controversial [12], it is generally believed that the number of follicles held within the primordial follicle (PMF) pool is finite. PMFs form from 17 weeks of gestation, and consist of an immature oocyte in meiotic arrest surrounded by a single layer of granulosa cells. During the reproductive age, both the oocyte and surrounding granulosa cells undergo stages of sequential growth and development, characterized by granulosa cell proliferation and oocyte growth, until resumption of meiosis by the ovulatory luteinizing hormone (LH) surge. PMFs are continuously being recruited out of the pool of germ cells and activated to grow until few remain. The presence of fewer than 1,000 remaining oocytes has been associated with menopause [13,14].

The risk of developing premature ovarian insufficiency (POI) after chemotherapy can vary depending on various factors. Some chemotherapy regimens are considered more gonadotoxic than others, with particularly strong evidence for the high toxicity of alkylating agents [14]. Dosage of the treatment used is also an important consideration, and previous studies have shown that early menopause occurs in a dose-dependent fashion [15]. In addition, the age of the patient at treatment is pivotal, as older women have a much higher reported incidence of POI during or immediately after treatment [5,16]. Chemotherapy undoubtedly has a detrimental effect directly on follicles [14], and previous studies have shown that chemotherapy acts on the ovary in a wide variety of ways (Table1).

1. Burnout theory

Chemotherapy-induced POI has been attributed to the loss of PMFs not only through the direct effects of chemotherapeutic agents, but also as a result of an increased rate of folliculogenesis to replace the damaged developing follicles. Meirow's group [17,18] proposed the "burnout theory" in an attempt to describe enhanced follicular demise owing to accelerated folliculogenesis in the ovary after gonadotoxic chemotherapy. In animal studies, increased phosphorylation of proteins through the phosphatidylinositol 3-kinase signaling pathway, which stimulates accelerated PMF activation in both oocytes and granulosa cells, was observed after administration of alkylating agents, resulting in the burnout effect and marked loss of the PMF pool during repeated cycles of anticancer treatment. [17,18] Furthermore, there is an extended version of the burnout theory explained by the reduced secretion of sex steroid hormones and the resulting mechanism of feedback. Gonadotoxic agents have been reported to induce acute loss of the growing follicle population, resulting in decreased secretion of sex steroid hormones and inhibin [19]. Low systemic concentrations of sex steroid hormones and inhibin then result in an inhibition of negative feedback on the pituitary gland and hypothalamus in order to increase gonadotropin secretion, mainly follicle-stimulating hormone (FSH). Increased FSH concentrations then enhance the rate of resting preantral follicle recruitment and maturation and the ability to enter the process of folliculogenesis. Due to the active metabolism of dividing cells during folliculogenesis, these growing follicles are subjected to the gonadotoxic effects of chemotherapy, resulting in an accelerated rate of follicular depletion [19,20].

2. Target of chemotherapy: germ cells or granulosa cells?

It has often been assumed that chemotherapy acts directly on the oocyte within immature follicles, initiating the death of germ cells. However, studies have thus far reported that chemotherapy targets not only oocytes [21-24], but also granulosa cells [25-27], and even ovarian stromal cells and blood vessels [14,28-30]. Oocytes are held in meiotic arrest within immature follicles, although they rapidly grow in developing follicles. Because chemotherapeutic agents are designed to act upon dividing cells, whether mitotically active granulosa cells are the target of chemotherapy drugs remains a subject of

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research. Granulosa cells have been reported to surround the oocyte and proliferate during follicle maturation. Considering the bidirectional interaction between the oocyte and the granulosa cells, with each regulating the growth and maturation of the other [31], damage to granulosa cells may result in indirect damage to the oocyte, which in turn can lead to germ-cell loss [14]. A study of ovarian biopsies from 10 girls who underwent treatment for leukemia found moderate to severe signs of stromal fibrosis and capillary changes [29]. In another human study, injury to blood vessels and focal ovarian cortical fibrosis were reported after exposure to chemotherapy, suggesting that local ischemia may be a potential additional mechanism by which follicles are lost [28].

3. Which stages of follicles are vulnerable to chemotherapy?

Follicles within the ovary are at various stages of maturation. It is possible that specific stages are more susceptible to damage after chemotherapy than others. Because previous studies have mainly examined the effects of different anticancer drugs on PMFs, there are limited data on this question [14]. Cyclophosphamide and its metabolites have reportedly decreased the number of PMFs and small primary follicles in rodent ovaries [32,33]. In humans, ovarian biopsies from patients treated with chemotherapy had significantly lower PMF counts [34]. Nonetheless, several studies have reported that more mature follicles, as well as PMFs, are affected by anticancer drugs [24,25,30,35]. Preantral follicles have been shown to be susceptible to chemotherapy, with deterioration in follicle quality after chemotherapy in humans [35]. In conclusion, there is evidence that chemotherapy causes damage to follicles at all stages, from primordial to antral follicles [14]. However, no experiment has conducted a comparative analysis to determine which follicle stage is more susceptible to anticancer drugs.

4. Different mechanisms of different chemotherapeutic agents

Direct cellular effects on various components of the ovary have been determined for various classes of chemotherapies that differ in their specific cellular targets. Doxorubicin is an anthracycline often used to treat lymphomas, leukemia, breast cancer, and sarcomas. It has been hypothesized to intercalate with DNA and prevent its replication and transcription [22]. Doxorubicin may interfere with the electron transport chain, leading to a release of cytochrome c into the cytosol. This activates the caspase family of proteins, in turn causing apoptosis and even cell death. There is evidence that In the cell nucleus, doxorubicin upregulates the expression of p53, a DNA repair protein that initiates apoptosis in the presence of high levels of DNA damage [36]. Doxorubicin could then affect the ovary by all of these mechanisms, but it primarily affects mitotically and metabolically active cells, as well as granulosa cells, rather than oocytes [37].

Cyclophosphamide is an alkylating agent that results in intra- and inter-strand crosslinking of DNA, which reportedly interferes with cell division [14]. The effects of alkylating agents have been examined in the granulosa cells of rat ovaries. As with doxorubicin, cyclophosphamide has also shown a mitochondrial effect, as it induces a reduction in mitochondrial transmembrane potential and an accumulation of cytochrome c in the cytosol, again leading to the activation of the caspase family and apoptosis [27].

Cisplatin interacts with DNA to form DNA adducts, primarily intra-strand crosslink adducts, which can activate several signal transduction pathways, including those involving ATR, p53, p73, and MAPK, culminating in the activation of apoptosis [38]. Cisplatin administration to neonatal mouse ovaries has been identified to cause an accumulation of Abl, a nonreceptor tyrosine kinase, and TAp63-a, a homologue of p53, which is expressed in the oocyte, leading to oocyte death [24,39]

5. Gonadotoxicity by high gonadotropin concentrations

High gonadotropin concentrations after chemotherapy have also been suggested to affect the resting PMF pool. An *in vivo* study using mice that produced chronically elevated levels of serum LH via expression of an LH β-subunit transgene reported a significant premature loss of their primordial and primary follicle pool 3 months after birth. These transgenic mice had a similar number of follicles as wild-type controls at birth. The finding that chronically elevated LH levels depleted the PMF pool implies that chronic LH elevation might hasten the onset of reproductive senescence [40]. It is believed that PMFs and primary follicles are gonadotropin-independent. However, several investigations have suggested that these follicles express mRNA for FSH and LH receptors [40-49]. These studies have also provided support for the possibility that even immature follicles, such as the primordial and primary follicles, may be gonadotropin-dependent [42-45,49,50]. Moreover, in adult sheep ovaries, FSH was found to modulate ovarian germinative stem cells and their progenitors via FSH-R3, an isoform of the FSH receptor, to undergo potential self-renewal, clonal expansion, and differentiation into oocytes [49].

Potential mechanisms through which GnRHa minimizes the ovarian toxicity of chemotherapy

Although there is still controversy regarding the efficacy of GnRHa on ovarian protection, recent studies have been able to better clarify the efficacy of temporary ovarian suppression with GnRHa during chemotherapy, especially in breast cancer patients (Table 1).

1. Simulating the prepubertal hormonal milieu

The administration of GnRHa has been identified to induce an ini-

tial release of gonadotropins, which desensitize the GnRH receptors on the pituitary gonadotropes, preventing pulsatile GnRH secretion, resulting in a hypogonadotropic, prepubertal hormonal milieu. It has been suggested that in this hypogonadotropic milieu, the follicles remain in the quiescent phase and are thus less vulnerable to chemotherapy-induced gonadotoxicity [51]. However, this mechanism is controversial because of the dogma that primordial and primary follicles are not gonadotropin-dependent. However, the growth of primordial and primary follicles is affected by many factors, such as the transforming growth factor superfamily, the bone morphogenetic proteins (-4, -7, and -9), and activin, which is secreted by gonadotropin-dependent antral follicles in a paracrine way [44,52,53]. These growth factors may also induce the exit of PMFs from the dormant inactive pool [44]. The rate at which PMFs leave the non-active resting pool is influenced by the presence or absence of the more advanced gonadotropin-dependent growing follicles [14]. Moreover, as described above, a few studies support the possibility that even immature follicles such as the primordial and primary follicles may be gonadotropin-dependent [42-45,49,50].

Therefore, GnRHa cotreatment, after the initial flare-up effect, decreases FSH levels through pituitary desensitization, preventing the secretion of growth factors by the more advanced FSH-dependent follicles, and secondarily preserves more PMFs in the dormant stage.

2. Interrupting the burnout effect

GnRHa may minimize POI by interrupting the burnout effect after gonadotoxic agents. Specifically, the administration of GnRHa may interfere with the accelerated follicle recruitment induced by chemotherapy by desensitizing the GnRH receptors in the pituitary gland, preventing an increase in FSH concentration despite low estrogen and inhibin levels [14,41,54]. However, there is no experimental support for this hypothesis; moreover, primordial and primary follicles are generally believed to be gonadotropin-independent, as mentioned above.

3. Decreased utero-ovarian perfusion

Another possible explanation for the beneficial effect of GnRHa cotreatment in reducing chemotherapy-associated gonadotoxicity is decreased utero-ovarian perfusion, which results from the hypoestrogenic milieu generated by pituitary-gonadal desensitization [55,56]. High estrogen levels increased ovarian perfusion in a rat model, and administration of GnRHa significantly inhibited this effect in a dose-dependent manner [55]. The decreased utero-ovarian perfusion in the hypoestrogenic milieu may result in a reduction of the total cumulative exposure of the ovaries to chemotherapeutic agents.

4. A possible direct effect mediated by ovarian GnRH receptors

Human gonads also contain GnRH receptors, similar to the ovaries of rodents, although at a lower concentration [47,48,57-59]. It has been observed that activation of the ovarian GnRH receptor may decrease apoptosis in ovarian cancer cells [57]. In another study, GnRHa slowed doxorubicin-induced granulosa cell damage through a GnRH receptor-mediated mechanism regardless of the hypogonadotropic milieu [60].

5. Possible upregulation of an antiapoptotic molecule

Sphingosine-1-phosphate (S1P) has been identified as a sphingolipid metabolite that inhibits ceramide-promoted cell death induced by radiation and chemotherapy in mice [21,61]. It has been speculated that GnRHa may upregulate ovarian S1P, thus reducing follicular demise [20]. Oocytes lacking the gene for acid sphingomyelinase, which degrades S1P and generates ceramide, resisted apoptosis induced by doxorubicin *in vitro* [61]. It has also been observed that S1P exposure prevented cyclophosphamide- and doxorubicin-induced oocyte death *in vivo* in mice [62]. In a primate study, when S1P or FTY720, an agonistic analog of S1P, was given by direct intraovarian cannulation for a week before ovarian irradiation, rapid resumption of menses and maintenance of ovarian follicles were observed [63]. Nevertheless, there is no experimental evidence regarding whether GnRHa activates the receptors in the ovaries and an intraovarian increase of S1P or other antiapoptotic factors [52].

6. Possible protection of ovarian germinative stem cells

Johnson et al. [12,64] demonstrated that rodent ovaries may have mitotically active germ cells that continuously replicate themselves. This finding contradicted the doctrine of reproductive medicine, whereby mammalian females are born with a fixed, determined, and non-increasing reserve of follicles and lose the capacity for germ-cell renewal during fetal life [12,64,65]. There is ongoing controversy regarding the existence of germinative stem cells and whether or not the ovaries of adult mammals can generate follicles de novo [66].

In patients undergoing chemotherapy, high menopausal FSH levels and undetectable anti-Müllerian hormone (AMH) levels have been observed. Approximately a year after the chemotherapeutic ovarian insult, FSH concentrations have been shown to decrease to normal levels. In addition, AMH has been found to increase in a large number of patients cotreated with GnRHa [66]. Based on these clinical findings, it has been speculated that administration of GnRHa may interact with these germ cells through some pathways essential for the cell growth and for the activation of PMFs after chemotherapy exposure [66].

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Table 1. Mechanisms through which chemotherapeutic agents damage the ovary and GnRHa could minimize ovarian damage

Mechanism through which chemotherapeutic agents damage the ovary		
	Mechanism	
1	Direct action on ovarian cells	Chemotherapy acts directly on various cells of the ovary, including oocytes, granulosa cells, ovarian stromal cells, and blood vessels. Various classes of chemotherapies differ in their specific cellular targets.
2	Burnout effect	Chemotherapy increases the rate of folliculogenesis to replace the damaged developing follicles. Repeated administration of anticancer drugs causes repetitive destruction of developing follicles, resulting in devastation of the primordial follicle pool.
3	Damage to various stages of follicles	Chemotherapy causes damage to follicles at all stages from primordial follicles to antral follicles.
4	Gonadotoxicity by high gonadotropin concentrations	Transgenic mice that produce chronically elevated levels of serum LH via expression of an LH β-subunit transgene reported a significant premature loss of their primordial and primary follicle pool after birth compared with wild-type mice.
Possible mechanisms through which GnRHa could minimize ovarian damage		
	Mechanism	Support	Limitation
1	Simulating the prepubescent hormonal milieu	Prepubescent girls are more resistant to the gonadotoxicity of anticancer drugs than adult women.	The relative resistance may be due to the significantly larger pool of primordial follicles in prepubescent girls.
2	Interrupting the "burnout effect"	GnRHa may interfere with the accelerated follicle recruitment induced by chemotherapy by desensitizing the GnRH receptors in the pituitary.	Primordial and primary follicles are not dependent on gonadotropin.
3	Decreased utero-ovarian perfusion	The decreased utero-ovarian perfusion in the hypoestrogenic milieu may reduce the total cumulative exposure of the ovaries to the chemotherapeutic agents.	In cases of ovarian metastasis, decreased ovarian exposure to chemotherapy may increase the risk of persistent ovarian disease. However, such cases have not been reported.
4	Possible direct effect by ovarian GnRH receptors	*In vitro*, GnRHa slowed doxorubicin-induced granulosa cell damage through a GnRH receptor-mediated mechanism, regardless of the hypogonadotropic milieu.	The exact mechanism by which GnRH receptors could minimize gonadotoxicity is unknown.
5	Possible upregulation of an antiapoptotic molecule	When S1P or an agonistic analog of S1P was given by direct intraovarian cannulation before ovarian irradiation, rapid resumption of menses, and maintenance of ovarian follicles were observed in primates.	There is no experimental evidence on whether GnRHa activates an intraovarian increase of S1P or other antiapoptotic factors.
6	Possible protection of the ovarian GSCs	After the end of chemotherapy, FSH decreases to normal and AMH increases in many patients coadministered GnRHa. Possibly, protected GSCs started growing and producing AMH and sex hormones after chemotherapy.	There is no experimental evidence indicating that GnRHa protects ovarian GSCs.
7	Antiapoptotic effect on cumulus cells	Coadministration of GnRHa inhibited the extrinsic pathway of apoptosis mediated by BCL2-associated X protein in cumulus cells.	There is no *in vivo* evidence supporting the hypothesis.

GnRHa, gonadotrophin-releasing hormone agonist; LH, luteinizing hormone; S1P, sphingosine 1-phosphate; FSH, follicle-stimulating hormone; AMH, anti-Müllerian hormone; GSC, germinative stem cell.

7. Antiapoptotic effects on cumulus cells

In a recent study, the effect of GnRHa and cyclophosphamide in the human oocytes and cumulus cell compartments was evaluated using a culture system of *ex vivo* immature cumulus cell-oocyte complexes [67]. Coadministration of GnRHa inhibited the extrinsic pathway of apoptosis mediated by BCL2-associated X protein in cumulus cells, whereas GnRHa did not directly act on oocytes, which do not express GnRH receptors. The authors suggested that GnRHa acts directly on cumulus cells to protect the oocytes from chemotherapy by an antiapoptotic effect.

Clinical experiences of GnRHa for fertility preservation

1. Recent guidelines for GnRHa to preserve fertility

In 2006, the ASCO and the American Society for Reproductive Medicine published the world's first guidelines on fertility preservation in patients with cancer, which was later updated in 2013 and 2018 [2-4]. The 2006 ASCO guidelines stated that cancer patients do not receive sufficient information about fertility preservation options; furthermore, it considered GnRHa as an experimental method of preserving fertility, stressing that cryopreservation of embryos is the standard fertility preservation therapy in women. In addition, the guidelines mentioned that resumption of menstruation does not always reflect maintenance of fertility, meaning that the presence of regular menstrual cycles after cancer treatment is not tantamount to fertility preservation. The basic stance of the ASCO guidelines was maintained through the 2006 and 2013 revisions.

However, there was a remarkable change in the 2018 ASCO guidelines regarding the role of GnRHa in fertility preservation. The 2018 ASCO guidelines recommended that GnRHa may be offered to patients in the hope of reducing the likelihood of chemotherapy-induced ovarian insufficiency, in case proven fertility preservation methods are not feasible, and in the setting of young women with breast cancer [4]. This change can be interpreted as partially acknowledging the effect of GnRHa on fertility preservation, at least in breast cancer patients.

Most of the guidelines released after 2018 have shown a tendency to follow the ASCO guidelines. In 2020, the Fourth International Consensus Guidelines for Breast Cancer in Young Women by the European School of Oncology and the European Society for Medical Oncology, stated that the concomitant use of GnRHa with adjuvant chemotherapy should be offered to all patients who wish to preserve ovarian function [10]. In this guideline, the limitations of GnRHa were also clearly described, stating that evidence regarding the efficacy of GnRHa for fertility protection remains insufficient and that GnRHa use during chemotherapy does not replace established fertility preservation methods. In 2018, the British Fertility Society recommended considering the possibility that ovarian suppression with GnRHa should be started immediately before and continued during chemotherapy, as it may partially preserve ovarian function in premenopausal women with early breast cancer. Furthermore, the British Fertility Society guideline recommended that GnRHa should also be advised to women with non-breast cancer because it is possible that there is a benefit of using GnRHa when other cancers are treated with gonadotoxic chemotherapy [11].

Nonetheless, other guidelines published just a few months before the 2018 ASCO guidelines remained conservative regarding the use of GnRHa in fertility preservation. The National Comprehensive Cancer Network Adolescent and Young Adult Oncology version 2.2018, which focuses on adolescents and young adults with cancer, did not recommend GnRHa as an option for fertility preservation because of insufficient evidence [68]. FertiPROTEKT, a network founded in 2006 for the German-speaking sphere, also published guidelines on fertility preservation for female cancer patients in 2017, stating that GnRH agonists appear to reduce the risk of premature ovarian failure by up to 50%, but the effect is may not be long-lasting [69].

Currently, perceptions of the role of GnRHa as a fertility preservation treatment are evolving. Attention has been drawn to what stance the upcoming Oncofertility Consortium guidelines and guidelines of the International Society for Fertility Preservation will take on GnRHa [70,71].

2. Recent large-scale RCTs on GnRHa in fertility preservation

To date, more than 17 RCTs have reported on more than 2,100 patients treated with GnRHa along with chemotherapy (Table 2) [8,72-87]. In the early days, the use of GnRHa in preserving fertility showed conflicting results. However, trials since 2013 have reported that the use of GnRHa was relatively helpful for preserving fertility. Crucially, three recent, large prospective RCTs in patients with breast cancer have shown results supporting the use of GnRHa cotreatment for fertility preservation, which influenced the 2018 ASCO guidelines to partially recognize the effectiveness of GnRHa in fertility preservation [4].

The first of these RCTs was the Italian PROMISE-GIM6 study, which attempted to evaluate the incidence of chemotherapy-induced early menopause, long-term ovarian function, pregnancy, and prognosis of breast cancer after GnRHa cotreatment with chemotherapy in patients with stage I to III breast cancer [82]. In total, 281 patients, most of whom were hormone receptor (HR)-positive (n = 226), were enrolled with a median follow-up of 7.3 years (range, 6.3–8.2 years). The 5-year cumulative incidence estimate of menstrual resumption was higher, with borderline significance, in the GnRHa group (hazard ratio, 1.28; 95% confidence interval [CI], 0.98–1.68; $p = 0.07$) and sig-

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Table 2. Randomized controlled trials of gonadotropin-releasing hormone agonists

Study (trial)	Enrolled (control)	Evaluable (control)	Type of GnRHa	Disease	Follow-up (yr)	Primary outcome	No. of pregnancies (%) (GnRHa/control)	*p*-value
Zhong et al. (2019) [87]	98 (47)	96 (45)	G 3.6 mg	Breast	1.25	POF	-	0.002
Zhang et al. (2018) [86]	216 (108)	170 (78)	G 3.75 mg, L 11.25 mg	Breast	4.7	POF	-	NS
Leonard et al. (2017) (OPTION) [85]	227 (121)	202 (107)	G 3.6 mg	Breast	5	POV	9 (9)/6 (6)	0.015
Demeestere et al. (2016) [84]	129 (64)	67 (35)	T 11.25 mg	Lymphoma	5.33	POF	17 (53.1)/14 (42.8)	NS
Moore et al. (2015) (POEMS) [83]	257 (131)	218 (113)	G 3.6 mg	Breast	4.1	POV	22 (21)/12 (11)	0.04
Lambertini et al. (2015) (PROMISE-GIM6) [82]	281 (133)	281 (133)	T 3.75 mg	Breast	7.3	POV	8 (5)/3 (2)	0.006
Karimi-Zarchi et al. (2014) [81]	42 (21)	42 (21)	D 3.75 mg	Breast	0.5	ROM	-	< 0.001
Elgindy et al. (2013) [80]	100 (50)	70 (37)	T 3.75 mg	Breast	1	ROM	1 (4)/1 (4)	NS
Song et al. (2013) [79]	220 (110)	183 (94)	L	Breast	1	POV	-	0.04
Munster et al. (2012) [78]	49 (22)	47 (21)	T	Breast	1.6	POV	0/2 (10)	NS
Gerber et al. (2011) (ZORO) [77]	61 (31)	60 (30)	G 3.6 mg	Breast	4	ROM	1 (3)/1 (3)	NS
Behringer et al. (2010) [76]	23 (12)	20 (10)	G 3.6 mg	Lymphoma	2.1	POF	-	NR
Sverrisdottir et al. (2009) [75]	285	260 (123)	G 3.6 mg	Breast	~3.0	ROM	-	0.006
Badawy et al. (2009) [74]	80 (40)	78 (39)	G 3.6 mg	Breast	0.7	ROM	-	0.001
Gilani et al. (2007) [73]	30 (15)	30 (15)	D3.75 mg	Ovary	0.5	FSH, LH, E_2	-	NR
Giuseppe et al. (2007) [72]	29 (15)	29 (15)	T 3.25 mg, 11.25 mg	Lymphoma	3.59	ROM	0 (14)/2 (15)	NR
Waxman et al. (1987) [8]	18 (10)	18 (10)	B (nasal)	Lymphoma	2	ROM	-	NR

GnRHa, gonadotrophin-releasing hormone agonist; G, goserelin; L, leuprorelin; T, triptorelin; D, Diphereline; B, buserelin; POF, premature ovarian failure; POV, preservation of ovarian function; ROM, resumption of menses; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E_2, estradiol; OPTION, Ovarian Protection Trial In Premenopausal Breast Cancer Patients; POEMS, Prevention of Early Menopause Study; PROMISE-GIM6, Prevention of Menopause Induced by Chemotherapy: A Study in Early Breast Cancer Patients-Gruppo Italiano Mammella 6; ZORO, Zoladex Rescue of Ovarian function; NS, not significant; NR, not reported.

nificantly higher after adjustment for age (hazard ratio, 1.48; 95% CI, 1.12–1.95; $p=0.006$). Eight pregnancies occurred in the GnRHa group and 3 in the control group, which was not a statistically significant difference (hazard ratio, 2.56; 95% CI, 0.68–9.60). Contrary to concerns, the 5-year disease-free survival rate in the GnRHa group was comparable to that of the control group (GnRHa vs. control: hazard ratio, 1.17; 95% CI, 0.72–1.92). A similar result was reported in a subgroup analysis of patients with HR-positive disease.

The Prevention of Early Menopause Study (POEMS)-The Southwest Oncology Group (SWOG)/S0230 study in the United States enrolled only HR-negative breast cancer patients, and demonstrated a statistically significant reduction in ovarian failure rate in the GnRHa arms 24 months after chemotherapy (odds ratio [OR], 0.30; 95% CI, 0.09–0.97; $p=0.04$). Remarkably, the POEMS-SWOG/S0230 study presented pregnancy rates [83]. In the GnRHa arm, 25 patients attempted pregnancy, 22 achieved clinical pregnancy, and 18 babies were born. In the control group, 18 patients attempted pregnancy, 12 achieved clinical pregnancy, and 12 babies were born. Significantly higher live birth and ongoing pregnancy rates were observed in the GnRHa arm (OR, 2.45; 95% CI, 1.09–5.51; $p=0.03$). Although this study included only patients with HR-negative breast cancer, women in the GnRHa group also showed improved disease-free survival (hazard ratio, 0.49; 95% CI, 0.24–0.97; $p=0.04$) and overall survival (hazard ratio, 0.43; 95% CI, 0.18–1.00; $p=0.05$).

Meanwhile, the third large, prospective study, the Anglo Celtic Group OPTION trial, examined the effect of GnRHa administration before and during chemotherapy to 202 stage I to IIIB breast cancer patients, including 95 HR-positive subjects [85]. The primary outcome was amenorrhea between 12 and 24 months after randomization, combined with elevated FSH levels. This RCT found that GnRHa reduced the prevalence of amenorrhea to 22% between 12 and 24 months (vs. 38% in the control group; $p=0.015$) and the prevalence of POI to 18.5% (vs. 34.8% in the control group; $p=0.048$). FSH concentrations were also found to be lower in all women treated with GnRHa at both 12 and 24 months ($p=0.027$ and $p=0.001$, respectively). An assessment of ovarian reserve using AMH showed a marked fall in both groups during treatment to median values of 5% of pretreatment levels in the control group and 7% in the goserelin group, which were not significantly different between groups. In subgroup analysis by age group, GnRHa demonstrated a significantly reduced risk of POI only in women younger than 40 years.

3. Meta-analyses and systematic reviews on GnRHa in fertility preservation

Comparing multiple RCTs through meta-analyses (MAs) or systematic reviews (SRs) will aid in determining directions of future research.

In recent years, several MAs and SRs have been published (Table 3), including the recently released RCTs described above. A number of recent RCTs have reported favorable effects of GnRHa on the prevention of chemotherapy-induced POI. Therefore, recent MAs and SRs, including the Cochrane review released in 2019, have also reported results in favor of adjuvant use of GnRHa with chemotherapy. In this section, we will review the areas that need to be clarified regarding the role of GnRHa in fertility preservation by comparing MAs and SRs published in the last 5 years.

Lack of a standardized definition of POI after chemotherapy

The lack of a standardized definition of chemotherapy-associated POI has been identified in several MAs as a limitation to the interpretation of findings in the literature regarding GnRHa. All MAs covered in this review analyzed the amenorrhea rate or resumption of menses as the primary outcome and the rate of spontaneous pregnancy as the secondary outcome, except for two studies [88,89]. Three studies assessed the results of the primary endpoint without setting a specific time point [90-92], one study analyzed the primary outcome 2 years after completion of treatment [88], and the remaining studies included analyses of the primary outcome at 1 year after the end of chemotherapy [89,93-96]. The rate of amenorrhea 1 year after the end of chemotherapy is a commonly adopted primary endpoint in several RCTs [79,80,82,86,87] because menopause is clinically defined as the absence of menstruation for a year, and resumption of menses is a clinically relevant and reproducible outcome [93].

However, resumption of menses does not necessarily translate into restoration of fertility [93]. Oktay and Turan [97] emphasized that menstruation or amenorrhea should not be considered surrogate markers of GnRHa efficacy in preserving the ovarian reserve. However, owing to the lack of data related to pregnancy, controversy remains as to whether GnRHa administration will really help future pregnancies in premenopausal women receiving chemotherapy. Ten SRs and MAs have been published in the past 5 years, seven of which have reported results on pregnancy outcomes. Five of the seven MAs found significantly higher rates of pregnancy in patients receiving chemotherapy and GnRHa than in those receiving chemotherapy alone [90,91,93-95]. In particular, studies that included only breast cancer patients showed clearer results for efficacy of GnRHa on the spontaneous pregnancy rate after chemotherapy [90,93-95]. However, as Munhoz et al. [94] noted in their MA, these analyses of pregnancy rates are closer to exploratory analyses than to a valid endpoint for a main analysis. The results of pregnancy outcomes in MAs might be affected by several biases. First, although pregnancy may be the most specific indicator of gonadal function, subfertility can be multifactorial, and inability to conceive does not necessarily indicate

Table 3. The past 5 years' meta-analyses and systematic reviews regarding the fertility preservation efficacy of GnRH agonists during chemotherapy

Study	No. of included studies	RCT addressing pregnancy	No. of patients	Disease	OR (95% CI)	*p*-value for POF, POV, ROM	OR (95% CI)	*p*-value for pregnancy
Sofiyeva et al. (2019) [89]	18	5	1,043	Breast, SLE, hematological malignancy	1.38[a] (1.18–1.63)	< 0.0001		-
Chen et al. (2019) [96]	12	7	1,369	Breast, ovary, lymphoma	0.44[a] (0.31–0.61)	< 0.00001	1.59[a] (0.93–2.70)	0.09
Hickman et al. (2018) [88]	10	7	1,051	Breast, ovary, lymphoma	1.83 (1.34–2.49)	NR		-
Senra et al. (2018) [91]	13	9	1,208	Breast, lymphoma	0.6[a] (0.45–0.79)	0.0004	1.43[a] (1.01–2.02)	0.04
Lambertini et al. (2018) [95]	5	3	847	Breast	0.38 (0.26–0.57)	< 0.001	1.83[b] (1.06–3.15)	0.03
Bai et al. (2017) [90]	15	5	1,540	Breast	1.36 (1.19–1.56)	< 0.00001	1.9 (1.06–3.41)	0.03
Munhoz et al. (2016) [94]	7	NR	1,047	Breast	2.41 (1.40–4.15)	0.002	2.41 (1.02–3.36)	0.04
Elgindy et al. (2015) [92]	10	8	427	Breast, ovary, lymphoma	1.12[a] (0.99–1.27)	NS	1.63[a] (0.94–2.82)	NS
Lambertini et al. (2015) [93]	12	5	359	Breast	0.36 (0.23–0.57)	< 0.001	1.83 (1.02–3.28)	0.04

GnRH, gonadotropin-releasing hormone; RCT, randomized controlled trial; OR, odds ratio; CI, confidence interval; POF, premature ovarian failure; POV, preservation of ovarian function; ROM, resumption of menses; SLE, systemic lupus erythematosus; NR, not reported; NS, not significant.
[a]Relative risk; [b]Incidence rate ratio.

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hypogonadism [88]. Second, limited information is available on the number of patients interested in future pregnancies at the time of randomization and on those who attempted to become pregnant [91]. Only one of the RCTs reported the number of women attempting pregnancy and calculated the pregnancy rate for this subgroup [83]. Therefore, most MAs used the number of the entire randomized population as the denominator for comparing pregnancy outcomes, rather than the number of women who actually attempted pregnancy. However, multiple biases are also possible when using the number of patients attempting pregnancy as a denominator for the pregnancy rate. For example, without blinding, intervention assignment can affect the likelihood of attempting pregnancy. In addition, because a significantly lower rate of participants developed POI in the group administered GnRHa, the intervention itself had an effect on the size of the group in which attempting pregnancy was possible. Furthermore, as observed in the POEMS-SWOG/S0230 study, some pregnancies might occur in women who did not attempt pregnancy [83]. Finally, the study period of the RCTs included in the MAs varied. A short median follow-up of the studies might be a possible explanation of the limited number of pregnancies observed, especially in trials including patients with HR-positive disease who received adjuvant endocrine therapy for at least 5 years [78,82,85], thus delaying attempts for pregnancy.

Gonadotoxicity by cancer type and chemotherapy regimen

GnRHa was recently reported to be effective in RCTs including breast cancer patients [74,75,79,81-83,85,87], whereas GnRHa was found to be ineffective in preserving fertility in a large-scale RCT targeting only lymphoma patients [84]. Conflicting results have been reported in MAs. Elgindy et al. [80] observed that GnRHa administration during chemotherapy did not protect the ovaries from gonadal toxicity regardless of cancer type. Senra et al. [91] reported that GnRHa had a significant benefit on the risk of POI in breast cancer patients (relative risk [RR], 0.57; 95% CI, 0.43–0.77), but not in lymphoma patients (RR, 0.70; 95% CI, 0.20–2.47). Meanwhile, Sofiyeva et al. [89] observed a gonadoprotective effect of GnRHa in both hematological malignancies (RR, 1.77; 95% CI, 1.15–2.74) and breast cancer (RR, 1.31; 95% CI, 1.05–1.62) .

