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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 ocial journal of Pacic Society for Reproductive Medicine, the Korean Society for Reproductive Medicine and Korean Society for Assisted Reproduction. Abbreviated title is *Clin Exp Reprod Med*. The aims of CERM are to publish the high quality articles that facilitate the improvement of the current diagnosis and treatment in couples with reproductive abnormalities through human or relevant animal model research. Its scope is the infertility, reproductive endocrinology, urology, andrology, developmental biology of gametes and early embryos, basic reproductive science, reproductive physiology, reproductive immunology, genetics and biology of stem cell.

Background

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

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REVIEW ARTICLE

ORIGINAL ARTICLES

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CERM

Female reproduction and abnormal uterine bleeding after COVID-19 vaccination

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Coronavirus disease 2019 (COVID-19) vaccines have been widely administered throughout the global community to minimize the morbidity and mortality caused by the COVID-19 pandemic. Although generally well-tolerated, these vaccines have generated some unwanted consequences, including thrombosis and menstrual irregularities. The effect of vaccination on female reproductive function has also been questioned. The aim of this review is to give readers a clear understanding of the effects of COVID-19 vaccines on thrombosis, reproductive function, and menstrual irregularities by systemically analyzing the available literature. The available evidence suggests that COVID-19 vaccines have a minimal impact on ovarian reserve. Furthermore, *in vitro* fertilization outcomes after COVID-19 vaccination remain unimpaired compared to those who did not receive the vaccines. Current evidence supports a certain degree of impact of COVID-19 vaccines on the menstrual cycle, with the most frequent alteration being menstrual irregularity, followed by menorrhagia. These changes are generally well-tolerated and transient, lasting less than 2 months. This review, by providing information with up-to-date references on this issue, may enhance readers' understanding of the impact of COVID-19 vaccines on female reproductive function and the menstrual cycle.

Keywords: COVID-19; Fertilization *in vitro*; Menstruation; Menstruation disturbances; Vaccination

Introduction

Since its outbreak in 2019, the coronavirus disease 2019 (COVID-19) pandemic has swept through the globe with catastrophic consequences. According to the World Health Organization (WHO), as of December 23, 2022, there have been 660.75 million confirmed cases of COVID-19 and 6.69 million deaths worldwide [1]. The deleterious effects of COVID-19 have led to the development of vaccines to halt the disease's rapid spread worldwide and minimize its impact. COVID-19 vaccination began in December 2020, almost a year after the pandemic began. Several COVID-19 vaccines have been validated for use by the WHO. The most prominent makers of vaccines are Pfizer-BioNTech, Moderna, Oxford-AstraZeneca, and Johnson & Johnson (J&J)/Janssen.

Consequently, the scientific community has expanded its scope of interest from the pandemic itself to the adverse effects of vaccines. There have been many investigations regarding the relationship between COVID-19 vaccination and female reproductive health. This research is critical, as it serves as a basis for altering the health-related behaviors of the general population, with potential impacts on overall health outcomes, and as evidence for many governmental policies.

The Korean Specialized Committee for the compensation of loss after COVID-19 vaccination has decided to include abnormal uterine bleeding (AUB) as a "suspected related symptom" after COVID-19 vaccination on August 16, 2021, for all vaccine types, including those manufactured by Oxford-AstraZeneca, Moderna, Pfizer-BioNTech, and J&J. This decision has enabled those who developed AUB after vaccination for COVID-19 to claim compensation from the committee and receive support. The scientific basis for this decision was the analysis by the COVID-19 Vaccine Safety Committee of the National Academy of Medicine of Korea. After a comprehensive analysis of

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domestic and international data on adverse reactions reported after COVID-19 vaccination, the committee has announced the discovery of a statistically significant association between AUB and COVID-19 vaccination, which is sufficient evidence to establish a causal relationship.

This article specifically focuses on the effect of COVID-19 vaccines on female reproductive health, including thrombosis, ovarian function, *in vitro* fertilization (IVF) outcomes, and the menstrual cycle.

COVID-19 vaccination and thrombosis

In 2021, Schultz et al. [2] reported five healthcare workers who experienced thrombosis after administration of the ChAdOx1 nCov-19 vaccine (AstraZeneca). In the same year, Scully et al. [3] reported thrombosis in 23 patients after receiving the same vaccine.

The adverse events of special interest after the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccine include deep vein thrombosis, disseminated intravascular coagulation, and pulmonary embolism. The reported thrombotic conditions mainly include venous thrombosis, with the most common location being cerebral venous sinus thrombosis and cerebral venous thrombosis. Other conditions include arterial thrombosis, organ thrombosis, infarction, thrombophlebitis, thrombotic microangiopathy, and transient ischemic attack [4].

1. Incidence

The reported incidence of thrombosis after vaccination is very low (0.00006% to 0.005%). No significant difference in incidence has been reported according to the type of vaccine [5-8]. However, inconsistencies exist in this regard; for instance, in another report, thrombotic events occurred most frequently with the Pfizer vaccine (55.4%) than with the Moderna (10.5%) and AstraZeneca (29.6%) vaccines [8]. In a systemic review by Al-Ali et al. [9], a higher incidence of thrombosis as a vaccine adverse event was noted for the AstraZeneca vaccine. That study analyzed 460 thrombotic events, and found that 9.8% were from the Pfizer-BioNTech vaccine, 81.5% from the AstraZeneca vaccine, and 8.7% from the J&J Janssen vaccine [9].

There is no apparent trend according to sex in the occurrence of thrombotic events after vaccination among studies; however, in a study by Tobaiqy et al. [7], more female patients than male patients experienced thrombotic events. In another meta-analysis investigating the relationship between COVID-19 vaccines and thrombosis, a combined analysis of the population that received the AstraZeneca vaccine showed a female preponderance compared to those who received other vaccines [10].

2. Mechanisms

One of the proposed mechanisms of thrombosis after vaccination is the formation of a complex between vaccine-induced antibodies and platelet factor 4, which may lead to a hypercoagulable state with platelet depletion. Other possible mechanisms include adenoviral vectors from vaccines acting as a possible aggregating agent with platelets, causing thrombosis [2,10].

COVID-19 vaccination and ovarian function

Concerns have been raised, albeit without scientific evidence, about the negative impact of COVID-19 vaccines on female fertility. The COVID-19 pandemic has changed the behaviors of the fertile population trying to conceive. However, there is a lack of scientific evidence proving the negative impact of the COVID-19 vaccine on pregnancy outcomes [11]. To date, there is no evidence that the COVID-19 vaccine affects female infertility. The American Society for Reproductive Medicine issued an updated a guidance document in December 2020 stating that individuals who are planning to conceive are not recommended to refrain from receiving COVID-19 vaccination [12].

1. Animal studies

An experiment using 44 female rats that were administered the BNT162b2 mRNA vaccine (Pfizer-BioNTech) was conducted to evaluate its safety regarding reproductive function and pregnancy. The vaccine showed no adverse effect on reproductive function, pregnancy, delivery, or the development of offspring. The pregnancy rate of rats in both groups was similar, with a 95% pregnancy rate in the vaccine group and a 98% rate in the control group [13]. Nonetheless, the nature of an animal study necessitated further research on the direct effect of COVID-19 vaccination on human ovarian function.

2. Human studies

Ovarian function in humans refers to the ability to produce a mature ovum to be fertilized with sperm for the production of offspring, as well as the ability to produce sex hormones. For the former definition, the term "ovarian reserve" is used to assess and predict the remaining ovarian function in women of reproductive-age. Anti-Müllerian hormone (AMH), a glycoprotein of the transforming growth factor-beta family produced by granulosa cells of the ovary, best serves the purpose of estimating the functional ovarian reserve, as noted by many researchers [14,15]. Another powerful tool for assessing the ovarian reserve is the antral follicle count (AFC), which, like AMH, is also closely correlated with age and declines with impending menopause [16,17].

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The AFC of the vaccinated and unvaccinated populations has been reported to be similar in most studies. In a small cohort study that investigated the effect of the Pfizer-BioNTech vaccine on reproductive function, the mean AFC of the vaccinated, COVID-19-positive, and control groups before the start of IVF were all similar [18]. Another study with a larger population showed that the AFC of the population vaccinated with either Pfizer or Moderna vaccines was comparable to that of the unvaccinated population, with no statistically significant difference [19]. For CoronaVac or Sinopharm, which are inactivated vaccines, the vaccinated group had an AFC of 14.5 while the unvaccinated group had an AFC of 16, which was a statistically significant difference [20]. However, that research was subject to limitations since it was a retrospective observational study of different groups, without adjustment for age or surgical history. Moreover, in the same study, the IVF outcomes, including the ovarian response and retrieved oocytes, were comparable after propensity score matching. In contrast to previous research that reported differences in the AFC, another study investigating the effects of inactivated vaccines demonstrated that the AFC was similar in the vaccinated and unvaccinated populations after propensity score matching [21]. Lastly, Requena et al. [22] evaluated the effects of various types of vaccines in comparison with unexposed groups. The AFC was comparable across populations vaccinated with different types of vaccines. The parameters of the same patients before and after vaccine administration were also compared, and the pre-vaccination and post-vaccination AFCs were consistently similar.

Three studies investigated differences in AMH levels between vaccinated and unvaccinated patients. Aharon et al. [19] observed no difference in AMH values between vaccinated (with either the Pfizer or Moderna vaccine) and unvaccinated populations (2.9 ± 2.9 vs. 2.7 ± 2.6, $p = 0.38$). A study by Wu et al. [20] also found no significant difference in AMH levels between vaccinated and unvaccinated populations. In this study, the AFC of the vaccinated group was slightly lower than that of the unvaccinated group, as previously mentioned. However, AMH levels, which constitute a more accurate measure of ovarian reserve, were similar, highlighting that vaccine administration was not associated with impaired ovarian reserve. Lastly, a more recent publication by the same group that investigated frozen embryo transfers reported comparable AMH levels between vaccinated and unvaccinated populations [23].

COVID-19 vaccination and IVF outcomes

1. Anti-SARS-CoV-2 immunoglobulin G level

Positive results were found for the serum anti-SARS-CoV-2 immunoglobulin G in those who had received vaccination or were infected with COVID-19, and the levels correlated with follicular fluid. Odeh-Natour et al. [24] analyzed the impact of the Pfizer-BioNTech vaccine on IVF treatment outcomes. Patients were classified based on their anti-spike (S) and anti-nucleotide (N) levels. After controlled ovarian hyperstimulation (COS), vaccinated, previously infected, and all-negative patients had similar numbers of follicles and mature oocytes, as well as comparable fertilization, cleavage, and pregnancy rates [24].

2. COS outcomes

Details of the previous studies are summarized in Table 1. Several studies have investigated the effect of the Pfizer and Moderna vaccines, and no statistically significant differences were observed in the oocyte number, mature oocyte number, fertilization rate, or blastocyst formation rate [19,22,25,26]. Likewise, no significant differences were found in follicle- stimulating hormone (FSH) or human menopausal gonadotropin (hMG) doses and the length of stimulation. For adenoviral vaccines such as Janssen and AstraZeneca, there were no significant differences in total doses of FSH or hMG, stimulation days, oocyte numbers, mature oocyte numbers, fertilization rates, or blastocyst formation rates [22]. For inactivated vaccines such as CoronaVac manufactured by Sinovac, no differences were observed in ovarian stimulation profiles and IVF outcomes [20,21,23].

Two studies used historical controls to compare IVF outcomes—in other words, the same population was studied before and after vaccination [22,25]. Both studies showed no differences in patients' IVF outcomes before and after vaccinations Vaccine administration did not increase the aneuploidy rate. A study investigating the effect of preimplantation genetic testing for aneuploidy in the vaccinated and unvaccinated populations found that the euploidy rate was similar between vaccinated and unvaccinated populations after multivariable linear regression and adjusted analysis [19].

3. Pregnancy outcomes

Studies with Pfizer, AstraZeneca, Janssen, Moderna, and Sinopharm vaccines have shown similar pregnancy rates between vaccinated and unvaccinated patients [19-21,24,27,28]. The implantation rates and miscarriage rates were also similar. The vaccine did not increase complication rates after IVF and embryo transfer. Two studies investigated the ectopic pregnancy rate, which was similar among the two groups [20,23]. A study found no significant difference in the incidence of ovarian hyperstimulation syndrome (OHSS) between vaccinated and unvaccinated groups [20].

Furthermore, the interval between vaccine administration and embryo transfer did not affect pregnancy outcomes. Pregnancy rates were similar when patients were classified based on their time from

Table 1. Studies investigating the effects of COVID-19 vaccines on IVF-ET

Study	Country	Study design	No. of patients	Vaccine type	Dosage, interval between vaccination and IVF-ET	No. of oocytes	No. of mature oocytes	Fertilization rate (%)	Blastulation rate (%)	Implantation rate (%)	Clinical pregnancy rate (%)	Miscarriage rate (%)
Aharon et al. (2022) [19]	USA	Retrospective cohort study	COS: Vaccinated (n = 222) Unvaccinated (n = 983) ET: Vaccinated (n = 214) Unvaccinated (n = 733)	Pfizer-BioNTech, Moderna	2 doses of vaccines 14 days before start of IVF	15.9 (14.4–17.5) vs. 15.0 (14.4–15.6) (NS) (vaccinated vs. unvaccinated)	12.2 (11.0–13.3) vs. 11.2 (10.7–11.7) (NS)	80.7 (78.4–83.0) vs. 78.7 (77.5–80.0) (NS)	62.9 (59.4–66.4) vs. 60.0 (58.2–61.7) (NS)	NA	59.5 (52.7–66.3) vs. 63.7 (60.2–67.3) (NS)	18.0 (11.1–24.9) vs. 12.0 (9.0–15.0) (NS)
Avraham et al. (2022) [26]	Israel	Retrospective cohort study	Vaccinated (n = 200) Unvaccinated (n = 200)	Pfizer-BioNTech	2 doses, at least 2 weeks before ovarian stimulation	8.47 (7.52–9.42) vs. 8.32 (7.38–9.27) (NS) (vaccinated vs. unvaccinated)		64.81 (60.6–68.93) vs. 61.98 (57.37–66.60)			32.8 vs. 33.1 (NS)	
Bentov et al. (2021) [18]	Israel	Prospective cohort study	Recovering from COVID-19 (n = 9) Vaccinated (n = 9) Uninfected and non-vaccinated (n = 14)	Pfizer-BioNTech	1–2 doses, mean 32.2 days (to oocyte retrieval day)	12.4 ± 8.7 vs. 10.89 ± 4.8 vs. 11.2 ± 6.7 (NS) (vaccine vs. COVID-19 vs. control)	7.25 ± 2.77 vs. 8.37 ± 4.1 vs. 7.75 ± 4.7 (NS)					
Brandao et al. (2022) [28]	Spain	Retrospective cohort study	Vaccinated (n = 890 ETs) Non-vaccinated (n = 3,272 ETs)	Pfizer-BioNTech, Moderna	1–2 doses, median 3.2 months (vaccination to ET)						70.4 vs. 70.6 (NS)	
Cao et al. (2022) [23]	China	Retrospective cohort study	Vaccinated (n = 502) Non-vaccinated (n = 1,589)	Inactivated vaccines (Sinopharm, Sinovac)	1–2 doses, median time interval 117.5 days (vaccination and FET)						54.7 vs. 54.2 (NS)	11.3 vs. 11.4 (NS)
Huang et al. (2022) [21]	China	Retrospective cohort study	Vaccinated (n = 146) Unvaccinated (n = 584)	Sinopharm or Sinovac	2 doses, mean time interval 72.4 days	9.9 ± 7.1 vs. 9.9 ± 6.7 (NS) (vaccinated vs. unvaccinated)	8.3 ± 6.1 vs. 7.9 ± 5.6 (NS)	71.1 ± 23.3 vs. 70.2 ± 23.9 (NS)	74.5 ± 30.5 vs. 71.6 ± 31.3 (NS)	45.4 vs. 46.7 (NS)	59.1 vs. 63.6 (NS)	NA
Odeh-Natour et al. (2022) [24]	Israel	Prospective cohort study	Vaccinated (n = 37) Unvaccinated (n = 22)	Pfizer-BioN-Tech	2 doses, 2–8 weeks after the second vaccination	10.05 ± 7.6 vs. 12.3 ± 9.11 vs. 11.89 ± 9.67 (NS) (positive anti-S/ positive anti-N/ negative anti S, N)	6.13 ± 4.66 vs. 4.66 ± 3.70 vs. 8.2 ± 6.5 (NS)	49/60/58 (NS)			44/33/50 (NS)	

(Continued to the next page)

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Table 1. Continued

Study	Country	Study design	No. of patients	Vaccine type	Dosage, interval between vaccination and IVF-ET	No. of oocytes	No. of mature oocytes	Fertilization rate (%)	Blastulation rate (%)	Implantation rate (%)	Clinical pregnancy rate (%)	Miscarriage rate (%)
Orvieto et al. (2021) [25]	Israel	Observational study	36 (historical control of same patients)	Pfizer-BioNTech	2 doses, 7–85 days from second vaccine to IVF treatment	9.7 ± 6.7 vs. 10.1 ± 8 (NS) (pre-vaccination vs. post-vaccination)	7.94 ± 5.7 vs. 8.0 ± 6.5 (NS)					
Requena et al. (2023) [22]	Spain	Retrospective observational Study	Vaccinated (n = 510) (Janssen n = 31, AstraZeneca n = 38, Pfizer-BioNTech n = 336, Moderna n = 105) Unexposed (n = 1,190)	Janssen, AstraZeneca, Pfizer-BioNTech, Moderna	2 doses, average of 2 months after the second dose	9.2/7.7/9.8/8.8/10.2 (NS) (AstraZeneca/Janssen/ Moderna/Pfizer/ Unvaccinated)	6.7/5.8/8.3/ 7.2/8.5 (NS)	80/78/70/81/75 (NS)	41.1/45.5/40.9/ 42.0/45.2 (NS)			
Wu et al. (2022) [20]	China	Retrospective cohort study	Vaccinated (n = 239) Unvaccinated n = 928 (after PSM)	Sinopharm CoronaVac	1–2 doses, median 31–60 days to ovarian stimulation	8 (5–12) vs. 9 (5–12) (NS) (vaccinated vs. unvaccinated)	NA	80.0 vs. 79.7 (NS)	48.9 vs. 49.3 (NS)	35.4 vs. 38.3 (NS)	44.4 vs. 47.4 (NS)	15.0 vs. 12.1 (NS)
Zhao et al. (2023) [27]	China	Retrospective cohort study	Vaccinated (n = 781) Unvaccinated (n = 1,851)	Inactivated vaccines	Varies (≤ 3 or > 3 months)						47.5 vs. 47.7 (NS)	Clinical pregnancy loss rate: 12.6 vs. 11.8 (NS)

COVID-19, coronavirus disease 2019; IVF, *in vitro* fertilization; ET, embryo transfer; COS, controlled ovarian hyperstimulation; NS, not significant; NA, not available; PSM, propensity score matching.

vaccination to embryo transfer into four quartiles, with a median of 3.2 months [28]. A recent study by Zhao et al. [27] compared pregnancy rates among patients classified based on the time interval between vaccination completion and embryo transfer. Individuals vaccinated with time intervals of less than 3 months and more than 3 months had similar clinical pregnancy and clinical pregnancy loss rates [27].