In fact, the difference in the efficacy of GnRHa may not be attributable to the type of cancer, but rather to the regimen of chemotherapy used for each cancer type. It is well known that gonadotoxicity varies depending on the type of chemotherapeutic agent and the duration of administration [2]. To overcome this heterogeneity, some early RCTs enrolled only those who received the same anticancer regimen to evaluate the efficacy of GnRHa [74-76]. However, unlike in RCTs, it is difficult to control the different anticancer regimens and different administration periods of the studies included in MAs. Several MAs have reported inconsistent results for risk of POI after administering different regimens and agents. A recent MA found that women exposed to taxanes demonstrated a lower rate of menstrual recovery than those treated with chemotherapy regimens that did not contain taxanes (OR, 0.49; 95% CI, 0.30–0.80; $p = 0.004$) [98]. In another MA that included only early breast cancer patients who were administered GnRHa for the purpose of fertility preservation, whether or not a taxane was included in the anticancer regimen was not an independent variable associated with the risk of developing chemotherapy-induced POI [95]. In the same MA, the duration of chemotherapy (> 4 months) was also not associated with the risk of POI [95].

In another MA including breast cancer and lymphoma patients [91], when all patients were subdivided according to chemotherapy regimen into high toxicity and low to medium toxicity groups, the benefit of GnRHa therapy in preventing POI appeared to be of a greater magnitude among women treated with low- to medium-toxicity chemotherapy (RR, 0.49; 95% CI, 0.29–0.84); this is in comparison to women treated with highly toxic chemotherapy (RR, 0.66; 95% CI, 0.45–0.96). The major mechanism of ovarian protection by GnRHa is believed to be the downregulation of pituitary GnRH receptors with a drastic reduction of serum gonadotropin levels and blocking of follicular recruitment [91,99]. The authors assumed that the reason for a weaker protection of GnRHa against highly toxic chemotherapy was that these agents cause ovarian damage through multiple mechanisms beyond follicle destruction, such as cortical fibrosis, vascular lesions, and accelerated atresia [91,100].

Time to recovery of ovarian function after chemotherapy

To date, there is a paucity of data available on the appropriate follow-up period to observe recovery of ovarian function after anticancer treatment. The difference in follow-up duration between groups [101] and between studies is a another possible source of heterogeneity and bias in the studies selected for MAs, which in turn could impact the chance of POI diagnosis and pregnancy outcomes [93]. Although there are several RCTs with a follow-up duration of 1 year after the end of chemotherapy, recent large-scale RCTs tended to have a follow-up duration of 2 years or more [75,78,82-84]. Recently, as RCTs have included HR-positive breast cancer patients, the study period has also been growing longer. In a trial by Lambertini et al. [82], because patients with HR-positive breast cancer were included, patients were followed up for more than 5 years to avoid endocrine treatment masking resumption of menses (mean follow-up, 7.3

years) . Moreover, an RCT showed different results within 2 years of follow-up compared with follow-up for only 1 year. For example, Sverrisdottir et al. [75] have observed that the proportion of menstruating women in the GnRHa group showed a statistically significant increase between 24 and 36 months, in contrast to all the other groups in which menses were unchanged or decreasing in the study. A short follow-up period may be responsible for discrepancies between studies, leading to premature conclusions [102].

The time at which ovarian function was evaluated has varied widely [88]. Therefore, several MAs have analyzed the outcomes at various time points, except for three studies that did not set a specific time point for assessing outcomes [90-92]. Eight MAs out of the nine studies in listed in Table 3 found a positive impact on preservation of ovarian function with GnRHa treatment compared with chemotherapy alone. Meanwhile, three of the eight MAs reported that the benefits of GnRHa administration may change over time [88,95,96]. Hickman et al. [88] reported that the effect of GnRHa treatment on ovarian protection was not valid after 2 years (OR, 0.53; 95% CI, 0.22–1.30). Chen et al. [96] also reported no difference during a follow-up period longer than 12 months between the GnRHa group and controls (RR, 1.08; 95% CI, 0.95–1.22). Meanwhile, the incidence of menstrual recovery was significantly higher in the GnRHa group during a follow-up period no longer than 12 months, with an overall effect favoring treatment with GnRHa (RR, 1.60; 95% CI, 1.14–2.24; $p=0.006$). However, an MA by Lambertini et al. [95] presented a non-significantly reduced risk of amenorrhea at 1 year after chemotherapy (OR, 0.92; 95% CI, 0.66–1.28; $p=0.623$). However, they found a significantly reduced risk at 2 years (OR, 0.51; 95% CI, 0.31–0.85; $p=0.009$) . Thus far, the duration of benefits from GnRHa cotreatment appears to be unclear and requires further study.

Age of the patient at the time of treatment

Age has been identified as a major determinant of the risk of POI after chemotherapy [98,103]. This age-related difference is most likely a result of the reduction of the PMF pool with aging, resulting in an increase in the risk of developing ovarian failure and infertility in older women after cytotoxic treatment, even at smaller doses [104]. According to recent MAs, this tendency is prominent in patients 40 years and older [89,90,95,98]. Patient age for eligibility varies considerably across trials, and only four RCTs set age limitations for eligibility to 40 years [74,76,80,85]. In the studies by Song et al. [79] and Munster et al. [78], the mean patient age was 41 and 45 years, respectively, which may have contributed to diminished recovery. Future clinical trials verifying the efficacy of GnRHa for ovarian protection should be designed considering the patient's age.

Hormonal receptor status in breast cancer patients

There have been safety concerns regarding the potential negative effect of the concurrent use of tamoxifen with chemotherapy based on preclinical and clinical evidence [93,105-107]. However, GnRHa has been found to have different pharmacodynamic properties from those of tamoxifen. Since the 1990s, there have been randomized studies that investigated the impact of concurrent ovarian function suppression (obtained pharmacologically or with surgery or radiotherapy) with chemotherapy, and these findings did not demonstrate differences in patients' prognoses [108-110]. Moreover, in recently published trials, excellent survival outcomes were reported with the use of GnRHa concomitantly with chemotherapy in patients, including HR-positive patients [82,83,86,111]. In an MA by Lambertini et al. [82], no difference was noted in the prognosis of breast cancer according to HR status when GnRHa was co-administered. An analysis according to HR status showed no significant interaction ($p_{interaction}=0.762$); and the adjusted hazard ratios for disease-free survival were 0.79 (95% CI, 0.24–2.59) and 0.65 (95% CI, 0.39–1.07) in patients with HR-positive and HR-negative disease, respectively. According to these favorable results, the OPTION trial amended its protocol to allow enrollment of women with HR-positive tumors [85]. The original protocol of the OPTION trial restricted enrollment to only those with HR-negative tumors.

It has also been speculated that GnRHa will not have an ovarian-protective effect on HR-positive breast cancers [112]. However, recent trials that included HR-positive breast cancer patients demonstrated favorable outcomes in preventing POI through the concurrent use of GnRHa with chemotherapy [75,79,82,85,86]. In an MA by Senra et al. [91], HR status was determined not to influence the effect of GnRHa on the risk for POI, which was comparable among women with receptor-positive (RR, 0.69; 95% CI, 0.48–1.00) and receptor-negative (RR, 0.62; 95% CI, 0.33–1.14) tumors.

However, opinions still vary among guidelines. Although the St. Gallen International Expert Consensus [113] and the National Comprehensive Cancer Network Guidelines [112] have recommended the use of GnRHa only for patients with HR-negative breast cancer, the Breast Cancer in Young Women-2 Panel concluded that a protective effect was likely in both HR-negative and HR-positive patients, without an increased risk of breast cancer recurrence [114]. Recent findings further support the position that GnRHa has an ovarian protection effect in breast cancer patients regardless of HR status.

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AMH as a surrogate marker for fertility in patients who have undergone chemotherapy

Several studies have reported that AMH was an accurate biomarker for assessing the extent of ovarian damage after chemotherapy [115-120]. Dunlop and Anderson [115] found that pretreatment AMH can predict POI or ongoing ovarian activity after chemotherapy. Despite the fact that the values of AMH fluctuate before 25 years of age in women, there are reports that AMH may be of value for assessing ovarian function in prepubertal girls after cancer treatment [116,117]. Thus, some trials have analyzed AMH as a secondary outcome to examine the ovary-protective effect of GnRHa [72,76,77,80,85].

However, some researchers are skeptical regarding the role of AMH in predicting chemotherapy-associated ovarian dysfunction. It is well known that AMH does not predict spontaneous pregnancy. A recent prospective time-to-pregnancy cohort study including 750 women between the ages of 30 and 44 found no association between AMH levels and rates of spontaneous conception [121]. Similarly, a retrospective study showed that AMH levels did not accurately predict pregnancy in breast cancer patients who underwent chemotherapy [122]. As in the study of Demeestere et al. [84], in which five patients with protocol-defined POI became pregnant during follow-up, low AMH levels do not necessarily indicate infertility. In addition, patients who have undergone ovarian tissue cryopreservation often have nearly undetectable AMH levels, but decent pregnancy rates have been reported [123].

Currently, no MAs have analyzed AMH levels as an outcome. The RCTs by Giuseppe et al. [72], Elgindy et al. [80], and Leonard et al. [85] reported that there was no difference in changes of AMH levels after chemotherapy between the GnRHa group and controls, and Gerber et al. [77] reported incomplete data regarding AMH. Meanwhile, an RCT by Leonard et al. [85] reported that AMH levels before chemotherapy were a predictor of posttreatment amenorrhea ($p < 0.001$). However, after adjustment for age, the effect of pretreatment AMH was no longer significant.

Conclusion

In the early days, the use of GnRHa for preserving fertility showed conflicting results. However, recent trials have reported that the use of GnRHa is helpful in preventing POI following chemotherapy, at least in breast cancer patients, owing to the overall increase in the number of patients enrolled in trials and the longer follow-up periods. In our opinion, all young women (even patients with high-risk leukemia) facing gonadotoxic chemotherapy should be counselled about and offered various options for fertility preservation, including both GnRHa and cryopreservation of embryos, oocytes, and ovarian tissue.

However, it remains unclear to what extent GnRHa coadministration could provide benefits for achieving pregnancy after chemotherapy. In general, attempting pregnancy after cancer treatment must be delayed until chemotherapy is out of the patient's system and the patient is at a low risk of recurrence. Experts suggest that the timing of pregnancy should be individualized [88,124]. In patients with HR-positive breast cancer who wish to become pregnant, it may be more difficult to decide when to attempt pregnancy, because these patients are typically treated with tamoxifen for 5 to 10 years [88,124]. The POSITIVE study is currently underway to determine the safety of an interruption in endocrine therapy to allow childbearing, and the results of this ongoing research have attracted significant attention.

Conflict of interest

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

ORCID

Jae Hoon Lee https://orcid.org/0000-0003-4223-1395
Young Sik Choi https://orcid.org/0000-0002-1157-4822

Author contributions

Conceptualization, data curation, Formal analysis: all authors. Writing–original draft: JHL. Writing–review & editing: YSC.

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Protective effects of curcumin on chromatin quality, sperm parameters, and apoptosis following testicular torsion-detorsion in mice

Abbas Shahedi[1], Ali Reza Talebi[1,2], Aghdas Mirjalili[1], Majid Pourentezari[1]

[1]Department of Biology and Anatomical Sciences, [2]Department of Reproductive Biology, Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

Objective: The chief outcome of testicular torsion in clinical and experimental contexts is testicular ischemia. Curcumin, a compound with anti-inflammatory and antioxidant properties, has fascinated researchers and clinicians for its promise in the treatment of fertility diseases.
Methods: Thirty-five fully grown male mice were randomly classified into five groups: control, sham, testicular torsion, treatment group 1 (testicular torsion+short-term curcumin), and treatment group 2 (testicular torsion+long-term curcumin). Thirty-five days later, spermatozoa from the right cauda epididymis were analyzed with regard to count and motility. Toluidine blue (TB), aniline blue (AB), and chromomycin A3 (CMA3) staining assays were used to evaluate the sperm chromatin integrity. In addition, the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) test was used to assess apoptosis.
Results: Treatment group 1 exhibited a remarkably elevated sperm count compared to the testicular torsion group. Additionally, notably lower sperm motility was found in the testicular torsion group compared to the control, treatment 1, and treatment 2 groups. Staining (CMA3, AB, and TB) and the TUNEL test indicated significantly greater testicular torsion in the torsion group compared to the control group ($p<0.05$). The data also revealed notably lower results of all sperm chromatin assays and lower apoptosis in both treatment groups relative to the testicular torsion group ($p<0.05$). Significantly elevated ($p<0.05$) AB and TB results were noted in treatment group 1 compared to treatment group 2.
Conclusion: Curcumin can compensate for the harmful effects of testicular ischemia and improve sperm chromatin quality in mice.

Keywords: Sperm; Spermatic cord torsion

Introduction

Infertility is a common problem that affects approximately 15% of couples who try to conceive [1]. In more than 50% of couples having difficulty conceiving, the problem is at least partially related to male reproductive issues [1,2]. A number of factors can cause male infertility or subfertility, including testicular torsion [3], which is one of the most prevalent urological issues affecting young men. In 1988, Anderson and Williamson [4] reported the annual incidence of testicular torsion as 1 in 4,000 individuals younger than 25 years old. While this condition can occur at any age, it usually occurs in young males, with a bimodal incidence in the pediatric population: during the first year of life and between the ages of 13 and 16 years [2]. Therefore, a high rate of testicular torsion can be regarded as a crucial contributor to male infertility. Testicular torsion and torsion repair induce an ischemia-reperfusion injury to the testis that can render the testis aspermatogenic [5]. The injuries caused by testicular torsion are severe enough to lead to ipsilateral damage, which results from the significant increase in blood flow after detorsion [6]. A 90% chance of res-

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Corresponding author: **Majid Pourentezari**
Department of Biology and Anatomical Sciences, Shahid Sadoughi University of Medical Sciences, Alem Sq, Yazd 8916978477, Iran
Tel: +98-3538203410 Fax: +98-3538203414 E-mail: m.pourentezari@gmail.com

cuing the testes exists if surgery is performed within the first 6 hours. This chance drops to 0% after 24 hours [7]. The treatment success rate is based on the duration of torsion and on early diagnosis as part of routine management for the preservation of spermatogenesis and fertility [8]. After the torsion event, the blood circulation is severed, which damages the tissue. After detorsion, blood flow is restored, which leads to events such as the production of reactive oxygen species (ROS) and reactive nitrogen species, lipid peroxidation, and the release of proinflammatory cytokines such as interleukin 6 and tumor necrosis factor alpha, resulting in further tissue injury [9]. Rapid reperfusion is vital for ischemic tissue survival and can limit the generation of oxygen-derived free radicals involved in pathophysiological cascades [10].

Since the 1940s, ROS have been presented in the literature as a potential contributor to male infertility [11]. Since then, major advances have been made in our understanding of the effects of ROS on infertility and sperm function. A recent study stated that ROS may be an important contributing factor affecting 30%–80% of infertile men [12]. Sperm cells are well-equipped against ROS attack with a robust defense mechanism involving antioxidants, but lack of balance between the production of ROS and the accessible antioxidant defenses results in oxidative stress [13]. A variety of antioxidants have been examined based on their capacity to either counteract ROS directly or impact the counter-ROS toxicity in the semen of different mammalian species.

Curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione) is a major phytochemical commonly found in turmeric (*Curcuma longa*). In fact, the rhizome of the *C. longa* Linn plant contains curcumin. The role of curcumin as a free radical scavenger has been demonstrated in numerous studies [14,15]. Particularly in the testicular tissue, it can constrain the generation of ROS and nitric oxide in macrophages [16]. The antioxidant mechanism of curcumin comes from its specific conjugated structure, which includes the enol form of a β-diketone and two methoxylated phenols. This structure has the capability to trap free radicals as a chain-breaking antioxidant [17]. Remarkably, curcumin can prevent the generation of ROS both *in vitro* and *in vivo*. The associated improvement of sperm mobility and the decrease in spermatozoa defects have been claimed to potentially be due to the cessation of peroxidative alterations in the sperm and testicular membrane [18,19].

In this regard, several *in vivo* and *in vitro* studies have emphasized the energy-promoting and protective properties of curcumin on male reproductive structures. In contrast, other reports have suggested that curcumin plays adverse roles in cellular signaling linked to spermatogenic processes [20,21]. This study therefore investigated the protective effects of curcumin on sperm count, chromatin condensation, motility, apoptosis, and DNA integrity following testicular torsion-detorsion in mice.

Methods

1. Animals

All animal experiments were approved by the Animal Ethics Committee at Shahid Sadoughi University of Medical Sciences (No. ir.ssumedicine.REC.1394412). Thirty-five adult NMRI mice 8–11 weeks in age and 35–40 g in weight were purchased from the Infertility Clinical Center of Shahid Sadoughi University of Medical Sciences in Yazd, Iran. During the experimental period, the mice were kept under standard laboratory conditions (12 hours:12 hours light-dark cycle, 25°C ± 2°C, and mean relative humidity of 50% ± 5%). They were fed with standard commercial laboratory chow and water. The mice were kept in separate cages for approximately 35 days (roughly equal to 1 period of spermatogenesis) [22].

2. Experimental design

The animals were randomly classified into five groups, each containing seven mice. The groups included (1) a control group, which was composed of mice that underwent no surgical procedure and were used for the determination of basal values of the parameters; (2) a sham group, which contained mice that underwent a sham operation for each surgical procedure, involving a midline scrotal incision and physical manipulation of the testis before placement back into the scrotum; (3) a testicular ischemia group; (4) treatment group 1 (testicular torsion+short-term curcumin), and (5) treatment group 2 (testicular torsion+long-term curcumin) [23].

The animals were first anesthetized via intraperitoneal injection of 100 mg/kg of ketamine and 10 mg/kg of xylazine, and an incision was made in the left inguinal region to access the testis. Then, after incising the scrotum, the left testis was extracted and rotated 720° counterclockwise using a 0-6 suture thread to the scrotal wall. After 2 hours, the testicle was restored, and the surgical site was closed. In the testicular torsion+short-term curcumin group, a single 100 mg/kg dose of curcumin was injected intraperitoneally 30 minutes before reperfusion. In the testicular torsion+long-term curcumin group, curcumin was injected intraperitoneally at a daily dose of 100 mg/kg from 30 minutes before reproduction until day 35. All surgical procedures were performed under sterile conditions. After 35 days, the animals were anesthetized with ketamine and xylazine (150 mg/kg and 10 mg/kg, respectively). One milliliter of prewarmed Ham F10 medium was added to the dissected cauda epididymis, and then delicate tearing was performed to allow the swim-out of spermatozoa into the culture medium. Finally, the dishes were incubated at 37°C and 5% CO_2 for 15 minutes.

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3. Sperm analysis

Sperm parameters, including count (10^6/mL) and motility, were assessed using 200 spermatozoa from each mouse. Sperm count and motility were investigated using a Makler chamber. Motility was stated as the percentages of rapid motility, slow motility, non-progressive motility, total motility, and immotile sperm.

4. Sperm count

The dissected epididymis from each mouse was transferred into 1 mL of Ham F10 medium and cut into small slices to allow swim-out of the spermatozoa into the medium. After 10 minutes of incubation, 10 µL of the sample was used in a Makler chamber, and a light microscope (Olympus, Tokyo, Japan) was used to perform a sperm count based on World Health Organization guidelines. Data were stated as the number of spermatozoa per milliliter [24].

5. Sperm motility

Sperm motility evaluation was implemented in line with the World Health Organization protocol. In brief, 10 µL of the sperm suspension was placed on a microscopic slide and a coverslip. At least 5 microscopic fields were investigated for each animal in order to assess sperm motility in a minimum of 200 spermatozoa [24].

6. Sperm chromatin/DNA evaluation

Standard cytochemical methods, including chromomycin A3 (CMA3), aniline blue (AB), and toluidine blue (TB), were used to assess chromatin condensation and DNA integrity. The dyes and chemicals used were obtained from Sigma-Aldrich (St. Louis, MO, USA). The dyes' effectiveness was carefully examined using standard samples with and without acid denaturation, which were reflected as positive and negative controls, respectively [25].

1) AB staining

AB selectively stains lysine-rich histones. This stain can also indicate anomalies in sperm chromatin condensation, which are germane to the residual histones. For this staining protocol, air-dried smears from the samples of washed semen were placed in 0.2 M phosphate buffer (pH 7.2) containing 3% buffered glutaraldehyde for 30 minutes at room temperature. Each of the smears was stained in 4% acetic acid (pH 3.5) with 5% aqueous AB stain for 7 minutes. Under light microscopic assessment, 200 spermatozoa were meticulously counted in various parts of each slide with ×100 eyepiece magnification [26].

2) TB staining

TB is a metachromatic dye. In sperm, it can be used as an indicator of the nuclear chromatin condensation and the DNA fragmentation quality and quantity by binding to the phosphate groups of DNA strands. In brief, 96% ethanol and acetone (1:1) were used to fix the air-dried sperm smears for 30 minutes at a temperature of 4°C. The slides were then incubated in 0.1 N HCl for 5 minutes at 4°C. Afterwards, the slides were thoroughly washed with distilled water three times for 2 minutes each time. Ultimately, they were stained for 10 minutes at room temperature using 0.05% TB in 50% citrate phosphate. In each sample, a minimum of 200 spermatozoa were counted using light microscopy with ×100 eyepiece magnification [26].

3) CMA3 staining

CMA 3 (Sigma, St. Louis, MO, USA) is a fluorochrome. It is used specifically for the detection of cytosine-rich and guanosine sequences and the evaluation of the degree of protamination of the chromatin in sperm. In this procedure, the sperm cells were fixed at 48°C for 10 minutes in Carnoy solution, which contains a 3 to 1 ratio of methanol:glacial acetic acid. Then, the slides were stained with CMA3 solution (0.25 mg/mL in McIlvaine buffer; 7 mL of 0.1 M citric acid+32.9 mL of 0.2 M $Na_2HPO_4 \cdot 7\ H_2O$, pH 7.0, containing 10 mM $MgCl_2$) for 20 minutes in a dark room. A fluorescence microscope (Olympus BX51) was used to count a minimum of 200 spermatozoa with a filter of 460 nm and ×100 eyepiece magnification [27].

7. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling assay

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) test is based on the detection of single- or double-stranded DNA fragments marked by dUTP due to a reaction catalyzed by the TdT enzyme. The TUNEL test generally consists of the fixation and permeabilization of sperm, the marking of DNA fragments, staining, and the evaluation of the stages of sperm. In the evaluation process, the sperm can be assessed subjectively under light and fluorescence microscopy or by flow cytometry [28]. The TUNEL assay can also be used to identify apoptotic cells in the tissues during histological examinations.

In this study, the TUNEL assay was used to detect DNA fragmentation in the spermatozoa. This was done utilizing an *in situ* cell death detection kit along with tetramethylrhodamine red-labeled dUTP (Roche Diagnostics, Basel, Switzerland). To determine the TUNEL-positive spermatozoa proportion, as previously described, a fluorescence microscope (Olympus BX50) was used for the final assessment. Using the appropriate filter, at least 200 spermatozoa per slide were counted [29].

8. Statistical analysis

The data were stated as mean ± standard deviation. One-way analysis of variance was used to evaluate the significance of the dif-

ferences among the four groups, and the Tukey post hoc test was performed to determine the differences between pairs of groups. Statistical significance was indicated by a two-sided *p*-value <0.05 for the sperm parameters and cytochemical tests.

Results

Table 1 presents the sperm parameters of the five groups. This table demonstrates that for the majority of the sperm parameters, significant differences ($p<0.05$) were present between different groups. Rapid motility and total motility were significantly lower in the testicular torsion group than in the control and sham groups. Significantly greater total motility was also noted in the treatment 1 and treatment 2 groups compared to the testicular torsion group, and significantly greater rapid motility was observed in the treatment 2 group compared to the testicular torsion group.

Table 2 presents the data on sperm chromatin condensation, DNA integrity, and apoptosis. Statistically significant differences ($p<0.05$)

Table 1. Results of sperm parameters analysis by group

Variable	Control	Sham	Testicular torsion	Treatment 1	Treatment 2	*p*-value
Count ($\times 10^6$)	18.33 ± 1.66	16.66 ± 3.07	8.33 ± 1.05	21.66 ± 5.01	15.83 ± 2.71	0.015[d]
Rapid motility (%)	25 ± 2.88	25 ± 3.65	0.83 ± 0.83	7.5 ± 2.14	15 ± 3.65	0.000[a]
						0.002[b]
						0.013[e]
Slow motility (%)	24.16 ± 3	21.66 ± 1.66	5 ± 1.29	15.83 ± 3	20.83 ± 1.53	0.000[a,e]
						0.016[d]
Non-progressive motility (%)	18.33 ± 1.66	16.66 ± 3.07	8.33 ± 1.05	21.66 ± 4.01	50.83 ± 2	0.017[c]
						0.01[d]
						0.002[f]
Immotile sperm (%)	32.5 ± 4.23	36.66 ± 3.57	85.83 ± 2.38	55 ± 8.85	58.33 ± 4.4	0.000[a]
						0.037[b]
						0.013[c]
						0.002[d]
						0.008[e]
Total motility	67.5 ± 4.23	67.5 ± 2.81	14.16 ± 2.38	43.33 ± 8.02	41.66 ± 4.4	0.000[a]
						0.012[b]
						0.007[c]
						0.002[d]
						0.004[e]

Values are presented as mean± standard deviation.
[a]Difference between control and testicular torsion group; [b]Difference between control and treatment 1 group; [c]Difference between control and treatment 2 group; [d]Difference between testicular torsion and treatment 1 group; [e]Difference between testicular torsion and treatment 2 group; [f]Difference between treatment 1 group and treatment 2 group.

Table 2. Results of sperm chromatin/DNA integrity and apoptosis in different groups

Variable	Control	Sham	Testicular torsion	Treatment 1	Treatment 2	*p*-value
CMA3	2.83 ± 0.6	3 ± 0.51	13.66 ± 0.66	7.66 ± 0.49	5.83 ± 0.77	0.000[a,b,d,e]
						0.006[c]
AB	3.16 ± 1.07	3 ± 1.03	36.33 ± 2.75	20.33 ± 2.77	5.16 ± 0.79	0.000[a,b,d,e,f]
TB	3.33 ± 1.08	5.83 ± 1.55	36 ± 3.38	20.83 ± 3.31	8.66 ± 2.38	0.000[a,b,e]
						0.002[d]
						0.017[f]
TUNEL	1.33 ± 0.21	1.5 ± 0.22	22.66 ± 3.09	9.33 ± 0.71	7.33 ± 1.57	0.000[a,d,e]
						0.006[b]

Values are presented as mean± standard deviation.
CMA3, chromomycin A3; AB, aniline blue; TB, toluidine blue; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.
[a]Difference between control and testicular torsion group; [b]Difference between control and treatment 1 group; [c]Difference between control and treatment 2 group; [d]Difference between testicular torsion and treatment 1 group; [e]Difference between testicular torsion and treatment 2 group; [f]Difference between treatment 1 group and treatment 2 group.

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present between different groups are indicated. Sperm chromatin damage and apoptosis were observed to increase after testicular torsion. However, in the curcumin groups (treatment groups 1 and 2), the extent of damage was lower than in the testicular torsion group, with particularly remarkable results in the treatment 2 group.

Discussion

The present study was intended to investigate the effects of curcumin on sperm damage in animals after exposure to testicular torsion. Testicular torsion, which is associated with severe scrotal pain and ischemia, is an emergent pathologic state requiring immediate surgery [30]. A few hours of testicular torsion may harm the testicle and can sometimes necessitate the removal of the impaired testicles [31]. After the surgical procedure, it may not be apparent whether the function of the testes has been entirely preserved. Therefore, finding new strategies and therapeutic applications to serve as adjuvant therapies to surgical detorsion seems necessary [3]. Surgery is aimed to restore blood flow and correct the perfusion of the testis. After surgical detorsion, overproduction of free radicals such as ROS and overexpression of proinflammatory cytokines, such as interleukin 1-beta and tumor necrosis factor alpha, occurs. Proinflammatory cytokines are responsible for the recruitment of neutrophils to the testicular tissue [5]. The disruption of spermatogenesis can be triggered by ischemic reperfusion injury on testicular torsion, as demonstrated by Ikebuaso et al [32]. Those researchers also showed that the short-term suppression of the secretion of testosterone could be permanent. The adverse effects of certain levels of ROS vary across semen samples. Sperm vulnerability, which varies among individuals and is related to the integrity of the sperm chromatin, may explain the occurrence of severe ROS sequelae [33].

Moreover, the effect of testicular torsion on DNA damage via the induction of oxidative stress and the generation of ROS has been demonstrated [34]. According to numerous studies, ischemia-reperfusion of the testis initiates several mechanisms, including the activation of inflammatory mediators such as cytokines, the infiltration of neutrophils, and the production of NO and ROS, damaging the cell membrane and DNA [3,35]. Our study also showed that testicular torsion decreased the parameters and the quality of sperm chromatin compared to the control group.

Sperm DNA integrity is an indispensable factor involved in the success of fertilization as well as in the healthy development of the embryo [36]. The molecular mechanism by which curcumin aids in proper sperm function remains unknown; however, the trapping of free radicals using β-diketone, methoxy, and phenolic functional groups is an antioxidant mechanism by which curcumin likely enhances sperm parameters [36].

Curcumin inhibits the generation of superoxide anions and hydroxyl radicals by preventing the oxidation of Fe^{2+} and Fe^{3+} through the Fenton reaction. However, another reason for the improvement of sperm motility may be the increase in the concentrations of ROS-scavenging molecules as a result of supplementation with curcumin [37]. Curcumin can also have preventive effects on oxidative stress via the suppression of nuclear factor-κB (NF-κB) DNA-binding activity and, by extension, of the expression of genes that require NF-κB for their activation [38]. Some reports in the literature claim that curcumin improves the sperm parameters and DNA integrity [13,18,19]. Our results seem to corroborate this. We found that curcumin had a beneficial effect on sperm count, motility, sperm chromatin condensation, and apoptosis in mice subjected to testicular torsion, especially over an extended period of supplementation. This result supports the potential application of curcumin in boosting sperm quality. Many studies have reported that curcumin decreases malondialdehyde levels and oxidative stress in various tissues [39]. Thus, it can be assumed that curcumin, in its antioxidant capacity, inhibits ROS and induces antioxidant responses in the cell and thereby reduces the adverse effects of testicular torsion and mitigates sperm damage.

An ischemia-reperfusion injury to the testis may be induced by testicular torsion followed by torsion repair, and such an injury can impact the spermatogenic capacity of the testis. Study results have shown that the loss of spermatogenic capacity may be due to germ cell apoptosis induced by oxidative stress [5]. Previous reports have demonstrated the occurrence of damage after testicular torsion repair, most likely due to germ cell apoptosis and severe seminiferous impairment. Our data showed that curcumin could reduce sperm apoptosis in a group of mice affected by testicular torsion. In 2018, Zha et al. [40] claimed that curcumin reduced testicular apoptosis caused by diabetes by "regulating apoptotic proteins and markedly inhibiting oxidative stress levels by downregulating malondialdehyde expression, decreasing NADPH activity, and restoring antioxidant enzymes". Wei et al. [16] conducted a study on the effect of curcumin on ischemia-reperfusion injury in rat testes by rotating the left testicle 720° for 2 hours after opening the scrotum and providing curcumin to the treatment group intravenously. The results showed that testicular torsion-detorsion significantly increased xanthine oxidase activity and malondialdehyde levels. In contrast, the group treated with curcumin exhibited reduced xanthine oxidase activity and malondialdehyde levels [16]. However, in our study, we focused on the parameters and DNA quality of sperm.

This study reported the deleterious effects of testicular torsion on sperm parameters and sperm chromatin quality. Curcumin can not only compensate for the destructive effects of testicular ischemia, but can also improve sperm chromatin quality in mice. In other

words, the administration of curcumin after reperfusion surgery in mice played a protective role in cases of testicular torsion. Moreover, ischemia/reperfusion sperm injury was reduced through the decrease of oxidative stress and reduction in anti-inflammatory agents. Other advantages of curcumin are its inexpensiveness and its availability under typical conditions.

Conflict of interest

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

ORCID

Abbas Shahedi https://orcid.org/0000-0002-9445-3806
Ali Reza Talebi https://orcid.org/0000-0003-4883-3020
Aghdas Mirjalili https://orcid.org/0000-0002-5532-7452
Majid Pourentezari https://orcid.org/0000-0001-7549-4672

Author contributions

Conceptualization: ART, AS, MP. Data curation: AM. Formal analysis: AS, MP, ART. Methodology: all authors. Project administration: ART. Visualization: AS. Writing–original draft: AS, MP, AM. Writing–review & editing: all authors.

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CERM

The effects of sesame oil and different doses of estradiol on testicular structure, sperm parameters, and chromatin integrity in old mice

Masoomeh Mohammadzadeh[1], Majid Pourentezari[2], Hadi Zare-Zardini[3,4,5], Ali Nabi[1], Saeed Ghasemi Esmailabad[1], Ali Khodadadian[6], Ali Reza Talebi[1]

[1]Department of Reproductive Biology, Yazd Reproductive Sciences Institute, Research and Clinical Center for Infertility, [2]Department of Biology and Anatomical Sciences, and [3]Hematology and Oncology Research Center, Shahid Sadoughi University of Medical Sciences, Yazd; [4]Department of Sciences, Farhangian University, Isfahan; [5]Medical Nanotechnology and Tissue Engineering Research Center, Yazd Reproductive Sciences Institute and [6]Department of Medical Genetics, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

Objective: Studies of the effects of estrogens on the male reproductive system have emphasized the role of these hormones in male fertility. Sesame oil has many phytoestrogenic compounds and may improve male fertility. This study investigated the effects of sesame oil and different concentrations of estrogen on sperm parameters and DNA integrity in male mice.

Methods: Twenty old NMRI (The Naval Medical Research Institute) male mice (40 weeks; weight, 30–35 g) were treated with sesame oil or different concentrations of estrogen (estradiol, 1 and 10 μL/kg/ day) or received no treatment (controls). After 35 days, sperm parameters and DNA integrity were assessed and analyzed.

Results: Sperm count, progressive motility, and morphology were decreased in the group that received 10 μL/kg of estradiol. A remarkably lower percentage of DNA fragmentation and protamine deficiency were detected in the group that received 1 μL/kg of estradiol. In the groups that received sesame oil and 1 μL/kg of estradiol, the numbers of spermatogonia and Leydig cells were higher than in controls. The combination of sesame oil and 1 μL/kg of estradiol led to improved sperm parameters and chromatin and testicular structure.

Conclusion: Based on this study, consumption of sesame oil and a low concentration of estradiol may improve testicular function in older mice.

Keywords: Chromatin; DNA fragmentation; Estradiol; Gene expression; Sesame oil; Testis

Introduction

Spermatogenesis is an essential process in the male reproductive system [1]. Associations of sex hormones with spermatogenesis, sperm survival, and DNA fragmentation have been demonstrated in previous research [2,3]. Sesame oil is an antioxidant agent containing large amounts of polyunsaturated fatty acids, lignin, and vitamin E. It has been proven that sesame oil can prevent DNA oxidative damage in an *in vivo* system [4]. The phytoestrogenic properties of sesame oil, similar to those of estradiol, may have additional effects on the improvement of sperm parameters and improve spermatogenesis through increasing epithelial proliferation and tubular thickening

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Corresponding author: **Ali Reza Talebi**
Department of Reproductive Biology, Yazd Reproductive Sciences Institute, Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Bo-Ali Ave, Safayeh, Yazd 8916877391, Iran
Tel: +98-3531834234 Fax: +98-3531834234 E-mail: prof_talebi@hotmail.com

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[5,6]. Aging has significant effects on sperm quality through the reduction of semen quality and an increase in DNA damage [7]. It has been observed that the increase in age has a detrimental effect on sperm DNA [8]. The production of reactive oxygen species (ROS) increases significantly in response to reduced production of steroidogenic enzymes. An increase in ROS also affects testicular morphology and reduces sperm parameters such as motility, concentration, and morphology [9,10]. These events may be associated with decreasing levels of estrogen and antioxidant agents associated with aging [11]. Estrogen affects the proliferation and arrangement of Sertoli cells, and it is also important for regulating the expression of the genes associated with cell adhesion [3,9,12]. The proteins that form adherent junctions include β-catenin and E-cadherin, which modulate intercellular junctions and stimulate hormones [13]. Furthermore, cadherins and catenins have effects on biological processes including intracellular messengers, signal transmission, and gene transcription. These actions may be associated with the regulatory function of steroids in reproductive tissues [14]. In addition, cadherins comprise a family of calcium-dependent glycoproteins that mediate cell-cell adhesion and sperm-oocyte interactions [15]. The presence of β-catenin is important for the adhesion of Sertoli cells, attachment to spermatids, and testicular-brain barrier function [16,17]. Many studies have been performed separately on the effect of sesame oil or estradiol on the testis; however, no study has yet compared the estrogenic effects of sesame or different concentrations of estradiol in old male mice. The aim of this study was to compare the estrogenic effects of sesame oil and different concentrations of estradiol on testicular structure, sperm parameters, chromatin/DNA integrity, and expression of *E-cadherin* and *β-catenin* genes in old mouse testis.

Methods

The study was approved by the Animal Ethics Committee of the Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran (IR.SSU.RSI. REC.1394.5). All protocols were performed according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

1. Sample collection

Twenty old NMRI (The Naval Medical Research Institute) male mice (mean age, 40 weeks; weight, 30–35 g) were purchased from the Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. All animals were kept in optimal housing and feeding conditions with a controlled temperature (22°C ± 2°C) and a 12-hour light/dark cycle. The mice were divided into four groups (n = 5): the E2-1 group (1 μL/kg/day of estradiol was intraperitoneally injected for 35 days), the E2-10 group (10 μL/kg/day of estradiol was intraperitoneally injected for 35 days), the sesame oil group (10 μL/kg/day of sesame oil was intraperitoneally injected for 35 days), and the control group (no treatment was done).

After 35 days (one cycle of spermatogenesis in mice), animals were sacrificed by cervical dislocation. The left cauda epididymis was removed and cut with a pair of syringes to transferred into Ham's F10 medium for the analysis of sperm parameters and DNA integrity. The left testicular tissue samples were used for the analysis of *E-cadherin* and *β-catenin* expression by molecular assays. To evaluate histological changes, the right testes were fixed in 4% paraformaldehyde solution.

2. Sperm parameters

After 30 minutes of incubation, sperm count, motility, and morphology were analyzed. A Makler chamber was used for the sperm count. Sperm motility was categorized as progressive, nonprogressive, and immotile spermatozoa. The percentage of sperm cells with normal morphology in the head, neck/mid-piece, and tail were obtained by Diff-Quik staining using light microscopy (× 1,000 magnification) (Figure 1A) [18].

3. Sperm chromatin integrity

Aniline blue (AB) staining was applied to evaluate sperm chromatin integrity based on the residual histones in the chromatin structure. Briefly, slides were prepared by smearing, air-drying, and fixing a sperm sample. Then, the sample was incubated for 30 minutes in 3% glutaraldehyde in phosphate-buffered saline (PBS) at room temperature. The smears were stained in 5% aqueous AB solution (pH 3.5) for 10 minutes. Afterward, the slides were rinsed and evaluated at × 1,000 magnification. Immature and/or abnormal spermatozoa with additional histones were seen in dark blue and mature nuclei were detected as light blue (Figure 1B) [19].

4. Sperm DNA fragmentation

The percentage of sperm apoptosis was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using a commercially available kit (In Situ Cell Death Detection Kit, fluorescein, Roche, USA). Spermatozoa with normal DNA show the background fluorescent color, while sperm with high DNA fragmentation has many 3-OH ends, resulting in a strong fluorescent color. Firstly, the smears were fixed in methanol solution for 4 minutes. The slides were washed with PBS for 5 minutes three times. Later, they were incubated with blocking solution for 15–20 minutes at 15°C–25°C in a dark room. Samples were incubated with 0.1% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate for 10 minutes on ice.

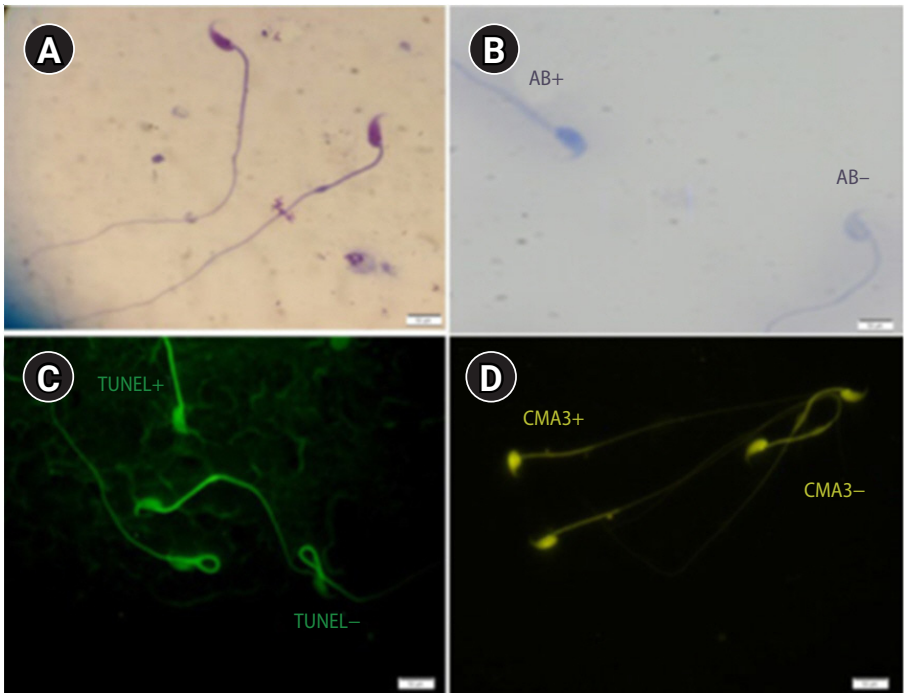

Figure 1. (A) Sperm morphology determined using Diff-Quik staining (×1,000). (B) AB staining assessing sperm chromatin status. Sperm heads with immature nuclear chromatin were shown as dark blue (AB+) and those with mature nuclei (AB–) were detected as light blue (×1,000). (C) TUNEL assay: apoptosis-positive cells are brilliant fluorescent green (TUNEL+) and apoptosis-negative cells are pale and opaque green (TUNEL–) (×1,000). (D) CMA3-positive cells (CMA3+) were seen as bright yellow, whereas cells with no protamine defects stained dark yellow (CMA3–) (×1,000). AB, aniline blue; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; CMA3, chromomycin A3.

Slides were again washed three times with PBS for 5 minutes and were stained with 50 µL of TUNEL reaction mixture for 1 hour at 37°C in a dark and humidified atmosphere. Then, they were examined under a fluorescent microscope at ×1,000 magnification (BX51; Olympus, Tokyo, Japan) (Figure 1C) [20].

5. Sperm protamine deficiency

Protamine deficiency in sperm was analyzed by chromomycin A3 (CMA3), which is bright yellow. The smears were fixed immediately with Carnoy solution for 10 minutes at 4°C. Each slide was treated with 100 µL of CMA3 solution for 10 minutes in a dark room (Sigma-Aldrich, St. Louis, MO, USA). The slides were rinsed in McIrvin buffer and air-dried. The slides were analyzed using fluorescent microscopy with suitable filters (×1,000 magnification) (Figure 1D) [20].

6. Gene expression

The testis tissue of each mouse was used for RNA extraction. Total RNA was extracted by the QuantiTect, RNeasy Micro kit (Qiagen, Hilden, Germany), following a slight modification of the manufacturer's protocol in a total volume of 14 µL. Concentrations of extracted RNA were measured by a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Subsequently, 1,000 ng/µL of extracted total RNA was reverse-transcribed using the Revert Aid First Strand cDNA synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. For negative control samples, the reverse transcriptase enzyme or the RNA template was removed from the reactions. Synthetic cDNA was stored at –80°C until quantitative real-time polymerase chain reaction was performed to assess the relative gene expression levels of the genes encoding *E-cadherin* and *β-catenin* in testis tissue from all groups, and the *β-actin* gene was considered as a reference gene for normalization. Relative expression of the genes was calculated using the QuantiTect SYBER Green RT-PCR kit (Applied Biosystems, Foster City, CA, USA) by an RT-PCR thermocycler (ABI 7500 Step One, Applied Biosystems). Primer sequences for genes are listed in Table 1. Amplification of all runs was performed in duplicate by an expert laboratory assistant blinded to the study design.

7. Testicular histology

Testicular sections were prepared with a 5-µm thickness. Furthermore, hematoxylin and eosin staining was performed to analyze the diameter of seminiferous tubules using an optical microscope (BX51, Olympus). In each section, three fields and at least 20 tubules were

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randomly selected. In this regard, large and small diameters were measured in each tubule and the average diameter was recorded. The thickness of the germinal epithelium layer was also calculated by subtracting the inner diameter of the tubule from the overall diameter of the seminiferous tubule [21]. Sertoli cells, spermatogonia, primary spermatocytes, spermatids, and Leydig cells were counted in each testis.

8. Statistical analysis

One-way analysis of variance was used to compare the data and Pearson correlation coefficients were used to quantify the relationships between the variables, with *p*-values < 0.05 considered to indicate statistical significance. The data were analyzed using IBM SPSS ver. 20 (IBM Corp., Armonk, NY, USA) and GraphPad software (GraphPad Inc., La Jolla, CA, USA) was used to draw the charts.

Results

As shown in Table 2, the percentage of progressive motility was significantly lower in the E2-10 group than in the control group ($p < 0.05$). There was no significant difference in the proportion of nonprogressive sperm across all groups. The percentage of immotile sperm was higher in the E2-10 and sesame oil groups than in the control group ($p < 0.05$). Normal morphology (Figure 1A) was significantly lower in the E2-10 group than in the control group ($p < 0.05$) (Table 2). The rate of abnormal chromatin in AB staining in the E2-10 and sesame oil groups was higher than in the control group and the low-dose estradiol group ($p < 0.001$) (Table 2, Figure 1B). As shown in Table 2, a difference was observed between the E2-10 and control groups in the TUNEL assay ($p < 0.05$) (Figure 1C). The results of CMA3 staining showed higher percentages of abnormal sperm in the E2-10 and sesame oil groups than in the control group ($p < 0.001$) (Table 2, Figure 1D). The expression of *β-catenin* and *E-catenin* was quantified in all groups (Table 1). The relative expression of *β-catenin* mRNA in the E2-1 ($p = 0.002$) and E2-10 ($p = 0.012$) groups was higher than in the control group (Figure 2A), but the relative expression of *E-catenin* mRNA did not significantly differ across all groups (Figure 2B). The number of spermatogonia showed no significant difference in any group compared to the control group (Figure 3A). The E2-10 group displayed higher primary spermatocyte, spermatid cell, and Sertoli cell counts compared to the controls at 35 days ($p < 0.05$) (Figure 3B-D). The Leydig cell count was significantly higher in the E2-1 and E2-10 groups ($p < 0.001$) (Figure 3E). In the E2-10 group (Figure 4A), the lumen diameter was significantly lower than that of the control animals ($p < 0.05$), and the cellular diameters of the seminiferous tubules were also significantly higher in the E2-1 ($p < 0.05$) and E2-10

Table 1. The primers used in real-time PCR

Accession number	Gene	Primer sequence (5'-3')	PCR product (bp)
NM_009864.3	*E-cadherin*	F: AGCCATTGCCAAGTACATCC	133
		R: AAAGACCGGCTGGGTAAACT	
NM_001165902.1	*β-catenin*	F: TCCCATCCACGCAGTTTGAC	166
		R: TCCTCATCGTTTAGCAGTTTTG	
NM_007393.5	*β-actin*	F: GTACTCTGTGTGGATCGGTGG	144
		R: AACGCAGCTCAGTAACAGTCC	

PCR, polymerase chain reaction ; F, forward; R, reverse.

Table 2. Comparison sperm parameters and sperm function tests between control and experimental groups

Variable	Control	E2-1	E2-10	Sesame oil
Sperm count ($\times 10^6$/mL)	27.8 ± 1	10.8 ± 1	2.6 ± 1	21 ± 2
Progressive motility (%)	41 ± 20	54.2 ± 1	11 ± 1	30.2 ± 1
Non-progressive motility (%)	29 ± 9	17 ± 6	15 ± 8	12 ± 5
Immotile (%)	34 ± 14	33.8 ± 12	80 ± 14[b]	62.2 ± 24
Normal morphology (%)	57 ± 3	57 ± 16	30 ± 0[a]	42 ± 6
AB (%)	19 ± 3	33 ± 3[b]	34 ± 5[b]	33 ± 6[b]
TUNEL assay (%)	11 ± 2	10 ± 2	22 ± 13	15 ± 2
CMA3 (%)	20 ± 1	22 ± 3	29 ± 2	28 ± 11

Values are presented as mean±standard deviation. The E2-1, E2-10 and sesame oil group were intraperitoneally injected with 1 μL/kg/day of estradiol, 10 μL/kg/day of estradiol, and 10 μL/kg/day of sesame oil for 35 days, respectively.
AB, aniline blue; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; CMA3, chromomycin A3.
Compared to the control group: [a]$p<0.05$, [b]$p<0.001$.

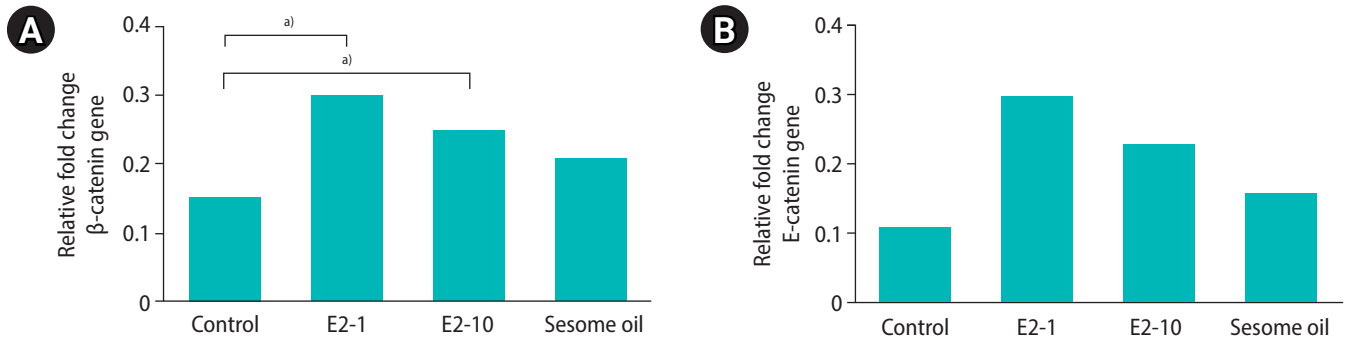

Figure 2. (A) Evaluation of mRNA levels of the *β-catenin* gene. (B) Evaluation of mRNA levels of the *E-cadherin* gene. The E2-1, E2-10 and sesame oil group were intraperitoneally injected with 1 μL/kg/day of estradiol, 10 μL/kg/day of estradiol, and 10 μL/kg/day of sesame oil for 35 days, respectively. [a]Significant mRNA levels of *β-catenin* and *E-cadherin* ($p<0.05$) when compared to the control group.

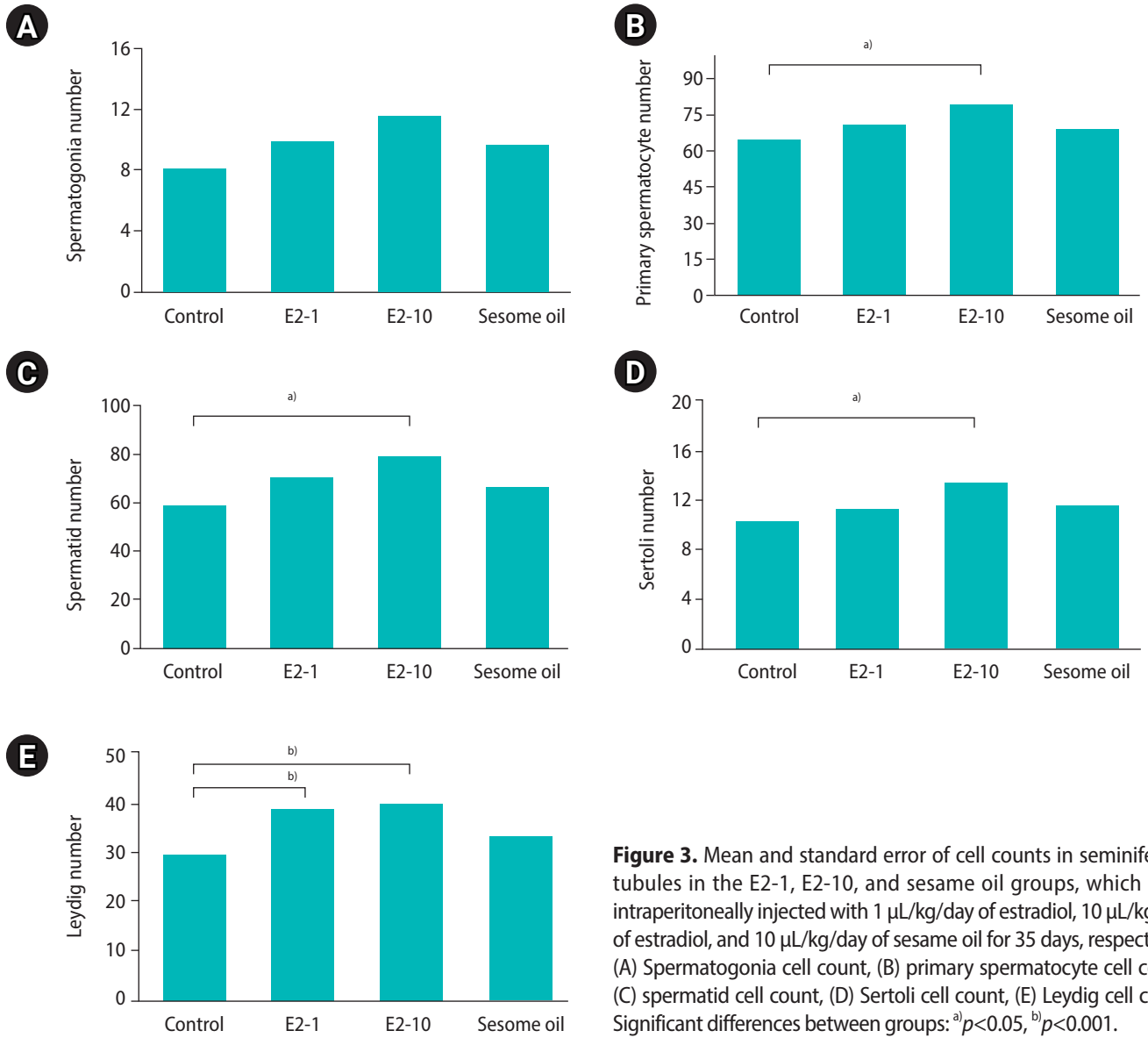

Figure 3. Mean and standard error of cell counts in seminiferous tubules in the E2-1, E2-10, and sesame oil groups, which were intraperitoneally injected with 1 μL/kg/day of estradiol, 10 μL/kg/day of estradiol, and 10 μL/kg/day of sesame oil for 35 days, respectively. (A) Spermatogonia cell count, (B) primary spermatocyte cell count, (C) spermatid cell count, (D) Sertoli cell count, (E) Leydig cell count. Significant differences between groups: [a]$p<0.05$, [b]$p<0.001$.

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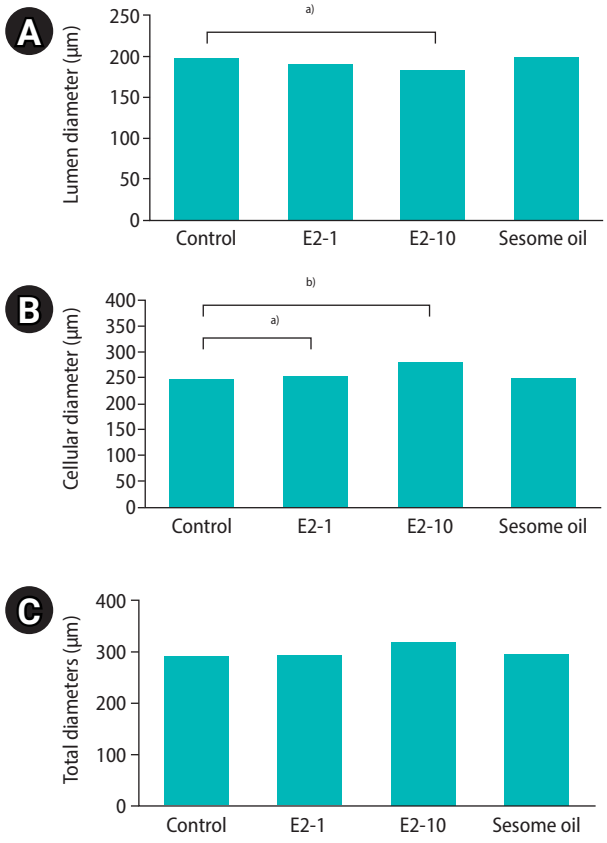

Figure 4. Mean and standard error of stereological indices of seminiferous tubules in the E2-1, E2-10, and sesame oil groups, which were intraperitoneally injected with 1 µL/kg/day of estradiol, 10 µL/kg/day of estradiol, and 10 µL/kg/day of sesame oil for 35 days, respectively. (A) Lumen diameter (µm), (B) cellular diameter (µm), (C) total diameters (µm) of the tubule. Significant differences between groups: [a)] $p<0.05$, [b)] $p<0.001$.

($p < 0.05$) groups (Figure 4B). The total diameter did not significantly vary across all groups (Figure 4C).

Discussion

Spermatogenesis decreases daily with a rate of 30% in men above 50 years old [22-24], and aging affects sperm parameters such as sperm motility and viability, as well as sperm chromatin status. Sesame oil is important source of phytoestrogens and has estrogenic properties [25]; furthermore, it can improve sperm count and motility. Thus, it has been suggested that sesame oil could be considered as an effective agent for improving the condition of epididymal spermatozoa [26]. An appropriate dose of sesame oil for its high antioxidant activity may have effective anti-aging results due to its ability to neutralize physiological ROS. Increased ROS levels in semen cause sperm dysfunction and DNA damage by oxidative stress, accounting for 25% of cases of induced male infertility [27,28]. This study showed that sesame oil was more effective than estradiol at 10 µL/kg in improving sperm parameters such as progressive motility and morphology. The more favorable effects of sesame oil may be due to its antioxidant properties and ability to bind to antioxidant enzymes within the cell [29]. In contrast, Abbasi et al. [30] reported that sesame oil could improve sperm parameters in diabetic rats, potentially due to hormonal imbalance and the presence of abundant ROS in those animals. Lubbert et al. [31] reported that a high dose (60 µg/day) of estrogen and a long duration of treatment could impact sperm motility and count [9]. Our results demonstrated the effects of an injection of 1 µL/kg/day of estradiol on sperm motility. In addition, we observed that a higher concentration of estradiol (10 µL/kg/day of estradiol) had a negative effect on sperm motility, but did increase the sperm count. Lubbert et al. [31] found that although a high dose of estrogen (60 µg/day) reduced the sperm count, low doses (20 µg/day) did not have a negative effect on the sperm count in adult men. The effect of a high dose of estrogen on the sperm count was observed a few days after injection. Steroid hormones play vitally important roles in the maintenance of male reproductive function [32]. and sperm DNA fragmentation and progressive motility are important factors for the evaluation of fertility [33-35]. Based on our data, a low dose of estradiol may lead to improvement in sperm parameters, especially chromatin quality and DNA fragmentation, rather than a high dose of estradiol. In this study, CMA3, TUNEL, and AB assays were used to evaluate chromatin and DNA status. The results of CMA3, TUNEL, and AB tests showed that low concentrations of estradiol and sesame oil were more appropriate than higher concentrations of estradiol. In another study, Ebrahimi et al. [36] detected that sesame oil had anti-apoptotic effects on sperm. Our data showed that 1 µL/kg/day of estradiol led to the most favorable results in the TUNEL assay. The CMA3 assay is a suitable method for detecting protamine deficiency in sperm chromatin [37-40]. Although a few studies have been conducted on the effect of estrogen on the gene expression of Sertoli-spermatid binding proteins [17], to the best of our knowledge, no study has reported the effects of different exogenous estrogen doses on the expression of *E-cadherin* and *β-catenin*. Our study showed that a low concentration of estradiol downregulated the *β-catenin* gene, which plays a role in germ cell-to-Sertoli cell attachment and mediates proteins in cellular connections, but there was no effect on the expression of the gene coding for *E-cadherin*, which also plays an important structural role [41]. A higher dose of estradiol (10 µL/kg/day) improved spermatogenesis in mice. This finding was confirmed by a significantly higher number of spermatogonia cells in the animals that received 10 µL/kg/day of estradiol than was found in the control group. Furthermore, the numbers of primary spermatocytes, spermatids, Sertoli cells, and Leydig cells were significantly higher in the E2-10 group than in the

control group. Toyama et al. [42] studied six different doses of estradiol and they reported that the effects of estradiol on the male reproductive system were dose-dependent [43]. It has also been found that estradiol synthesis by seminiferous cells plays an important role in tubal hormonal regulation and spermatogenesis improvement. and estradiol and platelet-derived growth factor likely induce proliferation of seminiferous cells in both a dose-dependent and dose-insensitive manner [44]. Moreover, incubation of the seminiferous tubules with estradiol inhibits apoptosis and produces germ cells in these tubules. Therefore, estradiol is a very important hormone for the survival of germ cells [45]. In addition, the estradiol beta receptor is present in Sertoli cells and estradiol exerts its effects through this receptor [46]. Our results showed an a higher cellular diameter of seminiferous tubules in mice that received 10 μL/kg/day of estradiol than was found in the control group. Moreover, MacCalman et al. [47] showed that estradiol increased the stimulating effects of follicle-stimulating hormone (FSH) and N-cadherin (a protein that is essential for adhesion and internal cell adhesion in the seminal epithelium) mRNA levels. The interaction between FSH and estradiol in Sertoli cells stimulates the mitotic activity of these cells [48-50]. Therefore, the increase of the cellular diameter was probably due to the interaction of injected estradiol with FSH. Furthermore, changes in tubal diameter could occur due to an increase in the number of germ lineage cells within the seminiferous tubules [51]. According to Shittu et al. [52], sesame oil raises testosterone levels and testosterone increases spermatogenesis in male animals. The aqueous extract of sesame leaves has an antioxidant effect and significantly increases the number of spermatogonia, seminiferous tubules, and testosterone levels. According to other researchers, sesame phytoestrogens bind to testicular estrogen receptors and stimulate spermatogenesis through the proliferation of epithelial cells and sex cells [52,53]. In the sesame oil and high-dose estradiol groups, a higher rate of chromatin and DNA damage was observed than in the control group and the low-dose estradiol group. Low doses of estradiol had a greater effect on sperm motility, in addition to exerting less chromatin and DNA damage. Therefore, it is recommended to use a combination of a low dose of estradiol and sesame oil. This combination may lead to a reduction of age-related ROS by balancing the oxidants and antioxidants in the cell. These are novel findings.

Despite the beneficial effects of high-dose estradiol on testicular function, we recommend that low doses of estradiol or sesame oil may play an important role on optimizing sperm parameters and chromatin quality in older mice.

Conflict of interest

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

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ORCID

Masoomeh Mohammadzadeh	https://orcid.org/0000-0003-2245-4700
Majid Pourentezari	https://orcid.org/0000-0001-7549-4672
Hadi Zare-Zardini	https://orcid.org/0000-0002-1501-2560
Ali Nabi	https://orcid.org/0000-0002-3794-6073
Saeed Ghasemi Esmailabad	https://orcid.org/0000-0002-9652-8699
Ali Khodadadian	https://orcid.org/0000-0001-9192-2016
Ali Reza Talebi	https://orcid.org/0000-0001-7753-0710

Author contributions

Conceptualization: ART, HZZ. Funding acquisition: MM, MP. Methodology: MM, ART. Project administration: AN, SGE. Writing–original draft: ART. Writing–review & editing: AK.

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Effects of different intensities of exercise on folliculogenesis in mice: Which is better?

Fitri Kurnia Rahayu[1], Sri Ratna Dwiningsih[2], Ashon Sa'adi[2], Lilik Herawati[3]

[1]Midwifery Education Program, Faculty of Medicine, Universitas Airlangga, Surabaya; [2]Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Airlangga, Dr. Soetomo Hospital, Surabaya; [3]Department of Physiology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

Objective: Exercise is a risk factor for infertility in women. However, research on the effects of different intensities of exercise on folliculogenesis has not yielded clear results. This study was conducted to analyze the effects of differences in the intensity of exercise on folliculogenesis in mice.
Methods: Nineteen female BALB/c mice (age, 3–4 months; weight, 13–25 g) were randomly divided into four groups: control, mild exercise, moderate exercise, and high-intensity exercise. The mice in the exercise groups engaged in swimming, with additional loads of 3%, 6%, or 9% of body weight, respectively. There were five swimming sessions per week for 4 weeks, with a gradually increasing duration every week. At the end of the treatment, ovarian extraction was carried out and hematoxylin and eosin staining was performed to identify folliculogenesis.
Results: There were significant differences in the number of total follicles between the control and moderate-exercise groups (p=0.036) and between the mild- and moderate-exercise groups (p=0.005). The mean number of primary follicles was higher in the moderate-exercise group than in the mild-exercise group (p=0.006). The mean number of secondary, tertiary, and Graafian follicles did not differ significantly among groups (p≥0.05). However, the number of total follicles and follicles in each phase tended to increase after exercise, especially moderate-intensity exercise.
Conclusion: Exercise of different intensities affected the total number of follicles and primary follicles. The number of follicles of each phase tended to increase after exercise. Moderate-intensity exercise had better effects than other intensities of exercise.

Keywords: Exercise; Health; Mice; Ovarian follicle

Introduction

Over the past few decades, more women have participated in competitive sports and become athletes [1,2]. Exercise has positive effects on the body and improves health in various ways, but it can also increase the risk of infertility in women [3,4]. Different intensities of physical exercise have different effects on the female reproductive system [3]. The female reproductive system is very sensitive to physiological changes, and 6%–79% of women involved in athletic activity experience menstrual disorders such as amenorrhea [1]. Exercise can increase levels of β-endorphins and dopamine, which can interfere with gonadotropin-releasing hormone (GnRH) pulses, thereby inhibiting follicle-stimulating hormone (FSH) production [5,6]. Exercise also triggers oxidative stress, which results in apoptosis in granulosa cells, including ovarian follicles [7,8]. Both of these mechanisms can cause folliculogenesis disorders, with important implications for female reproductive physiological function, which is closely related to folliculogenesis.