In summary, the IVF outcomes after vaccination with mRNA vaccines (Pfizer and Moderna), adenoviral vaccines (Janssen and AstraZeneca), or inactivated vaccines (Sinopharm) are comparable with those of unvaccinated individuals. The pre- and post-vaccination IVF outcomes are also similar. According to currently available data, rates of euploidy, OHSS, and ectopic pregnancy all seem comparable between vaccinated and unvaccinated populations. The time interval between vaccine administration and embryo transfer does not seem to interfere with pregnancy outcomes.

COVID-19 vaccination and the menstrual cycle

1. Incidence

The incidence of menstruation-related changes after COVID-19 vaccination varies significantly among published studies. A retrospective study in Saudi Arabia investigated patients who received either the Pfizer or AstraZeneca vaccine. An abnormal menstrual cycle was reported by 0.69% of those vaccinated with the Pfizer vaccine and 0.45% of those who received the AstraZeneca vaccine [29]. In an African study where individuals were vaccinated with mostly AstraZeneca (77.8%, followed by Pfizer [9.1%]), menstrual disorders were reported in 0.5% of patients [30]. A Chinese study that investigated the side effects of healthcare workers after administration of the inactivated vaccine (Sinopharm) showed menstrual changes in 2.1% [31]. The most frequent type of menstrual change was menstrual delay, followed by early menstruation. In some reports, the percentage tended to be higher when the reproductive-age population was targeted. One study showed that when the population was narrowed down to menstruating, reproductive-age women, 4.8% of patients reported menstruation-related symptoms [32].

2. Mechanisms

A proposed mechanism for COVID-19 vaccination-induced alterations in the menstrual cycle is that vaccination may function as a potential stressor to the human body, disrupting the hypothalamic-pituitary-ovarian axis [33]. Furthermore, immunological or inflammatory reactions following vaccine administration may play a role, interfering with menstrual homeostasis and creating hormonal disruptions [34]. Heavy menstrual bleeding may be due to the increased bleeding tendency after COVID-19 vaccination [35]. The specific mechanisms underlying the clinical symptoms may need further investigation and validation.

3. Menstrual irregularities

Studies have reported a higher incidence of menstrual irregularities after COVID-19 vaccination. A prospective cohort study by Edelman et al. [36] recruited volunteers to investigate the effects of COVID-19 vaccines on menstrual symptoms (n = 545) and found that 25% of patients reported changes in their menstrual cycle. Another similar study reported that about 50% to 60% of women of reproductive-age had menstrual irregularities [34]. An Israel survey-based study that enrolled only pre-menopausal non-pregnant patients (n = 7,904) reported that 47.2% of patients had changes in menstrual patterns [35]. These discrepancies in the incidence of menstrual side effects after the vaccine may be because larger population-based studies include all female patients regardless of their menstrual status or reproductive function, and specific information on incidence in tailored populations is unavailable. The short duration of surveillance, up to about a week in a larger population study, may have also had an impact. Additionally, there is a selection bias for recruiting those who participate in questionnaires related to menstrual irregularities; reproductive-age women with a keen interest in their menstrual patterns are more likely to be included in the questionnaire cohort.

COVID-19 vaccines influence the interval between cycles, the duration of the cycle, and the severity of symptoms, including menorrhagia and dysmenorrhea. Among various features presented as menstruation-related side effects of COVID-19 vaccines, the most commonly reported symptom is menstrual irregularity. In a study by Wong et al. [37] that analyzed 5,975,363 text responses entered into the V-safe surveillance application administered by the government, 1% (n = 62,679) reported menstrual irregularities or vaginal bleeding as a complication after COVID-19 vaccination. The most common theme was the timing of menstruation (83.6%), followed by menstrual symptom severity (67.0%). The reported symptoms were mainly within 0 to 7 days after vaccination [37]. In another study by Farland et al. [38], 25% of 545 individuals reported menstrual cycle changes after vaccination, and the most common change was irregular menstruation (43%). Rodriguez Quejada et al. [39] reported that among 184 patients with menstrual alterations after vaccination of various types, 42.9% had menstrual irregularities.

Regarding the quantification of menstrual cycle irregularities, Edelman et al. [36] reported that the change was less than a day. In a prospective cohort study including 3,959 patients, the authors showed that the cycle length increased by 0.71 days after the first dose and 0.91 days after the second dose, with statistical significance. There was no significant change in the duration of menstruation [36]. In a survey study of 164 women by Lagana et al. [34], the

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participants reported varying frequencies and duration of menstruation. After the first dose of the COVID-19 vaccine, 18% to 33.3% of women reported menstruation 1 to 5 days earlier than expected. Regarding the length of menstruation, 16% to 67% of women reported that menstruation lasted for more than 7 days, while 11% to 37% reported that it lasted for less than 3 days. After the second dose, the trend of the answers was similar; although variation existed, the participants tended to have earlier (12% to 38%), longer (21% to 50%), and heavier (28% to 62%) menstruation [34].

4. Menstrual severity

Menorrhagia has also been commonly reported after COVID-19 vaccination. An Israeli survey of 7,904 pre-menopausal non-pregnant women by Issakov et al. [35] reported that 80.6% of those who reported menstrual alterations had menorrhagia, the most common menstrual problem encountered by the COVID-19 vaccinated population. In a study by Wong et al. [37], menstrual severity, or heavy bleeding, was the second most commonly reported event (67.0%). Another retrospective study reported heavy menstrual bleeding among 41.8% of 184 patients with menstrual alterations [39]. Other reported problems include a higher frequency of premenstrual symptoms in 34% of the population and increased dysmenorrhea in 30%. Amenorrhea was less common but reported in 3% to 11% of the population [39].

5. First dose vs. second dose

Conflicting results have been reported on whether menstruation-related side effects are more severe with the first or second dose. Farland et al. [38] and Lagana et al. [34] reported a higher frequency of menstruation-related side effects after the second dose. In the former study, 56% of participants reported alterations after the second dose and 18% after the first dose. In the latter study, 60% to 70% of women reported menstrual irregularities after the second dose, whereas only 50% to 60% reported menstrual irregularities after the first dose. However, Muhaidat et al. [40] reported more frequent symptoms after the first dose, with 46.7% reporting symptoms after the first dose and 32.4% after the second dose. The type of vaccine seems unrelated, as studies comparing menstruation-related symptoms in populations with different vaccines did not show inter-group differences [34,39].

Interestingly, according to a cross-sectional survey by Muhaidat et al. [40], menstrual alterations after the COVID-19 vaccine did not seem to be affected by underlying diseases such as polycystic ovarian syndrome, thyroid disease, myomas, endometriosis, and adenomyosis. Several studies have shown that menstrual changes are only transient, usually resolving within 2 months [34,40].

Conclusion

Although COVID-19 vaccines have been proven to be generally safe, they may produce unwanted consequences. The main findings of this review are as follows:

Although rare, thrombosis may occur after vaccine administration (incidence, 0.00006% to 0.005%).

The impact of COVID-19 vaccines on ovarian reserve (AMH and AFC) is minimal.

IVF outcomes are not impaired after COVID-19 vaccine administration.

COVID-19 vaccines certainly seem to affect the menstrual cycle; however, the effects are generally well-tolerated and transient. The most frequently reported problems are menstrual irregularities, followed by menorrhagia. However, the findings of recent studies are generally reassuring, as symptoms resolve within about 2 months.

It remains unclear whether certain groups are particularly vulnerable to menstruation-related adverse events following COVID-19 vaccination. The natural pregnancy rates following vaccination also remain unclear. Further investigations may help the scientific community understand the remaining questions regarding the effects of COVID-19 vaccines on female reproductive function and menstruation.

Conflict of interest

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

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References

1. World Health Organization. WHO coronavirus (COVID-19) dashboard [Internet]. WHO; 2023 [cited 2023 May 3]. Available from: https://covid19.who.int/
2. Schultz NH, Sorvoll IH, Michelsen AE, Munthe LA, Lund-Johansen

F, Ahlen MT, et al. Thrombosis and thrombocytopenia after ChAdOx1 nCoV-19 vaccination. N Engl J Med 2021;384:2124–30.
3. Scully M, Singh D, Lown R, Poles A, Solomon T, Levi M, et al. Pathologic antibodies to platelet factor 4 after ChAdOx1 nCoV-19 vaccination. N Engl J Med 2021;384:2202–11.
4. Center for Biologics Evaluation and Research Office of Biostatistics and Epidemiology. CBER Surveillance Program: background rates of adverse events of special interest for COVID-19 vaccine safety monitoring protocol 2021 [Internet]. FDA; 2021 [cited 2023 May 3]. Available from: https://bestinitiative.org/wp-content/uploads/2021/02/C19-Vaccine-Safety-AESI-Background-Rate-Protocol-FINAL-2020.pdf
5. Pawlowski C, Rincon-Hekking J, Awasthi S, Pandey V, Lenehan P, Venkatakrishnan AJ, et al. Cerebral venous sinus thrombosis is not significantly linked to COVID-19 vaccines or non-COVID vaccines in a large multi-state health system. J Stroke Cerebrovasc Dis 2021;30:105923.
6. Pottegard A, Lund LC, Karlstad O, Dahl J, Andersen M, Hallas J, et al. Arterial events, venous thromboembolism, thrombocytopenia, and bleeding after vaccination with Oxford-AstraZeneca ChAdOx1-S in Denmark and Norway: population based cohort study. BMJ 2021;373:n1114.
7. Tobaiqy M, Elkout H, MacLure K. Analysis of thrombotic adverse reactions of COVID-19 AstraZeneca vaccine reported to EudraVigilance database. Vaccines (Basel) 2021;9:393.
8. Smadja DM, Yue QY, Chocron R, Sanchez O, Lillo-Le Louet A. Vaccination against COVID-19: insight from arterial and venous thrombosis occurrence using data from VigiBase. Eur Respir J 2021;58:2100956.
9. Al-Ali D, Elshafeey A, Mushannen M, Kawas H, Shafiq A, Mhaimeed N, et al. Cardiovascular and haematological events post COVID-19 vaccination: a systematic review. J Cell Mol Med 2022;26:636–53.
10. Bilotta C, Perrone G, Adelfio V, Spatola GF, Uzzo ML, Argo A, et al. COVID-19 vaccine-related thrombosis: a systematic review and exploratory analysis. Front Immunol 2021;12:729251.
11. Flynn AC, Kavanagh K, Smith AD, Poston L, White SL. The impact of the COVID-19 pandemic on pregnancy planning behaviors. Womens Health Rep (New Rochelle) 2021;2:71–7.
12. American Society for Reproductive Medicine. Patient management and clinical recommendations during the coronavirus (COVID-19) pandemic [Internet]. ASRM; 2020 [cited 2023 May 3]. Available from: https://www.asrm.org/ globalassets/asrm/asrm-content/news-and-publications/covid-19/covidtaskforceupdate11.pdf
13. Bowman CJ, Bouressam M, Campion SN, Cappon GD, Catlin NR, Cutler MW, et al. Lack of effects on female fertility and prenatal and postnatal offspring development in rats with BNT162b2, a mRNA-based COVID-19 vaccine. Reprod Toxicol 2021;103:28–35.
14. Moolhuijsen LM, Visser JA. Anti-Mullerian hormone and ovarian reserve: update on assessing ovarian function. J Clin Endocrinol Metab 2020;105:3361–73.
15. Cedars MI. Evaluation of female fertility-AMH and ovarian reserve testing. J Clin Endocrinol Metab 2022;107:1510–9.
16. van Rooij IA, Broekmans FJ, Scheffer GJ, Looman CW, Habbema JD, de Jong FH, et al. Serum antiMullerian hormone levels best reflect the reproductive decline with age in normal women with proven fertility: a longitudinal study. Fertil Steril 2005;83:979–87.
17. Bentzen JG, Forman JL, Larsen EC, Pinborg A, Johannsen TH, Schmidt L, et al. Maternal menopause as a predictor of anti-Mullerian hormone level and antral follicle count in daughters during reproductive age. Hum Reprod 2013;28:247–55.
18. Bentov Y, Beharier O, Moav-Zafrir A, Kabessa M, Godin M, Greenfield CS, et al. Ovarian follicular function is not altered by SARS-CoV-2 infection or BNT162b2 mRNA COVID-19 vaccination. Hum Reprod 2021;36:2506–13.
19. Aharon D, Lederman M, Ghofranian A, Hernandez-Nieto C, Canon C, Hanley W, et al. In vitro fertilization and early pregnancy outcomes after coronavirus disease 2019 (COVID-19) vaccination. Obstet Gynecol 2022;139:490–7.
20. Wu Y, Cao M, Lin Y, Xu Z, Liang Z, Huang Q, et al. Inactivated COVID-19 vaccination does not affect in vitro fertilization outcomes in women. Hum Reprod 2022;37:2054–62.
21. Huang J, Xia L, Lin J, Liu B, Zhao Y, Xin C, et al. No effect of inactivated SARS-CoV-2 vaccination on in vitro fertilization outcomes: a propensity score-matched study. J Inflamm Res 2022;15:839–49.
22. Requena A, Vergara V, Gonzalez-Ravina C, Ruiz ME, Cruz M. The type of SARS-CoV-2 vaccine does not affect ovarian function in assisted reproduction cycle. Fertil Steril 2023;119:618–23.
23. Cao M, Wu Y, Lin Y, Xu Z, Liang Z, Huang Q, et al. Inactivated Covid-19 vaccine did not undermine live birth and neonatal outcomes of women with frozen-thawed embryo transfer. Hum Reprod 2022;37:2942–51.
24. Odeh-Natour R, Shapira M, Estrada D, Freimann S, Tal Y, Atzmon Y, et al. Does mRNA SARS-CoV-2 vaccine in the follicular fluid impact follicle and oocyte performance in IVF treatments? Am J Reprod Immunol 2022;87:e13530.
25. Orvieto R, Noach-Hirsh M, Segev-Zahav A, Haas J, Nahum R, Aizer A. Does mRNA SARS-CoV-2 vaccine influence patients' performance during IVF-ET cycle? Reprod Biol Endocrinol 2021;19:69.
26. Avraham S, Kedem A, Zur H, Youngster M, Yaakov O, Yerushalmi GM, et al. Coronavirus disease 2019 vaccination and infertility treatment outcomes. Fertil Steril 2022;117:1291–9.
27. Zhao Y, Zhao Y, Ai A, Jin L. Association of inactivated COVID-19