Kelley et al. [9] conducted a 3-week exercise study on mares, in-

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Corresponding author: **Lilik Herawati**
Department of Physiology, Faculty of Medicine, Airlangga University, Mayjen Prof. Dr. Moestopo 47, Surabaya, Jawa Timur 60131, Indonesia
Tel: +62-8123181104 Fax: +62-31-5022472 E-mail: lilik_heraw@fk.unair.ac.id

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volving running, to identify changes in the number of follicles based on their diameter, and found no significant differences in the number of large follicles. A similar study was performed by Seyed Saadat et al. [4], who reported a decrease in the number of secondary follicles and Graafian follicles, but no significant differences in primary and primordial follicles in mice forced to swim after being placed in water with temperatures of 10°C and 23°C. The effects of differences in the intensity of exercise on folliculogenesis are unknown. The aim of this study was therefore to analyze the effects of exercise of different intensities on folliculogenesis using a histological technique (hematoxylin and eosin [H&E] staining). Folliculogenesis in this study was observed indirectly, by calculating the total follicle number and the number of each type of follicle. It is hoped that the data obtained from this study could provide insights into the effects of exercise at various intensities on women's reproductive health.

Methods

This animal study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Airlangga, Indonesia (43/EC/KEPK/FKUA/2019), and conduct was given to conduct the study at the Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia. This study was a randomized, cross-sectional study with a post-test–only control group design.

1. Animals and exercise procedure

The research was conducted at the Faculty of Veterinary Medicine, Universitas Airlangga. In this study, 19 female BALB/c (age, 3–4 months; weight, 13–25 g) were housed under standard laboratory condition (12-hour light/dark cycle) with free access to tap water and chow. The treatment flow is summarized in Figure 1. The mice were randomly divided into four groups: control (no exercise), mild exercise (swimming with an additional load of 3% of body weight), moderate exercise (swimming with an additional load of 6% of body weight), high-intensity exercise (swimming with an additional load of 9% of body weight). The intensities of swimming were determined following the workload levels described by Bompa and Carrera [10]. The choice of different intensities based on the percentage load of body weight was based on the findings reported by Gobatto et al. [11], who showed that, in rats subjected to different intensities of swimming, a 6% load corresponded to the maximal lactate steady state, while an 8% load represented higher exercise intensity. This study therefore compared intensities below the anaerobic threshold (3%), around the anaerobic threshold (6%), and above the threshold (9%).

The swimming protocol was adapted from Herawati et al. [12]. We

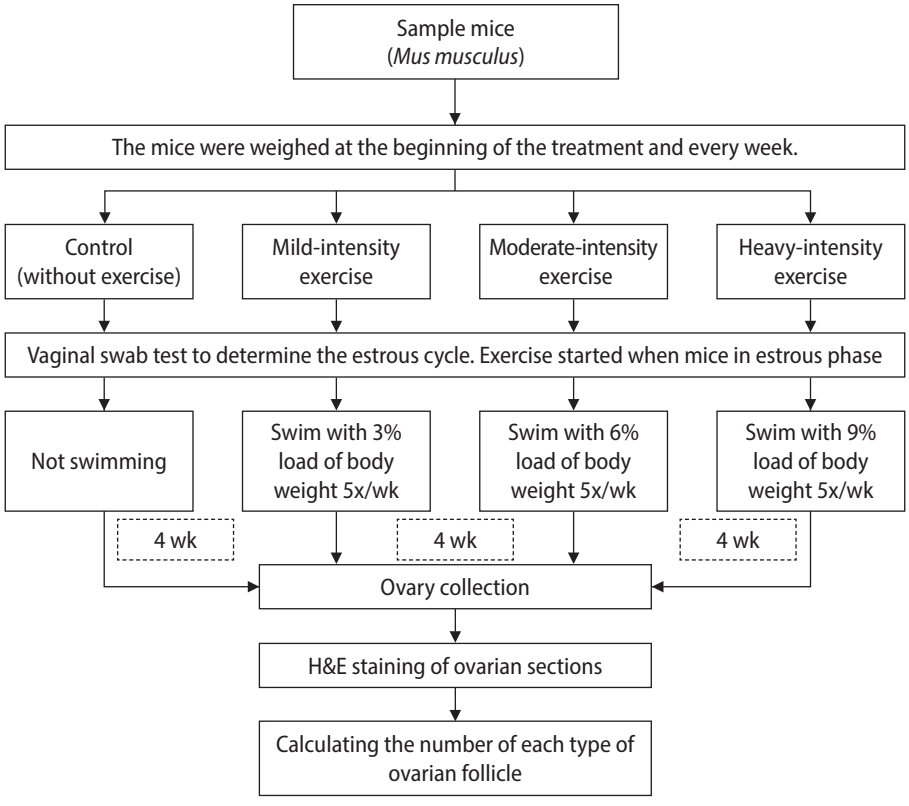

Figure 1. Methodological flowchart of the study.

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used a rectangular polyethylene tank (50 cm long × 30 cm deep × 25 cm wide) with 18-cm-deep water at 20°C ± 5°C. The swimming sessions each lasted for 3 minutes in the first week and increased gradually in length; the sessions in the second week lasted for 5 minutes, those in the third week lasted for 7 minutes, and those in the final week lasted for 9 minutes a day. Gradual improvement is the principle of exercise, and this design also aimed to avoid death in mice [13]. Treatment was given five times per week (Monday to Friday) for 4 weeks [12]. The swimming treatments in all groups were carried out during the day. To ensure that the mice were aligned in terms of the reproduction phase, all mice started the swimming program when they were in the estrus phase, as seen from the results of vaginal swabs.

At the end of the treatment, the next morning, the mice were sacrificed using 70% ether anesthesia and the medial part of the abdomen was surgically removed to retrieve the ovary. The ovaries then were processed for histology preparation with H&E staining. Before H&E staining, the ovaries were embedded in paraffin or wax blocks to enable clean thin-section cutting. A microtome was used to cut the paraffin blocks into approximately 2-μm-thick samples.

2. Folliculogenesis in the ovary

Folliculogenesis was observed with a light microscope with ×100 magnification (Figure 2). Folliculogenesis was quantified by calculating the total number of primary, secondary, tertiary, and Graafian follicles, classified based on their morphology, in five visual fields for each preparation. Follicles were classified as primary if they showed a single layer of cuboidal granulosa cells. Secondary follicles possessed more than one layer of granulosa cells with no visible antrum. Tertiary follicles possessed generally only one or two small areas of follicular fluid (antrum). Graafian follicles had a rim of cumulus cells surrounding the oocyte [14]. The follicle calculations were made by two observers (FKR and BAP) and the data were presented as averages for each group.

3. Statistical analysis

Data were analyzed using IBM SPSS ver. 21.0 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) was used when the data were normally distributed, and the Kruskal-Wallis test when the data were not normally distributed. Once a significant difference was found, it was confirmed using the post-hoc least significance difference or Mann-Whitney test. The significance level used was 5%.

Results

1. Characteristics of subjects

The body weight of the mice increased during treatment in all groups (Table 1). The highest increase was found in the control group

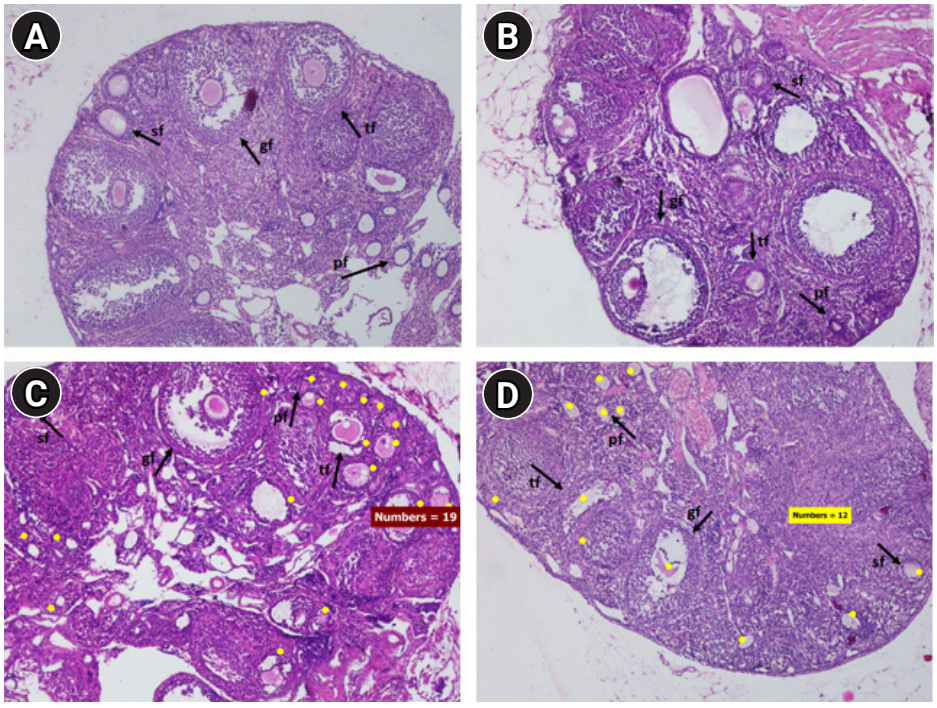

Figure 2. Light photomicrographs of mouse ovaries with H&E staining (×100). (A) Control group, (B) mild-intensity group, (C) moderate-intensity group, (D) high-intensity group. Swimming increased the number of ovarian follicles, especially in the moderate-intensity group. sf, secondary follicle; gf, Graafian follicle; tf, tertiary follicle; Pf, primary follicle. Yellow dot, follicle count.

and the lowest increase was found in the moderate-intensity group. However, according to ANOVA, the body weight increase was not significantly different among the groups ($p=0.784$).

2. Total follicles

There were significant differences in the number of total follicles between the control group and the moderate-exercise group ($p=0.036$) and between the mild-exercise group and the moderate-exercise group ($p=0.005$) (Table 2). Compared to the control group, a higher intensity of exercise corresponded to a higher total number of follicles. However, the peak number of total follicles was in the moderate-exercise group (Figure 1).

3. Primary follicles

The number of primary follicles differed significantly between the mild-exercise group and the moderate-exercise group ($p=0.006$) (Table 2). More intense exercise was associated with a higher number of primary follicles than in the control group. The highest number of primary follicles was in the moderate-exercise group.

4. Secondary follicles

The number of secondary follicles was higher in the groups that received exercise training, although there were no significant differences between the control and treatment groups ($p=0.096$) (Table 2).

5. Tertiary follicles

More tertiary follicles were found in the treatment groups, but the difference compared to the control group was not significant ($p=0.414$) (Table 2).

6. Graafian follicles

The Graafian follicle count was higher in the groups that received exercise training, but there were no significant differences among the groups ($p=0.714$) (Table 2).

Table 1. Body weight of mice before and after treatment

Group	n	Body weight (g)	
		Before treatment	After treatment
Control	4	15.5	22.8
Mild-intensity exercise	4	16.3	22.8
Moderate-intensity exercise	6	18.1	23.7
High-intensity exercise	5	15.0	21.8

No significant difference was found among groups (analysis of variance).

Discussion

In this study, no significant differences among groups were found in the increase in mice body weight before and after treatment. The greatest increase in body weight occurred in the control group, and the lowest increase in the moderate-intensity group. Regular exercise is an important component of supporting the long-term reduction of overweight. Changes in body weight occur as a result of compensatory mechanisms by the body. Increased energy expenditure due to exercise also increases food intake, as mediated by the hormone leptin. Low leptin levels can increase the risk of infertility due to increased body weight [1,15]. Leptin levels tend to be stable when there is a balance between energy expenditure and energy intake.

During exercise, there is a reciprocal relationship between the balance of energy and the hypothalamus-pituitary-gonadal axis, triggering proper development of ovarian follicles [15,16]. The present study showed a tendency for the number of follicles to be higher in the treatment groups than in the control group. This aligns with the findings of Kiranmayee et al. [17] regarding the effects of moderate-intensity exercise on markers of ovarian reserve in women with normal weight. The average antral follicular count was also higher in the exercise groups, although the difference was not significant. The study also revealed that moderate exercise had a positive influence on ovarian reserve profile, as the number of apoptotic follicles was lower in the groups that underwent exercise training. This implies that exercise improves the maturation of follicles, with positive impacts on women's fertility [18].

This study found that moderate-intensity exercise had better effects on the development of ovarian follicles than mild or high-inten-

Table 2. The average number of total follicles and each follicle type

Group	n	Follicle				
		Total	Primary	Secondary	Tertiary	Graafian
Control	4	26±7.9[a]	15.3±6.2	4.3±1.3	4.8±2.1	1.8±0.5
Mild-intensity exercise	4	19±5.5[a]	7.8±4.9	5±1.4	5±3.6	1.3±0.5
Moderate-intensity exercise	6	42.7±16.4[b]	25.8±12.4	8±2.7	7.3±3.1	1.5±1.5
High-intensity exercise	5	29.6±8.3[a,b]	16.6±7	6.4±2.9	5.4±1.1	1.2±0.8

Values are presented as mean±standard deviation. Data were analyzed with analysis of variance and the post-hoc least significant difference test, except for the data on Graafian follicles (Kruskal-Wallis test).
[a,b]Different superscripts showed significant differences ($p<0.05$).

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sity exercise. Generally, exercise may inhibit the reproductive axis through direct or indirect inhibition of GnRH by corticotropin-releasing hormone, β-endorphin, and glucocorticoids, thereby reducing the secretion of luteinizing hormone (LH) and FSH, as well as the hormonal secretion of the gonads, and rendering the target tissues of sex steroids resistant to these hormones [19]. However, some studies have reported that exercise of various intensities had different effects on ovarian follicle. In addition to our study, Kiranmayee et al. [17] conducted a study on 162 women at ages between 19 and 42 and found that moderate-intensity physical activity was associated with higher levels of anti-Müllerian hormone and FSH. Physical activity may influence the neurohumoral modulation of the metabolic pathways involved in energy metabolism and reproduction [20]. Moderate exercise may increase the responsiveness and sensitivity of the follicle to FSH and LH with a concomitant improvement in ovulatory status in young women [21]. Therefore, follicles developed to a greater extent in response to moderate-intensity exercise than in response to mild or high-intensity exercise.

The acute response of cortisol, which is associated with increased adrenocorticotropic hormone concentrations, was greater during high-intensity exercise than during moderate-intensity exercise [22]. Increased cortisol concentrations can suppress the synthesis of steroid hormones and can even impair the preovulatory LH surge, resulting in ovulation failure. Suppression of the LH surge can impair follicle development and estrogen synthesis, as well as increasing the formation of follicular atresia [23]. An increase in the plasma cortisol concentration initiates negative feedback of estrogen, which then suppresses the frequency or amplitude of GnRH, reducing the secretion of gonadotropins, ultimately inhibiting ovulation and triggering the formation of small ovarian cysts [24]. In contrast, low-intensity exercises may lead to a decrease in blood cortisol levels by increasing clearance [25]. Therefore, follicular development is less disturbed by mild-intensity exercise than by high-intensity exercise. However, mild-intensity exercise was still associated with less favorable follicular development than moderate-intensity exercise, which may be because moderate-intensity exercise elicits a better response to FSH and LH [21].

Primary follicles are independent of gonadotropins and therefore do not depend on FSH [26]. Consequently, reduced FSH levels due to physical exercise will not affect primary follicles. In this study, the number of primary follicles was significantly higher in the exercise groups than in the control group. This result aligns with those reported by Kelley et al. [9], who investigated outcomes in mares in response to a 3-week running intervention and showed significant differences in the number of small-diameter (primary) follicles compared to the control group. Environmental factors, including nutrition, also have a strong influence on the development of primary follicles. Primordial and primary follicles require a considerable amount of nutrients to continue development; therefore, the nutrients available affect the development of these follicles [27,28]. It can be assumed that there is a balance between the nutrient intake and the energy expenditure of physical exercise. Good nutrition can increase the number of developing primary follicles.

The folliculogenesis stage, in which primary follicles become secondary follicles, is the most common stage at which follicles undergo apoptosis. This is because these follicles are mainly controlled by intrafollicular factors such as growth factors, cytokines, and gonadal steroids, and have not been influenced by FSH. This stage reflects a transitional state from the gonadotropin-independent phase to the gonadotropin-dependent phase [29]. In this study, fewer secondary than primary follicles were observed, because only high-quality follicles were selected for the subsequent stage. Seyed Saadat et al. [4] investigated the effects of forced swimming training with cold and fresh water for 2 weeks on ovarian and uterine parameters in mice, and found fewer secondary follicles in the exercise group than in control group. In contrast, the present study observed higher numbers of secondary follicles in the exercise groups than in the control groups, which may reflect differences in the increase in reactive oxygen species (ROS) in response in different intensities and durations of exercise. ROS play a regulatory role in oocyte maturation, folliculogenesis, ovarian steroidogenesis, and luteolysis. A balance between ROS and antioxidant enzymes in the ovary can protect oocytes [7,30-32].

Tertiary follicles and Graafian follicles are gonadotropin-dependent stages. Hence, their development depends strongly on the production of gonadal hormones such as FSH. Exercise is known to trigger disordered GnRH pulses that can interfere with FSH levels. Folliculogenesis is not only related to the growth of granulosa cells, but also related to the intracrine, paracrine, and autocrine systems. Tertiary and Graafian follicles have more potent defense mechanisms against oxidative stress than primary and secondary follicles. Therefore, even if FSH deficiency occurs, these follicles can still survive until maturation [30,32,33]. This study revealed that the number of tertiary and Graafian follicles tended to increase, although no significant between-group differences were found. This result does not align with the findings of Seyed Saadat et al. [4], who reported significant differences in tertiary and Graafian follicles in mice in response to different water temperatures when swimming. Swimming in cold water (or other types of cold exposure) in mice can affect the hypothalamic-pituitary-adrenal axis, inhibiting the release of FSH and thereby inducing estrus cycle irregularities and some alterations in the morphology of the ovary [34,35]. As a result, our study, which had a longer duration of swimming (4 weeks) and a longer swimming time per session than Seyed Saadat's study [4], reported a different pat-

tern of ovarian follicles.

Physiologically, the number of follicles decreases from the primary to the Graafian stage, until the follicles become mature, because only follicles that are well developed and highly sensitive to gonadotropins will persist [18,33]. In this study, the decline did not differ significantly among groups, although different patterns were found in each group. The treatment groups showed more favorable development of follicles from the primary to the Graafian stage, with the highest numbers of follicles found in response to moderate-intensity exercise.

Conflict of interest

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

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ORCID

Fitri Kurnia Rahayu https://orcid.org/0000-0002-8213-3012
Sri Ratna Dwiningsih https://orcid.org/0000-0002-3952-2020
Ashon Sa'adi https://orcid.org/0000-0003-2682-3139
Lilik Herawati https://orcid.org/0000-0002-8014-6952

Author contributions

Conceptualization: LH, FKR. Data curation: FKR, LH, SRD. Formal analysis: FKR, LH. Funding acquisition: LH. Methodology: all authors. Project administration: LH, SRD. Visualization: FKR. Writing-original draft: FKR, LH. Writing-review & editing: LH, SRD, AS.

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High mRNA expression of GABA receptors in human sperm with oligoasthenoteratozoospermia and teratozoospermia and its association with sperm parameters and intracytoplasmic sperm injection outcomes

Paweena Kaewman[1,2], Sutisa Nudmamud-Thanoi[1,2], Patcharada Amatyakul[3,4], Samur Thanoi[1,2]

[1]Department of Anatomy and [2]Centre of Excellence in Medical Biotechnology, Faculty of Medical Science, Naresuan University, Phitsanulok; [3]Department of Obstetrics and Gynecology and [4]Naresuan Infertility Centre, Faculty of Medicine, Naresuan University, Phitsanulok, Thailand

Objective: This study investigated the mRNA expression of gamma-aminobutyric acid (GABA) receptors in the sperm of oligoasthenoteratozoospermic (OAT) and teratozoospermic (TER) men compared to normozoospermic (NOR) men, as well as the relationships between GABA receptor expression and sperm parameters, fertilization rate, and embryo quality.
Methods: The mRNA expression of GABA A-α1 and GABA B-R2 receptors in sperm was examined using reverse transcription–polymerase chain reaction in three groups of patients: NOR (n=32), OAT (n=22), and TER (n=45). The fertilization rate and embryo quality were assessed in 35 patients undergoing intracytoplasmic sperm injection (ICSI; 10 NOR, 10 OAT, and 15 TER men).
Results: OAT men had significantly higher mRNA expression of GABA A-α1 and GABA B-R2 receptors in sperm than NOR men; however, the difference between TER and NOR men was not significant. High levels of these receptors were significantly correlated with low sperm concentration, motility, and morphology, as well as the rate of good-quality embryos (GQEs) at the cleavage stage after ICSI. Patients whose female partners had a >50% GQE rate at the cleavage stage had significantly lower levels of GABA A-α1 receptor expression than those whose partners had a ≤50% GQE rate.
Conclusion: Our findings indicate that mRNA levels of GABA receptors in human sperm are correlated with poor sperm quality and associated with embryo development after ICSI treatment. The GABA A-α1 receptor in sperm has a stronger relationship with embryo quality at the cleavage stage than the GABA B-R2 receptor.

Keywords: GABA receptors; Intracytoplasmic sperm injection; Oligospermia; Semen analysis; Spermatozoa; Teratozoospermia

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Corresponding author: **Samur Thanoi**
Department of Anatomy, Faculty of Medical Science, Naresuan University, Phitsanulok 65000, Thailand
Tel: +66-55-964700 Fax: +66-55-964770 E-mail: samurt@nu.ac.th

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Introduction

Gamma-aminobutyric acid (GABA) is known to be a major inhibitory neurotransmitter in the central nervous system. Moreover, GABA has also been reported to play functional roles in peripheral organs such as the testis, ovary, uterus, pancreas, and adrenal glands [1]. It plays a role in the regulation of testicular and sperm function through its receptors (especially GABA A and B receptors). Several subunits of GABA A and B receptors have been detected in sperm

[2-5]. The GABA A receptor alpha 1 (α1) subunit is localized on the head of sperm, as well as the GABA B receptor R2 subunit [6,7]. Although the specific localization of the GABA receptor is not clearly defined, there is evidence that the GABA receptor alpha subunit is localized in the equatorial segment of the human sperm head [8]. Activation of the GABA A receptor, a ligand gated-chloride ion channel receptor, induces hyperpolarization of the cell membrane, whereas the GABA B receptor, a G-protein-coupled receptor, is involved in the opening of calcium and potassium channels [7]. Many studies investigated the role of GABA and its receptors in sperm functions that are important for fertilization. The GABA A and B receptors have been reported to be involved in the modulation of sperm kinetic parameters, including sperm motility [9], the stimulation of sperm capacitation [7,10,11], hyperactivation [9,12-14], and the acrosome reaction [2,7,5-18]; however, their physiological relevance remains elusive. Indeed, both GABA and progesterone can act through GABA receptors to promote those sperm functions by triggering the increase of intracellular calcium and chloride ions, cyclic 3',5' adenosine monophosphate (cAMP), and protein tyrosine phosphorylation in the sperm head [10,17]. Nevertheless, no studies have yet investigated levels of GABA receptors in poor-quality sperm.

Several terms are used to refer to men with poor sperm quality. Oligoasthenoteratozoospermic (OAT) men are defined as men with low levels of three sperm parameters (sperm concentration, motility, and morphology), whereas teratozoospermic (TER) men are defined as those with only low levels of normal sperm morphology. OAT and TER men commonly present to infertility clinics [19]. Intracytoplasmic sperm injection (ICSI) is an assisted reproductive technology for the treatment of infertility, which is defined the inability to conceive after 12 months of regular sexual intercourse without contraception [20]. Currently, up to 50% (reported range, 20%–70%) of the infertile population, corresponding to roughly 50–80 million people worldwide, is affected by male factor infertility, which mostly involves low sperm quality [21,22]. The ICSI procedure not only bypasses the processes of natural sperm selection for fertilization that naturally occur within the female reproductive tract such as capacitation, hyperactivation, and the acrosome reaction, but it also omits the process of sperm-oocyte plasma membrane fusion [23]. Nevertheless, failures of fertilization and embryo development from ICSI are also found, especially in men with poor sperm quality [24]. A major cause of those failures is delayed oocyte activation, which occurs after triggering intracellular calcium release and oscillations of sperm-borne oocyte activating factors (SOAFs) [25]. During ICSI, the timing of disintegration of the sperm plasma membrane and acrosome, which occurs within the oocyte, has been reported to influence the likelihood of successful ICSI outcomes because it controls the release of SOAFs [26-29]. Morozumi et al. [26] showed that removing the sperm plasma membrane and acrosome before ICSI could improve the timing of oocyte activation and the first cleavage division. These findings lead to the hypothesis that the remaining components of the intact sperm plasma membrane and acrosome in the ooplasm following ICSI, including GABA receptors, might have an impact on fertilization and embryo development.

Therefore, the present study aimed to determine the levels of mRNA expression of GABA receptors, including GABA A-α1 and GABA B-R2 receptors, in the sperm of men with poor sperm quality (OAT and TER men) compared to normozoospermic (NOR) men. The fertilization rate and embryo quality were compared between groups in patients undergoing ICSI. Moreover, to provide further support for our hypothesis, we also evaluated the correlations of mRNA expression of these receptors with sperm parameters, fertilization rate, and embryo quality.

Methods

1. Semen samples

Semen samples were provided from 113 volunteer patients at the Naresuan Infertility Center, Faculty of Medicine, Naresuan University, Thailand. The experimental protocols were approved by the Institutional Review Board of Naresuan University (IRB No. 0549/60). A signed consent form was obtained from all volunteers. The semen samples were collected by masturbation, following 3–4 days of sexual abstinence, and allowed to liquefy for 30–60 minutes at room temperature. The semen samples were then assessed for liquefaction, appearance, viscosity, pH, volume, and sperm quality (including sperm concentration, motility, and morphology) following the World Health Organization (2010) guidelines [30]. An overview of the sample composition is shown in Figure 1. Ninety-nine semen samples with normal appearance, liquefaction, and pH values along with a sperm concentration $\geq 5 \times 10^6$/mL were included in this study, while 14 semen samples with a sperm concentration $< 5 \times 10^6$/mL were excluded. The included samples were divided into three groups: NOR (n = 32), OAT (n = 22), and TER (n = 45). The volunteer patients in the NOR group had normal sperm concentrations ($\geq 15 \times 10^6$/mL), progressive motility (PR; ≥ 32%) and morphology (normal form ≥ 4%), while those in the OAT group had abnormal sperm concentrations ($< 15 \times 10^6$/mL), PR (< 32%), and morphology (normal form < 4%). Volunteer patients with normal sperm concentration and PR, but abnormal sperm morphology, were defined as TER men.

2. Semen analysis

A drop (~10 μL) of the semen sample was loaded into a Makler counting chamber. Sperm concentration and motility were recorded

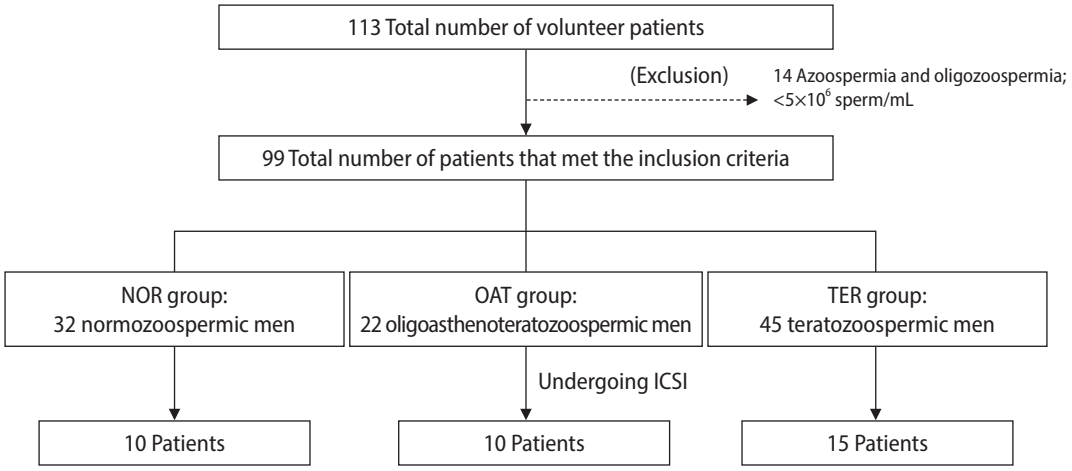

Figure 1. Schematic overview of the sample composition. ICSI, intracytoplasmic sperm injection.

using an Optikam microscope camera and software (OPTIKA Microscopes, Ponteranica, Italy) under a bright-field microscope at ×200 magnification. The average number of sperm per 10 squares was defined as the sperm concentration (×10^6 sperm/mL). The sperm were also graded into PR, non-progressive motility (NP), and immotility. The total of motile sperm was defined as the sum of PR and NP. For sperm morphology analysis, a drop of semen was smeared on a glass slide, allowed to air-dry, fixed, and stained using the Diff-Quick stain. Approximately 200 sperm were examined for sperm morphology under a bright-field microscope at ×1,000 magnification. The percentage of sperm with normal and abnormal morphology was calculated. The label of each slide for the sperm morphology analysis was blinded. For the study of gene expression, all semen samples were stored at –80°C until used.

3. Reverse transcription-polymerase chain reaction analysis

Total RNA was isolated from 5–10 ×10^6 sperm in each sample using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and chloroform. The RNA was converted to complementary DNA (cDNA) using a qScript XLT cDNA Supermix (Quanta Biosciences, Beverly, MA, USA) according to the manufacturer's protocol. The synthesized cDNA was used as a template for reverse transcription-polymerase chain reaction. Oligonucleotide primers for GABA receptors, GABA A-α1 and GABA B-R2 receptors, and GAPDH (reference gene used as an internal control) genes were used following previous studies [31-33], as shown in Table 1. Complementarity with other sequences in the human genome was searched using BLAST on the NCBI website (freely available at https://blast.ncbi.nlm.nih.gov/Blast.cgi). The oligonucleotide primers were synthesized by Macrogen (Macrogen Inc., Seoul, Korea). Each PCR product was amplified from 2 ng of cDNA template using 2×SensiFAST SYBR No-Rox (BIO-98005, Bioline, London, UK) and performed on the LineGene 9600 Plus QPCR system (Bioer, Hangzhou, China). The expression levels of GABA receptor genes were normalized to the GAPDH gene and represented as relative mRNA expression values [34].

4. ICSI procedure, fertilization rate, and embryo quality assessments

In total, 35 volunteer patients received ICSI treatment at the Naresuan Infertility Centre, Faculty of Medicine, Naresuan University, Thailand, including 10 of the 32 patients in the NOR group, 10 of the 22 patients in the OAT group, and 15 of the 45 patients in the TER group (Figure 1). The female partner of each patient undergoing ICSI underwent controlled ovarian stimulation using a standard protocol (a gonadotropin-releasing hormone antagonist protocol), and oocyte retrieval, as previously described [35]. After incubation for 3 hours, oocyte denudation was performed using a hyaluronidase solution (80 IU/mL hyaluronidase in FertiCult Flushing Medium; FertiPro NV, Beernem, Belgium). Sperm were prepared using two-layer density gradient centrifugation, with 45% and 90% of Sil-Select solutions (FertiPro NV), following the standard swim-up method. After ICSI, the fertilization rate and embryo quality were assessed. The fertilization of the injected oocyte was monitored on day 1 (~16–18 hours after ICSI). An embryo that showed two polar bodies (first and second polar bodies) and two pronuclei was defined as a fertilized oocyte. Embryo quality in each stage of embryo development was then observed from day 2 to 5 after ICSI, including the cleavage stage on day 3; the morula stage on day 4; and the blastocyst stage on day 5. The cell number, symmetry, and fragmentation were used to verify the embryo quality at the cleavage stage. A good-quality embryo was defined as having 6–8 cells and less than 10% cytoplasmic fragmentation [36]. At the morula stage, an embryo with evidence of compaction was defined as a good-quality embryo. Blastocyst formation, the inner cell mass (ICM), and the trophectoderm (TE) were

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Table 1. Sequences of oligonucleotide primers for gene expression analysis in human sperm

Gene	Primer sequence (5′-3′)	Annealing temperature (℃)	Product size (bp)	Reference
GABA A-α1	F: AGAAAAACAACACTTACGCTCCA	57	119	[31]
	R: GGGCTTGACCTCTTTAGGTTC			
GABA B-R2	F: GGAAGAGGTCACCATGCAG	66	101	[32]
	R: AGTTTCCCAGGTTGAGGATG			
GAPDH (reference)	F: GCCTCAAGATCATCAGCAATGCCT	63	104	[33]
	R: TGTGGTCATGAGTCCTTCCACGAT			

F, forward; R, reverse.

used to assess embryo quality at the blastocyst stage according to the guideline of Gardner et al. [37]. Blastocyst formation was evaluated using 6 grades based on the degree of expansion and hatching status, as follows: grade 1, early blastocyst; grade 2, blastocyst; grade 3, full blastocyst; grade 4, expanded blastocyst; grade 5, hatching blastocyst; and grade 6, hatched blastocyst. The ICM was categorized as grade A, tightly packed with many cells; grade B, loosely grouped with several cells; and grade C, very few cells. The TE scores were grade A, many cells forming a cohesive epithelium; grade B, few cells forming a loose epithelium; and grade C, very few cells [37]. In this study, the embryo quality at the blastocyst stage was divided into good (3–6 with AA, AB, BA, or BB), moderate (3–6 with BC, CB, or CC), and poor (1–2 regardless of ICM and TE grades and arrested embryos; defective at reaching the blastocyst stage). The fertilization rate and number of embryos of good, moderate, and poor quality in each stage of embryo development were recorded.

To investigate the relationship between the mRNA expression of GABA A-α1 and GABA B-R2 receptors and the embryo quality on cleavage stage, all patients undergoing ICSI were divided into two groups based on the percentage of good-quality embryos at the cleavage stage: good (having a $>50\%$ proportion of good-quality embryos, n = 22) and poor (having a $\leq 50\%$ proportion of good-quality embryos, n = 13).

5. Statistical analysis

The normality of the data distribution was determined using the Shapiro-Wilk test. The statistical significance of differences between the two groups was analyzed using the Student *t*-test (for parametric data) and the Mann-Whitney test (for nonparametric data). The statistical significance of differences among three groups was analyzed using one-way analysis of variance followed by the Dunnett post hoc test (for parametric data) and the Kruskal-Wallis test followed by the Dunn multiple comparison test (for nonparametric data). Moreover, the Pearson correlation coefficient was used to investigate the relationships of the relative mRNA expression of GABA receptors with sperm parameters, fertilization rate, and embryo quality. The chi-square test was used to compare the categorical variables of groups of volunteer patients regarding the rate of fertilization and embryo quality. Statistical significance was considered to be indicated by *p*-values < 0.05.

Table 2. Comparison of semen parameters in each group of volunteer patients

Variable	NOR (n = 32)	OAT (n = 22)	TER (n = 45)
Male age (yr)	36.0 ± 0.8	37.5 ± 1.3	37.7 ± 1.1
Sperm concentration ($\times 10^6$/mL)	104.5 ± 14.0	10.2 ± 0.5[a]	91.7 ± 11.2
Progressive motility (%)	57.8 ± 2.9	20.6 ± 1.9[a]	52.8 ± 1.8
Total motility (%)	72.3 ± 1.9	41.6 ± 3.4[a]	67.2 ± 1.9
Normal morphology (%)	8.6 ± 0.4	1.0 ± 0.2[a]	1.2 ± 0.2[a]
Semen volume (mL)	3.0 ± 0.4	2.9 ± 0.3	2.6 ± 0.2

Values are presented as mean±standard error of the mean.
NOR, normal; OAT, oligoasthenoteratozoospermic; TER, teratozoospermic.
[a]Statistically significant compared to NOR group ($p<0.0001$); Kruskal-Wallis test followed by the Dunn multiple comparison test.

Results

1. Semen analysis

The results of the semen analysis for all parameters are shown in Table 2. There were no significant differences in the age of the male patients or semen volume in the OAT and TER groups compared to the NOR group. The OAT group had significantly lower sperm concentration, PR, total motility, and normal morphology than the NOR group. A significantly lower percentage of sperm with normal sperm morphology was found in the TER group than in the NOR group.

2. The mRNA expression of GABA A-α1 and GABA B-R2 receptors

Significantly higher mRNA expression of the GABA A-α1 and GABA B-R2 receptors was found in the OAT group than in the NOR group, as shown in Figure 2A (GABA A-α1: 1.7 ± 0.2 vs. 1.0 ± 0.1 for OAT vs. NOR) and Figure 2B (GABA B-R2: 2.2 ± 0.3 vs. 1.0 ± 0.2 for OAT vs. NOR); however, the differences between the TER and NOR groups were not significant.

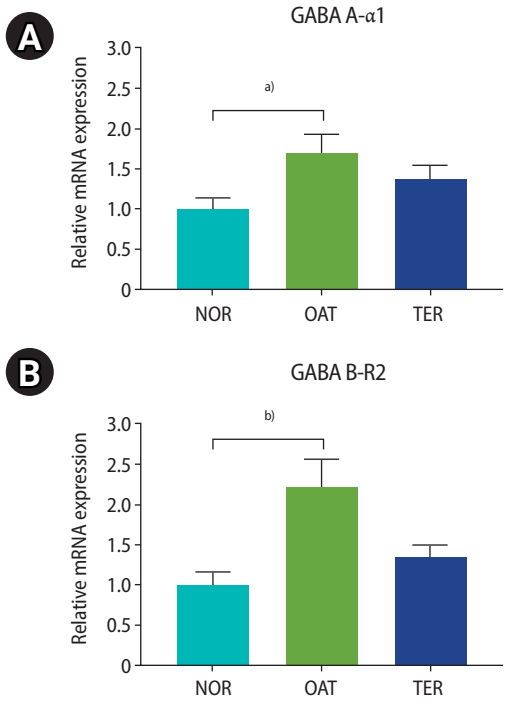

Figure 2. Relative mRNA expression of gamma-aminobutyric acid (GABA) receptors ([A] GABA A-α1, [B] GABA B-R2 receptors) in the oligoasthenoteratozoospermic (OAT) and teratozoospermic (TER) groups compared to normal (NOR) group. Values are presented as mean±standard error of the mean. [a]$p<0.05$, [b]$p<0.01$; the Kruskal-Wallis test followed by Dunn multiple comparison.

3. Fertilization rate and embryo quality after ICSI

As shown in Table 3, a significantly lower proportion of good-quality embryos at the cleavage stage was found in the OAT and TER groups than in the NOR group. There were no significant differences between groups in the age of the female partner, fertilization rate, or embryo quality in the morula and blastocyst stages.

4. Correlations of mRNA expression of GABA A-α1 and GABA B-R2 receptors with sperm parameters, fertilization rate, and embryo quality

High expression of both GABA A-α1 and GABA B-R2 receptors was significantly correlated with low sperm concentration, PR, total motility, and normal morphology (Figure 3). Patients in the good group had significantly lower levels of GABA A-α1 receptor expression than patients in the poor group (1.00±0.16 vs. 2.24±0.40), but a significant difference was not found for GABA B-R2 receptor expression (1.32±0.27 vs. 1.72±0.40), as shown in Figure 4. A strong negative correlation was present between the expression of the GABA A-α1 receptor and the percentage of good-quality embryos at the cleavage stage ($r=-0.464$, $p=0.005$) (Figure 5A); however, a significant correlation was not found for GABA B-R2 receptor expression ($r=-0.227$, $p=0.191$) (Figure 5B). Moreover, the percentage of good-quality embryos at the cleavage stage exhibited a positive correlation with normal sperm morphology ($r=0.468$, $p=0.005$)

Table 3. Comparison of baseline characteristics of the female partners of the volunteer patients undergoing ICSI and clinical outcomes after ICSI including fertilization rate and embryo quality

Variable	NOR	OAT	*p*-value	TER	*p*-value
Number of patients with ICSI	10	10		15	
Female age (yr)	34.3±1.5 (29–40)	36.5±1.3 (32–44)	NS[a]	36.7±1.1 (30–46)	NS[a]
Number of cycles	10	10		15	
Number of retrieved oocytes	98	66		155	
Number of MII oocytes injected	94	63		147	
Fertilization rate	87.2 (82/94)	82.5 (52/63)	NS[b]	84.4 (124/147)	NS[b]
Embryo at cleavage stage			0.01[b]		<0.001[b]
GQE	76.8 (63/82)	55.8 (29/52)		42.9 (63/124)	
PQE	23.2 (19/82)	44.2 (23/52)		57.1 (61/124)	
Embryo at morula stage			NS[b]		NS[b]
GQE	48.8 (40/82)	46.2 (24/52)		40.3 (50/124)	
PQE	51.2 (42/82)	53.8 (28/52)		59.7 (74/124)	
Embryo at blastocyst stage			NS[b]		NS[b]
GQE	22.0 (18/82)	19.2 (10/52)		22.6 (28/124)	
MQE	9.8 (8/82)	5.8 (3/52)		11.3 (14/124)	
PQE	68.3 (56/82)	75.0 (39/52)		66.1 (82/124)	

Values are presented as mean±standard error of the mean (range) or percent (number).
ICSI, intracytoplasmic sperm injection; NOR, normal; OAT, oligoasthenoteratozoospermic; TER, teratozoospermic; NS, not significant; MII, metaphase II; GQE, good-quality embryo; PQE, poor-quality embryo; MQE, moderate-quality embryo.
[a]One-way analysis of variance followed by the Dunnett post hoc test; [b]Chi-square test.

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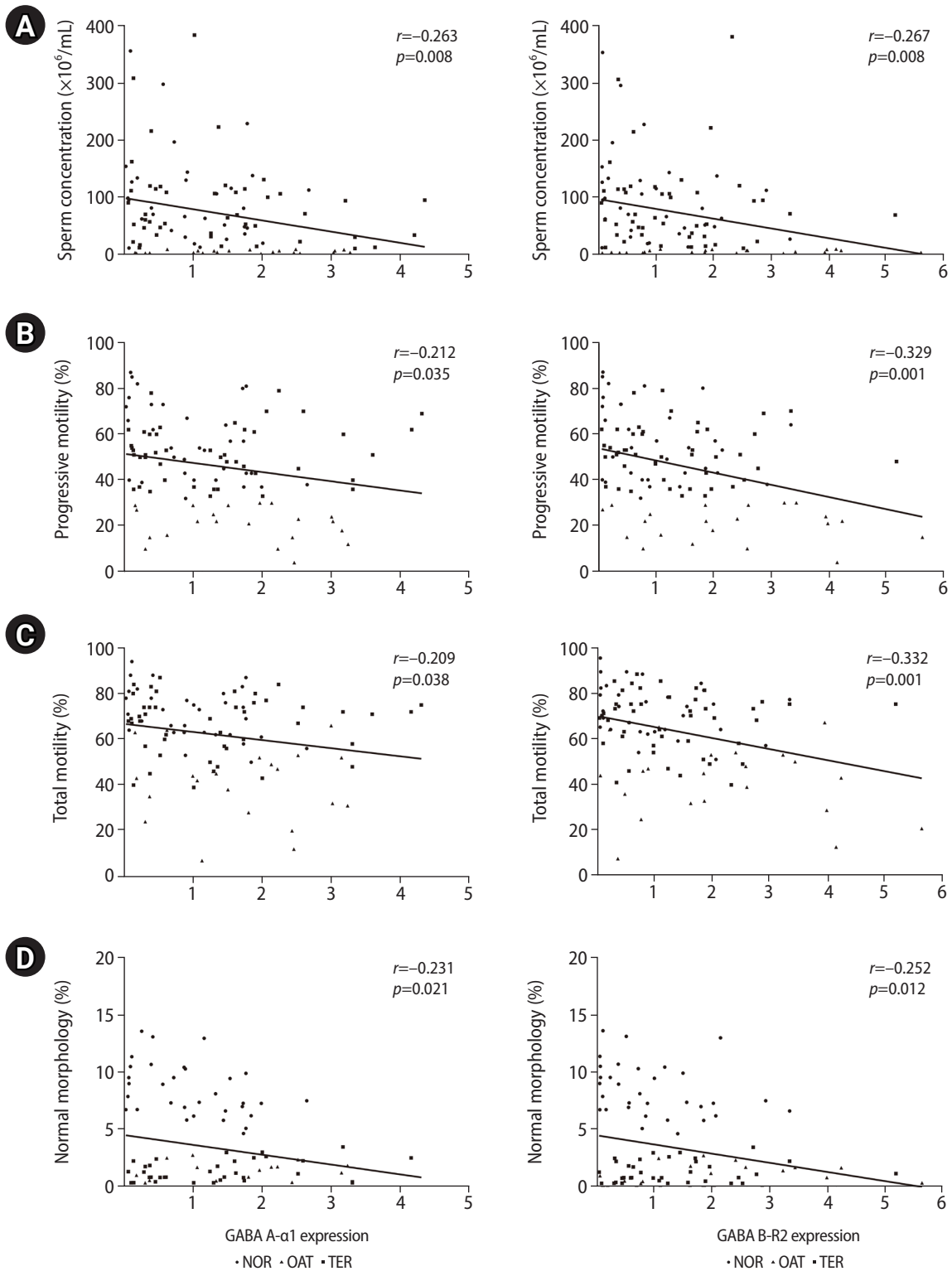

Figure 3. Correlations of mRNA expression of GABA A-α1 (left) and GABA B-R2 (right) receptors with sperm parameters: (A) sperm concentration, (B) progressive motility, (C) total motility, and (D) normal morphology. All data points in the normal (NOR; circles), oligoasthenoteratozoospermic (OAT; triangles) and teratozoospermic (TER; squares) groups are shown. Linear regression line (black line) fitted to all data points. GABA, gamma-aminobutyric acid.

(Figure 6). There were no significant correlations between the expression of GABA receptors and other outcomes, including fertilization rate and embryo quality at the morula and blastocyst stages (data not shown).

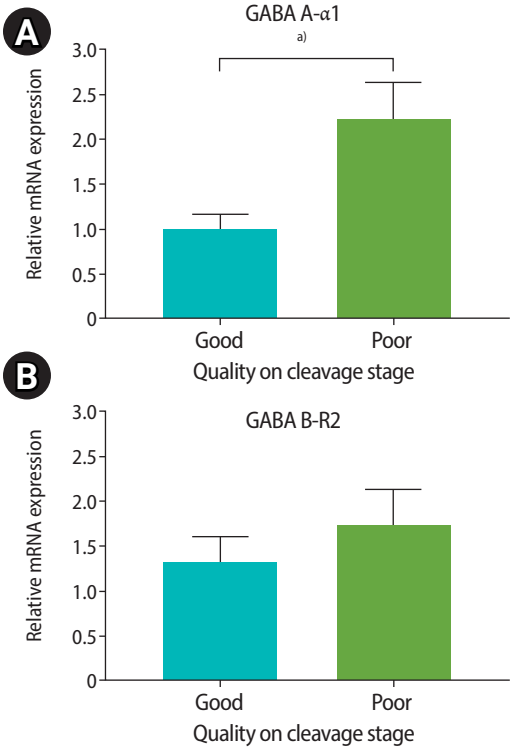

Figure 4. The relative mRNA expression of gamma-aminobutyric acid (GABA) receptors ([A] GABA A-α1, [B] GABA B-R2 receptors) in patients who had the female partner with a good (>50% GQE) and poor (≤50% GQE) proportion of embryos at the cleavage stage. Values are presented as mean±standard error of the mean. GQE, good-quality embryo. [a] $p<0.01$, Student *t*-test.

Discussion

The present study demonstrated high mRNA expression of GABA receptors in the sperm of OAT and TER patients. This result is consistent with our previous study in an animal model of poor sperm quality (in methamphetamine-administered rats), which found significantly higher GABA concentrations as and mRNA expression of the GABA A-α1 receptor and a GABA-synthesizing enzyme in the testis of methamphetamine–administered rats [38]. Additionally, significantly higher GABA A-α1 receptor expression in epididymal sperm was also found in those rats (unpublished data). These results indicate that the expression of GABA receptors changes both in the testis and sperm under conditions of poor sperm quality. GABAergic function in the testis has been reported, including the stimulation of spermatogenesis, Leydig cell proliferation, and testosterone production [39,40]. Therefore, it should be noted that the increase of GABA receptors is expected to occur earlier in the testis to compensate for sperm impairment and to maintain the homeostasis of testicular

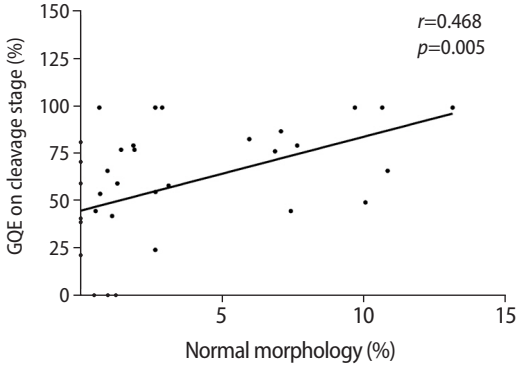

Figure 6. Correlation between the percentage of good-quality embryos (GQEs) at the cleavage stage and normal sperm morphology.

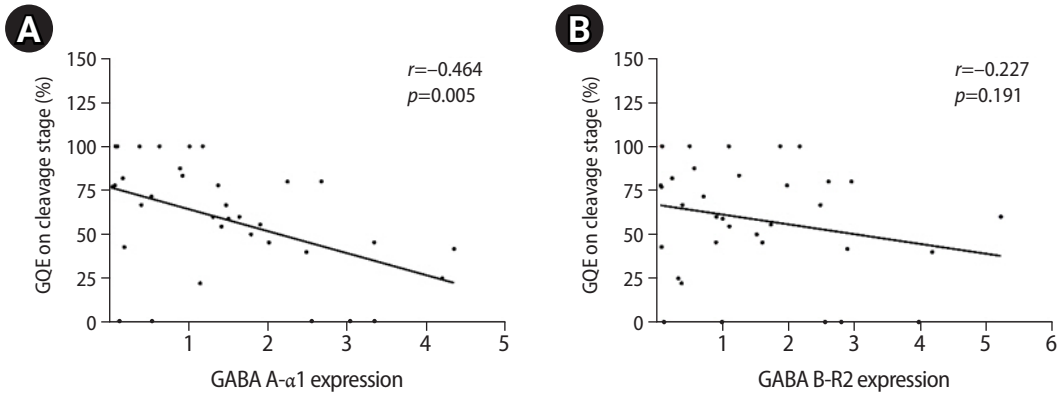

Figure 5. Correlations of mRNA expression of (A) GABA A-α1 and (B) GABA B-R2 receptors with the percentage of good-quality embryos (GQEs) at the cleavage stage. GABA, gamma-aminobutyric acid.

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function; moreover, those receptors remain expressed in epididymal sperm after spermiation. Similarly, changes in GABA receptors were also found in the ejaculated sperm of OAT and TER men. As mentioned above, significantly higher mRNA expression of GABA receptors was found only in OAT men, but not in TER men. Moreover, significant correlations between high levels of GABA receptor expression and low sperm parameters were observed. These results demonstrate that the mRNA levels of GABA receptors depend on the severity of sperm impairment. However, the results of this study only focused on alterations of the transcription process in GABA receptors as measured by mRNA expression, not on the protein expression of GABA receptors in human sperm; the need to clarify the translation process remains a limitation.

Our results for ICSI outcomes are consistent with those of previous studies. Loutradi et al. [41] and Li et al. [42] showed that OAT and TER patients had significantly lower embryo quality at the cleavage stage after ICSI than NOR patients, but no significant difference was found in the fertilization rate. Moreover, Loutradi et al. [41] revealed that there was no significant difference in the embryo quality at the blastocyst stage of OAT patients undergoing ICSI compared to NOR patients. The present study confirmed that the impairment of embryo development in OAT and TER men undergoing ICSI occurs at the cleavage stage. The positive correlation between the percentage of good-quality embryos at the cleavage stage and normal sperm morphology indicates that abnormal sperm morphology might be a major cause of embryo developmental impairment in these patients. There is evidence that OAT and TER men had a high level of sperm DNA fragmentation and chromatin condensation abnormalities [43]. Moreover, several studies have suggested that sperm from those men isolated by density gradient centrifugation still have higher sperm DNA fragmentation and aneuploidy levels than sperm from NOR men, with implications for embryonic cleavage after ICSI [44-48]. A review study on the effects of sperm DNA fragmentation on ICSI outcomes found that sperm DNA fragmentation had adverse effects on the pregnancy rate and the timing of blastocyst-stage embryo development, but not on cleavage-stage embryos; however, those relationships were not found in some studies [49,50].

Interestingly, this is the first report on the correlation between GABA receptor expression in sperm and ICSI outcomes. In this study, we showed that significantly higher levels of GABA A-α1 receptor mRNA expression in sperm were found in patients whose female partner had a low percentage of good-quality embryos at the cleavage stage after ICSI. These findings support the hypothesis that the GABA receptors, especially the GABA A-α1 receptor, in human sperm are involved in embryonic cleavage after ICSI. Although sperm immobilization is performed immediately before ICSI to induce sperm plasma membrane disruption, the sperm plasma membrane and acrosome remain intact for several hours in the oocyte [51]. As mentioned earlier, during ICSI, the intracellular calcium level in the ooplasm increases in response to triggering of SOAFs from the acrosome after the disintegration of the sperm plasma membrane. Interestingly, it is important for oocyte activation, which has an impact on fertilization and embryo development (from early embryonic development to the blastocyst stage) [23,52]. Taken together, the levels of remaining GABA receptors on the intact sperm head may be associated with delayed oocyte activation because it is known that the activation of GABA receptors results in an increase of intracellular calcium levels in sperm. Using sperm with high levels of GABA receptors in ICSI might disturb the pattern of intracellular calcium oscillations within the ooplasm and cause delays in oocyte activation and cleavage division of the embryo. However, the functional role of GABA receptors in those processes remains unknown.

In summary, the findings of the present study suggest that the mRNA levels of GABA receptors, GABA A-α1 and GABA B-R2 receptors, in sperm can be used as biomarkers to predict male infertility. The finding of high-level mRNA expression of these receptors in sperm reflects poor sperm quality. Moreover, our results demonstrate that the mRNA levels of GABA receptors in human sperm, especially the GABA A-α1 receptor, are associated with embryo quality at the cleavage stage after ICSI.

Conflict of interest

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

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ORCID

Paweena Kaewman https://orcid.org/0000-0001-7431-4422
Sutisa Nudmamud-Thanoi https://orcid.org/0000-0001-9356-1162
Patcharada Amatyakul https://orcid.org/0000-0003-1523-1584
Samur Thanoi https://orcid.org/0000-0002-6227-7721

Author contributions

Conceptualization: ST, SNT. Data curation & Formal analysis: PK, SNT, ST. Methodology: all authors. Project administration: ST, SNT. Visualization: PK, SNT, ST. Writing–original draft: PK. Writing–review &

editing: SNT, PA, ST.

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Semen parameters on the intracytoplasmic sperm injection day: Predictive values and cutoff thresholds of success

Alaa El din-Abdel Aal Moubasher[1], Emad Abdelrehim Taha[1], Ehab Mohamed Elnashar[2], Ahmed Abdel Aal Abdel Maged[1], Asmaa Mohamed Zahran[3], Heba Hassan Sayed[1], Hisham Diab Gaber[1]

Departments of [1]Dermatology, Venereology and Andrology and [2]Obstetrics and Gynecology, Faculty of Medicine, Assiut University, Assiut; [3]Department of Clinical Pathology, South Egypt Cancer Institute, Assiut University, Assiut, Egypt

Objective: This study was conducted to investigate the relationship of semen parameters in samples used for intracytoplasmic sperm injection (ICSI) with fertilization and pregnancy rates in infertile couples.
Methods: In this prospective study of Infertile couples with male factor infertility that had undergone ICSI, fractions of the same semen samples obtained for microinjection (to ensure the best predictability) were evaluated to determine the semen parameters and sperm DNA fragmentation index (DFI) on the day of oocyte recovery.
Results: In total, 120 couples completed the study and were subdivided into fertilized (n=87) and non-fertilized couples (n=33). The fertilized couples were further classified into pregnant (n=48) and non-pregnant (n=39) couples. Compared to non-fertilized and non-pregnant couples, fertilized and pregnant couples showed statistically significantly higher sperm viability and percentage of normal sperm morphology, as well as significantly lower sperm DFI values. A receiver operating characteristic curve analysis of data from the 120 ICSI cycles showed that sperm viability, normal sperm morphology percentages, and sperm DFI were significant prognostic indicators of fertilization at cutoff values of 40%, 7%, and 46%, respectively. A sperm DFI of 46% showed sensitivity and specificity of 95% and 90%, respectively, for predicting fertilization, and no clinical pregnancies occurred in couples with a sperm DFI above 46%.
Conclusion: Semen parameters from the ICSI day sample, especially sperm viability, normal morphology, and DFI, had an impact on fertilization and pregnancy outcomes in ICSI cycles.

Keywords: Intracytoplasmic sperm injection; Fertilization; Pregnancy rate; Semen; Sperm DNA fragmentation index

Introduction

The introduction of intracytoplasmic sperm injection (ICSI) has been heralded as one of the major breakthroughs in the field of reproductive medicine. Since the first live births with ICSI were reported in 1992, this technique has been deployed as a powerful tool to treat almost all forms of male infertility, as well as to overcome fertilization failure. ICSI, in conjunction with *in vitro* fertilization (IVF), has been integral in millions of advanced reproductive treatments, resulting in the birth of over 5 million babies so far [1].

Following several refinements, ICSI has become a powerful tool for overcoming suboptimal semen parameters and fertilization defects, thereby allowing infertile men to reproduce at rates that previously would have been impossible [2]. Since ICSI bypasses many steps of the normal fertilization process, conventional sperm parameters such as morphology, motility, and viability have received little attention regarding their relationships with ICSI outcomes, with some

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Corresponding author: **Hisham D Gaber**
Department of Dermatology, Venereology and Andrology, Faculty of Medicine, Assiut University, Assiut 17515, Egypt
Tel: +20-88-2340680 Fax: +20-88-2332278 E-mail: hishamdiab@aun.edu.eg

conflicts and no consensus on cutoff values predictive of successful ICSI outcomes [3].

More importantly, sperm DNA parameters are recognized as objective, independent measures of sperm quality that may have better diagnostic and prognostic capabilities than standard sperm parameters, especially with assisted reproductive techniques [4]. Although research into the importance of sperm DNA fragmentation as a predictive factor for ICSI outcome has been conducted for more than 10 years, there is lack of consensus among studies about its precise role in predicting ICSI outcomes in terms of fertilization, embryo development, pregnancy, miscarriage, and live birth [5]. These inconsistencies may be attributed to study design, the use of different assays of sperm DNA fragmentation, and a lack of standardization.

Another important limitation is that the great majority of these studies, with few exceptions, obtained semen samples before assigning the studied men to ICSI cycles, rather than testing the sample obtained and used on the day of ICSI [6]. Additionally, most of these studies measured ICSI outcomes in terms of fertilization and chemical pregnancy rates, with no comment on clinical pregnancy [7]. Therefore, it is a pressing necessity to evaluate the effect of conventional semen parameters and sperm DNA fragmentation of the semen sample used for microinjection on ICSI outcomes in terms of fertilization, embryo quality, and clinical pregnancy.

Methods

This prospective controlled study was conducted at the Andrology Unit of the Dermatology, Venereology and Andrology Department, Assiut University Hospitals, in collaboration with the assisted conception unit at a women's health hospital and the Department of Clinical Pathology at South Egypt Cancer Institute, in the period from April 2018 to December 2019. The study was approved by the Institutional Review Board of Assiut University Hospital (IRB No. 17200054) and all patients signed an informed consent form. Privacy and confidentiality of all data were assured.

The study included infertile couples who presented to the assisted conception unit at a women's health hospital for ICSI procedure based on the diagnosis of male factor infertility with normal female partners. We excluded men taking medications affecting spermatogenesis or who had pyospermia, clinical varicocele, systemic diseases, a history of cryptorchidism, or azoospermia. Men with inadequate semen volume on the ICSI day were also excluded. We excluded female partners aged >38 years or with any gynecological diseases that could affect oocyte quality or endometrial receptivity, such as endometriosis, adenomyosis, pelvic inflammatory disease, pyosalpinx, or subendometrial fibroids, or systemic diseases that may impair reproductive capacity, such as hepatic, renal, or endocrine conditions, including diabetes mellitus and autoimmune diseases.

All female patients received ovarian stimulation using a standard luteal down-regulation regimen (long protocol) or a flare-up short regimen (short protocol) [8]. On the day of oocyte retrieval, semen samples were obtained from the male partners by masturbation. After complete liquefaction in a 37°C incubator, a small fraction (0.5 mL) was taken from the ICSI sample for evaluation according to the World Health Organization (WHO) 2010 criteria [9] and sperm DNA fragmentation assessment, and the rest was used for microinjection of the selected sperm into retrieved oocytes according to the procedure reported by Palermo et al. [10].

1. Sperm DNA fragmentation assessment

Sperm DNA fragmentation was assessed using flow cytometry (Beckman Coulter, Fullerton, CA, USA) based on the fluorescence emission from individual spermatozoa stained with propidium iodide (PI) and excitation with a 488-nm argon laser. Flow cytometric detection of sperm DNA chromatin damage was carried out according to the method described by Martinez-Soto et al. [11]. A 100-µL fraction of the semen sample was diluted with phosphate-buffered saline to 2×10^6 sperm/mL. Fifty microliters of the semen sample was directly stained with 50 µg/mL PI, using the cycle test kit (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) and analyzed immediately by FACSCalibur flowcytometry with Cell Quest software (Becton Dickinson Biosciences). Ten thousand events were measured for each specimen; this permitted the state of condensation of the sperm chromatin to be analyzed, as DNA condensation is directly related to PI uptake. The percentage of sperm cells with DNA damage was automatically calculated and the result was expressed as the sperm DNA fragmentation index (DFI).

2. Fertilization assessment

The oocytes were assessed for fertilization at 16–18 hours after the microinjection. Fertilization was confirmed to be normal if two pronuclei and two polar bodies were identified, and pronuclei size and position, as well as the size, distribution, and number of nucleoli, were evaluated. Oocytes without obvious pronuclei were considered unfertilized. Oocytes with a single pronucleus or more than two pronuclei were cancelled due to abnormal fertilization. The percentage of fertilization was calculated as the number of fertilized oocytes divided by the total number of injected metaphase II oocytes [12]. The included couples were classified according to the fertilization rate into fertilized couples versus non-fertilized couples.

3. Embryo assessment and transfer

Embryos were assessed according to their number, symmetry, blastomeres, type, and percentage of fragmentation according to

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the Istanbul consensus of embryo assessment [13]. Fresh embryo transfer was performed on day 3 or 5 after oocyte retrieval using the best-quality embryos among a cohort of resultant embryos. The grading criteria were as follows: grade I or good embryo quality: < 10% fragmentation with equal-sized cells and no multinucleation; grade II or fair embryo quality: 10%–25% fragmentation with equal-sized cells and no evidence of multinucleation; and grade III or poor embryo quality: severe fragmentation (> 25%) with unequal-sized cells and evidence of multinucleation. No more than three surviving embryos were transferred into the uterine cavity. The extra embryos were cryopreserved for subsequent embryo transfers. The luteal phase was routinely supported with progesterone (40–60 mg, intramuscular) per day for 14 days and continued for another 4 weeks if pregnancy was established.

4. Pregnancy determination and follow-up

Serum human chorionic gonadotropin levels were measured 14 days after embryo transfer. Clinical pregnancy was confirmed by observing a fetal heartbeat in the uterine cavity on ultrasound 4 weeks after embryo transfer. All pregnant women were followed up regularly in the prenatal clinic every 4 weeks until the 28th week of pregnancy. After the 28th week, the follow-up became every 2 weeks until the 36th week, and then, once a week until the end of pregnancy and delivery of the babies. On this basis, the live birth rate was analyzed.

5. Statistical analysis

Data entry and data analysis were done using IBM SPSS ver. 22 (IBM Corp., Armonk, NY, USA). Data were presented as number, percentage, mean, median and standard deviation. The chi-square test was used to compare qualitative variables.

The Student *t*-test was used to compare quantitative variables between groups for parametric data, while the Mann-Whitney test was used for non-parametric data. Spearman correlation coefficients were calculated to measure correlations between quantitative variables. MedCalc (Ostend, Belgium) was used to calculate sensitivity, specificity, positive and negative predictive values, and receiver operating characteristic (ROC) curves. For all tests, *p*-values < 0.05 were considered to indicate statistical significance.

Results

The study initially included 134 couples, of whom 14 were excluded due to either inadequate semen samples or cancellation as a result of ovarian hyperstimulation or abnormal fertilization. The remaining 120 couples completed the study and were classified according to fertilization into fertilized (n = 87) and non-fertilized (n = 33) groups. Among the included couples, 50 were diagnosed with idiopathic male factor infertility, 40 were diagnosed with bilateral tubal blockage, and 30 had unexplained infertility. There were no significant differences in the mean ages of the male partners (35.27 ± 4.78 vs. 34.98 ± 3.5 years) and female partners (28.55 ± 3.51 vs. 29.13 ± 3.24 years) between the fertilized and non-fertilized groups. The mean infertility duration was not significantly between the two groups (5.93 ± 1.38 years for the fertilized group vs. 6.83 ± 2.61 years for the non-fertilized group). There was also no significant difference in the mean retrieved oocyte number between the two groups (8.36 ± 1.86 for the fertilized group vs. 7.96 ± 1.96 for the non-fertilized group). The fertilization rate was 72% and the pregnancy rate was 55%.

In the fertilized couples, statistically significantly higher values were found for total sperm motility, progressive motility, viability (using the hypo-osmotic swelling test [HOST]), and the percentage of normal morphology than in non-fertilized couples ($p = 0.001$, $p = 0.001$, $p = 0.001$, and $p = 0.001$, respectively), while the sperm DFI was significantly lower ($p = 0.001$). No statistically significant differences in semen volume or sperm concentration and count were noted between the fertilized and non-fertilized couples, as shown in Table 1.

The fertilized couples were further classified according to clinical pregnancy into pregnant (n = 48) and non-pregnant couples (n = 39). Significantly higher values were found for sperm viability and percentage of normal sperm morphology in the pregnant couples than in the non-pregnant couples ($p = 0.002$ and $p = 0.045$, respectively). A significantly lower sperm DFI was found in the pregnant couples than in the non-pregnant couples ($p = 0.001$). No statistically significant differences were found in semen volume, total sperm motility, progressive motility, concentration, and count between pregnant and non-pregnant couples, as shown in Table 2.