vaccination with in vitro fertilization and early pregnancy outcomes. J Med Virol 2023;95:e28432.
28. Brandao P, Pellicer A, Meseguer M, Remohi J, Garrido N, Garcia-Velasco JA. COVID-19 mRNA vaccines have no effect on endometrial receptivity after euploid embryo transfer. Reprod Biomed Online 2022;45:688–95.
29. Alghamdi AN, Alotaibi MI, Alqahtani AS, Al Aboud D, Abdel-Moneim AS. BNT162b2 and ChAdOx1 SARS-CoV-2 post-vaccination side-effects among Saudi vaccinees. Front Med (Lausanne) 2021;8:760047.
30. Anjorin AA, Odetokun IA, Nyandwi JB, Elnadi H, Awiagah KS, Eyedo J, et al. Public health surveillance for adverse events following COVID-19 vaccination in Africa. Vaccines (Basel) 2022;10:546.
31. Cheng Y, Li T, Zheng Y, Xu B, Bi Y, Hu Y, et al. Self-reported adverse events among Chinese healthcare workers immunized with COVID-19 vaccines composed of inactivated SARS-CoV-2. Hum Vaccin Immunother 2022;18:2064134.
32. Dar-Odeh N, Abu-Hammad O, Qasem F, Jambi S, Alhodhodi A, Othman A, et al. Long-term adverse events of three COVID-19 vaccines as reported by vaccinated physicians and dentists, a study from Jordan and Saudi Arabia. Hum Vaccin Immunother 2022;18:2039017.
33. Girardi G, Bremer AA. Scientific evidence supporting coronavirus disease 2019 (COVID-19) vaccine efficacy and safety in people planning to conceive or who are pregnant or lactating. Obstet Gynecol 2022;139:3–8.
34. Lagana AS, Veronesi G, Ghezzi F, Ferrario MM, Cromi A, Bizzarri M, et al. Evaluation of menstrual irregularities after COVID-19 vaccination: results of the MECOVAC survey. Open Med (Wars) 2022;17:475–84.
35. Issakov G, Tzur Y, Friedman T, Tzur T. Abnormal uterine bleeding among COVID-19 vaccinated and recovered women: a national survey. Reprod Sci 2023;30:713–21.
36. Edelman A, Boniface ER, Male V, Cameron ST, Benhar E, Han L, et al. Association between menstrual cycle length and covid-19 vaccination: global, retrospective cohort study of prospectively collected data. BMJ Med 2022;1:e000297.
37. Wong KK, Heilig CM, Hause A, Myers TR, Olson CK, Gee J, et al. Menstrual irregularities and vaginal bleeding after COVID-19 vaccination reported to v-safe active surveillance, USA in December, 2020-January, 2022: an observational cohort study. Lancet Digit Health 2022;4:e667–75.
38. Farland LV, Khan SM, Shilen A, Heslin KM, Ishimwe P, Allen AM, et al. COVID-19 vaccination and changes in the menstrual cycle among vaccinated persons. Fertil Steril 2023;119:392–400.
39. Rodriguez Quejada L, Toro Wills MF, Martinez-Avila MC, Patino-Aldana AF. Menstrual cycle disturbances after COVID-19 vaccination. Womens Health (Lond) 2022;18:17455057221109375.
40. Muhaidat N, Alshrouf MA, Azzam MI, Karam AM, Al-Nazer MW, Al-Ani A. Menstrual symptoms after COVID-19 vaccine: a cross-sectional investigation in the MENA region. Int J Womens Health 2022;14:395–404.

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The effects of purslane (*Portulaca oleracea*) and fennel (*Foeniculum vulgare Mill*) hydroalcoholic extracts on the functional parameters of human spermatozoa after vitrification

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Objective: Reactive oxygen species (ROS) are produced during cryopreservation of human sperm and impair sperm function. Antioxidant compounds, such as fennel and purslane, reduce the damaging effects of ROS. This study aimed to evaluate motility parameters, plasma membrane integrity (PMI), mitochondrial membrane potential (MMP), intracellular ROS, and DNA damage to determine the optimum concentrations of hydroalcoholic extracts of fennel and purslane for human spermatozoa cryopreservation.
Methods: Twenty human sperm samples were used and divided into seven equal groups consisting of fennel hydroalcoholic extract (5, 10, and 15 mg/L), purslane hydroalcoholic extract (25, 50, and 100 mg/L), and no additive.
Results: Supplementation of 25 mg/L and 50 mg/L purslane extract and 10 mg/L fennel extract in cryopreservation extender significantly increased the motility and PMI of sperm with a significant reduction in intracellular ROS compared to control groups ($p<0.05$). A 50 mg/L concentration of purslane extract elevated progressive motility and MMP compared to the control group ($p<0.05$). No significant differences were seen for motion patterns and DNA damage of frozen-thawed human sperm in extender containing these extracts.
Conclusion: The results showed that supplementation of 50 mg/L purslane extract and 10 mg/L fennel extract in semen cryopreservation extender has the potential to decrease intracellular ROS and subsequently elevate the motility and PMI of human sperm.

Keywords: Fennel; Human sperm; Purslane; Vitrification

Introduction

Previous studies have investigated the effect of using different antioxidants in sperm cryopreservation extenders for animal and human species to reduce cryopreservation damage [1-5]. Despite the significant scientific evidence published on the effects of synthetic antioxidants to improve sperm parameter functions, the use of less toxic medicinal plants with antioxidant properties and bioactive compounds has also received much attention from researchers [6-8]. Previous studies have reported improvements in the quality of cryopreserved sperm cells with the addition of plant extracts such as fennel (*Foeniculum vulgare*), rosemary, and green tea to the cryopreservation medium [7,9-11]. Portulaca olerace, also known as purslane, is an edible green herb that can be used raw or cooked [12]. This medicinal plant was named a "global panacea" by the World Health Organization (WHO) [13]. Purslane contains compounds such as flavonoids, terpenoids, phenolic acids, alkaloids, saponins, omega-3 fatty acids, carotene, vitamins, glutathione, and melatonin [14-16]. Some of the biologically active compounds existing in this plant have ex-

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hibited antibacterial, anti-inflammatory, and antioxidant activities [17,18]. Previous research has attributed the antioxidant effect of purslane to three phenolic alkaloids (oleracein A, B, and E), which lead to free radical scavenging and prevent lipid peroxidation in rats [19]. In Nigeria, the plant has been used by traditional medicine practitioners to control infertility in women. In southern Nigeria, in the Niger Delta region, the leaves of *P. oleracea* are used by women to enhance fertility. In eastern Nigeria, the aerial parts of the plants are crushed to extract juice, which is taken with or without raw egg for the purpose of improving fertility in both males and females [20]. Many *in vitro* and *in vivo* studies have documented the antioxidant and protective effects of different extracts of purslane against oxidative stress [21]. The main compounds of purslane (phenols and flavonoids) might be responsible for its antioxidant effects [21]. In other studies, purslane extract increased activity levels of superoxide dismutase (SOD) and decreased levels of malondialdehyde (MDA) in an ulcerative colitis mouse model [22]. *F. vulgare* mill, also known as fennel, is an aromatic Mediterranean plant that has received much attention in traditional medicine [23,24]. This medicinal plant has diuretic, analgesic, anti-inflammatory, antipyretic, and antioxidant activity [25,26]. The therapeutic properties of fennel can be attributed to the presence of volatile compounds such as phenols and flavonoids in fennel extract. Among other compounds identified in this plant are terpenes, terpenoids, coumarin, anethole, and fenchone [27-29]. Fennel is an important source of antioxidants. It can significantly increase the activity of SOD and catalase. Malo et al. [10] showed that fennel decreased MDA levels in boar semen cryopreservation, thereby inhibiting lipid peroxidation. The cryopreservation of spermatozoa is widely used in infertility treatments [30]. In artificial insemination, sperm cryopreservation and its timely use have helped to increase the fertility rate of infertile couples, though still not as successfully as artificial insemination with fresh sperm [31]. The process of semen cryopreservation can lead to cell death and may reduce the rate of fertility post artificial insemination by triggering oxidative stress reactions [32,33]. Sperm viability is reduced under the influence of ice crystals formed by cryopreservation [34]. One method of sperm cryopreservation, vitrification, is capable of reducing the damage caused by conventional cryopreservation methods by reducing intracellular ice crystals. Vitrification can also be implemented in less time and at less expense than other methods [35,36]. During the cryopreservation process, reactive oxygen species (ROS) are produced whose products can increase lipid peroxidation [37]. Sperm is normally protected by antioxidant systems existing in its cytoplasm and cell membrane [30], but this protection reduces during cryopreservation and sperm are largely deprived of antioxidant protection. This imbalance between ROS and the sperm antioxidant system leads to cryopreservation damage and affects sperm characteristics such as motility, plasma membrane integrity (PMI), mitochondrial membrane potential (MMP), and DNA structure [38,39]. Although several studies have investigated the effect of fennel and purslane extracts on the quality of sperm, no study has yet compared the antioxidant effect of the extracts of these two plants on human functional parameters during cryopreservation. Therefore, the aim of this study was to compare the antioxidant effect of hydroalcoholic extracts of fennel and purslane on sperm motility, PMI, MMP, DNA damage, and intracellular ROS parameters during cryopreservation.

Methods

This study was approved by the Ethics Committee of Tehran University of Medical Sciences and informed consent was obtained from participants.

1. Preparation of hydroalcoholic extracts of fennel and purslane

Fennel seeds and fresh purslane wild plants were prepared by a plant classification expert from the local medicinal plants market and identified by a pharmacognosy expert. Maceration was used to prepare the hydroalcoholic extract of fennel seeds. In this method, the seeds were first crushed into powder by an electric grinder for 6 minutes. Then, 100 g of the seed powder was mixed with 500 mL of 70% ethanol and placed at room temperature for 48 hours. During this period, the resulting suspension mixture was stirred intermittently. The materials were filtered using Whatman No. 1 filter paper. The solution was concentrated under vacuum conditions on a rotary operator, and then put in an oven at 40 °C for 48 hours to be dried. The prepared powder was kept at 4 °C until use [40]. The dried powder was dissolved in distilled water in order to obtain the required concentration of the extract [41].

To prepare hydroalcoholic purslane extract, the method described by Azimi et al. [38] was used. In this method, the plant was first crushed into powder using an electric mill for 10 minutes, then 100 g of the plant powder was mixed with 800 mL of methanol and the resulting suspension was kept at room temperature for 72 hours. The materials were then passed through a piece of fabric and the resulting filtered solution was concentrated using the vacuum system of a rotary operator. Finally, the solution was put in an oven at 30 °C for 48 hours to be dried and the prepared powder was kept at 4 °C until use [38]. The dried powder was dissolved in distilled water in order to obtain the required concentration of the extract [38].

2. Subject and semen collection

Our research was carried out in the embryology laboratory at the

Research Center of Tehran University of Medical Sciences. A total of 20 normal sperm samples were obtained from Aban Infertility Center from February 2020 to April 2020 and used for this study. Study samples were collected from patients referred to the Infertility Center who had obtained samples by masturbation after 4–6 days of sexual abstinence. Sperm parameters were assessed according to the WHO (2010) guidelines. Sperm motility and concentration were assessed using the CASA system sperm class analyzer version 5.1 (Microptic). The criteria for inclusion of each sample were: progressive motility of 70%, volume of 2–6 mL, and concentration greater than 1×10^9 sperm/mL.

3. Vitrification and warming procedure

A micro-droplet technique was used to freeze sperm samples [42]. For cryopreservation, the spermatozoa samples were first added to the human tubal fluid (HTF; Sigma) solution. The resulting solution was then diluted with 5% human serum albumin (HSA; Sigma) and 0.5 mol/L of sucrose. The solution was then divided into seven equal parts for the experimental groups and fennel extract (5, 10, and 15 mg/L) or purslane extract (25, 50, and 100 mg/L) was added to each group, respectively. Finally, this suspension (at 30 µL/drop) was incubated in a nitrogen tank for 1 week. In the thawing phase, 5 mL of HTF solution was heated at 37 °C for 2 hours. In this phase, samples and –1% HSA were immersed in HTF solution. The resulting suspension was then incubated at 37 °C and 5% CO_2 for 5 minutes. Finally, samples were centrifuged at 500 ×*g* for 5 minutes and suspended in 50 µL of HTF [39].

4. Assessment of sperm motion characteristics

First, 10 µL of a sperm sample was placed on a Makler slide and the details were examined using the CASA system. The investigated parameters included motility (%), progressive motility (%), average path velocity (VAP; µm/sec), curvilinear velocity (VCL; µm/sec), linearity (LIN; %), straight-line velocity (VSL; µm/sec), straightness (STR; %), wobble (WOB; %), amplitude lateral head displacement (ALH; %), and beat cross frequency (BCF; %). Finally, five microscopic fields for 400 spermatozoa were selected to be assessed.

5. Assessment of sperm PMI

To determine the PMI, the hypo-osmotic swelling (HOS) test was used. To this purpose, 500 µL of a sperm sample was mixed with 500 µL of HOS solution (1.35 g of fructose [Merck], 0.73 of g sodium citrate [Merck], and 100 mL of distilled water; osmolality ~190 mOsmol/kg) at 37 °C for 30 minutes. A total volume of 10 µL of the suspension was placed on a dry slide. Finally, evaluations were performed using phase contrast microscopy (Olympus BX20) [43].

6. Assessment of mitochondrial membrane potential

Lipophilic cationic dye, JC-1 (T4069, Sigma-Aldrich), was used to investigate the MMP. During this phase, samples were centrifuged (500 ×*g* for 5 minutes) and then dissolved in phosphate-buffered saline (PBS) up to a concentration of 1×10^6 sperm/mL. After that, 1 µg of JC-1 dye was added to 1 mL of sample solution. Finally, samples were evaluated using flow cytometry techniques with orange (FL1, 585 nm) and red fluorescence (FL2, 530 nm) [44].

7. Assessment of DNA damage

Acridine orange (AO) fluorescence was used to investigate DNA damage. After centrifugation (500 ×*g* for 5 minutes), samples were added to Tris-null-EDTA buffer solution (1 mmol of EDTA, 10 mmol of Tris, and 0.15 mol of NaCl). The resulting solution was mixed with 400 µL of detergent acid and 1.2 mL of AO solution. The final assessment was performed using the flow cytometry technique for intact chromatin (FL1, 500–530 nm) and altered chromatin (FL2, 620 nm) [43].

8. Assessment of intracellular ROS

Dihydroethidium solution was used to evaluate the intracellular ROS. The reaction between dihydroethidium and anion superoxide leads to its oxidation. Then, it intercalates into DNA and emits red fluorescence. Samples, each with a concentration of 1×10^6 sperm/mL, were suspended with PBS solution, and 10 µL of dihydroethidium solution (Sigma-Aldrich Co.) was then added to the suspension and incubated at 25 °C for 20 minutes. Finally, red fluorescence (FL1, 525–625 nm) was assessed using the flow cytometry technique [45].

9. Flow cytometric analysis

The flow cytometric analysis was conducted using FACS Calibur (BD Immunocytometry Systems) with excitement by an argon laser at 488 nm. After eliminating debris, 10,000 spermatozoa were evaluated using flow cytometry (Flowing Software version 2.5.1; Cell Imaging and Cytometry Core).

10. Statistical analysis

The Kolmogorov-Smirnov test was used to confirm the normality of data distribution. Additionally, the differences between experimental groups were assessed using analysis of variance and the Tukey test. Data were analyzed using IBM SPSS ver. 20.0 (IBM Corp.). The results were shown as mean ± standard error, and $p < 0.05$ was considered to indicate statistical significance.

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Table 1. Motility parameters of post-thawed human spermatozoa supplemented with different concentrations of fennel and purslane hydroalcoholic extracts

Group	Control	Purslane			Fennel		
		25 mg/L	50 mg/L	100 mg/L	5 mg/L	10 mg/L	15 mg/L
Motility (%)	60.14 ± 1.59[c),d)]	67.43 ± 1.99[a),b)]	71.13 ± 1.42[a)]	57.40 ± 2.48[d)]	64.95 ± 1.59[b),c)]	66.75 ± 1.90[a),b)]	58.92 ± 1.50[d)]
Progressive motility (%)	31.73 ± 1.40[b),c)]	35.89 ± 1.43[a),b)]	37.30 ± 1.15[a)]	30.30 ± 1.65[c)]	33.87 ± 1.12[a),b),c)]	35.36 ± 1.29[a),b)]	29.93 ± 1.30[c)]
VCL (μm/sec)	48.90 ± 0.80	51.35 ± 0.72	52.62 ± 0.98	48.19 ± 0.81	50.13 ± 0.79	50.46 ± 0.85	47.99 ± 0.81
VSL (μm/sec)	27.25 ± 0.55	28.31 ± 0.55	28.88 ± 0.56	26.23 ± 0.51	28.10 ± 0.71	27.78 ± 0.52	27.06 ± 0.62
VAP (μm/sec)	33.68 ± 0.66	34.52 ± 0.81	35.95 ± 0.64	32.44 ± 0.62	34.41 ± 0.66	34.37 ± 0.61	32.62 ± 0.75
LIN (%)	55.60 ± 0.67	54.83 ± 0.62	55.20 ± 0.61	54.31 ± 0.69	55.63 ± 0.66	55.32 ± 0.67	56.23 ± 0.61
STR (%)	82.30 ± 0.37	82.05 ± 0.34	81.75 ± 0.50	82.03 ± 0.39	82.36 ± 0.45	82.25 ± 0.45	83.31 ± 0.45
WOB (%)	69.81 ± 0.66	69.06 ± 0.66	69.77 ± 0.56	68.35 ± 0.68	69.33 ± 0.65	69.51 ± 0.71	69.74 ± 0.59
ALH (%)	6.45 ± 0.05	6.55 ± 0.05	6.61 ± 0.06	6.50 ± 0.05	6.46 ± 0.07	6.52 ± 0.05	6.41 ± 0.05
BCF (%)	9.84 ± 0.08	9.82 ± 0.08	9.86 ± 0.06	9.96 ± 0.07	9.95 ± 0.04	9.85 ± 0.04	9.75 ± 0.04

Values are presented as mean±standard error.
VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude lateral head displacement; BCF, beat cross frequency.
[a),b),c),d)]Means with different letters in the same row demonstrate significant differences ($p<0.05$).

Table 2. PMI, MMP, DNA damage, and intracellular ROS of post-thawed human spermatozoa supplemented with different concentrations of fennel and purslane hydroalcoholic extracts

Group	Control	Purslane			Fennel			*p*-value
		25 mg/L	50 mg/L	100 mg/L	5 mg/L	10 mg/L	15 mg/L	
PMI	65.15 ± 0.99[c)]	69.74 ± 1.05[b)]	74.96 ± 0.83[a)]	68.30 ± 0.84[b),c)]	68.34 ± 1.45[b),c)]	69.59 ± 1.35[b)]	65.09 ± 0.95[c)]	< 0.05[e)]
MMP	9.12 ± 0.18[b),c)]	45.85 ± 2.17[a),b),c)]	49.53 ± 1.89[a)]	44.26 ± 2.50[a),b),c)]	44.97 ± 2.07[a),b),c)]	48.50 ± 2.86[a),b)]	41.45 ± 2.31[c)]	> 0.05[f)]
DNA damage	91.80 ± 2.03	7.95 ± 0.15	7.90 ± 0.16	8.49 ± 0.18	8.91 ± 0.16	8.71 ± 0.16	9.22 ± 0.19	< 0.05[e)]
ROS (intracellular)	41.85 ± 1.20[a),b)]	75.76 ± 1.98[c),d)]	72.35 ± 1.78[d)]	79.40 ± 1.52[b),c)]	79.14 ± 1.61[b),c)]	75.10 ± 1.45[c),d)]	85.32 ± 0.85[a)]	< 0.05[e)]

Values are presented as mean±standard error.
PMI, plasma membrane integrity; MMP, mitochondrial membrane potential; DNA damage; ROS, reactive oxygen species.
[a),b),c),d)]Means with different letters in the same column demonstrate significant differences; [e)]$p<0.05$; [f)]No significant difference.

Results

The effects of fennel and purslane extracts on the motility of cryopreserved-thawed human sperm are shown in Table 1. Cryopreservation extender supplementation with 25 mg/L and 50 mg/L purslane extract and 10 mg/L fennel extract improved sperm speed compared to the cryopreserved control group ($p < 0.05$). In addition, 50 mg/L purslane extract had the highest progressive motility, which was statistically significant compared to the cryopreservation control group ($p < 0.05$). The motility characteristics (VCL, VSL, VAP, LIN, BCF, ALH, STR, and WOB) showed no significant difference in the presence of both extracts compared to the cryopreserved control group ($p \geq 0.05$). Human sperm cryopreservation extender supplemented with 25 mg/L and 50 mg/L purslane extract and 10 mg/L fennel extract significantly increased PMI in sperm and cause a significant reduction in ROS compared to the cryopreserved control group ($p < 0.05$). Additionally, MMP increased with the addition of 50 mg/L purslane extract to the cryopreservation extender ($p < 0.05$). No significant difference was observed in sperm DNA damage between groups ($p \geq 0.05$) (Table 2).

Discussion

The main aim of this study was to assess the protective effects of two plant extracts on sperm functional parameters in a vitrification process. The analysis demonstrated that sperm speed as well as PMI and ROS production improved with 25 mg/L and 50 mg/L extracts of purslane and the 10 mg/L fennel extract. The highest increase in motility was observed with the 50 g/mL purslane extract, which also increased MMP. Sperm DNA damage and motility characteristics did not show significant relationships with the different doses of purslane and fennel in the vitrification groups.

Mammalian sperm is prone to the effects of increased intracellular ROS and lipid peroxidation products due to low levels of antioxidants

in the cytoplasm and high levels of saturated fatty acids in the plasma membrane. The presence of these products can disrupt the normal function of sperm. Though small amounts of ROS are required for sperm physiological activity, high amounts of ROS are toxic to sperm. Anion superoxide, the main source of ROS in sperm, is converted to hydrogen peroxide spontaneously or through the action of SOD. The results of our study are in agreement with a previous report on boar sperm, indicating that the presence of fennel extract causes a reduction in ROS levels compared to the control group. Moreover, Malo et al. [10] showed that fennel inhibits 5-lipoxygenase activity due to its antioxidant function. Fennel is also capable of stopping the release of the peroxidative chain reaction. Previous research has shown that purslane extract causes an increase in the activity of SOD [22]. Purslane extract can thus be effective in converting anion superoxide to hydrogen peroxide and thereby reducing intracellular ROS products. Therefore, the observation of a decrease in intracellular superoxide anion in this study can probably be attributed to the increased enzymatic capacity of antioxidants, which leads to the removal of ROS within sperm cells. Sperm mitochondria are the main site of ROS production due to their oxidative phosphorylation activity [46]. Koohestanidehaghi et al. [39] showed a negative relationship between intracellular ROS in human sperm and mitochondrial membrane activity after sample thawing. An increase in sperm MMP in the presence of purslane extract can be related to the high potential of this extract to reduce and eliminate intracellular ROS, and consequently protect the activity of the mitochondrial membrane. Sperm motility is one of the main characteristics of successful fertility. The findings of this study with regard to sperm motility align with those of previous studies in which fennel and purslane extracts increased the motility of boar and goat sperm post thawing [38,10]. Research evidence shows that phenols and flavonoids, the main components of purslane extract, are mainly responsible for its antioxidant and healing activity [19]. Previous studies have shown that goat and rat semen cryopreservation, supplemented with 50 mg/L purslane extract and 100 mg/kg and 200 mg/kg fennel extract significantly improved sperm motility [38,41,47] and Asadmobini et al. [30] showed the beneficial effects of fennel and purslane extracts, finding that they increased rat and human sperm motility [30,41]. Damage to the sperm axoneme structure and the reduction of adenosine triphosphate caused by ROS products are among the primary reasons for sperm motility reduction [48]. Several studies have shown a negative relationship between high levels of ROS and sperm motility in different animal species and humans [39,43,49]. In this study, superoxide anion levels were decreased in the 50 mg/L and 25 mg/L purslane extracts and 10 mg/L fennel extracts, mainly because of the increase in human sperm motility post-thawing. Additionally, motility characteristics (BCF, ALH, STR and VCL, VSL, VAP, LIN, and WOB) showed no significant differences between the experimental groups containing fennel and purslane extracts at all concentrations and the control group. The result of this part of our study is supported by the study of Topraggaleh et al. [49] on buffalo sperm in which the sperm motility characteristics were not different after an antioxidant was added to the cryopreservation extender. In addition to sperm motility, sperm fertility and survival post-thawing can be affected by PMI [50]. Malo et al. [10] reported that fennel extract in cryopreservation extender reduced MDA formation in the thawed sperm compared to the control group, indicating that this antioxidant is capable of inhibiting lipid peroxidation by inhibiting 5-lipoxygenase. Additionally, Azimi et al. [38] showed that the presence of purslane extract reduced the amount of MDA and lipid peroxidation. However, reports have suggested that the antioxidant abilities of plants depend on their phenolic compounds [51]. Perhaps the reduction of superoxide anion through antioxidant extract supplementation was the main reason for plasma membrane protection against ROS. In addition to the mentioned parameters, a healthy sperm chromatin structure is necessary for successful fertility and fetal growth [52]. In our study, cryopreservation supplementation with fennel and purslane extracts showed no significant effect on preventing DNA damage in thawed cryopreserved sperm. This finding might be attributable to the higher concentration of sperm chromatin during puberty, which promotes stability of the sperm DNA structure and protects against the ROS produced during cryopreservation, as well as the resultant DNA damage.

Supplementation of fennel and purslane extracts at concentrations of 10 mg/L and 50 mg/L, respectively, reduced intracellular ROS during cryopreservation and thawing of sperm, contributing to an increase in sperm motility and PMI compared to the control group. This study also emphasized the improvement of progressive motility and MMP with the addition of 50 mg/L purslane extract to cryopreservation extender. The addition of fennel and purslane extracts to cryopreservation extender had no significant negative effect on human sperm motility characteristics or DNA damage compared to the control group. Therefore, we recommend the addition of 10 mg/L fennel and 50 mg/L purslane extracts to cryopreservation extender to improve human sperm quality post-thawing, although further research is recommended to improve the *in vitro* and *in vivo* fertility rates of thawed cryopreserved human sperm using fennel and purslane extracts.

Conflict of interest

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

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References

1. de Oliveira RA, Wolf CA, de Oliveira Viu MA, Gambarini ML. Addition of glutathione to an extender for frozen equine semen. J Equine Vet Sci 2013;33:1148–52.
2. Farshad A, Hosseini Y. The cryoprotective effects of amino acids supplementation on cooled and post-thaw Markhoz bucks semen quality. Small Rumin Res 2013;114:258–63.
3. Merati Z, Farshad A, Farzinpour A, Rostamzadeh J, Sharafi M. Anti-apoptotic effects of minocycline on ram epididymal spermatozoa exposed to oxidative stress. Theriogenology 2018;114:266–72.
4. Motemani M, Chamani M, Sharafi M, Masoudi R. Alpha-tocopherol improves frozen-thawed sperm quality by reducing hydrogen peroxide during cryopreservation of bull semen. Span J Agric Res 2017;15:15.
5. Zhu Z, Fan X, Pan Y, Lu Y, Zeng W. Trehalose improves rabbit sperm quality during cryopreservation. Cryobiology 2017;75:45–51.
6. Kohestani Y, Kohestani B, Shirmohamadi Z, Roushandeh AM, Faghani M. The protective properties of hydro-alcoholic extract of Nigella sativa on male reproductive system in type 2 diabetes rats. Health Biotechnol Biopharma 2019;3:45–56.
7. Mehdipour M, Daghigh Kia H, Najafi A, Vaseghi Dodaran H, Garcia-Alvarez O. Effect of green tea (Camellia sinensis) extract and pre-freezing equilibration time on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender. Cryobiology 2016;73:297–303.
8. Omogbadegun Z, Uwadia C, Ayo C, Mbarika V, Omoregbe N, Otofia E, et al. Multimedia-based medicinal plants sustainability management system. IJCSI Int J Comput Sci Issues 2011;8:arXiv:1111.6911.
9. Baghshahi H, Riasi A, Mahdavi AH, Shirazi A. Antioxidant effects of clove bud (Syzygium aromaticum) extract used with different extenders on ram spermatozoa during cryopreservation. Cryobiology 2014;69:482–7.
10. Malo C, Gil L, Cano R, Gonzalez N, Luno V. Fennel (Foeniculum vulgare) provides antioxidant protection for boar semen cryopreservation. Andrologia 2012;44 Suppl 1:710–5.
11. Motlagh MK, Sharafi M, Zhandi M, Mohammadi-Sangcheshmeh A, Shakeri M, Soleimani M, et al. Antioxidant effect of rosemary (Rosmarinus officinalis L.) extract in soybean lecithin-based semen extender following freeze-thawing process of ram sperm. Cryobiology 2014;69:217–22.
12. Uddin MK, Juraimi AS, Hossain MS, Nahar MA, Ali ME, Rahman MM. Purslane weed (Portulaca oleracea): a prospective plant source of nutrition, omega-3 fatty acid, and antioxidant attributes. ScientificWorldJournal 2014;2014:951019.
13. Dweck AC. Purslane (Portulaca oleracea): the global panacea. Personal Care Mag 2001;2:7-15.
14. Erkan N. Antioxidant activity and phenolic compounds of fractions from Portulaca oleracea L. Food Chem 2012;133:775–81.
15. Naeem F, Khan SH. Purslane (Portulaca oleracea L.) as phytogenic substance: a review. J Herbs Spices Med Plants 2013;19:216–32.
16. Yan J, Sun LR, Zhou ZY, Chen YC, Zhang WM, Dai HF, et al. Homoisoflavonoids from the medicinal plant Portulaca oleracea. Phytochemistry 2012;80:37–41.
17. Lee AS, Kim JS, Lee YJ, Kang DG, Lee HS. Anti-TNF-α activity of Portulaca oleracea in vascular endothelial cells. Int J Mol Sci 2012;13:5628–44.
18. Uddin MK, Juraimi AS, Ali ME, Ismail MR. Evaluation of antioxidant properties and mineral composition of Purslane (Portulaca oleracea L.) at different growth stages. Int J Mol Sci 2012;13:10257–67.
19. Sanja SD, Sheth NR, Patel NK, Patel D, Patel B. Characterization and evaluation of antioxidant activity of Portulaca oleracea. Int J Pharm Pharm Sci 2009;1:74–84.
20. Obinna VC, Kagbo HD, Agu GO. Effects of lipophilic and hydrophilic leaf extracts of portulaca oleracea Linn.(Purslane) on male reproductive parameters in albino rats. Am J Physiol Biochem Pharmaco 2019;9:1–12.
21. Yang Z, Liu C, Xiang L, Zheng Y. Phenolic alkaloids as a new class of antioxidants in Portulaca oleracea. Phytother Res 2009;23:1032–5.
22. Yang X, Yan Y, Li J, Tang Z, Sun J, Zhang H, et al. Protective effects of ethanol extract from Portulaca oleracea L on dextran sulphate sodium-induced mice ulcerative colitis involving anti-inflammatory and antioxidant. Am J Transl Res 2016;8:2138–48.
23. Chegini R, Soleimani P, Sadeghi M, Yosef RM, Zafari F. Investigating