According to the ROC curve analysis of data from the 120 ICSI cycles, sperm viability, normal sperm morphology percentage, and sperm DFI were statistically significant as a prognostic indicators of the fertilization rate, with areas under the curve (AUCs) of 0.745, 0.770, and 0.965 respectively (Figure 1). The values with the best ratio of sensitivity and specificity were evaluated and were found to be 40%, 7%, and 46%, respectively; these can be used as cutoff values for predicting fertilization. A pairwise comparison of the three ROC curves in Figure 1 showed significant differences between sperm DFI and sperm viability (difference between AUCs, 0.220; $p = 0.001$) and normal sperm morphology (difference between AUCs, 0.195; $p = 0.001$). However, there was no significant difference between the ROC curves for sperm viability and normal morphology (difference between AUCs, 0.0252; $p = 0.54$). Sperm DFI was also a statistically significant prognostic indicator of the clinical pregnancy rate, with

Table 1. Comparison between semen parameters and sperm DFI% of the fertilized and non-fertilized couples

Variable	Fertilized couple (n = 87)	Non-fertilized couple (n = 33)	*p*-value
Semen volume (mL)[a]			0.462
Mean ± SD	2.31 ± 0.60	2.18 ± 0.72	
Median (range)	2.0 (1.0–3.0)	2.0 (1.0–3.0)	
Total motility (%)[a]			0.001[c]
Mean ± SD	30.67 ± 6.79	22.00 ± 5.51	
Median (range)	30.0 (20.0–55.0)	20.0 (15.0–30.0)	
Progressive motility (%)[a]			0.001[c]
Mean ± SD	16.04 ± 6.47	8.33 ± 2.40	
Median (range)	15.0 (5.0–32.0)	10.0 (5.0–10.0)	
Viability by HOST (%)[a]			0.001[c]
Mean ± SD	51.11 ± 9.71	41.33 ± 8.60	
Median (range)	50.0 (10.0–65.0)	40.0 (30.0–60.0)	
Sperm concentration (million/mL)[b]			0.927[c]
Mean ± SD	13.87 ± 6.15	13.10 ± 4.56	
Median (range)	10.0 (8.0–38.0)	10.0 (5.0–20.0)	
Sperm count (million/mL)[b]			0.315[c]
Mean ± SD	31.04 ± 13.24	26.97 ± 8.97	
Median (range)	30.0 (10.0–76.0)	30.0 (10.0–40.0)	
Normal sperm morphology by spermac stain (%)[b]			0.001[c]
Mean ± SD	5.60 ± 3.27	2.67 ± 1.49	
Median (range)	4.0 (2.0–10.0)	2.0 (2.0–10.0)	
Sperm DFI (%)[a]			0.001[c]
Mean ± SD	30.89 ± 11.44	59.18 ± 10.38	
Median (range)	28.0 (4.0–67.0)	58.5 (40.0–83.0)	

DFI, DNA fragmentation index; SD, standard deviation; HOST, hypo-osmotic swelling test.
[a]Student *t*-test; [b]Mann-Whitney test; [c]Significant *p*-value <0.05.

an AUC of 0.788. The value with the best ratio of sensitivity and specificity was evaluated and was found to be 33%, which can be used as a cutoff value for predicting pregnancy (Supplementary Figure 1).

Using the cutoff value of DFI (46%) for predicting fertilization according to the ROC curve (Figure 1), the patients were divided into two groups, as shown in Table 3. We compared the DFI groups according to the outcomes of the ICSI procedure and we found that the fertilization rate and good-quality embryo rate were significantly higher in the low-DFI group than in the high-DFI group ($p = 0.001$ and $p = 0.026$, respectively). No clinical pregnancy was achieved in patients with a sperm DFI higher than 46%.

Discussion

Male infertility management has grown at a slower pace than female infertility management. Despite being revolutionized by ICSI, the success rate of the technique is much lower in patients with male infertility [14]. To improve the success rate, there is a continuing need for more research to unravel the mystery of male infertility parameters that may have predictive value for the different measures of ICSI outcomes [15].

The current study evaluated semen parameters and sperm DFI as predictors for ICSI outcome among 120 couples who were classified according to fertilization into fertilized and non-fertilized couples. In this study, statistically significantly higher values for total sperm motility, progressive sperm motility, sperm count, and the percentage of normal sperm morphology were found among fertilized couples than non-fertilized couples. Sperm viability, normal morphology, and DFI were significant prognostic factors of fertilization.

HOST has been shown to be an effective method for selecting live sperm for ICSI. In this study, sperm viability was significantly higher in the fertilized and pregnant couples than in the non-fertilized and non-pregnant couples. A cutoff level of 40% of sperm viability was found to be a predictor for the fertilization rate in subsequent analyses, with a sensitivity of 91% and a specificity of 52.5%. These findings are in accordance with those of Charehjooy et al. [16], who reported a significantly higher percentage of fertilization, embryos that had good quality, implantation, and pregnancy rates in a group of infertile men undergoing ICSI cycles in which HOST was used as a guide for sperm selection. Other studies have also noted higher rates

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Table 2. Comparison between semen parameters and sperm DFI% of the pregnant and non-pregnant couples

Variable	Pregnant couple (no = 48)	Non-pregnant couple (no = 39)	*p*-value
Semen volume (mL)[a]			0.586
Mean ± SD	2.36 ± 0.57	2.25 ± 0.64	
Median (range)	2.0 (1.0–3.0)	2.0 (1.0–3.0)	
Total motility (%)[a]			0.507
Mean ± SD	31.40 ± 7.57	29.75 ± 5.73	
Median (range)	30.0 (20.0–55.0)	30.0 (20.0–40.0)	
Progressive motility (%)[a]			0.475
Mean ± SD	16.88 ± 7.07	15.00 ± 5.62	
Median (range)	15.0 (10.0–32.0)	15.0 (5.0–25.0)	
Viability by HOST (%)[a]			0.022[c]
Mean ± SD	53.00 ± 11.81	48.75 ± 5.59	
Median (range)	50.0 (10.0–65.0)	50.0 (40.0–60.0)	
Sperm concentration (million/mL)[b]			0.09[c]
Mean ± SD	12.96 ± 5.95	13.25 ± 5.67	
Median (range)	11.0 (8.0–38.0)	12.0 (10.0–30.0)	
Sperm count (million/ejaculate)[b]			0.058[c]
Mean ± SD	28.08 ± 12.36	34.75 ± 13.66	
Median (range)	30.0 (10.0–76.0)	33.0 (15.0–60.0)	
Normal sperm morphology by spermac stain (%)[b]			0.045[c]
Mean ± SD	6.68 ± 3.56	4.25 ± 2.31	
Median (range)	8.0 (2.0–10.0)	3.0 (2.0–8.0)	
Sperm DFI%[a]			0.001[c]
Mean ± SD	26.26 ± 8.75	38.60 ± 11.35	
Median (range)	27.5 (4.0–46.0)	39.5 (21.0–67.0)	

DFI, DNA fragmentation index; SD, standard deviation.
[a]Student *t*-test; [b]Mann-Whitney test; [c]Significant *p*-value <0.05.

of fertilization in groups with higher sperm viability detected by HOST, although the results were not statistically significant [17].

A cutoff level (7%) of normal sperm morphology percentage, with a sensitivity of 68.75% and a specificity of 77.5%, was found to predict fertilization in ICSI procedures in our study. The WHO 2010 manual provides 4% as the lower limit of normal sperm morphology percentage; however, previous studies have reported different cutoff values for spontaneous and assisted conception [9]. Semen samples with an elevated proportion of abnormal sperm morphology usually have a high DFI, which may affect fertilization in response to ICSI [18].

Previous studies were contradictory and failed to identify a positive predictive value for routine semen parameters in ICSI outcomes, with no consensus on cutoff values predicting success [12,19]. This may be explained by differences among these studies in terms of the retrospective study design, etiology of infertility in the investigated infertile couples, or the edition of the WHO guidelines that was used.

Sperm DNA integrity is one of the most important objective parameters affecting ICSI outcomes, and it may even indicate male subfertility regardless of conventional semen parameters [20]. In our study, the DFI was significantly lower in both fertilized and pregnant couples than in non-fertilized and non-pregnant couples. Furthermore, sperm DFI was a significant prognostic indicator of both fertilization and pregnancy. This finding is in accordance with previous studies that reported negative correlations between the sperm DFI and fertilization, embryo grade, and pregnancy rates with ICSI cycles [14,20]. However, these results contradict the findings of some other studies that reported no correlation between the DFI and fertilization, embryo quality, or pregnancy rates in infertile patients undergoing ICSI treatment [5,6,12,14,21]. This may be due to a lack of standardization of sperm DNA fragmentation tests or the use of different methods of sperm selection techniques or different definitions of pregnancy outcomes. The variable ability of oocytes and early embryos to repair sperm DNA damage may be another explanation. Additionally, the paternal genome is switched on after the 4- to 8-cell stage, which further affects embryo development. In the stage of *in vitro* development, good-quality embryos may not necessarily develop to the blastocyst stage. Moreover, poor embryos might reach the blastocyst stage despite the higher level of DFI in the sperm used for fertilization [5,22].

In their recent study, Green and coauthors et al. [6] failed to find a

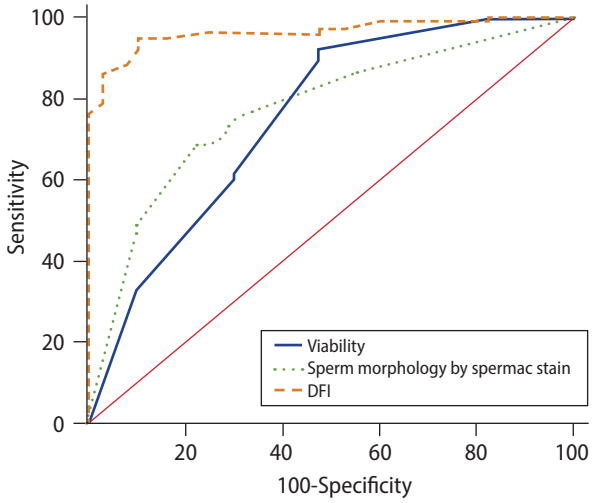

Figure 1. Receiver operating characteristic curve analysis. Sperm viability of the microinjection semen sample vs. fertilization rate; area under the curve (AUC) =0.745 (p<0.05; accuracy, 78.33%). The value with the best ratio of sensitivity (91.25%) and specificity (52%) was 40%, which was used as the cutoff value for predicting fertilization in subsequent analyses. Normal sperm morphology percentage of the microinjection semen sample vs. fertilization rate; AUC=0.770 (p<0.05; accuracy, 71.67%). The value with the best ratio of sensitivity (68.75%) and specificity (77.5%) was 7%, which was used as the cutoff value for predicting fertilization in subsequent analyses. Sperm DNA fragmentation index (DFI) vs. fertilization rate; AUC=0.965 (p<0.05; accuracy, 93.33%). The value with the best ratio of sensitivity (95%) and specificity (90%) was 46%, which was used as the cutoff value for predicting fertilization in subsequent analyses.

difference in the fertilization, blastulation, implantation, pregnancy and miscarriage rates with ICSI between the low (< 15%) and high (> 15%) sperm DFI groups. Although the authors performed the analysis using semen samples obtained on the ICSI day, as we did, they used a different technique (sperm chromatin structure assay) for assessing sperm DNA damage and they assayed the embryos for aneuploidy before transfer using trophectoderm blastocyst biopsy with real-time polymerase chain reaction or next-generation sequencing.

In this study, a sperm DFI threshold of 46% was used as the cutoff value for predicting fertilization, with high sensitivity (95%) and specificity (90%). A threshold of 33% was used as the cutoff value for predicting pregnancy, with a sensitivity of 84% and a specificity of 70%. The fertilization rate was statistically significantly lower in men with a sperm DFI > 46% than in those with a DFI ≤ 46%. Furthermore, a significantly higher good-quality embryo formation rate was found in the low-DFI group than in the high-DFI group. Previous studies used different cutoff values of DFI as assessed by various sperm DNA integrity assays, including 29% [20], 22.3% [23], and 15% [6].

Table 3. Comparison between the different outcomes of ICSI in relation to cutoff value of sperm DFI

Variable	Low DFI (≤ 46) (n = 87)	High DFI (> 46) (n = 33)	p-value
Fertilization rate			0.000[a]
Yes	83 (95.4)	4 (12.1)	
No	4 (4.6)	29 (87.9)	
Embryo quality			0.026[a]
Good	51 (61.4)	0	
Fair and poor	32 (38.6)	4 (100.0)	
Pregnancy rate			-
Yes	48 (57.8)	0	
No	35 (42.2)	4 (100.0)	
Abortion			-
No abortion	29 (60.4)	-	
First trimester abortion	12 (25)	-	
Second trimester abortion	7 (14.6)	-	
Live birth rate			-
Yes	29 (60.4)	-	
No	19 (39.6)	-	

Values are presented as number (%).
ICSI, intracytoplasmic sperm injection; DFI, DNA fragmentation index.
Chi-square test. [a]Significant p-value <0.05.

We were not able to compare miscarriage or live birth rates between the low-DFI and high-DFI groups in our study, as no patient achieved pregnancy in the high DFI group. Some studies reported no statistically significant difference in the abortion rate or live birth rate between low- and high-DFI groups [6,20,24,25]. However, two meta-analyses found that sperm DNA damage was associated with a significantly increased risk of pregnancy loss after ICSI, and they recommended evaluating sperm DNA damage prior to ICSI [26,27]. Nonetheless, a well-designed meta-analysis failed to confirm the predictive value of sperm DFI for ICSI outcomes in terms of clinical pregnancy and pregnancy loss [28]. The different findings again can be attributed to different patient selection criteria and ICSI protocols among studies.

In conclusion, our observations emphasize the importance of sperm viability, normal morphology, and DNA integrity of the sample used for ICSI to enhance the likelihood of good outcomes. This necessitates proper management of male partners and the elimination of any treatable risk factors that could impair these parameters prior to enrollment in ICSI. Further studies are recommended to achieve a consensus on standardized protocols for sperm DNA evaluation and selection with ICSI, especially for patients affected by male factor infertility.

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Conflict of interest

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

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ORCID

Alaa El din-Abdel Aal Moubasher https://orcid.org/0000-0002-0695-8215
Emad Abdelrehim Taha https://orcid.org/0000-0001-6549-2526

Supplementary material

Supplementary material can be found via https://doi.org/10.5653/cerm.2020.03965.

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CERM

Association of single-nucleotide polymorphisms in the *ESR2* and *FSHR* genes with poor ovarian response in infertile Jordanian women

Amer Mahmoud Sindiani[1], Osamah Batiha[2], Esra'a Al-zoubi[2], Sara Khadrawi[2], Ghadeer Alsoukhni[2], Ayesha Alkofahi[2], Nour Alhoda Alahmad[2], Sherin Shaaban[2], Eman Alshdaifat[3], Masood Abu-Halima[4]

[1]Department of Obstetrics and Gynecology, Faculty of Medicine and [2]Department of Biotechnology and Genetic Engineering, Jordan University of Science and Technology, Irbid; [3]Department of Obstetrics and Gynecology, Faculty of Medicine, Yarmouk University, Irbid, Jordan; [4]Institute of Human Genetics, Saarland University, Homburg, Germany

Objective: Poor ovarian response (POR) refers to a subnormal follicular response that leads to a decrease in the quality and quantity of the eggs retrieved after ovarian stimulation during assisted reproductive treatment (ART). The present study investigated the associations of multiple variants of the estrogen receptor 2 (*ESR2*) and follicle-stimulating hormone receptor (*FSHR*) genes with POR in infertile Jordanian women undergoing ART.
Methods: Four polymorphisms, namely *ESR2* rs1256049, *ESR2* rs4986938, *FSHR* rs6165, and *FSHR* rs6166, were investigated in 60 infertile Jordanian women undergoing ART (the case group) and 60 age-matched fertile women (the control group), with a mean age of 33.60±6.34 years. Single-nucleotide polymorphisms (SNPs) were detected by restriction fragment length polymorphism and then validated using Sanger sequencing.
Results: The *p*-value of the difference between the case and control groups regarding *FSHR* rs6166 was very close to 0.05 (p=0.054). However, no significant differences were observed between the two groups in terms of the other three SNPs, namely *ESR2* rs1256049, *ESR2* rs4986938, and *FSHR* rs6165 (p=0.561, p=0.433, and p=0.696, respectively).
Conclusion: The association between *FSHR* rs6166 and POR was not statistically meaningful in the present study, but the near-significant result of this experiment suggests that statistical significance might be found in a future study with a larger number of patients.

Keywords: Estrogen receptor; Follicle-stimulating hormone receptor; Ovarian stimulation; Single-nucleotide polymorphism

Introduction

About 72.4 million couples suffer from infertility worldwide; accordingly, almost three million children have been conceived through assisted reproductive treatment (ART) [1]. ART is a multistep process that involves oocyte collection, oocyte fertilization, and embryo implantation [2]. The first step of ART is the collection of oocyte-containing follicles after ovarian stimulation with follicle-stimulating hormone (FSH) to obtain high-quality oocytes [3]. The response to this hormonal stimulation varies among women. Women producing 6–15 oocytes are considered normal responders, while those with not more than 4–5 oocytes are referred to as poor responders and women producing more than 15 oocytes are classified as hyperresponders [4].

Several factors, such as age, hormonal status, and ovarian reserve, play a role in the prediction of ovarian response [5,6]. In addition to

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Corresponding author: **Amer Mahmoud Sindiani**
Department of Obstetrics and Gynecology, Faculty of Medicine, Jordan University of Science and Technology, Irbid 22110, Jordan
Tel: +962-796025538 Fax: +962-2-7095010 E-mail: amsindiani0@just.edu.jo

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previously identified predictors, various genetic polymorphisms have been proposed as markers predicting ovarian response. These variations have been observed in many genes, such as estrogen receptor 2 (*ESR2*) and follicle-stimulating hormone receptor (*FSHR*) [7,8]. It is believed that polymorphisms in the *FSHR* and *ESR2* genes cause differences in the ovarian response and folliculogenesis [9]. *FSHR* is a G protein-coupled receptor (GPCR) that leads to the activation of adenylate cyclase through its main signal transduction pathway by increasing intracellular levels of cyclic adenosine monophosphate [10,11].

It is well-known that single-nucleotide polymorphisms (SNPs) in genes that play a fundamental role in oogenesis and folliculogenesis have an impact on female reproduction. To date, two different mechanisms have been proposed for this effect. Specifically, this impact can be induced by changes in the biochemical properties of a protein or at the level of transcription, which subsequently affects the activity of the promoter of a specific gene [12,13]. *ESR2* and *FSHR* are known to influence the number of mature oocytes; therefore, they can affect the outcomes of *in vitro* fertilization (IVF). Boudjenah et al. [3] described that the variant of *FSHR* (*FSHR* 2039 A > G) with a G allele at position 2039 may have no effect on young people; however, it might affect people at an older age. It was also observed that patients with an A allele variant in *ESR2* (*ESR2* 1730 G > A) had a significantly higher number of mature oocytes.

Poor ovarian response (POR) can be precisely defined as occurring when two of the three clinical criteria proposed by the European Society of Human Reproduction and Embryology (ESHRE) are present. These criteria include advanced maternal age (≥ 40 years), a low antral follicle count (AFC; ≤ 3 oocytes with conventional stimulation), and abnormal ovarian reserve test results (i.e., an anti-Müllerian hormone [AMH] level of 0.5–1.1 ng/mL) [13]. As a part of ART, gonadotropin therapy is used to stimulate ovarian function. This therapy has been reported to be successful in several aspects. With this background in mind, the present study was conducted to investigate the association of multiple variants of *ESR2* and *FSHR* genes with POR among Jordanian women.

Methods

The current study was carried out according to the Declaration of Helsinki guidelines and approved by the Institutional Review Board of King Abdulla University Hospital in Jordan (IRB No. 2912015). Furthermore, written informed consent was obtained from all subjects or their guardians before enrollment.

1. Study population

The cohort analyzed in this study has been described in detail elsewhere [14]. To summarize, 60 female partners of selected couples undergoing ART for infertility were enrolled in the present study. The mean age of the participants was 33.60 ± 6.34 years (range, 20–46 years). The study population was selected from couples referred to different medical centers in Jordan (King Hussein Medical Center, Islamic Hospital, Prince Rashid Hospital, and Al-Amal Maternity Hospital) to undergo controlled ovarian stimulation for IVF/intracytoplasmic sperm injection during 2014–2017. Patients with a history of endometrioma, ovarian surgery, and chemotherapy were excluded from the study.

Ultrasonography was performed on the second day of the menstrual cycle to evaluate the anatomical characteristics of the female reproductive system and to identify the AFC. On the third day of the menstrual cycle, 5 mL of venous blood was collected from each participant in two tubes, including 2.5 mL in a plain tube and 2.5 mL in a tube containing tripotassium ethylenediaminetetraacetic acid (K3-EDTA). The samples in the plain tube were immediately centrifuged to separate the serum and then used for the assessment of FSH and AMH following the manufacturer's recommendations (Beckman Coulter, San Jose, CA, USA). In addition, the blood samples in the K3-EDTA tubes were utilized to investigate the SNPs located in *ESR2* and *FSHR* genes as shown in Table 1.

Women were included in the case group if they met two or more of the POR criteria defined by the ESHRE before the initiation of the study. The inclusion criteria were: (1) FSH level of > 10 mIU/mL on the third day of the menstrual cycle, (2) AFC of < 9, (3) AMH level of < 1.1 ng/mL, and (4) < 5 retrieved oocytes in metaphase II (MII). The subjects were divided into 10 groups based on these categories as summarized in Table 2.

Table 1. Summary of the four studied single-nucleotide polymorphisms

dbSNP-ID	Sequence variation	Position	Consequence
rs1256049	G > A	Chr 14:64257333(GRCh38.p12)	*ESR2*: synonymous variant
rs4986938	G > A	Chr 14:64233098(GRCh38.p12)	*ESR2*: noncoding transcript variant
rs6165	919 A > G	Chr 2:48963902(GRCh38.p12)	*FSHR*: missen se variant (p.Thr307Ala)
rs6166	2039 A > G	Chr 2:48962782(GRCh38.p12)	*FSHR*: missense variant (p.Asn680Ser)

dbSNP, single-nucleotide polymorphism database; Chr, chromosome; *ESR2*, estrogen receptor 2; *FSHR*, follicle-stimulating hormone receptor.

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In addition, 60 age-matched healthy volunteers were included in the study as controls. The participants of the control group were selected from female partners with proven fertility (i.e., with normal laboratory test results showing the potential for normal pregnancy without medical assistance). A comparison between the laboratory results of the cases and controls is presented in Table 3.

2. DNA extraction and polymerase chain reaction-restriction fragment length polymorphism detection of four SNPs

Genomic DNA was isolated from the peripheral blood samples of the case and control groups using the Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of the isolated DNA were measured by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Polymerase chain reaction (PCR) was used to identify the 4 different SNPs in *ESR2* and *FSHR*.

Two of the SNPs were located on *ESR2*, and the other two were on *FSHR*, as shown in Table 4. Each SNP was covered by its own set of primers, and all four sets of primers were designed to detect the target SNP using Primer3 Input (version 0.4.0) based on the sequences obtained from NCBI for *ESR2* (NC_000014.9) and *FSHR* (NC_000002.12). The primers were synthesized at the Princess Haya Biotechnology Center of Jordan. Table 4 presents the primer sequences, product size, DNA variation, sequence variation, and PCR conditions for each SNP.

PCR was performed in a monoplex fashion for each primer set as indicated in Table 4. Specifically, PCR was carried out in a 0.2-mL PCR

Table 2. Number of included women in each selected category

Category	No. of samples
1. AFC/AMH	4
2. MII/FSH	9
3. AFC/MII	13
4. FSH/AMH	6
5. MII/AMH	19
6. AFC/FSH/AMH	1
7. MII/FSH/AMH	1
8. AFC/MII/FSH	3
9. AFC/MII/AMH	3
10. AFC/MII/FSH/AMH	1

AFC, antral follicle count; AMH, anti-Müllerian hormone; MII, metaphase II; FSH, follicle-stimulating hormone.

Table 3. Laboratory results of cases and controls

Parameter	Infertile women with poor ovarian response (n = 60)	Control fertile women (n = 60)	*p*-value[a]
FSH (mIU/mL)	19.55 ± 13.7	5.3 ± 0.93	< 0.001
AMH (ng/mL)	0.344 ± 0.257	2.4 ± 0.47	< 0.001
AFC (< 9)	3.52 ± 1.64		
MII (< 5)	2.25 ± 1.27		

Values are represented as mean±standard deviation.
FSH, follicle-stimulating hormone; AMH, anti-Müllerian hormone; AFC, antral follicle count; MII, metaphase II.
[a]Nonparametric Mann-Whitney test; statistical significance, $p<0.05$.

Table 4. Summary of the studied single-nucleotide polymorphisms

Primer set	Primer sequence	Included polymorphism			PCR program
		Product size	DNA variation	Sequence variation ID	
Set 1	F: AGCTGAGGAGGAGGGGTG R: CCGGGGTGGTCAATTGAG	152 bp	rs1256049	G > A	97°C for 30 sec 55.6°C for 30 sec 72°C for 30 sec 35 Cycles
Set 2	F: CCAGAACCCACAGTCTCAGT R: GGTGGAGGGAAGGATGGTAC	169 bp	rs4986938	G > A	97°C for 30 sec 52°C for 30 sec 72°C for 30 sec 45 Cycles
Set 3	F: TCTGAGCTTCATCCAATTTGCA R: ACGTCAACCACTTCATTGCA	176 bp	rs6165	A > G	97°C for 30 sec 51°C for 30 sec 72°C for 30 sec 45 Cycles
Set 4	F: CCCCTCATCACTGTGTCC R: GCACTGTCAGCTCTTTGTGAC	374 bp	rs6166	A > G	97°C for 30 sec 59.5°C for 30 sec 72°C for 30 sec 35 Cycles

PCR, polymerase chain reaction; F, forward; R, reverse.

tube with a 20-μL reaction volume, containing 2 μL of template genomic DNA (~200 ng), 10 μL of 2X PCR Master Mix (New England Biolabs, Hitchin, UK), 2 μL (10 μmoL) of each primer, and 20 μL of nuclease-free water. The amplification reaction for each set was conducted in a programmable thermal cycler (Thermo Fisher Scientific) as shown in Table 4. Nuclease-free water was used instead of genomic DNA as a blank to check for any DNA contamination.

The generated PCR product was run on a 2% (w/v) agarose gel prepared in 1X Tris-borate-EDTA (Sigma-Aldrich, St. Louis, MO, USA), containing ethidium bromide (Promega Corp., Madison, WI, USA). Moreover, a 50-bp DNA ladder (GeneDireX Inc., Taoyuan, Taiwan) was concurrently applied with each electrophoretic run to confirm the product size. After electrophoresis at 120 V for 45 minutes, the results were visualized and recorded using the UVP GelDoc-It^2 310 Imaging System (Thermo Fisher Scientific).

Restriction fragment length polymorphism (RFLP) was carried out on the cleaned PCR products using restriction endonuclease enzymes (New England Biolabs). To determine the genotype for each sample, the PCR product was incubated with different restriction endonuclease enzymes overnight at 37°C (except for *Bsr*I, which was incubated overnight at 65°C), as shown in Table 5. The samples were then run on a 1% agarose gel at 90 V for 1 hour. Three samples of each polymorphism with different genotypes were sent to Macrogen Inc. in South Korea to be purified and sequenced for confirmation of the RFLP results.

3. Data analysis

Patients with *GG, GA*, and *AA* alleles were considered to be normal homozygous, heterozygous, and abnormal homozygous, respectively, except for the rs6165 substitution mutation, for which subjects with *AA, AG*, and *GG* genotypes were regarded as normal homozygous, heterozygous, and abnormal homozygous, respectively. GraphPad Prism software ver. 7.0 (GraphPad, La Jolla, CA, USA) was used for the statistical analysis of Hardy-Weinberg equilibrium (HWE) [15]. All other statistical analyses were performed using version 8 of this software. The chi-square test, the Fisher exact tests (used when cells had counts of less than 5), and odds ratios (ORs) (variant homozygotes were compared to the sum of the homozygotes for the wild-type alleles plus heterozygotes) were utilized to compare differences between genotype frequencies. A *p*-value less than 0.05 was considered to indicate statistical significance, and 95% confidence intervals (CIs) were used to describe the strength of associations.

Table 5. Restriction endonuclease enzymes utilized for restriction fragment length polymorphism

SNP ID	Enzyme name	Product size	
		Genotype	Band size
rs1256049	*Rsa*I	*GG*	152 bp
		GA	152, 72, and 80 bp
		AA	72 and 80 bp
rs4986938	*Alu*I	*GG*	169 bp
		GA	169, 107, and 62 bp
		AA	107 and 62 bp
rs6165	*CviKI*-1	*AA*	101, 69, and 6 bp
		AG	101, 69, 58, 43, and 6 bp
		GG	69, 58, 43, and 6 bp
rs6166	*Bsr*I	*AA*	374 bp
		GA	374, 239, and 135 bp
		GG	135 and 239 bp

SNP, single-nucleotide polymorphism.

Results

1. Study design and clinical/demographic characteristics

A total of 60 female partners of selected couples who underwent ovarian stimulation during IVF were included in the current study. The mean levels of AMH and FSH were 0.344 ± 0.257 ng/mL and 19.55 ± 13.7 mIU/mL, respectively. The mean AFC and number of MII oocytes were 3.52 ± 1.64 and 2.25+1.27 (range, 0–4) after stimulation, respectively.

2. Allele frequency distribution of the investigated genes

The SNPs of the two genes under investigation (*ESR2* [rs1256049 and rs4986938] and *FSHR* [rs6165 and rs6166]) were assessed in the case and control groups to determine whether the genotype frequencies of these polymorphisms were in HWE and to investigate the association of these polymorphisms with the development of POR. Table 6 tabulates the frequency of SNP genotypes in both the case and control groups.

The investigation of the genotype frequencies of the *ESR2* SNPs (rs1256049 and rs4986938) revealed that the majority of the infertile women (92.73%) were homozygotes for the wild-type allele (*GG*) of rs1256049, while 5.45% of the subjects were heterozygotes for the rs1256049 allele (*GA*) and 1.82% of the cases were homozygotes for the AA variant allele of rs1256049 (Table 6). The *AA* homozygous genotype was not observed in the control group. Regarding rs4986938, fewer than half of the subjects in the case (41.86%) and control (32.69%) groups were homozygotes for the wild-type allele (*GG*). Furthermore, 46.51% and 59.62% of the infertile and fertile women were heterozygotes for rs4986938 (*GA*), respectively. Additionally, 11.63% and 7.69% of the case and control groups were homozygotes for the *AA* variant allele of rs4986938, respectively.

Assuming random mating of the population in Jordan and applying the HWE for the distribution of the alleles, the observed genotype frequency of rs1256049 was significantly different from that

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Table 6. Hardy-Weinberg equilibrium analysis of the four studied SNPs

SNP ID	Infertile women with poor ovarian response					Control fertile women			
	Genotype	Observed (%)	Expected (%)	χ^2	*p*-value	Observed (%)	Expected (%)	χ^2	*p*-value
rs1256049	*GG*	92.73	91.12	13.83	0.001	93.22	93.33	0.12	0.941
	GA	5.45	8.68			6.78	6.55		
	AA	1.82	0.21			0	0.11		
	G	95.46				96.61			
	A	4.54				3.39			
rs4986938	*GG*	41.86	42.4	0.06	0.972	32.69	39.06	7.39	0.025
	GA	46.51	45.43			59.62	46.88		
	AA	11.63	12.17			7.69	14.06		
	G	65.11				62.5			
	A	34.89				37.5			
rs6165	*AA*	60.46	46.46	43.29	< 0.001	55.53	44.83	22.58	< 0.001
	GA	13.95	41.74			23.21	44.23		
	GG	23.25	9.35			21.42	10.9		
	A	69.05				66.97			
	G	30.95				33.03			
rs6166	*AA*	28.57	33.67	4.388	0.112	41.67	36.006	5.57	0.062
	GA	58.89	48.688			36.67	47.998		
	GG	12.5	17.6			21.66	15.996		
	A	58.04				60			
	G	41.96				40			

SNP, single-nucleotide polymorphism.

predicted by HWE ($p = 0.001$) (Table 6). However, there was no statistically significant difference among the infertile women in terms of rs4986938 ($p = 0.972$) (Table 6). The HWE analysis of the *FSHR* SNPs (rs6165 and rs6166) revealed that more than half of the subjects in the case (60.46%) and control (55.35%) groups were homozygotes for the wild-type allele (*AA*) of rs6165. Moreover, 13.95% and 23.21% of the infertile and fertile women were heterozygotes for the rs6165 allele (AG), respectively, and 23.25% and 21.42% of the case and control groups were homozygotes for the *GG* variant allele of rs6165, respectively.

With regard to rs6166, fewer than a third of the infertile (28.57%) and fertile (41.67%) women were homozygotes for the wild-type allele (*AA*). In addition, 58.89% and 36.67% of the case and control groups were heterozygotes for the rs6166 allele (*AG*), respectively, and 12.50% and 21.66% of the infertile and fertile women were homozygotes for the *GG* variant allele of rs6166, respectively. The observed genotype frequency of rs6165 was significantly different from that expected based on HWE ($p \leq 0.001$) (Table 6) for both infertile and fertile women.

To determine significance of the associations of SNP allele and genotype frequencies with POR, the chi-square test was performed for genotypes, and *p*-values were calculated for each SNP. The results revealed no significant associations ($p \geq 0.05$) (Table 7), although it should be noted that rs6166 had a *p*-value very close to 0.05 ($p = 0.054$) (Table 7). Furthermore, a comparison of allele frequency between the case and control groups demonstrated no significant difference in the four studied SNPs.

In addition, ORs were calculated for each polymorphism, with an OR of > 1 indicating an association between the homozygous variant of the allele and disease. Moreover, 95% CIs were calculated to indicate how reliable the ORs were in 95% of the occasions, with a wider interval indicating greater uncertainty. The ORs calculated for rs1256049, rs4986938, rs6165, and rs6166 were 1.07 (95% CI, 0.07–17.6), 1.58 (95% CI, 0.4–6.29), 1.15 (95% CI, 0.44–2.98), and 1.79 (95% CI, 0.82–3.87), respectively (Table 7).

Discussion

It is generally believed that the outcomes of ART depend on how a woman responds to the administered gonadotropin dose. In this study, genetic variants in *FSHR* and *ESR2* genes were investigated in infertile Jordanian women with POR and control fertile women using RFLP and Sanger sequencing (as a confirmative method). Out of the four investigated SNPs (*ESR2* rs1256049, *ESR2* rs4986938, *FSHR* rs6165, and *FSHR* rs6166), the *p*-value for the difference between the two groups regarding the rs6166 SNP in the *FSHR* gene was very close to 0.05 ($p = 0.054$).

Previous studies have investigated the associations of genetic vari-

Table 7. Association of poor ovarian response with *ESR2* rs1256049 and rs4986938 and *FSHR* rs6165 and rs6166 alleles, and genotype frequencies

SNP ID	Genotype	Poor ovarian response women	Control fertile women	χ^2	*p*-value	OR (95% CI)	Relative risk
		Frequency (%)	Frequency (%)				
rs1256049		(n=55)	(n=59)	1.16	0.561	1.07 (0.07–17.6)	
	GG	51 (92.73)	55 (93.22)				
	GA	3 (5.45)	4 (6.78)				
	AA	1 (1.82)	0				
	G	95.45	96.661	0.521	0.471	1.702 (0.433–6.556)	1.347
	A	4.55	3.389				
	GG	51 (92.72)	55 (93.32)	0.011	0.918	1.078 (0.301–3.861)	1.038
	GA+AA	4 (7.27)	4 (6.77)				
rs4986938		(n=43)	(n=52)	1.675	0.433	1.58 (0.4–6.29)	
	GG	18 (41.86)	17 (32.69)				
	GA	20 (46.51)	31 (59.62)				
	AA	5 (11.63)	4 (7.69)				
	G	65.11	62.5	0.087	0.768	0.917 (0.519–1.614)	0.958
	A	34.88	37.5				
	GG	18 (41.86)	17 (32.69)	0.850	0.357	1.482 (0.651–1.537)	1.201
	GA+AA	25 (58.14)	35 (67.31)				
rs6165		(n=43)	(n=56)	0.726	0.696	1.15 (0.44–2.98)	
	AA	26 (60.46)	31 (55.35)				
	GA	7 (13.95)	13 (23.21)				
	GG	10 (23.25)	12 (21.42)				
	A	68.6	66.96	0.092	0.762	1.096 (0.602–2.009)	1.047
	G	31.39	33.03				
	AA	26	31	0.260	0.610	0.811 (0.371–1.878)	0.914
	AG+GG	17	25				
rs6166		(n=56)	(n=60)	5.845	0.054	1.79 (0.82–3.87)	
	AA	16 (28.57)	25 (41.67)				
	GA	33 (58.89)	22 (36.67)				
	GG	7 (12.5)	13 (21.66)				
	A	58	0.6	0.083	0.774	0.921 (0.526–1.644)	0.960
	G	42	0.4				
	AA	16	25	2.174	0.140	1.786 (0.834–3.711)	1.307
	GA+GG	40	35				

ESR2, estrogen receptor 2; *FSHR*, follicle-stimulating hormone receptor; SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

ants in *ESR2* and *FSHR* with response to ovarian stimulation in various populations and presented variable and conflicting results. A review of the literature was carried out to identify studies that focused on the relationship of these genetic variants with various clinical parameters. Table 8 presents a summary of the collected data in this regard. Estrogen receptors (ERs) are nuclear receptors that bind to estrogen and act as transcription factors to induce follicle growth, oocyte maturation, and oocyte release, in addition to their role in uterine endometrial thickening and preparation for implantation [16,17]. Two ERs are known in humans: ERα, encoded by the *ESR1* gene, and ERβ, encoded by the *ESR2* gene [16,17]. Several genetic variants in ER genes have been linked to different ovarian dysfunctions [18,19]. The genetic variants of rs1256049 in *ESR2* have not been extensively studied; however, this variant was reported to be associated with the amount of recombinant FSH administered and time of medication use [7]. In another study, no significant difference was observed between recurrent spontaneous abortion and rs1256049 genetic variants [20]. The rs4986938 variant occurs in a noncoding region of the *ESR2* gene, and its clinical significance is not reported in ClinVar [17,18].

Two recent studies addressing the role of rs4986938 variants in ovarian response in Middle Eastern populations came to different conclusions. In a study conducted in Egypt, it was observed that women homozygous for the rs4986938 A allele variant had a lower

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number of retrieved oocytes after stimulation and a lower rate of clinical pregnancy [21]. However, in another study carried out in Iran, no association was reported between these SNP genotypes and response to ovarian stimulation [8]. The results of the present study are in line with the findings of the Iranian study, in which no association was observed between genetic variants in the rs4986938 SNP and

Table 8. Summary of allele frequencies and types of associations between the four studied SNPs and clinical measurements obtained in previous studies

SNP ID	Reference	Country	No. of samples		Allele frequency (%)		*p*-value	Association
			Association					
			Control	Case	*G*	*A*		
ESR2 rs1256049	[8]	Brazil	-	136	94	6	0.001	Women with the *GG* genotype needed more days of medication use
							0.011	The *GG* group used a higher amount of rFSH
	[16]	China	182	196	68	32		Recurrent spontaneous abortion was not significantly associated with SNP genotypes.
ESR2 rs4986938	[16]	China	182	196	86	14		Recurrent spontaneous abortion was not significantly associated with SNP genotypes.
	[19]	Egypt	111	105	55	45	< 0.001	Duration of stimulation, total dose of applied gonadotrophins, number of retrieved oocytes, number of transferred embryos, and clinical pregnancy rate were lower.
							< 0.001	Mean AMH level and number of oocytes were lower in *AA* genotype patients.
	[9]	Iran	106	92	66	34	> 0.05	No association was found between SNP genotypes and response to ovarian stimulation.
FSHR rs6165	[22]	China	-	450	33	67	< 0.05	Basal FSH level was higher in *GG* genotype patients than in *AA* and *AG* genotype patients on the third day of the menstrual cycle.
							0.009	*AA* genotype cases needed a longer time of stimulation than other groups.
	[23]	Germany	-	148	51	49	< 0.01	A significant difference was observed between anovulatory patients and normoovulatory controls regarding SNP genotype frequencies.
	[24]	Italy		149	47	53	0.037	Heterozygotes had a higher number of embryos.
	[19]	Egypt	111	105	51	49	< 0.001	The *GG* genotype was 2.5-fold more common in poor responders than in good responders.
	[9]	Iran	104	90	54	46	< 0.05	Total number of oocytes and levels of hormones (i.e., LH, FSH, and AMH) were significant in patients with the *AA* genotype than in those with other genotypes.
FSHR rs6166	[22]	China	-	450	31	69	< 0.05	Basal FSH level was higher in *GG* genotype patients than in *AA* and *AG* genotype patients on the third day of the menstrual cycle.
							0.009	*GG* genotype patients required a longer time of stimulation than other groups.
	[25]	Italy	25	17	58	42	0.02	A significant difference was observed between hyporesponders and controls regarding *GG* and *GA* genotypes.
							0.04	
	[21]	Greece	33	41	45	55	< 0.05	Total amount of gonadotropins needed in patients with the *AA* genotype was higher than needed for *GA* and *GG* genotypes.
							0.057	AA genotype women needed more stimulation days.
	[26]	Germany	-	93	53	47	< 0.05	Women with a *GG* genotype required higher FSH stimulation to overcome lower E_2 than women with the *AA* genotype.
	[27]	Spain	83	19	43	57	0.04	Frequency of the *G* allele was higher among the poor responders.
	[28]	China	-	1,250	37	63	< 0.01	Basal FSH level and dose of exogenous FSH were higher in *GG* poor responders.
							< 0.05	Follicular fluid E_2 level (on the day of hCG administration) and number of retrieved oocytes were lower in *GG* genotype individuals.
	[29]	Italy	87	140	52	48	< 0.05	Basal E_2 was significantly higher in women with the *AA* genotype than in those with the *AG* genotype.
							0.03	The *AG* genotype was significantly associated with the highest number of collected oocytes.

(Continued to the next page)

Table 8. Continued

SNP ID	Reference	Country	Number of samples		Allele frequency (%)		*p*-value	Association
			Association					
			Control	Case	*G*	*A*		
	[30]	South Korea	-	263	35	65	0.001	Third-day basal FSH levels were significantly higher in the *GG* group than in the *GA* and *AA* groups.
							0.013	Clinical pregnancy rate per embryo transfer was significantly higher in the *AA* group than in the *GA* or *GG* group.
	[31]	The Netherlands	-	105	40	60	0.003	Pregnancy rate and implantation rate in *GG* patients were three times higher than those in *AA* patients.
	[23]	Germany	-	148	61	39	< 0.05	The FSH serum concentration was significantly higher in *GG* patients than those in *GA* and *AA* subjects.
	[32]	Spain	-	145	62	38	< 0.001	The number of retrieved eggs was higher in *AA* genotype patients than in *GG* and *GA* genotype subjects.
							< 0.001	Patients with the *GG* genotype required higher gonadotropin doses than those with *AA* and *AG* genotypes.
							< 0.001	Women with *AG* and *AA* genotypes needed less time for stimulation than *GG* women.
	[33]	Greece	46	79			< 0.05	Gonadotropin dose correlated significantly with the observed levels of third-day FSH and was higher in *GG* and *AA* genotype women than in women with the *AG* genotype.
							< 0.01	Estrogen levels on the day of hCG administration were higher in the *AG* group.
							< 0.01	The number of preovulatory follicles and collected oocytes in the *AG* genotype group was significantly higher than in groups with other genotypes.
	[34]	UK	-	212			> 0.05	No statistically significant differences were observed in the number of mature retrieved oocytes, oocyte output rates, or fertilization rates among patients with different rs6166 genotypes; no significant difference was noted in the clinical pregnancy rate per transfer.
	[35]	UK	-	73	49	51	0.045	*AA* genotype patients produced higher concentrations of E_2 than *GG* genotype patients.
							0.005	Peak E_2 correlated with the mean cycle length in *AA* genotype patients
							0.002	Basal FSH was correlated with basal LH in *AA* genotype patients.
							0.002	Age at menarche was correlated with the mean days of stimulation in *AA* genotype patients.
							0.001	Peak E_2 concentration was correlated with the number of retrieved oocytes in *AA* genotype patients, and it showed a weak correlation in *GG* genotype patients.
	[36]	Germany	-	161	49	51	< 0.01	Basal levels of FSH on the third day were significantly different among three genotypes.
							< 0.01	The dose of FSH ampoules required for stimulation was different among the three genotypes.
	[4]	Iran	-	108	47	53	0.022	The number of retrieved oocytes in the *AA* group was higher than in the other groups.
	[37]	Japan	-	522	36	64	< 0.05	Basal FSH levels in *AG* and *GG* patients were significantly higher than in *AA* patients.
							< 0.05	The *AG* group needed a lower dose of hMG to achieve adequate follicular growth.
							< 0.05	The *AA* and *AG* groups showed significantly higher levels of serum E_2 than the *GG* group.
	[24]	Italy	-	149	42	58	-	No significant difference was observed among different genotypes in terms of FSH and E_2 serum levels and ovarian response.
	[22]	China	-	450	31	69	< 0.05	The *GG* group needed more days of induction.

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Table 8. Continued

SNP ID	Reference	Country	Number of samples		Allele frequency (%)		*p*-value	Association
			Association					
			Control	Case	*G*	*A*		
							< 0.05	Patients with the *GG* genotype had significantly higher rates of poor response to stimulation.
	[38]	Poland	-	22	36	64	-	*AA* homozygotes had higher rhFSH-induced expression of *FSHR* than carriers of the *GG* genotype.

SNP, single-nucleotide polymorphism; *ESR2*, estrogen receptor 2; rFSH, recombinant follicle-stimulating hormone; AMH, anti-Müllerian hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; E_2, estradiol; hCG, human chorionic gonadotropin; hMG, human menopausal gonadotropin; rhFSH, recombinant human follicle-stimulating hormone.

ovarian response.

FSHR, a member of the GPCR family, is expressed in the granulosa cells of the ovary and is considered essential for proper FSH action [28]. The rs6165 *A* > *G* SNP is a missense variant that causes a p. Thr307Ala amino acid substitution in the *FSHR* protein. All published studies have confirmed the association between this genetic marker and ovarian response. Specifically, it has been demonstrated that different rs6165 genotypes were associated with basal FSH levels, duration of stimulation [30], number of obtained embryos [23], and total number of retrieved oocytes [9,19].

The rs6166 A > G SNP is another well-studied SNP in the *FSHR* gene that causes a p.Asn680Ser missense variation. In a number of studies, rs6166 showed an association with basal FSH levels, the time required for stimulation [21-23,28,30], the number of retrieved oocytes [4,28,29,32,39], and implantation and pregnancy rates [40]. On the contrary, in other studies, rs6166 was reported to have no association with ovarian response, especially oocyte retrieval, pregnancy rate, and FSH levels [24,34,41]. The results of the present study are consistent with the findings of the majority of previous studies regarding the important role of this SNP in determining the response of women to ovarian stimulation.

The current study was the first attempt to investigate polymorphisms in *FSHR* and *ESR2* genes in a subset of the Jordanian Arab population. The allele and genotype frequencies were determined among women with POR and their normal counterparts. Minor allele frequencies (MAFs) were calculated for the studied SNPs in the control group, including rs1256049 MAF (A = 0.039), rs4986938 MAF (A = 0.375), rs6165 MAF (G = 0.33), and rs6166 MAF (G = 0.4). A comparison of the rs6165 MAFs obtained in this study with those reported in other studies revealed that our values were similar to those reported for populations of European and Asian origins. However, they were different from the values obtained for populations of African origin (Table 8) [25].

The rs6166 MAF was also similar to those reported for many European and Asian populations (Table 8) [42,43]. Nevertheless, only 1 study could be found regarding the Middle Eastern Arab population, which was conducted in Bahrain and reported a MAF of almost 0.5 [27]. Discrepancies among the results of various studies could be due to differences in cohort sample size, ethnicity, population stratification, and frequency of consanguinity. There are no isolated communities in Jordan; however, this country has a high rate of consanguineous marriage (20%–59%) [33]. This high consanguinity rate could explain why the genotype frequencies of the studied SNPs were out of HWE (Table 5).

The limited number of the study population and heterogeneous patients with POR are the major limitations of this study. Every year, millions of couples seek medical assistance due to infertility problems. In many ART cycles, the lack of a normal response to stimulation affects fertilization and pregnancy outcomes. Although ART is a very common therapeutic procedure in Jordan, studies on infertility and the causes of POR remain limited. The present study assessed the role of four genetic variants in two important genes, namely *ESR2* and *FSHR*. Based on the results, only one of these variants (*FSHR* rs6166) should be further studied and evaluated as a marker of POR in Jordanian women. In the present study, the association between *FSHR* rs6166 and POR was not statistically meaningful, but the present results suggest that statistical significance may be observed in a further study with a larger number of patients.

Conflict of interest

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

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ORCID

Amer Mahmoud Sindiani https://orcid.org/0000-0001-5115-7372
Osamah Batiha https://orcid.org/0000-0003-2415-324X
Esra'a Al-zoubi https://orcid.org/0000-0003-3093-2143
Sara Khadrawi https://orcid.org/0000-0001-6655-0855
Ghadeer Alsoukhni https://orcid.org/0000-0001-7743-9917
Ayesha Alkofahi https://orcid.org/0000-0002-8831-7509
Nour Alhoda Alahmad https://orcid.org/0000-0001-5829-1267
Sherin Shaaban https://orcid.org/0000-0001-5834-8243
Eman Alshdaifat https://orcid.org/0000-0003-2096-3165
Masood Abu-Halima https://orcid.org/0000-0001-9048-4958

Author contributions

Conceptualization: AMS, OB, EHA. Data curation: OB. Formal analysis: MAH. Funding acquisition: OB. Methodology: EA, SK, GA, AA, NA, SS. Project administration: AMS, OB. Visualization: AMS, OB, EHA. Writing–original draft: AMS, OB, MAH. Writing–review & editing: AMS, OB, EHA.

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Are women with small endometriomas who undergo intracytoplasmic sperm injection at an elevated risk for adverse pregnancy, obstetric, and neonatal outcomes?

Fatma Ferda Verit[1], **Ayse Seyma Ozsuer Kucukakca**[2]

[1]Department of Obstetrics and Gynecology, University of Health Sciences, Istanbul Training and Research Hospital, Istanbul; [2]Department of Obstetrics and Gynecology, Bezmialem Foundation University, Istanbul, Turkey

Objective: The aim of the study was to investigate pregnancy, obstetric, and neonatal outcomes in women with small (<4 cm) unilateral endometriomas.
Methods: This retrospective study included 177 patients: 91 patients with small endometriomas and 86 controls with unexplained or tubal factor infertility who were treated at the Süleymaniye Gynecology and Maternity Training and Research Hospital Infertility Unit between January 2010 and July 2015. The groups were matched with regards to demographic characteristics such as age, body mass index, and infertility duration. All of the women in this study conceived via intracytoplasmic sperm injection. We compared pregnancy, obstetric, and neonatal outcomes between these groups.
Results: Women with endometriomas had a higher biochemical pregnancy rate, but lower clinical pregnancy and live birth rates than women with unexplained and tubal factor infertility ($p<0.05$ for all). However no significant differences were found in terms of obstetric and neonatal complications between the two groups ($p>0.05$ for all).
Conclusion: In this study, we found that women with endometriomas less than 4 cm were more prone to early pregnancy complications. We also showed that this group did not have any increased risks of late pregnancy, obstetric, and neonatal complications.

Keywords: Endometrioma; Neonatal; Obstetric outcome; Pregnancy

Introduction

Endometriosis is a chronic, estrogen-dependent, inflammatory disease that is associated with pelvic pain and infertility. Approximately 30%–50% of women with endometriosis have infertility problems, most of whom seek to achieve pregnancy through assisted reproductive technology (ART) [1]. Although the exact pathogenesis of infertility in these patients remains unclear, studies have proposed that endometriosis may be associated with some potential etiological factors, such as poor ovarian reserve, poor oocyte and embryo quality, endometrial dysfunction, implantation defects, increased inflammation in the peritoneal fluid, distorted pelvic anatomy, and adhesions.

Studies have also demonstrated that the eutopic endometrium of women with endometriosis has some problems such as stem cell content, hormonal sensitivity, junctional zone defects, and the pres-

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Corresponding author: **Fatma Ferda Verit**
Department of Obstetrics and Gynecology, Istanbul Training and Research Hospital, Kasap Ilyas Mah. Org. Abdurrahman Nafiz Gurman Cd., TR-34098 Fatih/Istanbul, Turkey
Tel: +90-212-4596000 Fax: +90-212-4596230 E-mail: fverit@gmail.com

ence of proliferation and adhesion-inducing molecules that may impair implantation and decidualization [2]. Although it has been suggested that ovarian endometrioma, peritoneal endometriosis, and deep infiltrating endometriosis may have distinct molecular pathologies, similar endometrial changes are observed in all three of these conditions [3]. However, the outcomes may be worse at more advanced stages of the disease [4].

Conflicting data have been reported regarding the relationship between endometriosis and poor pregnancy outcomes. Some studies suggested that the incidence of placentation defects due to impaired decidualization such as preeclampsia, preterm birth, small for gestational age, and placenta previa may be higher in women with endometriosis [5,6], but other studies reported no such associations [7-9]. These discrepancies may be due to considerable variation across studies in methodology, design, sample size, study population, the subtype of endometriosis, and diagnostic accuracy. Confounders such as coexisting adenomyosis cannot be totally excluded in observational studies. The selection of control groups and subgroup analyses are also matters of debate. Moreover, pregnancies in these women are mostly achieved through ART, which may have negative effects on pregnancy and neonatal outcomes.

Some studies have investigated the relationships between ovarian endometrioma and pregnancy outcomes; however the endometriomas included in these studies were heterogeneous in size and subgroup analyzes were not performed [9,10]. It is unknown whether the size of the endometrioma may also be an important factor affecting these outcomes. It is possible that outcomes may be worse in patients with more advanced disease, and patients must be informed of this possibility before conception [4]. The aim of the study was to investigate pregnancy, obstetric, and neonatal outcomes in pregnancies achieved through intracytoplasmic sperm injection (ICSI) in women with endometriomas smaller than 4 cm.

Methods

The study was approved by the Institutional Review Board of Süleymaniye Gynecology and Maternity Training and Research Hospital (IRB No. 02-2015). Owing to the retrospective design, the requirement for informed consent was waived.

This retrospective study was performed at the Süleymaniye Gynecology and Maternity Training and Research Hospital Infertility Unit and included patients who met our eligibility criteria between January 2010 and July 2015. The study included 177 patients: 91 with small (< 4 cm) unilateral endometriomas and 86 who did not have endometriomas and were diagnosed with unexplained or tubal factor infertility. Controls were matched to endometrioma patients in terms of demographic characteristics such as age, body mass index (BMI), and infertility duration. We compared pregnancy, obstetric, and neonatal outcomes in these groups. Data were collected using patients' charts from our *in vitro* fertilization (IVF), obstetric, and neonatal intensive care unit. Patients were also contacted by telephone to gather any missing data about the outcomes. Women who were managed throughout their pregnancy by other obstetric units were not included.

Ovarian endometrioma was suspected based on the presence of a round cystic mass with a minimum diameter of 1 cm with thick walls, homogeneous fluid, and scattered internal echoes on ultrasonography [11]. The presence of suspected endometriomas was documented on at least one previous ultrasound examination at least 2 months before the ART cycle. The diameters of the suspected endometriomas were calculated using the mean values of three perpendicular layers. Doubtful and atypical cases were excluded. Conception was achieved by ICSI in all of the women in the study. The patients were treated using the standardized clinical protocol of our IVF unit. Only cases with fresh embryo transfers were included. Pregnancy was confirmed by a plasma beta-human chorionic gonadotropin (β-hCG) level of > 20 IU/L at 14 days after oocyte retrieval.

The pregnancy outcomes included chemical pregnancy, miscarriage (spontaneous pregnancy loss at < 20 weeks), pregnancy-induced hypertension (gestational hypertension, preeclampsia), preterm birth (delivery at < 37 weeks of gestation), small for gestational age (birth weight < 10th percentile), placenta previa (placenta covering the cervix partially or completely), gestational diabetes, intrauterine growth restriction (birth weight < 10th percentile because of a pathological process; the fetus has not attained its biologically determined growth potential).

Biochemical pregnancy was defined as a very early miscarriage with a transient increase in serum hCG levels before the fifth week of gestation and before the fetus was visible on ultrasound. Preeclampsia was defined as a blood pressure reading ≥ 140/90 mmHg after 20 weeks of gestation with proteinuria in formerly normotensive women. Gestational hypertension was defined as an elevated blood pressure reading (≥ 140/90 mmHg) after 20 weeks of gestation without proteinuria in previously normotensive women. Gestational diabetes was defined as carbohydrate intolerance with onset or recognition in pregnancy based on a positive oral glucose tolerance test. Data about neonates were collected after their admission to the neonatal intensive care unit.

The primary exclusion criteria were age above 37 years; endometrioma larger than 4 cm; prior cystectomy or any surgery that may affect ovarian reserve; bilateral endometriomas; adenomyosis; intramural myomas; uterine malformations; polycystic ovarian disease; obesity (BMI ≥ 30 kg/m^2); chronic diseases such as hypertension, cardiovascular disease, diabetes mellitus, insulin resistance, hyperlip-

idemia, liver or kidney disease, and neoplastic disease; a history of venous thromboembolism, antiphospholipid syndrome, or poor obstetric outcomes; ectopic pregnancy; hydrosalpinx; multiple pregnancies; autoimmune disease; smoking; and alcohol consumption. The neonatal outcomes analyzed were factors associated with increased neonatal morbidity and mortality such as perinatal asphyxia, necrotizing enterocolitis, congenital abnormalities, hypoglycemia, jaundice, hypothermia, and sepsis.

1. Statistical analysis

Data are presented as percentages for categorical variables and means for continuous variables. Proportions were compared using the Fisher exact test or chi-square test. Continuous variables were analyzed using the Student *t*-test or the Mann-Whitney *U*-test. For all tests, *p*-values of < 0.05 were considered to indicate statistical significance.

Results

The baseline characteristics of the study and control groups are summarized in Table 1. There were no differences in demographic characteristics such as age, BMI, duration of infertility, and previous pregnancies between the groups. However, day 3 follicle-stimulating hormone and anti-Müllerian hormone levels were significantly different in the endometrioma group.

Treatment cycle characteristics are shown in Table 2. Women with endometriomas received higher doses of gonadotropins, the duration of stimulation was longer, and fewer oocytes were retrieved. No significant differences were found in terms of transferred embryos between the groups.

Pregnancy and neonatal outcomes are shown in Table 3. There were no differences in terms of the number of pregnancies, miscarriage, obstetric complications such as preterm birth, small for gestational age births, intrauterine growth restriction, preeclampsia, placenta previa, and gestational diabetes. No significant differences were also found in neonatal complications such as asphyxia, necrotizing enterocolitis, hyperbilirubinemia, and stillbirth. Nonetheless, the endometrioma group had a higher biochemical pregnancy rate and lower clinical pregnancy and live birth rates.

Discussion

The aim of the study was to evaluate the relationship between unilateral small ovarian endometriomas (< 4 cm) and pregnancy, obstetric, and neonatal outcomes. We demonstrated that women with endometriomas less than 4 cm who underwent ICSI had poor pregnancy outcomes, such as a higher biochemical pregnancy rate and lower clinical pregnancy and live birth rates than ICSI control patients with unexplained or tubal factor infertility. However, women

Table 1. Baseline characteristics of the patients

Variable	Endometrioma (n = 91)	No endometrioma (n = 86)	*p*-value
Age (yr)	31.4 ± 3.8	30.4 ± 3.5	0.071
BMI (kg/m^2)	24.3 ± 3.7	24.3 ± 3.2	0.95
Duration of infertility (yr)	6.9 ± 3.3	6.2 ± 3.3	0.17
Previous pregnancy	11 (12.1)	14 (16.3)	0.42
Day 3 FSH (mIU /mL)	7.3 ± 3.1	6.2 ± 2.0	0.005
AMH (ng/mL)	2.1 ± 1.3	3.0 ± 2.3	0.002

Values are presented as mean±standard deviation or number (%).
BMI, body mass index; FSH, follicle-stimulating hormone; AMH, anti-Müllerian hormone.

Table 2. Treatment cycle characteristics

Variable	Endometrioma (n = 91)	No endometrioma (n = 86)	*p*-value
Total FSH dose (IU)	2,685.5 ± 798.5	2,076.0 ± 752.3	< 0.001
Day of stimulation	10.3 ± 1.7	9.8 ± 1.1	0.028
Number of oocytes retrieved	6.0 ± 4.3	8.4 ± 4.5	< 0.001
Number of transferred embryos	1.3 ± 0.5	1.3 ± 0.4	0.71

Values are presented as mean±standard deviation.
FSH, follicle-stimulating hormone.

Table 3. Pregnancy and neonatal outcomes

Variable	Endometrioma (n = 91)	No endometrioma (n = 86)	*p*-value
Number of pregnancies	45 (49.5)	46 (53.5)	0.60
Biochemical pregnancy	14 (31.1)	2 (4.3)	< 0.001
Clinical pregnancy	31 (68.9)	44 (95.7)	0.001
Miscarriage	5 (11.1)	6 (13.0)	0.77
Live birth	26 (57.8)	38 (82.6)	0.01
Obstetric complication	6 (23.1)	4 (10.5)	0.17
Preterm birth	6 (23.1)	9 (23.7)	0.95
SGA birth	2 (7.7)	2 (5.3)	0.69
IUGR	1 (3.8)	0	0.35
Preeclampsia	1 (3.8)	0	0.22
Placenta previa	2 (7.7)	1 (2.6)	0.34
Gestational diabetes	1 (3.8)	0	0.22
Neonatal complication	3 (11.5)	2 (5.3)	0.35
Asphyxia	1 (3.8)	1 (2.6)	0.78
Necrotizing enterocolitis	1 (3.8)	0	0.22
Hyperbilirubinemia	1 (3.8)	1 (2.6)	0.78
Stillbirth	0	0	

Values are presented as number (%).
SGA, small for gestational age; IUGR, intrauterine growth restriction.

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with endometriomas did not have higher risks of obstetric and neonatal complications in the study.

In our study, we demonstrated that endometriomas smaller than 4 cm were associated with poor pregnancy outcomes. The rate of biochemical pregnancies was higher, while the clinical pregnancy and live birth rates were lower in patients with endometriomas; however, the miscarriage rate did not differ between the endometrioma and control groups. Although many studies have investigated the effect of endometriosis on pregnancy outcomes, the evidence is still conflicting [12-16]. Some studies reported that women with endometriosis did not have different clinical pregnancy, miscarriage, and live birth rates from their healthy counterparts [12,13] while others found lower clinical pregnancy and live birth rates in endometriosis patients [14-16]. Omland et al. [17] also demonstrated that abortions prior to 6 weeks were more common and live birth rates were lower in women undergoing IVF with endometriosis than in those with unexplained infertility. However, the miscarriage rate after 6 weeks was not significantly different between the groups [17]. The low live birth rates in our study were associated with a higher biochemical pregnancy rate and a lower clinical pregnancy rates, but not with obstetric and perinatal complications.

What is the relationship between poor pregnancy outcomes and small endometriomas? There is considerable evidence that endometriosis may negatively affect follicle, oocyte, and embryo quality, as well as embryogenesis and embryo viability [12]. We suggest that endometriosis has a significant adverse environmental impact, which oocytes and embryos may not be able to tolerate in the early stages of pregnancy. Inflammatory mediators such as macrophages, cytokines, and vasoactive substances and oxidative stress cannot be blocked by IVF or ICSI in patients with endometriosis. It was reported that the fertilization capacity of oocytes and the developmental potential of embryos decreased when oocytes and embryos were cultured in media with peritoneal fluid obtained from women with endometriosis [18]. Inflammation within the ovary and endometriotic peritoneal fluid may impair oocyte and embryo development by blocking embryo growth factor receptor/signal transduction [19]. The inflammatory peritoneal fluid of women with endometriosis may also be toxic to the endometrium and the preimplantation embryo [20]. It has been also demonstrated that the eutopic endometrium of women with endometriosis is different from that of healthy women in terms of stem cell content, hormone sensitivity, cellular proliferation, adhesion, angiogenesis, and immunity. Progesterone resistance and defective endometrial receptivity impair the processes of implantation, decidualization, and placentation in women with endometriosis [21,22].

We did not find a higher rate of late pregnancy/obstetric complications and neonatal complications in women with small endometriomas. Benaglia et al. [9] reported that late pregnancy/obstetric and neonatal outcomes did not differ in women with small endometriomas (the mean diameter was 22 mm in their study) compared with women without endometriomas. Another study conducted in Japan likewise showed no relationship between endometriosis and poor obstetric outcomes in women who did not conceive using IVF/ICSI in order to exclude the negative effects of IVF on pregnancy outcomes [7].

However, recent systematic reviews and meta-analyses reported that obstetric complications such as preterm birth, placenta previa, cesarean section, and neonatal complications were more common in women with endometriosis [6,19]. The findings of previous studies have varied because all types of endometriosis were included. Moreover, earlier studies were not adjusted for confounders such as maternal age, poor obstetric history, parity, BMI, chronic disease, and socioeconomic status. The diagnosis of endometriosis was not uniform; in small studies, the diagnosis was made surgically, but in epidemiological studies the diagnosis was made using International Classification of Diseases codes, which have the potential of misclassification. The control groups were also heterogeneous in prior studies.

There are some limitations of this study; first, it was retrospective, which may have reduced the reliability of the findings. Second, the study size was relatively small. Third, we did not perform laparoscopy to confirm the endometriosis diagnosis histologically; instead, the endometriosis diagnosis was made by ultrasonography, and the patients were suspected to have endometrioma. However, the cases were small endometriomas and there was no need to perform surgery in order to improve pregnancy outcomes in these patients. Furthermore, we did not want to affect ovarian reserve negatively and increase patients' surgical and anesthetic risks. Fourth, the control group also did not undergo laparoscopy, so we cannot totally exclude the possibility that some women in the control group may have had mild endometriosis.

In conclusion, we showed that women with small endometriomas had higher rates of biochemical pregnancy and lower rates of clinical pregnancy and live birth; however, obstetric and neonatal complications did not differ between the groups. The findings suggest that women with endometriomas are more prone to early pregnancy complications than women with unexplained and tubal factor infertility. Women with small endometriomas undergoing ART should be counseled about these adverse aspects of their prognosis. Further prospective studies with larger groups are needed.

Conflict of interest

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

ORCID

Fatma Ferda Verit https://orcid.org/0000-0002-7104-4532
Ayse Seyma Ozsuer Kucukakca https://orcid.org/0000-0002-7464-0591

Author contributions

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

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Sperm retrieval by conventional testicular sperm extraction for assisted reproduction in patients with Zinner syndrome

Gianmartin Cito[1], Luca Gemma[1], Claudia Giachini[2], Elisabetta Micelli[2], Andrea Cocci[1], Rossella Fucci[2], Rita Picone[2], Simone Sforza[1], Gabriella Nesi[3], Raffaella Santi[3], Andrea Minervini[1], Lorenzo Masieri[1], Marco Carini[1], Maria Elisabetta Coccia[2,*], Alessandro Natali[1,*]

[1]Department of Urology, Careggi Hospital, [2]Assisted Reproductive Technology Centre, Careggi Hospital, and [3]Pathology Section, Department of Health Sciences, University of Florence, Florence, Italy

We present data from three Caucasian men with Zinner syndrome who attended our center for the treatment of primary couple's infertility. Each patient was scheduled for conventional testicular sperm extraction (cTESE) and cryopreservation. Sperm analysis confirmed absolute azoospermia. Patient 1 had right and left testis volumes of 24 mL and 23 mL, respectively; left seminal vesicle (SV) agenesis, severe right SV hypotrophy with right renal agenesis. Follicle-stimulating hormone (FSH) was 3.2 IU/L. Patient 2 exhibited right and left testis volumes of 18 mL and 16 mL, respectively; a left SV cyst of 32 × 28 mm, ipsilateral kidney absence, and right SV agenesis. FSH was 2.8 IU/L. Patient 3 showed a testicular volume of 10 mL bilaterally, a 65 × 46 mm left SV cyst, right SV enlargement, and left kidney agenesis. FSH was 32.0 IU/L. Sperm retrieval was successful in all patients. Nevertheless, cTESE should be performed on the day of oocyte retrieval.