the effect of fennel and cinnamon combined extract on spermatogenesis and testis tissues in busulfan induced infertile rats. J Appl Biotechnol Rep 2019;6:96–100.
24. Helal EG, AL Jalaud NA, El-Aleem MA, Ahmed SS. Effect of phytoestrogen (fennel) on some sex hormones and other physiological parameters in male albino rats. Egypt J Hosp Med 2019;74:1616–20.
25. Choi EM, Hwang JK. Antiinflammatory, analgesic and antioxidant activities of the fruit of Foeniculum vulgare. Fitoterapia 2004;75:557–65.
26. Shah AH, Qureshi S, Ageel AM. Toxicity studies in mice of ethanol extracts of Foeniculum vulgare fruit and Ruta chalepensis aerial parts. J Ethnopharmacol 1991;34:167–72.
27. Csupor-Loffler B, Hajdu Z, Zupko I, Rethy B, Falkay G, Forgo P, et al. Antiproliferative effect of flavonoids and sesquiterpenoids from Achillea millefolium s.l. on cultured human tumour cell lines. Phytother Res 2009;23:672–6.
28. Dehghani F, Panjehshahin MR, Mirzaee Z, Mehrabani D. Effect of Foeniculum vulgare organic extract on blood sex hormones and reproductive tissues of male rats. J Appl Anim Res 2005;27:17–20.
29. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972;18:499–502.
30. Asadmobini A, Bakhtiari M, Khaleghi S, Esmaeili F, Mostafaei A. The effect of Tribulus terrestris extract on motility and viability of human sperms after cryopreservation. Cryobiology 2017;75:154–9.
31. Miah AG, Bathgate R, Hamano KI, Salma U. Effects of pre-freeze Nigella sativa oil supplementation on cryosurvival of ovine spermatozoa. Reprod Domest Anim 2018;53:1424–33.
32. Ozturk C, Gungor S, Ataman MB, Bucak MN, Baspinar N, Ili P, et al. Effects of arginine and trehalose on post-thawed bovine sperm quality. Acta Vet Hung 2017;65:429–39.
33. Peris SI, Bilodeau JF, Dufour M, Bailey JL. Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation, and functional parameters in ram sperm. Mol Reprod Dev 2007;74:878–92.
34. Tomas RM, Bailey TL, Hasan M, Gibson MI. Extracellular antifreeze protein significantly enhances the cryopreservation of cell monolayers. Biomacromolecules 2019;20:3864–72.
35. Isachenko V, Todorov P, Seisenbayeva A, Toishibekov Y, Isachenko E, Rahimi G, et al. Vitrification of human pronuclear oocytes by direct plunging into cooling agent: non sterile liquid nitrogen vs. sterile liquid air. Cryobiology 2018;80:84–8.
36. Spis E, Bushkovskaia A, Isachenko E, Todorov P, Sanchez R, Skopets V, et al. Conventional freezing vs. cryoprotectant-free vitrification of epididymal (MESA) and testicular (TESE) spermatozoa: three live births. Cryobiology 2019;90:100–2.
37. Partyka A, Rodak O, Bajzert J, Kochan J, Nizanski W. The effect of L-carnitine, hypotaurine, and taurine supplementation on the quality of cryopreserved chicken semen. Biomed Res Int 2017;2017:7279341.
38. Azimi G, Farshad A, Farzinpour A, Rostamzadeh J, Sharafi M. Evaluation of used Purslane extracts in Tris extenders on cryopreserved goat sperm. Cryobiology 2020;94:40–8.
39. Koohestanidehaghi Y, Torkamanpari M, Shirmohamadi Z, Lorian K, Vatankhah M. The effect of cysteine and glutamine on human sperm functional parameters during vitrification. Andrologia 2021;53:e13870.
40. Mansouri E, Asadi-Samani M, Kooti W, Ghasemiboroon M, Ashtary-Larky D, Alamiri F, et al. Anti-fertility effect of hydro-alcoholic extract of fennel (Foeniculum vulgare Mill) seed in male Wistar rats. J Vet Res 2016;60:357–63.
41. Najafi A, Daghigh Kia H, Mehdipour M, Shamsollahi M, Miller DJ. Does fennel extract ameliorate oxidative stress frozen-thawed ram sperm? Cryobiology 2019;87:47–51.
42. Seify M, Zarabadipour M, Ghaleno LR, Alizadeh A, Rezazadeh Valojerdi M. The anti-oxidant roles of taurine and hypotaurine on acrosome integrity, HBA and HSPA2 of the human sperm during vitrification and post warming in two different temperature. Cryobiology 2019;90:89–95.
43. Kadirvel G, Kumar S, Kumaresan A. Lipid peroxidation, mitochondrial membrane potential and DNA integrity of spermatozoa in relation to intracellular reactive oxygen species in liquid and frozen-thawed buffalo semen. Anim Reprod Sci 2009;114:125–34.
44. Minervini F, Guastamacchia R, Pizzi F, Dell'Aquila ME, Barile VL. Assessment of different functional parameters of frozen-thawed buffalo spermatozoa by using cytofluorimetric determinations. Reprod Domest Anim 2013;48:317–24.
45. Mahfouz RZ, du Plessis SS, Aziz N, Sharma R, Sabanegh E, Agarwal A. Sperm viability, apoptosis, and intracellular reactive oxygen species levels in human spermatozoa before and after induction of oxidative stress. Fertil Steril 2010;93:814–21.
46. Thuwanut P, Chatdarong K, Bergqvist AS, Soderquist L, Thiangtum K, Tongthainan D, et al. The effects of antioxidants on semen traits and in vitro fertilizing ability of sperm from the flat-headed cat (Prionailurus planiceps). Theriogenology 2011;76:115–25.
47. Nejatbakhsh R, Riyahi S, Farrokhi A, Rostamkhani S, Mahmazi S, Yazdinezhad A, et al. Ameliorating effects of fennel and cumin extracts on sperm quality and spermatogenic cells apoptosis by inducing weight loss and reducing leptin concentration in diet-in-

https://doi.org/10.5653/cerm.2021.04805

duced obese rats. Andrologia 2017;49:e12748.
48. Bochenek M, Smorag Z. The level of sperm DNA fragmentation in bulls of different breeds. Ann Anim Sci 2010;10:379–84.
49. Topraggaleh TR, Shahverdi A, Rastegarnia A, Ebrahimi B, Shafiepour V, Sharbatoghli M, et al. Effect of cysteine and glutamine added to extender on post-thaw sperm functional parameters of buffalo bull. Andrologia 2014;46:777–83.
50. Mehmood A, Anwar M, Naqvi SM. Motility, acrosome integrity, membrane integrity and oocyte cleavage rate of sperm separated by swim-up or Percoll gradient method from frozen-thawed buffalo semen. Anim Reprod Sci 2009;111:141–8.
51. Al-Bishri WM, Abdel-Reheim ES, Zaki AR. Purslane protects against the reproductive toxicity of carbamazepine treatment in pilocarpine-induced epilepsy model. Asian Pac J Trop Biomed 2017;7:339–46.
52. Tuncer PB, Bucak MN, Buyukleblebici S, Sariozkan S, Yeni D, Eken A, et al. The effect of cysteine and glutathione on sperm and oxidative stress parameters of post-thawed bull semen. Cryobiology 2010;61:303–7.

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Human sperm parameter improvement associated with *Ceratonia siliqua* extract as a cryopreservation supplement after vitrification

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Objective: Given the destructive effects of oxidative stress on sperm structure, this study was conducted to investigate the antioxidant effects of different concentrations of *Ceratonia siliqua* plant extract on human sperm parameters after the freezing-thawing process.
Methods: A total of 20 normozoospermic samples were frozen. Each sample was divided into two control groups (fresh and cryopreservation) and three cryopreservation experimental groups (containing *C. siliqua* extract at concentrations of 20, 30, and 40 μg/mL in the freezing extender). Motility, intracellular levels of reactive oxygen species (ROS), plasma membrane integrity (PMI), mitochondrial membrane potential (MMP), viability, and acrosome reaction parameters were evaluated.
Results: Statistical analysis showed that the highest motility, viability, and PMI were associated with the 20 μg/mL concentration of *C. siliqua* extract. At all concentrations, intracellular ROS levels were significantly lower and the levels of MMP and the acrosome reaction were significantly higher than in the cryopreservation control group ($p \leq 0.05$).
Conclusion: *C. siliqua* extract supplements at concentrations of 20, 30, and 40 μg/mL improved sperm motility, viability, PMI, MMP, intracellular ROS, and the acrosome reaction.

Keywords: Antioxidants; Carob; Cryopreservation; Fabaceae; Sperm motility

Introduction

Freezing human sperm is an effective and beneficial strategy in the field of male fertility [1]. This method can be used for men with cancer who are undergoing radiotherapy or chemotherapy treatment, in sperm donation programs, or in conjunction with surgical procedures that endanger male fertility [2-5]. The use of cryopreservation to create a sperm bank for healthy men who are exposed to ionizing radiation, biological contaminants, or toxins at work is another related goal [6]. Despite these benefits of sperm cryopreservation, we now understand that cryopreservation and thawing cause irrecoverable changes in sperm function and structure [7]. In the cryopreservation process, sperm are exposed to stressors such as osmotic pressure change, pH change, dehydration, the creation of ice crystals, and the generation of free radicals [8]. These factors can impair sperm motility, cell membrane and mitochondrial structure, chromatin structure, and sperm viability [9]. In recent years, extensive efforts have been made to improve cryopreservation and reduce the harmful effects of the freezing-thawing process on human sperm [10]. These include changes to cryopreservation methods, composition of the cryopreservation medium, cryopreservation and thawing times, and packaging of samples [11,12]. In general, freezing media include cryoprotectants, ionic or non-ionic materials to maintain the pH, and an energy substrate, as well as fatty acids, antibiotics, and antioxidants [11]. According to previous studies, the complementa-

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tion of freezing-thawing environments with various factors, including antioxidants, is an effective approach to improve the quality of frozen-thawed sperm [13]. Many studies have focused on plants with antioxidant capacities that can counteract the damaging effects of free radicals due to oxidative stress [14]. *Ceratonia siliqua* is an evergreen plant native to the Mediterranean region. *C. siliqua* contains compounds such as vitamins (B, C, D, and E), polyphenols, and minerals (iron, phosphorus, potassium, sodium, and calcium) [15,16]. The phenolic components of this plant improve oxidative stress conditions [17] and can act as a powerful source of antioxidants. As such, the use of phenolic antioxidants is recommended to improve oxidative damage, with minimal side effects and ease of use [12,18]. For the first time, in the current study, we simultaneously evaluated the effects of *C. siliqua* extract on human sperm parameters including motility, viability, cell membrane and mitochondrial potential, intracellular levels of reactive oxygen species (ROS), and the acrosome reaction using computer-assisted sperm analysis, fluorescence, hypoosmotic swelling (HOS) testing, JC-1 staining, flow cytometry, and fluorescent thiocyanate, respectively.

Methods

1. Extraction

1) Preparation of C. siliqua hydroalcoholic extract

The *C. siliqua* plant was prepared by a medicinal plant expert from the local plant market and approved by a pharmacognosy expert. First, 100 g of fresh *C. siliqua* plant matter was dried at room temperature and then ground in a blender, yielding a homogeneous powder. Then, 10 g of *C. siliqua* powder was mixed with 1,000 mL of 96% ethanol (Merck). The resulting solution was added to distilled water in a 50:50 ratio and kept at room temperature for 72 hours. This extract was then filtered (filter paper no. 4; Whatman PLC). The filtered solution was concentrated at 50 ℃ using a rotary apparatus. Finally, a brown extract was obtained, dried in an oven at 40 ℃, and kept at −20 ℃ until use [19].

2. Participation and semen collection

Verbal informed consent was obtained from each patient for the use of their semen samples (IR.ACECR.HAMEDAN.1400.140) were approved by the ethics committee of the Hamadan Branch of Azad University. A total of 20 normal sperm samples from men referred to the Aban Infertility Center between April 29, 2021 and August 17, 2021 were used. Samples were prepared during 3 to 5 days of abstinence. These normal samples had normal morphology (> 4%), normal motility (< 40%), and a concentration above 15 million/mL and were collected according to the 2010 World Health Organization guidelines.

3. Sperm processing

The swim-up method was used to process the semen samples [20]. In this method, the samples were first centrifuged for 5 minutes at 300 ×g; then, the supernatant was removed, and the pellets were precipitated with preheated human tubal fluid (HTF) and 2.5% human serum albumin (HSA) (Vitrolife) [21]. These samples were poured into a Falcon tube and incubated at a 45° angle in an incubator (5% CO_2, 37 ℃) for 1 hour. The supernatant was poured into a microtube, analyzed, and then frozen by vitrification.

4. Cryopreservation and thawing

After the preparation of sperm, the samples were frozen via the microdroplet technique [22]. For the cryopreservation control group, the sperm solution and HTF were mixed with a solution containing 5% HSA and 0.5 mol/L sucrose (Vitrolife) at a 1:1 ratio. *C. siliqua* extract was prepared at several concentrations (20, 30, and 40 µg/mL). For the cryopreservation experimental groups, the sperm solution was mixed with HSA and sucrose solution along with 20, 30, or 40 µg/mL of *C. siliqua* extract.

Droplets of 30 µL of the micropipette-prepared suspensions were poured into a metal strainer and placed in liquid nitrogen, and the frozen samples were kept in nitrogen storage tanks for 1 week [23]. For the thawing process, we first incubated the HTF medium for 2 hours at 37 ℃ and then immersed the frozen samples in 5 mL of heated HTF supplemented with 1% HSA.

Next, the sperm suspensions were incubated at 37 ℃ and 5% CO_2 for 5 minutes. Finally, the samples were centrifuged for 5 minutes at 1,800 rpm, the pellets were resuspended in 50 µL HTF, and the sperm were evaluated [23].

5. Assessment of sperm parameters

1) Motion characteristics

A computer-aided sperm analyzer system (Sperm Class Analyzer version 6; Microptic) was used to assess motility and the motility indices. In this study, 5-µL samples from each group were placed in a preheated Makler chamber (Proiser), and general motility, progressive movement and motor indices, average path velocity (µm/sec), curvilinear velocity (µm/sec), straight-line velocity (µm/sec), mean linearity (µm/sec), straightness (µm/sec), amplitude of lateral head displacement (µm), and beat cross frequency (Hz) were assessed for 500 sperm.

2) Viability

To assess sperm viability, a LIVE/DEAD sperm kit was used according to the manufacturer's instructions (L-7011; Molecular Probes). To accomplish this, 0.1 µL of SYBR14 working solution (containing 150 mM sodium chloride, 10 mM HEPES, 10% bovine serum albumin,

and SYBR14) was added to 50 µL of sperm suspension. The resulting solution was incubated at 37 °C for 10 minutes. Then, 1 µL of propidium iodide solution was added to the previous suspension and incubated for another 5 minutes in an incubator at 37 °C, and 10 µL of Hancock solution was added to the solution to immobilize the sperm. Finally, to assess viability, 6 µL of this final suspension was placed on a glass slide, covered, and examined using a fluorescence microscope (CX21; Olympus; excitation at 450–490 nm, emission at 520 nm) with a magnification of ×1,000. In this protocol, cells were stained with propidium iodide and SYBR14 on each slide. Dead and living spermatozoa were evaluated based on red and green color emissions, respectively. The LIVE/DEAD sperm ratio is depicted in Figures 1 and 2 [21].

3) Plasma membrane integrity

The HOS test was used to evaluate membrane integrity. We mixed 10 µL of the sperm sample, 100 µL of HOS solution containing 0.73 g of sodium citrate and 1.35 g of fructose (Merck), and 100 mL of distilled water and incubated the mixture (5% CO_2, 37 °C). Then, 15 µL of this solution was added to 5 µL of eosin Y solution (2%), and a smear was prepared from the resulting mixture on a glass slide. Finally, 200 sperm were assessed using a light microscope (ECLIPSE 50i; Nikon) with a magnification of ×1,000. Coiled-tail sperm were considered to have intact plasma membranes (Figure 3) [24].

4) Mitochondrial activity

JC-1 is a lipophilic cationic dye (T4069; Sigma-Aldrich) used to as-

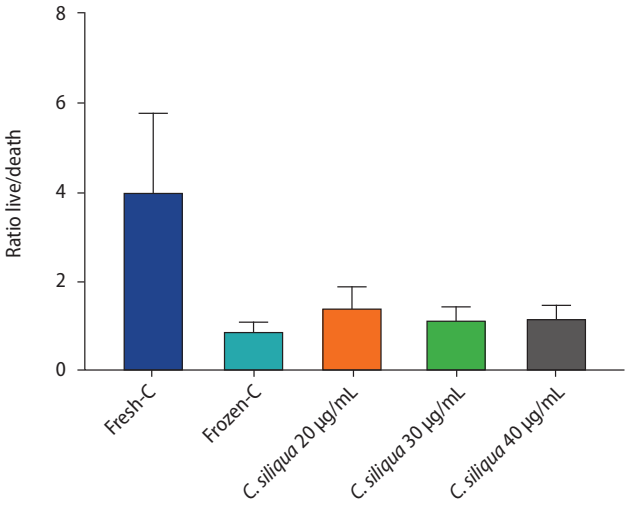

Figure 2. The graph shows the ratio of live/death sperms in the control and experimental groups. The most positive effect on the ratio of live/death sperms is related to the group containing 20 µg/mL cryopreservation supplement of siliqua extract. *C. siliqua, Ceratonia siliqua.*

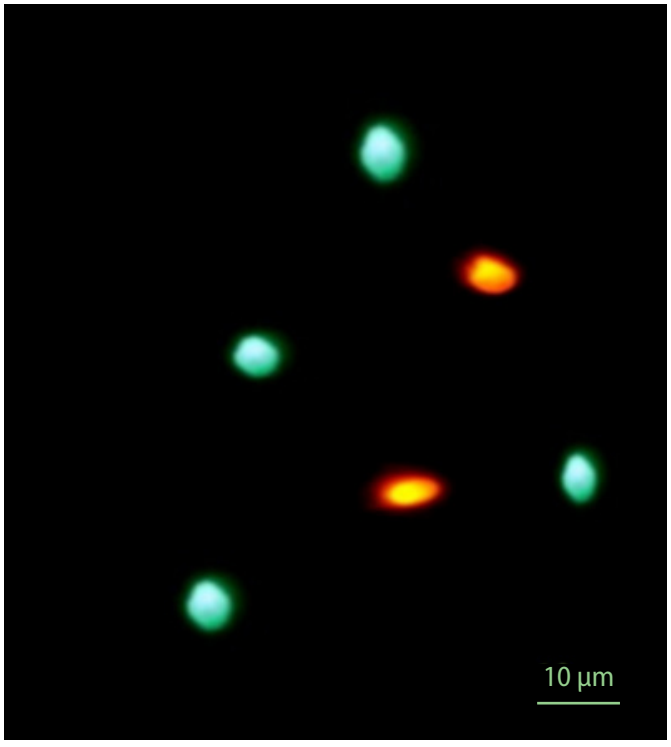

Figure 1. This shows dead and live sperm based on the fluorescence emitted. Green sperm stained with SYBR14 are alive and orange sperm stained with propidium iodide are dead.

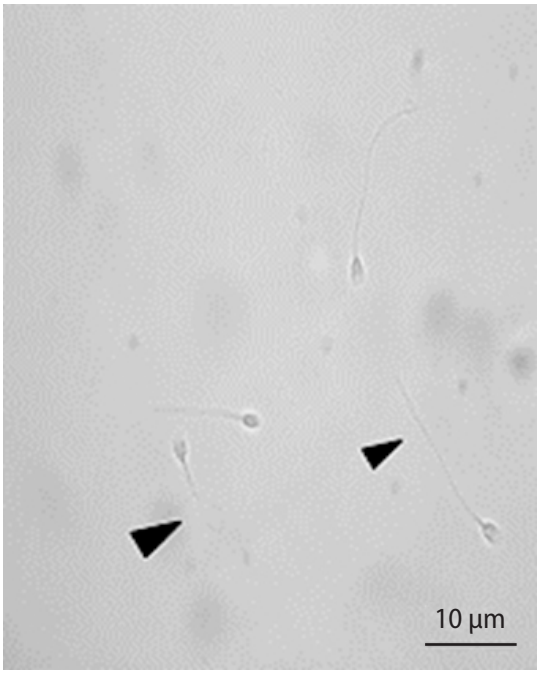

Figure 3. This figure shows the integrity of the plasma membrane based on hypoosmotic swelling tests. Sperms with straight tails are considered dead and sperms with coiled tails are considered alive.

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sess sperm mitochondrial activity. For this assessment, sperm samples were centrifuged for 5 minutes at 500 ×g, after which the pellet was diluted with phosphate-buffered saline. Then, 500 µL of this solution was mixed with 1 µL of JC-1 stock solution and incubated at 37 °C for 40 minutes. Next, 10 µL of Hancock solution was added to the previous solution to immobilize the sperm. Finally, 2.5 µL of the prepared sample was placed on a glass slide, covered, and assessed under a BX51 fluorescence microscope (Olympus; excitation at 450–490 nm, emission at 520 nm) at a magnification of ×1,000, under which 200 sperm per slide were examined. Sperm with yellow/orange fluorescence at the midline were considered to display high mitochondrial activity, while sperm with green fluorescence were considered to exhibit low mitochondrial activity (Figure 4) [25].

5) Acrosome integrity

To evaluate the integrity of the acrosome, fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA, L0770; Sigma-Aldrich) was used. Initially, a 30-µL smear was prepared on a glass slide for each sample. After drying, the samples were fixed at room temperature with methanol for 30 minutes. Then, 50 µL of FITC-PSA solution was poured on each slide and incubated for 30 minutes. Finally, the stained slides were washed with distilled water and were evaluated using a BX51 fluorescence microscope (Olympus; excitation at 450–490 nm, emission at 520 nm) with a magnification of ×1,000. For each slide, 200 sperm were examined. Sperm with bright green fluorescence in the acrosome area were identified as having intact acrosomes, while sperm with no green or pale green fluorescence near the equator were identified as acrosome-reacted (Figure 5) [21].

6) Intracellular ROS

To assess intracellular ROS, sperm samples were first suspended after melting with phosphate-buffered saline. Then, 10 µL of dihydroethidium solution was added. The samples were incubated at room temperature for 30 minutes. For this measurement, flow cytometry was used. Red fluorescence with an FL2 detector (525–625 nm) was used to indicate intracellular ROS [26,27].

7) Flowcytometric analysis

Flowcytometric analysis was conducted using Cyflogic version

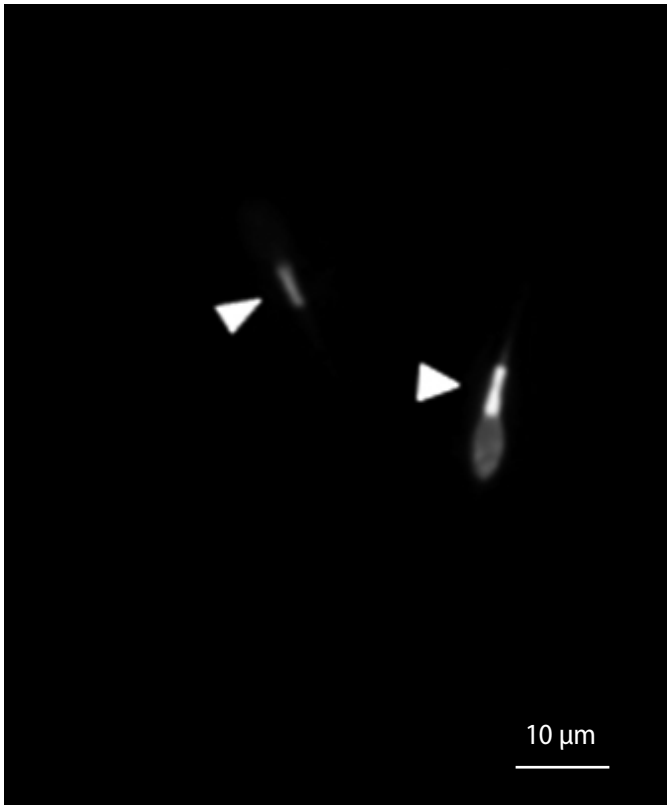

Figure 4. This figure shows the activity of sperm mitochondria. Sperms with yellow fluorescence in the midpiece region show high activity of the mitochondrial membrane and sperms with green fluorescence show low activity of the mitochondrial membrane.

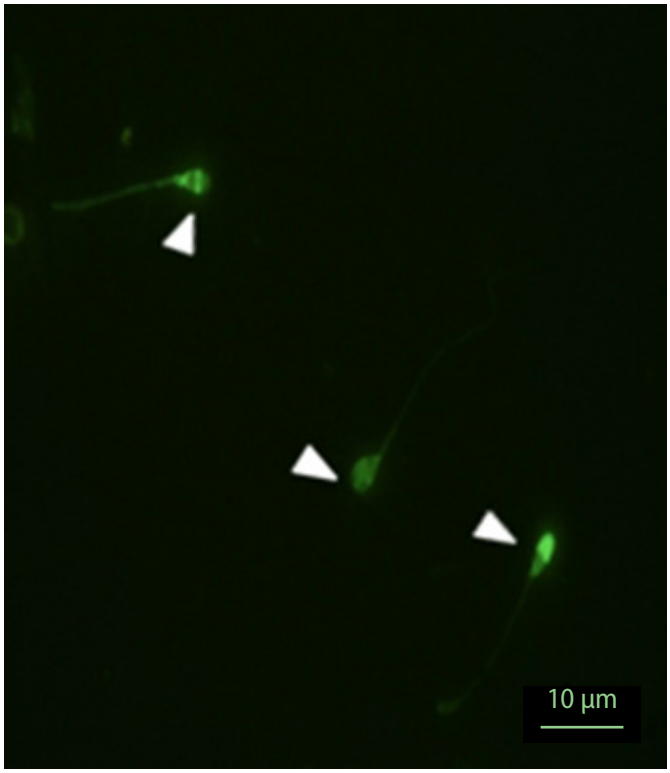

Figure 5. This figure shows the acrosome reaction based on the emission of different colors from the acrosome region using a fluorescence microscope. Sperms with clear green fluorescence as intact acrosomes and the sperms with pale green fluorescence were determined as acrosome reaction.

2.5.1 (CyFlo Ltd.). This analysis was performed using a FACSCalibur flow cytometer (BD Biosciences) and an argon laser with a wavelength of 488 nm [28].

Results

As shown in Table 1, when either 30 or 40 μg/mL of *C. siliqua* extract was added to the sperm freezing extender, no significant differences in total or progressive motility were observed relative to the cryopreservation control group. In contrast, significantly greater motility was observed in the group treated with 20 μg/mL extract ($p \leq 0.05$). No significant difference was observed in the other motility characteristics for any of the extract concentrations relative to the cryopreservation control group (Table 1).

As shown in Table 2, the intracellular ROS level was significantly reduced among sperm treated with any concentration of *C. siliqua* extract relative to the cryopreservation control group ($p \leq 0.05$). Additionally, by increasing the concentration of *C. siliqua* extract from 20 μg/mL to 30 or 40 μg/mL, viability and plasma membrane integrity (PMI) were significantly improved in the cryopreservation experimental groups relative to the cryopreservation control group. The greatest increase relative to the control was associated with the 20 μg/mL extract concentration, and no significant difference was observed between 30 and 40 μg/mL ($p \geq 0.05$). Levels of both mitochondrial membrane potential (MMP) and the acrosome reaction showed a significant increase at all three concentrations compared to the cryopreservation control group. However, no significant difference was seen between the experimental groups (Table 2).

Discussion

Sperm cryopreservation can involve the formation of ice crystals, osmotic imbalance, and oxidative stress, potentially causing irreparable damage to the structure and ultimately the function of the cell [29]. One method to prevent the formation of ice crystals and the associated damage is cryopreservation by vitrification [30]. In this process, sperm cryopreservation is performed more quickly and with greater safety than in other methods [31,32]. The cryopreservation process plays a role in the production of ROS by accelerating the conversion of anion superoxide to hydrogen peroxide [33]. Although a

Table 1. Effect of *Ceratonia siliqua* concentrations 20, 30, and 40 μg/mL added to cryopreservation/thawing media on motility parameters of human spermatozoa

Groups	Fresh-C	Frozen-C	*C. siliqua* 20 μg/mL	*C. siliqua* 30 μg/mL	*C. siliqua* 40 μg/mL
Motility	45.12±11.10	21.51±8.33[a]	31.75±7.13[b]	25.30±7.12	25.66±7.91
Progressive motility	40.52±5.21	16.63±5.45[a]	41.89±5.14[b]	20.55±5.71	21.09±5.41
VCL	63.12±1.16	56.62±0.71[a]	59.40±0.79	58.12±0.87	56.33±0.71
VSL	45.91±0.69	37.22±0.51[a]	38.17±0.64	36.59±0.58	35.12±0.50
VAP	50.45±0.69	43.64±0.60[a]	45.92±0.66	44.12±0.59	43.52±0.67
LIN	59.95±0.62	50.60±0.63[a]	51.25±0.57	49.44±0.60	49.75±0.69
SRT	70.30±0.35	55.72±0.31[a]	57.11±0.36	54.61±0.30	54.23±0.39
BCF	10.03±0.06	8.71±0.06[a]	8.91±0.05	8.77±0.06	8.62±0.06
ALH	9.73±0.05	6.25±0.05[a]	6.63±0.06	6.20±0.05	6.22±0.05

Values are presented as mean±standard error of the mean.
C, control; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; SRT, straightness; BCF, beat cross frequency; ALH, amplitude of lateral head displacement.
[a]$p<0.05$ significant differences vs. fresh control group; [b]$p<0.05$ significant differences vs. frozen control group.

Table 2. Effect of *Ceratonia siliqua* concentrations 20, 30, and 40 μg/mL added to cryopreservation/thawing media on ROS intracellular, PMI, viability, MMP, and acrosome reaction of human spermatozoa

Groups	Fresh-C	Frozen-C	*C. siliqua* 20 μg/mL	*C. siliqua* 30 μg/mL	*C. siliqua* 40 μg/mL
ROS intracellular	40.1±6.3	59.1±6.1[a]	52.0±6.5[b]	52.3±6.1[b]	51.3±6.0[b]
PMI	53.1±3.5	24.4±3.9[a]	55.1±3.4[b]	41.3±3.9[b]	41.9±3.5[b]
Viability	71.3±1.5	45.3±1.09[a]	51.6±1.8[b]	50.5±1.3[b]	50.9±1.3[b]
MMP	65.5±4.2	33.6±4.7[a]	42.5±4.9[b]	44.2±4.9[b]	42.9±4.5[b]
Acrosome reaction	77.2±5.5	56.5±5.1[a]	61.5±5.9[b]	60.3±5.2[b]	60.9±5.7[b]

Values are presented as mean±standard error of the mean.
ROS, reactive oxygen species; PMI, plasma membrane integrity; MMP, mitochondrial membrane potential; C, control.
[a]$p<0.05$ significant differences vs. fresh control group; [b]$p<0.05$ significant differences vs. frozen control group.

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moderate ROS level is required for optimal sperm function, high levels are associated with the impairment of function [34]. ROS produced as a result of cryopreservation can damage the lipid, protein, and DNA structures of the sperm [35].

Studies have shown that cryopreservation reduces the activity of the sperm antioxidant system, leading to impaired cell motility, integrity, and membrane fluidity [33]. *C. siliqua* extract can protect against oxidative stress [15,36]. Various studies have indicated that the protective effect of this plant extract is due to its strong antioxidant properties [12,19,37]. The results of the current study confirm the protective properties of this extract against oxidative stress, as the intracellular ROS level was significantly reduced at all concentrations of *C. siliqua* extract. Although no previous research had directly examined the intracellular ROS level in sperm treated with *C. siliqua* extract, the improvement of ROS-dependent parameters in other studies aligns with this result. Previous studies have shown that *C. siliqua* extract can increase the activity of antioxidant enzymes such as catalase and superoxide dismutase [15], so the reduction of intracellular ROS during cryopreservation can be explained by the capacity of *C. siliqua* extract to stimulate these enzymes.

Notably, mitochondrial activity in oxidative phosphorylation can be a source of ROS production [38]. At all three concentrations of *C. siliqua* extract, the sperm MMP was significantly increased relative to the cryopreservation control group, most likely by reducing the level of intracellular ROS. Motility is an essential sperm parameter that can have a positive or negative effect on fertility. In the current study, the total and progressive movement in the cryopreservation control group were significantly lower than in the fresh group, likely due to the sensitivity of the adenosine triphosphate-dependent sodium-potassium pump and the consequent leakage of ions related to movement [39]. In the sperm treated with the extract, the total and progressive movement were improved compared to the cryopreservation control group, most notably in the sperm treated with 20 µg/mL of the extract. These findings align with the results of Sabzeie et al. [19] and Faramarzi et al. [12]. Like the present study, those studies revealed not only a positive effect of *C. siliqua* extract on motility, but also an optimal extract concentration of 20 µg/mL. This is likely because *C. siliqua* extract has a greater capacity to reduce superoxide anions at 20 µg/mL than at other concentrations. However, for the other motor characteristics (average path velocity, curvilinear velocity, straight-line velocity, mean linearity, straightness, amplitude of lateral head displacement, and beat cross frequency), we found no significant differences between the concentrations of 20, 30, and 40 µg/mL or in comparison with the cryopreservation control group, which contradicts the study of Sabzeie et al. [19]. Motility is a known indicator of survival and of other health parameters such as PMI, mitochondria health, and even sperm DNA damage. Our results showed that the presence of *C. siliqua* extract in cryopreservation can help increase sperm viability. This finding supports the results of the Faramarzi et al. [12] study, which revealed a higher percentage of sperm survival in groups treated with the extract than in the control group; additionally, the survival rate of sperm treated with 20 µg/mL was significantly higher than among the control group or the sperm treated with 10 or 40 µg/mL. The improvement of this parameter can likely be attributed to the antioxidant contents of gallic acid, chlorogenic acid, cinnamic acid, and caffeic acid in *C. siliqua* extract. Generally, these compounds have been shown to reduce intracellular ROS and increase sperm viability. In the current study, the results of the HOS test indicated that the greatest membrane integrity was present in the group treated with 20 µg/mL of extract, while this parameter was not significantly different in the groups with concentrations of 30 and 40 µg/mL. This finding is completely consistent with the result of Sabzeie et al. [19]. In our study, the presence of *C. siliqua* extract at all concentrations (20, 30, and 40 µg/mL) led to a significant increase in the acrosome reaction compared to the cryopreservation control group. This result can be attributed to the antioxidant roles of this extract in countering the damage caused by oxidative stress as well as in osmotic balance regulation, membrane preservation, conjugation, and calcium regulation. Accordingly, the role of extracellular antioxidants in improving sperm membrane resistance can be explained by the reduction of lipid peroxidation [40].

In conclusion, *C. siliqua* extract supplements at concentrations of 20, 30, and 40 µg/mL improve sperm motility, viability, cell membrane and mitochondrial potential, intracellular ROS, and the acrosome reaction. A concentration of 20 µg/mL has a particularly high capacity to reduce intracellular ROS, oxidative stress, and its products, eventually improving ROS-affected parameters such as motility, PMI, MMP, and the acrosome reaction.

Conflict of interest

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

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Conceptualization: YK, ES. Data curation: FM. Formal analysis: TF. Funding acquisition: ES. Methodology: YK. Project administration: TF.

Visualization: TF. Writing-original draft: YK. Writing-review & editing: FM, YK.