Keywords: Azoospermia; Infertility; Testicular sperm retrieval; Wolffian duct anomalies; Zinner syndrome

Introduction

Zinner syndrome (ZS) is one of the rarest congenital anomalies of the urogenital tract. Due to its lack of symptoms, it is usually discovered and diagnosed in the second to fourth decades of life [1]. ZS can be considered a variant of the Wolffian duct anomalies, with a classic triad of seminal vesicle cyst, ipsilateral renal agenesis, and male infertility due to ejaculatory duct obstruction (EDO). The prevalence of cystic lesions in the pelvis with ipsilateral renal agenesis or dysplasia has been reported to be as high as 0.0046% [2]. Usually, this condition is also associated with other urogenital findings such as ureterocele, hypospadias, or abnormalities of the testes, epididymis, or adrenal glands [3,4]. The embryological origins of the kidneys and seminal vesicles are similar; the kidney is formed by the metanephric blastema, which is induced by the ureteral bud that originates from the dorsal aspect of the distal mesonephric duct. The mesonephric duct gives rise to most of the genital tract, including the epididymis, vas deferens, ejaculatory duct (ED), and seminal vesicles [5]. Thus, ZS can be considered the result of any malformation of the ureteral bud or mesonephric duct.

Patients with ZS are often asymptomatic, although aspecific signs may be present such as pelvic pain, painful ejaculation, urinary frequency, dysuria, urgency, constipation, recurrent urinary tract infections, or epididymitis [6]. As a consequence of the wide range of symptoms of this syndrome, the diagnosis of infertility can be de-

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Corresponding author: **Gianmartin Cito**
Department of Urology, Careggi Hospital, University of Florence, Largo Brambilla, 3, 50134 Florence, Italy
Tel: +39-0557949203 Fax: +39-0557949203 E-mail: gianmartin.cito@gmail.com

*These authors contributed equally to this work.

layed, and ZS requires prompt management upon proper diagnosis. Semen evaluation is unlikely to reveal absolute azoospermia, but commonly reveals obstructive azoospermia (OA), although secretory damage may also be present due to a long-lasting obstruction to sperm outflow [6]. While the surgical removal of seminal vesicle cysts [7] or transurethral resection of the ED (TURED) may improve sperm quality [8], testicular fine needle aspiration, epididymal percutaneous sperm aspiration, and testicular sperm extraction (TESE) may be crucial to obtain viable spermatozoa and allow a chance of conception.

The aim of this work was to describe the outcomes of surgical sperm retrieval (SSR) in infertile patients with ZS who underwent conventional TESE (cTESE) for assisted reproduction.

Case report

We collected data from three Caucasian men with ZS who attended our assisted reproductive technology (ART) center for the treatment of primary couple's infertility. Written informed consent to use their data anonymously was obtained. In accordance with the 2010 World Health Organization guidelines [9], two semen analyses revealed absolute azoospermia, low ejaculate volume (< 1 mL), and normal semen pH (Table 1) in all patients. The mean duration of infertility was 24 ± 3 months. No previous surgery, ongoing pathologies, or genetic abnormalities were reported. All patients were scheduled for multiple cTESE with cryopreservation.

Patient 1 was 27 years old. No history of cryptorchidism, delayed pubertal development, or scrotal-perineal discomfort was reported in his past medical records. His partner was 29 years old and did not report any gynecological diseases, except for a brief period of stress amenorrhea. Physical examination of the external genitalia detected a testicular volume of approximately 25 mL bilaterally, dilatation of the right vas deferens and epididymis, nonpalpable left vas deferens/epididymis structures, and no clinical signs of varicocele bilaterally. Scrotal and transrectal ultrasound (US) examinations showed right and left testis volumes of 24 mL and 23 mL, respectively; dilatation of the right caput epididymis, corpus, and cauda; and left seminal vesicle agenesis and right seminal vesicle severe hypotrophy. Transabdominal US revealed the absence of the right kidney as well as left nephroptosis. An abdominal contrast-enhanced computed tomography (CT) scan confirmed these findings.

In this patient, the follicle-stimulating hormone (FSH) level was 3.2 IU/L, the luteinizing hormone (LH) level was 5.2 IU/L, the total testosterone (TT) level was 22.3 nmol/L, the prolactin (PRL) level was 22 ng/mL, and the 17β-estradiol level was 0.12 nmol/L. SSR was successful: the sperm concentration was 0.01×10^6/mL, the sperm motility was 1%, and five straws were cryopreserved. Evaluation of the testicular histology showed a pattern of normal spermatogenesis, thickening of tubular membranes and interstitial edema (Figure 1), and no germ cell neoplasia *in situ* (GCNIS).

Patient 2 was 31 years old with no past medical history. His partner was 30 years old and had a history of ovarian endometriosis. Genital examination revealed testicular volumes of approximately 20 mL on the right and 18 mL on the left side, mild dilatation of the left vas deferens and epididymis, and marked dilatation of the right vas deferens and epididymis structures. US showed right and left testis volumes of 18 mL and 16 mL, respectively; a left seminal vesicle cyst with dimensions of 32 × 28 mm and fluid content; right seminal vesicle agenesis; and left renal agenesis.

The patient's levels of FSH, LH, TT, PRL, and 17β-estradiol were 2.8 IU/L, 4.2 IU/L, 14.3 nmol/L, 21 ng/mL, and 0.11 nmol/L, respectively. SSR was successful: the sperm concentration was 0.01×10^6/mL, the sperm motility was 1%, and four straws were cryopreserved. Histological assessment showed a pattern of normal spermatogenesis, without findings indicative of GCNIS.

Patient 3 was 27 years old, and his partner was 26 years old. Persistent scrotal-perineal pain and dysuria during the last 6 months were reported. The andrological examination showed a testicular volume of approximately 10 mL bilaterally. The patient's levels of FSH, LH, TT, PRL, and 17β-estradiol were 32.0 IU/L, 16.3 IU/L, 9.0 nmol/L, 21 ng/mL, and 0.12 nmol/L, respectively. US showed (1) an oval hypodense mass with dimensions of 65 × 46 mm and fluid con-

Table 1. Comparison of seminal parameters among patients

Variable	Patient 1	Patient 2	Patient 3	Normal range
Abstinence (day)	3	3	5	2–7
Color	Opalescent white	Opalescent white	Opalescent white	-
Viscosity	Normal	Normal	Normal	-
Liquefaction	Complete	Complete	Complete	-
Semen volume (mL)	0.2	0.3	0.6	> 1.5
Semen pH	7.4	7.2	7.6	7.2–7.8
Concentration (million/mL)	0	0	0	> 15
Leukocyte	< 1 million	< 1 million	< 1 million	< 1 million

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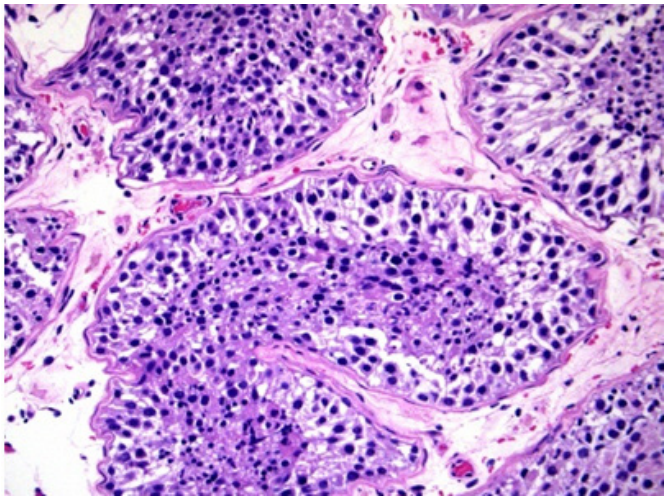

Figure 1. Cross-section of the seminiferous tubules with complete spermatogenesis surrounded by the tunica propria (H&E, ×200).

tent that impacted the bladder and the left paramedian prostatic region and was compatible with a left seminal vesicle pseudocyst; and (2) enlargement of the right seminal vesicle. Abdominal contrast-enhanced CT confirmed these data and also revealed left kidney agenesis (Figure 2). The patient underwent left robot-assisted laparoscopic vesiculectomy (Figure 3). At the 12-month follow-up visit, no sperm were found in the semen analysis. Therefore, conventional bilateral TESE was proposed. SSR was successful: the concentration was 0.001×10^6/mL, the sperm motility was 1%, and three straws were cryopreserved. Histological assessment indicated hypospermatogenesis without GCNIS.

Discussion

ZS is a rare condition that is usually diagnosed in adulthood during a couple's infertility assessment [10]. The diagnosis is mainly based on imaging examinations that reveal the typical cystic dilatations of the seminal vesicle and ipsilateral renal agenesis [11-14]. In our series, two patients had conditions resembling the classic ZS variant, while 1 (patient 1) had right seminal vesicle severe hypotrophy and an absence of the ipsilateral kidney. Given the presence of the triad of unilateral renal agenesis, ipsilateral seminal vesicle obstruction, and ipsilateral EDO, this clinical presentation can be considered a rare type of embryological malformation, included as a pseudo-ZS variant.

ZS is often asymptomatic until the age of peak sexual and reproductive activity, usually after repeated failure to achieve natural conception. Incomplete EDO, which occurs in approximately 4% of these patients, can cause seminal abnormalities, which create difficulties in diagnosis [15]. In our study, in two patients, the diagnosis was made

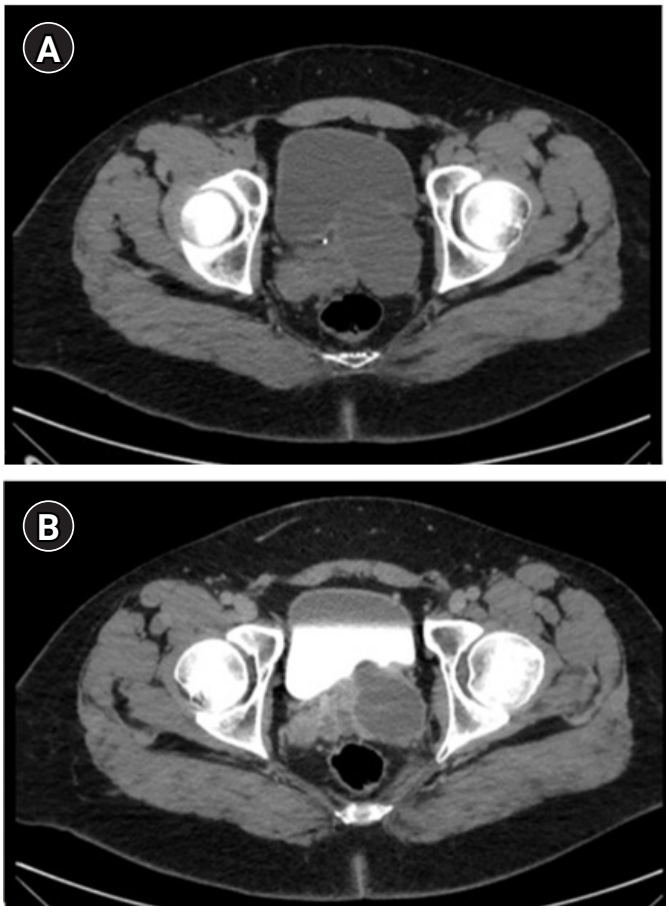

Figure 2. Abdominal contrast-enhanced computed tomography (CT) scan showing a left seminal vesicle pseudocyst. (A) Non-contrast CT. (B) CT urogram.

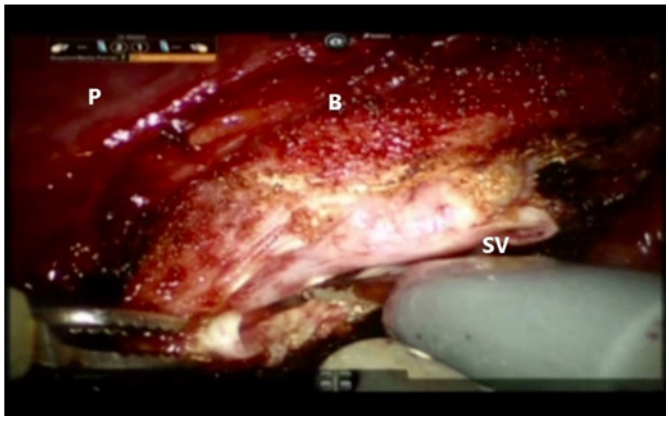

Figure 3. Robot-assisted laparoscopic vesiculectomy. A transversal incision of the peritoneum was performed, and the retrovesical region was sectioned using monopolar scissors and a bipolar Maryland dissector. A large cyst of the left seminal vesicle (SV) was isolated and liberated from the contiguous tissue. P, prostate; B, bladder.

based on the occurrence of infertility without any urological complaints. The findings of absolute azoospermia via seminal analysis required further diagnostic investigations, including abdominal, scrotal, and transrectal US. Only one patient (patient 3) reported scrotal-perineal pain and dysuria that required further examination. Given the broad spectrum of symptoms, the diagnosis of ZS can be delayed, which can further compromise the patient's reproductive potential.

From a pathophysiological standpoint, ZS can be associated with seminal tract obstruction, and secretory damage may occur due to the presence of a long-lasting blockage to sperm outflow [6]. Although TURED represents the first-line treatment in patients with EDO to restore fertility and improve sperm quality, testicular fine-needle aspiration, epididymal percutaneous sperm aspiration, or TESE are effective options to obtain viable spermatozoa. In the literature, the semen improvement and paternity rates have been recorded across multiple studies as 38% to 60% and 22% to 31%, respectively, after TURED [8]. Otherwise, SSR by testicular surgery in cases of OA usually has a success rate of around 90%–100% [16]. No patient described in this report underwent vesiculography and endoscopic procedures to enhance fertility. Robot-assisted laparoscopic vesiculectomy was performed in patient 3 to treat his clinical symptoms (chronic pelvic pain) due to the large seminal pseudocyst that affected the bladder. In that case, TESE was proposed at the 1-year follow-up visit, in the absence of semen quality improvement. Then, patients 1 and 2, according to their preference, decided to undergo TESE, given that they had already started an ART program with their partners.

Long-lasting obstruction (that is, that lasts for several years) has been proposed to potentially cause germ cell failure in the spermatogenetic tubules [6]. The elevated concentrations of macrophages and sperm fragments in the distal epididymis may affect the sperm quality [17]. Moreover, according to previous authors, higher levels of reactive oxygen species and sperm DNA fragmentation could mediate the reproductive toxicity [18]. In our patients, the presence of high levels of polyunsaturated fatty acids on the sperm membrane; the low quantities of glutathione peroxidase, superoxide dismutase, and cytoplasm; and the presence of inactive, highly condensed chromatin could have led to a reduction in fertilization competence, motility, and viability, as well as a loss of DNA repair capacity [19].

When we proposed cTESE to our patients, we questioned whether it was appropriate to carry out the procedure to obtain fresh sperm (on the same day as oocyte retrieval/intracytoplasmic sperm injection [ICSI]) or to plan sperm cryopreservation. The question arose from awareness that patients with ZS exhibit an embryological defect resulting from the abnormal development of the mesonephric or Wolffian duct with abnormal development of the ipsilateral upper urinary tract. We were not certain whether these patients may also exhibit spermatogenetic failure. Thus, although SSR in cases of OA usually has nearly a 100% success rate [20], we assumed that cases of ZS may also involve damage to the germ line associated with impaired spermatogenesis, which is why we preferred to schedule cryopreservation anyway.

The ART outcomes of surgically retrieved fresh and cryopreserved sperm for assisted reproduction remain a topic of debate. High fertilization and pregnancy rates have been reported in *in vitro* fertilization (IVF)/ICSI procedures using fresh sperm obtained by TESE in both patients with OA and those with non-OA [21,22]. Usually, cryopreservation decreases the number of viable sperm by at least 50% [23]. Moreover, many cryopreserved testis specimens from men with non-OA have been found to have insufficient motile sperm after thawing. Thus, the fertilization rate has been described to be higher when fresh motile sperm were used than when frozen-thawed sperm were used. Furthermore, higher spontaneous abortion rates after implantation have also been seen when immotile spermatozoa were used. According to the experience of practitioners at our center, using fresh rather than cryopreserved sperm optimizes the ICSI outcomes, as these outcomes may be influenced by the effects of freezing and thawing on the sperm membrane.

Several authors have demonstrated similar IVF/ICSI outcomes for both sperm-handling methods [24,25]. The rupture of the cell membrane due to osmotic stress and the formation of ice crystals in freeze-thaw cycles could facilitate the release, dissolution, and integration of DNA once the sperm is injected into the oocyte cytoplasm, leading to higher fertilization rates [24,26]. Given that diagnostic testicular biopsy may be considered obsolete, it is crucial to establish preoperatively all of the possible predictive factors of successful SSR. Although a fresh procedure could maximize the potential of retrieving viable spermatozoa, cryopreservation before ICSI could represent the treatment of choice, as it could obviate the possibility of unexpected testicular failure on the day of the IVF procedure.

This is the first study to describe the outcomes of SSR in patients with ZS. In our case series, sperm recovery by cTESE was successful in all patients. Although the coexistence of a defect in the germ line cells can be supposed, the histological findings demonstrated that spermatogenesis was normal in these patients. As the normal transport of sperm through the ductal system is altered by a congenital defect in ZS patients, surgical extraction remains an excellent option for the management of ZS.

In conclusion, in patients with ZS, it may be advisable to perform cTESE to obtain fresh sperm on the same day as oocyte retrieval. Sperm cryopreservation should be avoided, since the thawing procedure could further compromise the sperm quality. However, in the

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case of unsuccessful SR, oocyte freezing would avoid the nullification of controlled ovarian hyperstimulation and allow the use of the cryopreserved female gametes for a future IVF cycle via sperm donation. In this scenario, proven laboratory experience with oocyte thawing is key to successful ART outcomes.

Conflict of interest

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

ORCID

Gianmartin Cito https://orcid.org/0000-0001-7526-4025
Luca Gemma https://orcid.org/0000-0001-6576-3218

Author contributions

Conceptualization, Formal analysis, Methodology, & Data curation: GC, LG, CG, EM, AC, RF, RP, SS,GN, RS. Project administration: AM, LM, MC. Writing–original drafting: GC, LG. Writing–review & editing: MEC, AN.

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CASE REPORT
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CERM

Adenomyotic cyst mimicking a congenital Müllerian anomaly: Diagnosis and treatment with laparoscopy

Sangam Jha

Department of Obstetrics and Gynecology, AIIMS Patna, Bihar, India

A 28-year-old woman presented with a 1-year history of severe progressive dysmenorrhea following suction evacuation and tubal ligation. Sonography showed a bicornuate uterus with hematometra in the left horn. Hysteroscopy ruled out a diagnosis of a congenital Müllerian anomaly, as both ostia appeared normal. Under laparoscopy, a mass was seen on the left fundal region near the insertion of the round ligament, and needle aspiration of a chocolate-colored fluid confirmed the diagnosis of an adenomyotic cyst. The cyst was excised. The patient recovered well and has been symptom-free since surgery. Adenomyotic cyst is a rare entity in young women and must be differentiated from obstructive Müllerian anomaly. Laparoscopy is the preferred minimally invasive modality for managing this rare disorder.

Keywords: Adenomyotic cyst; Dysmenorrhea; Laparoscopy; Obstructive Müllerian anomaly

Introduction

Cysts in the myometrium are rare, and the differential diagnosis includes myoma with cystic degeneration, cystic adenomyoma, and obstructive Müllerian anomalies. Small cystic lesions (< 5 mm) can be associated with adenomyosis due to minor bleeding into the myometrium, but larger cysts (> 1 cm) are rare. In 1908, Cullen [1] first described cystic lesions filled with chocolate-colored fluid in the submucosa of patients with adenomyosis. These cysts were lined with endometrial glands. In 1990, Parulekar [2] first described adenomyotic cysts. These cysts are usually found in younger women with symptoms of chronic pelvic pain and/or dysmenorrhea. Due to the close clinical and radiological similarity of this condition to obstructive Müllerian anomalies, an accurate diagnosis of adenomyotic cysts poses challenges. Evidence from the literature supports surgical excision as the recommended treatment. We report a case of cystic adenomyosis that was diagnosed as bicornuate uterus with hematometra in the left horn on ultrasonography. The diagnosis was confirmed via hysteroscopy based on the visualization of two ostia; the presence of a normal uterine cavity ruled out a Müllerian anomaly, and laparoscopy showed a myometrial cyst located on the left cornu of the uterus. The cyst was laparoscopically excised with repair of the defect. The patient recovered well and has been symptom-free since surgery. This case report was written to explore the clinical features and treatment of an adenomyotic cyst in order to help facilitate the early diagnosis and appropriate treatment of this rare entity.

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Corresponding author: **Sangam Jha**
Department of Obstetrics and Gynecology, AIIMS Patna, Phulwarisharif, Patna, 801105 Bihar, India
Tel: +91-9827388001 E-mail: sangam.jha78@gmail.com

Case report

This study was performed in accordance with the principles of the Declaration of Helsinki. Ethical approval for this case report was waived by the Institutional Ethical Committee of AIIMS Patna. Informed consent was obtained from the patient for the publication of this report.

A 28-year-old woman (G4P2A2L2) presented with a 1-year history of severe progressive dysmenorrhea. She had experienced menarche at 14 years of age. The patient had no previous history of dysmenorrhea. Pain, localized at the left iliac region, typically started 2–3 days before the onset of menses and persisted for a week afterward. This

pain was refractory to analgesic drugs. The patient's menstrual cycle was regular (28 days/4–5 days). She had experienced two normal vaginal deliveries and one spontaneous abortion. The patient had a history of suction and evacuation (induced abortion) along with tubal ligation 1 year prior. Her symptoms appeared following that surgical intervention. Otherwise, the patient had no noteworthy past medical history.

Local examination revealed a mildly enlarged uterus with a small, slightly tender mass palpable on the left side of the uterus. Transabdominal ultrasound showed a bicornuate uterus with an anechoic collection measuring 3.3×1.2 cm in the left horn. The bilateral adnexa were normal (Figure 1). The patient refused magnetic resonance imaging (MRI) due to financial constraints. Thus, under a diagnosis of bicornuate uterus with left-horn hematometra, we proceeded with hysteroscopy and laparoscopic excision of the rudimentary horn. On hysteroscopy, the uterine cavity and the bilateral ostia appeared normal (Figure 2). On laparoscopy, a bulge of 3×3 cm was seen on the left cornu of the uterus (Figure 3A). The attachments of the bilateral Fallopian tubes and round ligament to the uterus were normal. To make a more precise diagnosis of the cystic collection, needle aspiration was performed, and approximately 7 mL of chocolate-colored fluid was aspirated from the mass (Figure 3B). The surgical procedure consisted of wide excision of the lesion followed by reconstruction of the defect (Figure 3C and D).

Intrauterine injection of methylene blue during the procedure revealed no communication between the lesion and the endometrial cavity. The patient was discharged on the day after surgery and was prescribed dienogest (2 mg) for 3 months. The patient did not experience dysmenorrhea upon menstruation. Biopsy of the lesion revealed a cyst lined by endometrial glands along with stroma and surrounding myometrial hyperplasia. Hemosiderin-laden macrophages were observed in the endometrial tissue. The findings were consistent with an adenomyotic cyst.

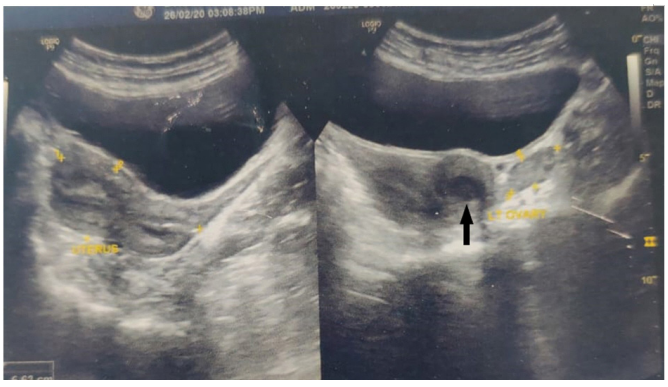

Figure 1. Sonographic finding of a bicornuate uterus with an anechoic collection measuring 3.3×1.2 cm in the left horn (black arrow).

Discussion

Adenomyosis is histologically defined as the invasion of the myo-

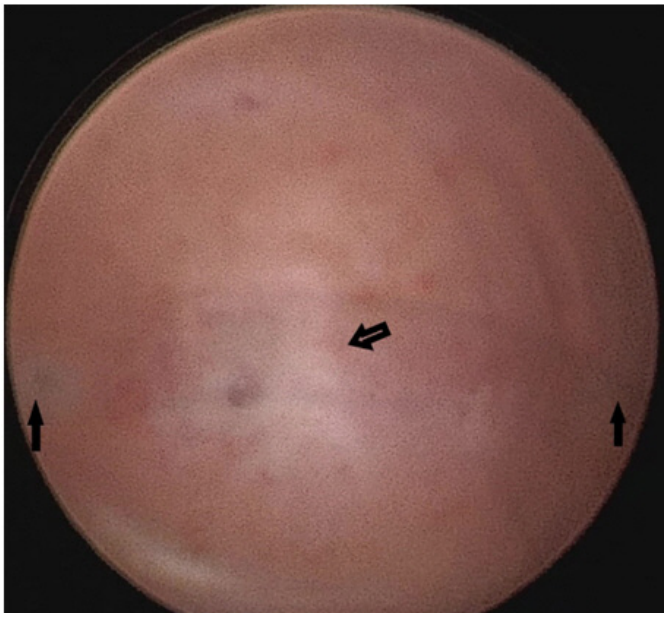

Figure 2. Hysteroscopic view showing bilateral ostia (solid arrows) with mild adhesion at the fundus (hollow arrow).

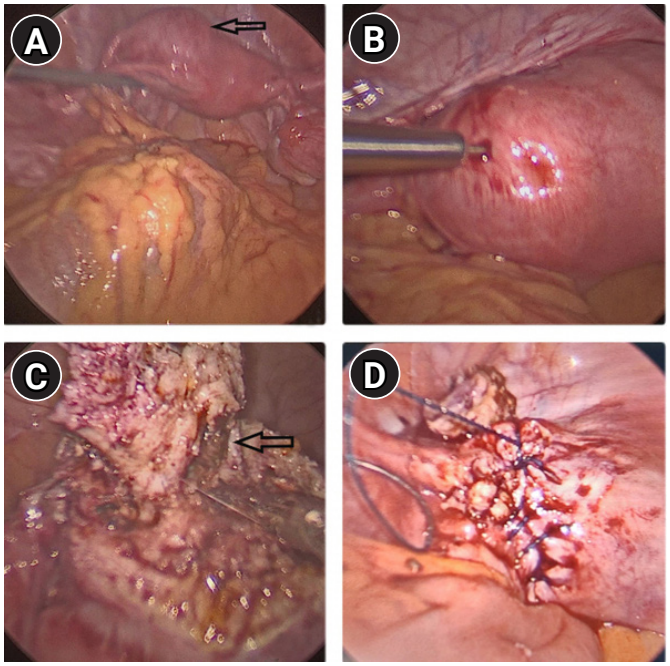

Figure 3. Laparoscopic view of the cystic lesion. (A) Uterus with an anterolateral adenomyotic cyst on the left side of the uterine fundus (arrow). (B) Aspiration of chocolate-colored fluid from the cyst. (C) At the time of cyst excision, a cystic cavity with chocolate-colored fluid was clearly seen (arrow). (D) The uterine myometrium was closed laparoscopically in two layers with continuous sutures.

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metrium by endometrial glands and/or stroma deeper than 2.5 mm from the endometrial-myometrial junction with accompanying surrounding myometrial hyperplasia. Adenomyosis is clinicohistologically classified into three categories: diffuse, with thickening of the junctional zone and diffuse myometrial involvement; focal, including well-circumscribed adenomyotic masses and cystic adenomyomas; and polypoid adenomyomas, both typical and atypical [3]. In 2015, Brosen et al. [4] classified adenomyotic cysts into five subtypes based on location. Subtype A1 includes submucous or intramural cystic adenomyosis, subtype A2 includes cystic polypoid lesions, subtype B1 includes subserous cystic adenomyosis, subtype B2 includes cases involving exophytic growth, and subtype C comprises uterine-like masses within the uterus also known as juvenile cystic adenomyomas (JCAs) or accessory and cavitated uterine masses. These cysts are lined by ectopic endometrium with thin stroma throughout the cyst, and the surrounding myometrium may exhibit hyperplasia [5]. The cysts are clinically characterized by intractable progressive dysmenorrhea owing to an increase in intracystic pressure as a result of estrogen-dependent cyclical bleeding and shedding of ectopic endometrium within the myometrium. The pathogenesis of these cystic myometrial lesions is uncertain. The juvenile form is considered a congenital anomaly that develops from the proliferation and persistence of Müllerian tissue at or near the insertion of the round ligament on the uterus and likely results from gubernaculum dysfunction [6].

In 2011, Chun et al. [7] proposed these diagnostic criteria for JCA: (1) an age of onset of < 18 years or severe dysmenorrhea developing within 5 years after the onset of menarche; (2) no history of uterine surgery; and (3) a diameter of the cystic cavity of > 5 mm. The adult form is hypothesized to result from injury to the endometrial-myometrial junction and invagination of the endometrial glands into the myometrium following uterine surgery [8]. However, a few investigators have described JCA as a cystic variant of adenomyosis rather than a congenital anomaly [9,10]. Based on the relevant literature, we consider the present case to be an acquired adenomyotic cyst, even though the location of the cyst was typical of JCA.

Diagnosis of this lesion may pose difficulties due to its clinical and radiological similarity to congenital Müllerian anomalies, especially the presence of a non-communicating rudimentary horn with unicornuate uterus or bicornuate uterus with segmental atresia. While adenomyosis is usually asymptomatic, adenomyotic cysts generally present with chronic pelvic pain or progressive dysmenorrhea. Transvaginal sonography is the first-line modality for the evaluation of intractable dysmenorrhea. However, the specific sonographic features of an adenomyotic cyst and its relationship with the endometrial cavity can be challenging to recognize. MRI, as a noninvasive modality with excellent tissue characterization capacity, is very useful for diagnosing such a lesion as well as differentiating it from complex uterine anomalies [11]. However, cases have been reported in which MRI failed to yield a correct diagnosis, so imaging also has its pitfalls [12]. Unfortunately, in the current case, we could not perform MRI due to financial constraints. Sonography indicated an obstructive Müllerian anomaly, but hysteroscopy was also performed to ascertain the accuracy of this diagnosis. Visualization of two ostia on hysteroscopy ruled out an obstructive Müllerian anomaly.

Zhou et al. [13] reported a case of a giant adenomyotic cyst with a levonorgestrel-containing intrauterine device present in the cyst cavity in a 46-year-old woman. The cyst had developed following myomectomy. The case was managed with laparoscopic excision of the mass along with removal of the intrauterine device. Similar cases have been reported in which patients developed cysts after some form of uterine surgery [14,15], hence supporting the hypothesis of endometrial-myometrial junctional injury as an adenomyotic cyst precursor. In the present case, too, the patient experienced progressive dysmenorrhea following suction and curettage.

The treatment of an adenomyotic cyst involves wide surgical excision, though the symptoms can be temporarily relieved by the use of gonadotropin-releasing hormone, cyclical oral contraceptives, or analgesics. As minimally invasive methods have many advantages, laparoscopy is the route of choice. Hysteroscopic resection of the lesion is the preferred mode of treatment for the submucosal subtype [14]. In the present case, we performed laparoscopic radical excision of the cyst.

To conclude, cystic adenomyosis of the uterus is rare. Diagnosis poses a challenge, as this condition is usually misdiagnosed as an obstructive Müllerian anomaly, a degenerated myoma, or (rarely) as an adnexal cyst. Previous uterine surgery and injury to the endometrial-myometrial junction are precursors of the disease pathology. Radical excision of the lesion is the definitive and preferred mode of treatment. No clinical data are available on whether postoperative medication can effectively prevent recurrence of this condition. Thus, research is urgently required regarding how to effectively detect and treat intrauterine cystic adenomyosis and to develop methods to prevent its recurrence.

Conflict of interest

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

ORCID

Sangam Jha https://orcid.org/0000-0002-4349-1589

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CERM

Acknowledgment to reviewers for 2020

On behalf of the editor and editorial board, we would like to express our sincere gratitude for the reviewers and contributors who have generously endowed with their time and efforts in appraising the manuscripts submitted to Clinical and Experimental Reproductive Medicine in 2020. It was their help that made it possible for the journal to achieve a new level of caliber and rigor.

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INSTRUCTIONS TO AUTHORS

CERM

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I. ABOUT THE JOURNAL

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

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.

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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.

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

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Editor-in-Chief, Clinical and Experimental Reproductive Medicine
Address: Department of Obstetrics and Gynecology, Seoul NationalUniversity 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 preiv pared with an MS-word or RTF format. The manuscript should be written in 11-point font with double-line spacing on A4 (21.0 × 29.7 cm) or letter (8.5 × 11.0 in) sized paper with 2.5 cm (1.0 in) margins.
- All manuscript pages are to be numbered at the upper right corner consecutively, beginning with the title page as page 1.
- 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.
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- Include presentation history at a meeting.

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

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Examples of references

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

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

(3) **Book**

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

(4) **In press**

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

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

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

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IX. MANUSCRIPT ACCEPTED FOR PUBLICATION

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Product Information

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