References

1. Oehninger S, Duru NK, Srisombut C, Morshedi M. Assessment of sperm cryodamage and strategies to improve outcome. Mol Cell Endocrinol 2000;169:3–10.
2. Braga DP, Setti AS, Figueira RC, Iaconelli A Jr, Borges E Jr. The negative influence of sperm cryopreservation on the quality and development of the embryo depends on the morphology of the oocyte. Andrology 2015;3:723–8.
3. Shufaro Y, Schenker JG. Cryopreservation of human genetic material. Ann N Y Acad Sci 2010;1205:220–4.
4. Tamburrino L, Cambi M, Marchiani S, Manigrasso I, Degl'Innocenti S, Forti G, et al. Sperm DNA fragmentation in cryopreserved samples from subjects with different cancers. Reprod Fertil Dev 2017;29:637–45.
5. Di Santo M, Tarozzi N, Nadalini M, Borini A. Human sperm cryopreservation: update on techniques, effect on DNA integrity, and implications for ART. Adv Urol 2012;2012:854837.
6. Gupta S, Agarwal A, Sharma R, Ahmady A. Recovery, preparation, storage and utilization of spermatozoa for fertility preservation in cancer patients and sub-fertile men. J Reprod Stem Cell Biotechnol 2010;1:150–68.
7. Amidi F, Pazhohan A, Shabani Nashtaei M, Khodarahmian M, Nekoonam S. The role of antioxidants in sperm freezing: a review. Cell Tissue Bank 2016;17:745–56.
8. Kopeika J, Zhang T, Rawson DM, Elgar G. Effect of cryopreservation on mitochondrial DNA of zebrafish (Danio rerio) blastomere cells. Mutat Res 2005;570:49–61.
9. Layek SS, Mohanty TK, Kumaresan A, Parks JE. Cryopreservation of bull semen: evolution from egg yolk based to soybean based extenders. Anim Reprod Sci 2016;172:1–9.
10. Hezavehei M, Sharafi M, Kouchesfahani HM, Henkel R, Agarwal A, Esmaeili V, et al. Sperm cryopreservation: a review on current molecular cryobiology and advanced approaches. Reprod Biomed Online 2018;37:327–39.
11. Mohammadi F, Varanloo N, Heydari Nasrabadi M, Vatannejad A, Amjadi FS, Javedani Masroor M, et al. Supplementation of sperm freezing medium with myoinositol improve human sperm parameters and protects it against DNA fragmentation and apoptosis. Cell Tissue Bank 2019;20:77–86.
12. Faramarzi A, Aghaz F, Golestan Jahromi M, Bakhtiari M, Khazaei M. Does supplementation of sperm freezing/thawing media with Ceratonia siliqua improve detrimental effect of cryopreservation on sperm parameters and chromatin quality in normozoospermic specimens? Cell Tissue Bank 2019;20:403–9.
13. Forouzanfar M, Sharafi M, Hosseini SM, Ostadhosseini S, Hajian M, Hosseini L, et al. In vitro comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen. Theriogenology 2010;73:480–7.
14. Faramarzi A, Aghaz F, Bakhtiari M, Roshankhah S, Rashidi Z, Khazaei M. Ceratonia siliqua (Carob) extract improved in vitro development of vitrified-warmed mouse germinal vesicle oocytes: assessment of possible mechanism. Cell Tissue Bank 2021;22:137–44.
15. Vafaei A, Mohammadi S, Fazel A, Soukhtanloo M, Mohammadipour A, Beheshti F. Effects of carob (Ceratonia siliqua) on sperm quality, testicular structure, testosterone level and oxidative stress in busulfan-induced infertile mice. Pharma Sci 2018;24:104–11.
16. Pan B, Yang H, Wu Z, Qazi IH, Liu G, Han H, et al. Melatonin improves parthenogenetic development of vitrified-warmed mouse oocytes potentially by promoting G1/S cell cycle progression. Int J Mol Sci 2018;19:4029.
17. Khazaei M, Pazhouhi M, Khazaei S. Evaluation of hydro-alcoholic extract of Trifolium pratens L. for its anti-cancer potential on U87MG cell line. Cell J 2018;20:412–21.
18. Sadeghzadeh F, Sadeghzadeh A, Changizi-Ashtiyani S, Bakhshi S, Mashayekhi FJ, Mashayekhi M, et al. The effect of hydro-alcoholic extract of Ceratonia Silique L. on spermatogenesis index in rats treated with cyclophosphamide: an experimental study. Int J Reprod Biomed 2020;18:295–306.
19. Sabzeie MM, Soleimanzadeh A, Ayen E. The effect of Ceratonia siliqua aqueous extract on canine semen quality on during storage at Refrigerator. Vet Res Biol Prod 2021;34:120–10.
20. Ghaleno LR, Valojerdi MR, Janzamin E, Chehrazi M, Sharbatoghli M, Yazdi RS. Evaluation of conventional semen parameters, intracellular reactive oxygen species, DNA fragmentation and dysfunction of mitochondrial membrane potential after semen preparation techniques: a flow cytometric study. Arch Gynecol Obstet 2014;289:173–80.
21. Taher-Mofrad SM, Topraggaleh TR, Ziarati N, Bucak MN, Nouri M, Seifi S, et al. Knockout serum replacement is an efficient serum substitute for cryopreservation of human spermatozoa. Cryobiology 2020;92:208–14.
22. Aitken RJ, Clarkson JS. Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. J Androl 1988;9:367–76.
23. Seify M, Zarabadipour M, Ghaleno LR, Alizadeh A, Rezazadeh Valojerdi M. The anti-oxidant roles of taurine and hypotaurine on acrosome integrity, HBA and HSPA2 of the human sperm during vitrification and post warming in two different temperature. Cryobiology 2019;90:89–95.

https://doi.org/10.5653/cerm.2022.05554

24. Ziarati N, Topraggaleh TR, Rahimizadeh P, Montazeri L, Maroufizadeh S, Sadighi Gilani MA, et al. Micro-quantity straw as a carrier for cryopreservation of oligozoospermic semen samples: effects of storage times and cryoprotectant. Cryobiology 2019;86:65–70.
25. Bucak MN, Bodu M, Baspinar N, Gungor S, Ili P, Acibaeva B, et al. Influence of ellagic acid and ebselen on sperm and oxidative stress parameters during liquid preservation of ram semen. Cell J 2019;21:7–13.
26. Koohestanidehaghi Y, Torkamanpari M, Shirmohamadi Z, Lorian K, Vatankhah M. The effect of cysteine and glutamine on human sperm functional parameters during vitrification. Andrologia 2021;53:e13870.
27. Torkamanpari M, Ghorbani F, Lorian K, Koohestanidehaghi Y. The effects of purslane (Portulaca oleracea) and fennel (Foeniculum vulgare Mill) hydroalcoholic extracts on the functional parameters of human spermatozoa after vitrification. Clin Exp Reprod Med 2023;50:78-85.
28. Nasiri Z, Ghorbani F, Seify M, Sharbati A. Effect of aqueous Nigella sativa extract on the functional parameters of post-thaw human spermatozoa during vitrification. Clin Exp Reprod Med 2022;49:110–6.
29. Tiwari A, Tekcan M, Sati L, Murk W, Stronk J, Huszar G. A new media without animal component for sperm cryopreservation: motility and various attributes affecting paternal contribution of sperm. J Assist Reprod Genet 2017;34:647–57.
30. Arav A, Natan Y, Kalo D, Komsky-Elbaz A, Roth Z, Levi-Setti PE, et al. A new, simple, automatic vitrification device: preliminary results with murine and bovine oocytes and embryos. J Assist Reprod Genet 2018;35:1161–8.
31. Horta F, Alzobi H, Jitanantawittaya S, Catt S, Chen P, Pangestu M, et al. Minimal volume vitrification of epididymal spermatozoa results in successful in vitro fertilization and embryo development in mice. Asian J Androl 2017;19:107–12.
32. Spis E, Bushkovskaia A, Isachenko E, Todorov P, Sanchez R, Skopets V, et al. Conventional freezing vs. cryoprotectant-free vitrification of epididymal (MESA) and testicular (TESE) spermatozoa: three live births. Cryobiology 2019;90:100–2.
33. Ghorbani F, Nasiri Z, Koohestanidehaghi Y, Lorian K. The antioxidant roles of L-carnitine and N-acetyl cysteine against oxidative stress on human sperm functional parameters during vitrification. Clin Exp Reprod Med 2021;48:316–21.
34. Lone SA, Prasad JK, Ghosh SK, Das GK, Balamurugan B, Verma MR. Study on correlation of sperm quality parameters with antioxidant and oxidant status of buffalo bull semen during various stages of cryopreservation. Andrologia 2018;50:e12970.
35. Bustamante Filho IC, Pederzolli CD, Sgaravatti AM, Gregory RM, Dutra Filho CS, Jobim MI, et al. Skim milk-egg yolk based semen extender compensates for non-enzymatic antioxidant activity loss during equine semen cryopreservation. Anim Reprod 2018;6:392–9.
36. Rtibi K, Selmi S, Grami D, Saidani K, Sebai H, Amri M, et al. Ceratonia siliqua L. (immature carob bean) inhibits intestinal glucose absorption, improves glucose tolerance and protects against alloxan-induced diabetes in rat. J Sci Food Agric 2017;97:2664–70.
37. Faramarzi A, Aghaz F, Bakhtiari M, Khazaei M. In vitro application of Ceratonia siliqua improved sperm parameters and chromatin quality after vitrifacation in normozoospermic aged men. Middle East Fertil Soc J 2020;24:6.
38. He B, Guo H, Gong Y, Zhao R. Lipopolysaccharide-induced mitochondrial dysfunction in boar sperm is mediated by activation of oxidative phosphorylation. Theriogenology 2017;87:1–8.
39. Saito K, Kinoshita Y, Kanno H, Iwasaki A. The role of potassium ion and extracellular alkalization in reinitiation of human spermatozoa preserved in electrolyte-free solution at 4 degrees C. Fertil Steril 1996;65:1214–8.
40. Dawson R Jr, Biasetti M, Messina S, Dominy J. The cytoprotective role of taurine in exercise-induced muscle injury. Amino Acids 2002;22:309–24.

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The effects of orchiectomy and steroid on fertility in experimental testicular atrophy

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Objective: Despite our understanding of Sertoli cell function and the state of spermatogenesis, the underlying mechanisms remain unclear. This study was conducted to compare the effects of orchiectomy and steroid treatment on fertility in testicular atrophy occurring after testicular torsion.

Methods: Thirty-three rats were divided into four groups. The atrophy, orchiectomy, and atrophy-steroid groups each contained nine rats, while the control group contained six. The left testes were rotated 720º, and atrophy was observed. In the atrophy-steroid rats, orchiectomy was performed after atrophy, and 1 mg/kg steroid was injected. Each male rat was housed with five female rats for 6 days. The fertility of the male rats was evaluated based on the pregnancy of the female rats. Left and right orchiectomies were performed to determine the tissue Johnsen score (JS) and the serum inhibin B (IB) level.

Results: JS values were significantly lower in the atrophy, orchiectomy, and atrophy-steroid groups than in the control group ($p<0.05$), while no significant difference was observed in JS between the atrophy and orchiectomy groups ($p>0.05$). Similarly, no significant differences in IB level or fertility percentage were found between the atrophy and orchiectomy rats ($p>0.05$).

Conclusion: In unilateral testicular atrophy, which can occur in the prepubertal period due to various causes, orchiectomy does not appear to benefit fertility, as indicated by IB, JS, and the fertility percentage.

Keywords: Atrophy; Fertility; Orchiectomy; Steroids; Testis

Introduction

Testicular torsion (TT) causes severe testicular injury through cell membrane lipid peroxidation, protein denaturation, and DNA impairment [1]. Even after the diagnosis and treatment of TT, problems with fertility and testicular atrophy persist. The severity of testicular atrophy is exacerbated by a delay in surgical intervention. Researchers have considered whether testicular damage after TT, which negatively impacts spermatogenesis and fertility, depends on testicular anomalies or autoimmune mediators. Along these lines, the decision of whether to excise an atrophic testis is controversial [2].

The degree of infertility in cases of TT and testicular atrophy can range from oligospermia to azoospermia. Torsion causes spermatogenesis and Sertoli cells to deteriorate in the testicular tissue, and these changes can be identified based on histopathological examination. Johnsen score (JS) conveys detailed information regarding germ and Sertoli cells [2]. Some studies have shown that serum inhibin B (IB) levels reflect Sertoli cell function and the state of spermatogenesis [2-4].

Steroids prevent the production of reactive oxygen species by blocking the phospholipase A2 enzyme and inhibiting leukocyte activation. In this manner, they preserve the integrity of the cell membrane, inhibit an increase in capillary permeability, and exert an anti-inflammatory effect [5].

A prior study from our unit demonstrated for the first time that melatonin is a potent antioxidant agent in preventing testicular ischemia-reperfusion injury, as indicated by increased IB levels and JS values [2]. Although this finding enhanced our knowledge of Sertoli

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cell function and the state of spermatogenesis, many questions remain unanswered.

The current study was designed to determine the effectiveness of orchiectomy and steroid treatment on fertility after TT and to identify the more effective approach.

Methods

1. Animals

Local ethics committee approval was received before this study. The experimental protocol was approved by the Ethical Committee of Necmettin Erbakan University Scientific Research Council (2012-077). Six pubertal (75-day-old) and 27 prepubertal (45-day-old) male Wistar rats were used in this study [6]. The rats were housed in cages with four animals per cage on sawdust bedding and provided with standard rodent chow and water *ad libitum*. A constant temperature of 21 ºC and humidity of 55% were maintained, with 12-hour periods of light-dark exposure. A 3-month acclimatization period was allowed.

2. Experimental design

The animals were divided into four groups. The control group contained six pubertal male rats, while each of the three experimental groups (atrophy, orchiectomy, and atrophy-steroid treatment) contained nine prepubertal male rats. All surgical procedures were performed under ketamine anesthesia (50 mg/kg intramuscularly) with sterile technique. Surgery was conducted through a left ilioinguinal incision.

3. Testicular torsion procedure

In the experimental groups, the left testis was rotated 720° clockwise and fixed within the hemiscrotum with 4–0 polyglactin suture. The skin was closed with 4–0 silk suture, and the torsion was left in place for 40 days [7]. Atrophy was observed via inspection. After the rats recovered from surgery and reached the pubertal period, each was housed with five fertile female rats for 6 days.

The rats in the control group were also kept with five fertile female rats for 6 days. Rats were sacrificed via high-dose anesthetic agents after the birth of the pups. Later, left and right orchiectomies were performed. The tissue JS and the serum IB level were evaluated after orchiectomy. The fertility of the male rats was evaluated by identifying births and determining the pregnancy rates among the female rats (duration of pregnancy, 21 ± 2 days).

In the atrophy group, the left testis was dissected from the scrotum, and the TT procedure was performed [7]. Atrophy was observed by inspection. After the rats recovered from surgery and reached the pubertal period, each was housed with five fertile female rats for 6 days.

In the orchiectomy group, the torsion procedure was applied exactly as in the atrophy rats. Then, atrophy was observed by inspection, and left orchiectomy was performed. After the rats recovered from surgery and reached the pubertal period, each was housed with five fertile female rats for 6 days.

In the atrophy-steroid group, the torsion procedure was applied exactly as in the prior experimental groups. Then, 1 mg/kg steroid (methylprednisolone) (Prednol L; Mustafa Nevzat) was injected intramuscularly after orchiectomy following atrophy. Injections of methylprednisolone were continued once daily for 7 days for the atrophy-steroid group. In all groups, left orchiectomy was performed 40 days after torsion.

In the experimental groups, the rats were sacrificed after these procedures. Histopathologic examination was performed, and the JS in the testicular tissue was evaluated. A biochemical examination was performed, and serum IB levels were measured. The fertility of all male rats was evaluated by identifying births and determining the pregnancy rates among the female rats.

4. Biochemical evaluation of the blood

After blood samples were taken, serum IB levels were measured with an IB enzyme-linked immunosorbent assay kit (MyBioSource Inc.; catalog no.: MBS-162795).

5. Processing of histologic samples and histopathologic evaluation

All testes were fixed in Bouin solution for 24 hours, postfixed in 70% alcohol, and embedded in paraffin. Then, 5-µm sections were cut and stained with hematoxylin and eosin. Using a light microscope, histologic evaluation was performed by a pathologist in a blinded, randomly-numbered fashion without knowledge of which testes were experimentally torsed.

Testicular injury and spermatogenesis were graded as described by Johnson et al. [8]. JS was determined by evaluating four areas on four separate slides for each animal. A score of 1 was used to indicate a lack of seminiferous epithelial cells along with tubular sclerosis. A score of 2 indicated no germ cells, only Sertoli cells. A score of 3 indicated spermatogonia only, while a score of 4 indicated no spermatids, few spermatocytes, and arrest of spermatogenesis at the primary spermatocyte stage. A score of 5 indicated no spermatids and many spermatocytes. A score of 6 indicated no late and few early spermatids, arrest of spermatogenesis at the spermatid stage, and disturbance of spermatid differentiation. A score of 7 indicated no late and many early spermatids, a score of 8 indicated few late spermatids, and a score of 9 indicated many late spermatids and disorganized tubular epithelium. Finally, a score of 10 indicated full spermatogenesis.

6. Fertility evaluation of the testes

The fertility capacities of all male rats were evaluated based on the rates of pregnancy among the female rats.

7. Statistical analysis

The Kruskal-Wallis *H* and Mann-Whitney *U* tests were used for statistical analysis of IB, JS, and fertility. *p*-values < 0.05 were considered to indicate statistical significance.

Results

The experimental model was very well tolerated in all groups, and no animals died during the experimental period. The mean IB activity levels for all groups are shown in Table 1. IB activity was significantly lower in the atrophy-steroid group than in the control, orchiectomy, and atrophy groups, whereas pairwise comparisons showed it was higher in the atrophy group than in the atrophy-steroid group ($p < 0.05$). However, no significant differences in IB values were observed between the atrophy and orchiectomy groups ($p > 0.05$).

Testicular injury and lower histologic scores were observed in rats in the atrophy, orchiectomy, and atrophy-steroid groups, but not in the control rats (Figure 1). The JS values in the three experimental groups (Figures 2-4) were significantly lower than in the control

Table 1. Serum inhibin B levels

Group	Inhibin B level (pg/mL protein)
Control	$34.96 \pm 0.53^{a),b)}$
Atrophy	$38.09 \pm 0.46^{a)}$
Orchiectomy	$35.83 \pm 0.39^{a)}$
Atrophy plus steroid	$31.79 \pm 0.39^{b)}$

Values are presented as mean±standard error.
[a),b)]Different symbols indicate a statistically significant difference between means ($p<0.05$).

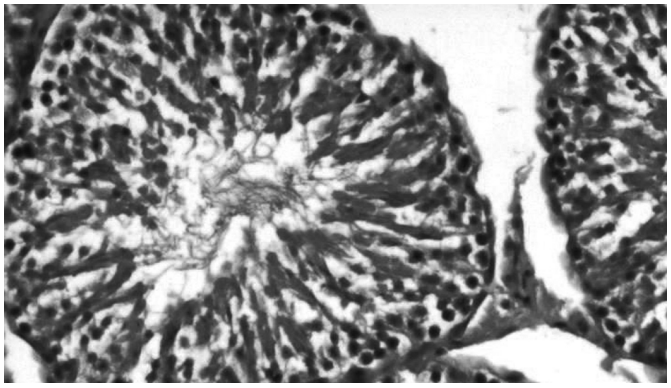

Figure 1. Photomicrograph of the control group showing the morphology of normal testicular tissue (Johnsen score, 9.500±0.091) (H&E, ×100).

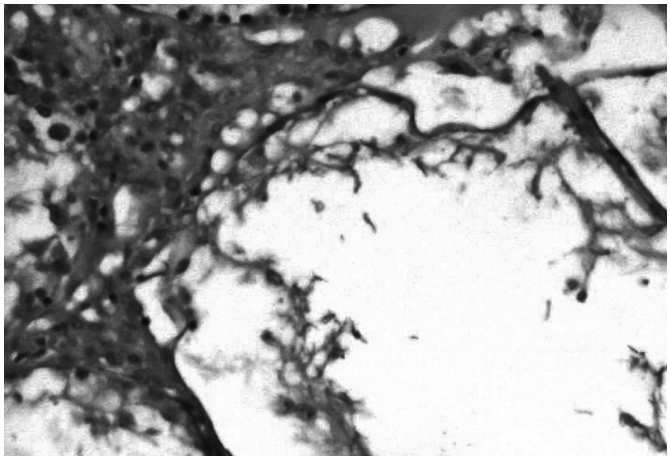

Figure 2. Photomicrograph of the atrophy group showing higher magnification of the morphology of atrophic testicular tissue (Johnsen score, 2.000±0.147) (H&E, ×100).

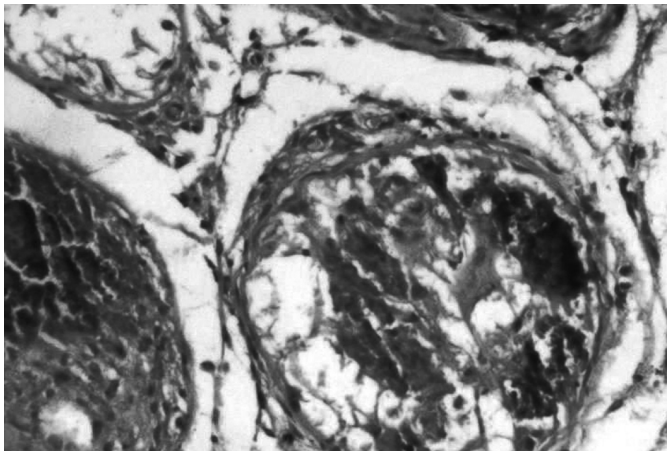

Figure 3. Photomicrograph of the orchiectomy group showing higher magnification of the morphology of testicular tissue (Johnsen score, 1.556±0.113) (H&E, ×100).

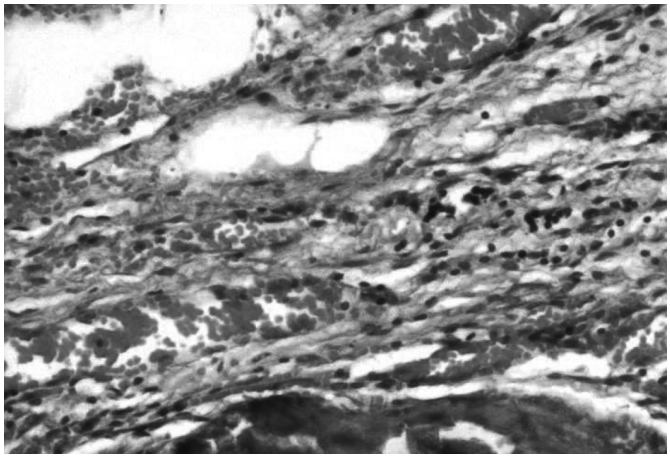

Figure 4. Photomicrograph of the atrophy-steroid group showing higher magnification of the morphology of testicular tissue (Johnsen score, 1.000±0.000) (H&E, ×100).

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group ($p < 0.05$). Testicular atrophy had developed in all testes in the three experimental groups, but JS was significantly higher in the control group than in the other groups ($p < 0.05$) (Table 2). The histopathologic evaluation is summarized in Table 2.

The fertility of all male rats was evaluated by identifying births and determining the pregnancy rates among the female rats. The fertility evaluation is summarized in Table 3. Fertility percentages were significantly lower in all three experimental groups than in the control group ($p < 0.05$); however, no significant differences were present between the fertility percentages of the atrophy and orchiectomy groups ($p > 0.05$).

Discussion

TT necessitates an aggressive surgical approach. Early surgical intervention increases the possibility of testicular salvage, but only 32% of testes can be saved [9].

The clinical applications of steroids include the treatment of hormone deficiency, inflammation suppression, immunosuppression, and the suppression of excess hormone secretion [10]. In an experimental study, Etensel et al. [11] reported that the contralateral testicular weight and volume in rats treated with torsion and detorsion, torsion with saline and detorsion, and dexpanthenol did not differ significantly from the control measurements. They suggested that dexpanthenol prevents testicular atrophy after 60 days of TT.

In our previous study, we found that once daily use of antioxidant agents for 7 days prevented testicular atrophy and was effective in testicular salvage [12]. Although this finding furthered our knowledge of the effects of antioxidants, many questions regarding infertility remained. To further clarify the effects of steroids, we chose to analyze testicular tissue specimens, blood, and fertility after steroid treatment.

We identified significantly lower JS values in the atrophy, orchiectomy, and atrophy-steroid groups compared to the control rats ($p < 0.05$), whereas we observed no significant difference in JS between the atrophy and orchiectomy groups ($p > 0.05$) in the ipsilateral testes. We also noted no significant differences in JS values among all groups ($p > 0.05$) in the contralateral testes. No significant differences were present in IB level between the atrophy and orchiectomy groups ($p > 0.05$). Fertility percentages were significantly lower in all three experimental groups than in the control group ($p < 0.05$), but no significant difference was observed in fertility percentage between the atrophy and orchiectomy groups ($p > 0.05$).

In unilateral testicular atrophy, which can occur in the prepubertal period due to various causes, orchiectomy does not appear to benefit fertility, as indicated by IB, JS, and fertility percentage.

Table 2. Tissue Johnsen scores

Group	Left testis
Control	$9.500 \pm 0.091^{a),b)}$
Atrophy	$2.000 \pm 0.147^{a)}$
Orchiectomy	$1.556 \pm 0.113^{a)}$
Atrophy plus steroid	$1.000 \pm 0.000^{b)}$

Values are presented as mean±standard error.
[a),b)]Different symbols indicate a statistically significant difference between means ($p<0.05$).

Table 3. Fertility results

Group	Fertility (%)
Control	$93.33 \pm 1.72^{a)}$
Atrophy	$62.22 \pm 2.82^{b)}$
Orchiectomy	$62.22 \pm 2.82^{b)}$
Atrophy plus steroid	$80.00 \pm 1.92^{a),b)}$

Values are presented as mean±standard error.
[a),b)]Different symbols indicate a statistically significant difference between means ($p<0.05$).

Conflict of interest

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

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Conceptualization: MY, RK. Data curation: MY, SK, RK. Formal analysis: SK. Methodology: MY, SK, RK. Writing-original draft: MY, RK. Writing-review & editing: MY, SK, RK.

References

1. Filho DW, Torres MA, Bordin AL, Crezcynski-Pasa TB, Boveris A. Spermatic cord torsion, reactive oxygen and nitrogen species and ischemia-reperfusion injury. Mol Aspects Med 2004;25:199–210.
2. Yurtcu M, Abasiyanik A, Avunduk MC, Muhtaroglu S. Effects of melatonin on spermatogenesis and testicular ischemia-reperfu-

sion injury after unilateral testicular torsion-detorsion. J Pediatr Surg 2008;43:1873–8.
3. Kubini K, Zachmann M, Albers N, Hiort O, Bettendorf M, Wolfle J, et al. Basal inhibin B and the testosterone response to human chorionic gonadotropin correlate in prepubertal boys. J Clin Endocrinol Metab 2000;85:134–8.
4. Balci T, Kocabas R, Cuce G, Akoz M. Inhibition of fatty acid binding protein 4 in obese male mice adversely affects reproductive parameters. J Reprod Infertil 2021;22:16–22.
5. Jansen NJ, van Oeveren W, van den Broek L, Oudemans-van Straaten HM, Stoutenbeek CP, Joen MC, et al. Inhibition by dexamethasone of the reperfusion phenomena in cardiopulmonary bypass. J Thorac Cardiovasc Surg 1991;102:515–25.
6. Havenaar R, Meijer JC, Morton DB, Ritskes-Hoitinga J, Zwart P. Biology and husbandry of laboratory animals. In: van Zutphen LFM, Baumans V, Beynen AC. editors. Principles of laboratory animal science. Revised ed. Elsevier; 2001. p. 19–77.
7. Aydın A, Sonmez MG, Ecer G, Kilinc F, Kocabas R, Atilgan AE, et al. The effect of intratesticular dexpanthenol on experimentally-induced testicular ischaemia/reperfusion injury. J Pediatr Urol 2021;17:440.e1–7.
8. Johnson L, Petty CS, Neaves WB. The relationship of biopsy evaluations and testicular measurements to over-all daily sperm production in human testes. Fertil Steril 1980;34:36–40.
9. Krarup T. The testes after torsion. Br J Urol 1978;50:43–6.
10. Brown DC. Adrenal corticosteroids, antagonists, corticotropin. In: Bennett PN, Brown MJ, Sharma Pankaj S. editors. Clinical pharmacology. 11th ed. Elsevier; 2012. p. 558–71.
11. Etensel B, Ozkisacik S, Ozkara E, Serbest YA, Oztan O, Yazici M, et al. The protective effect of dexpanthenol on testicular atrophy at 60th day following experimental testicular torsion. Pediatr Surg Int 2007;23:271–5.
12. Yurtcu M, Abasiyanik A, Bicer S, Avunduk MC. Efficacy of antioxidant treatment in the prevention of testicular atrophy in experimental testicular torsion. J Pediatr Surg 2009;44:1754–8.

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Seminal prolactin is associated with *HSP90* transcript content in ejaculated spermatozoa

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Objective: Evidence indicates that an imbalance between the production of reactive oxygen species and defense ability of antioxidants has clinical significance in the pathophysiology of male infertility. To investigate the role of seminal prolactin (PRL) in the fertilizing capacity of men, the present study evaluated the associations of seminal PRL levels with semen parameters and heat shock protein 90 (*HSP90*) transcript abundance in ejaculated spermatozoa.
Methods: We assessed seminal PRL levels and the abundance of *HSP90* transcripts in ejaculated spermatozoa from normozoospermic donors (n=18) and infertile men (n=18). The transcript content of *HSP90* in ejaculated spermatozoa was analyzed using real-time polymerase chain reaction.
Results: Seminal PRL concentrations in infertile patients were significantly lower (p=0.004) than in fertile controls. Seminal PRL showed relatively good diagnostic power for discriminating infertile men (area under the curve=0.776; 95% confidence interval, 0.568 to 0.934; p=0.005). Significant positive correlations were seen between seminal PRL levels and sperm count (r=0.400, p=0.016) and progressive motility (r=0.422, p=0.010). Infertile patients showed a significantly higher abundance of sperm *HSP90* than fertile controls (p=0.040). Sperm *HSP90* transcript abundance was negatively correlated with sperm progressive motility (r=0.394, p=0.018). Men with higher seminal PRL levels exhibited a lower abundance of sperm *HSP90* transcripts.
Conclusion: Our finding demonstrated associations among semen quality, seminal PRL levels, and the abundance of *HSP90* transcripts in ejaculated spermatozoa. Seminal PRL may contribute to male fertility by maintaining the seminal antioxidant capacity and may have the potential to act as a diagnostic and prognostic biomarker.

Keywords: HSP90; Male infertility; Oxidative stress; Prolactin; Spermatozoa

Introduction

Infertility is a public health concern with psychological, social, emotional, and financial implications that is estimated to affect 8% to 12% of reproductive-age couples worldwide [1]. It has been reported that a male factor contributes to infertility in approximately 50% of couples who fail to conceive [2]. Evidence shows that oxidative stress—an imbalance between reactive oxygen species (ROS) production and antioxidant defense ability—has clinical significance in the pathophysiology of male infertility [3]. Excessive generation of ROS and/or a decreased available antioxidant defense system leads to abnormal sperm function by affecting the plasma membrane and DNA integrity [4]. There are various enzymatic and non-enzymatic antioxidants in the seminal plasma that are essential for protecting spermatozoa from oxidative damage [5].

Prolactin (PRL), a 198-amino-acid polypeptide hormone, is synthesized by lactotroph cells and secreted from the anterior pituitary in a pulsatile manner. It weighs 23 kDa, and its secretion is affected by

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many factors, including stress, sleep, and food [6]. It has been shown that human PRL can increase the viability of breast cancer cells treated with DNA-damaging agents [7]. Receptors of PRL have been identified on spermatogenic, Leydig, and Sertoli cells, as well as on efferent duct epithelial cells, suggesting a potential role for this hormone in promoting secretory/adsorptive functions, steroidogenesis, and spermatogenesis in the male reproductive system [8].

In addition, PRL is present in human seminal plasma [9] and has been shown to affect sperm metabolism [10] and motility [11,12]. It also exerts a prosurvival effect on spermatozoa cells mediated by the suppression of apoptosis, inhibits DNA strand breaks, and suppresses their entry into a state of capacitation [13]. Moreover, the potential antioxidant activity of PRL in several cells and tissues has been suggested. Research has shown that PRL significantly reduces total lipid peroxidation (LPO) product levels in rat hippocampal neurons and prevents glutamate-induced mitochondrial dysfunction [14]. A significant increase in both the mRNA and protein of superoxide dismutase 1 (*SOD1*) and *SOD2* in luteinized granulosa cells exposed to PRL has been reported [15]. Evidence also exists that PRL prevents hydrogen peroxide-induced damage to human retinal pigment epithelial cells by reducing intracellular levels of ROS [16].

Heat shock proteins (HSPs) are described as chaperones with protective and anti-apoptotic roles in cells that are induced in response to both intrinsic and extrinsic stressors [17]. HSP90, the most abundant cytoplasmic chaperone, is upregulated in response to increased levels of free radicals and protects cells against oxidative stress [18]. HSP90 protects metastable regulatory molecules such as kinases and steroid hormone receptors and supports the maturation and folding of newly synthesized proteins [19]. Higher levels of sperm *HSP90* transcripts have been reported in couples experiencing recurrent idiopathic pregnancy loss [20]. It has also been reported that HSP90 plays an important role in the regulation of sperm motility [21,22].

The maturation or stability of the PRL receptor, as a client protein of HSP90, depends on HSP90, and inhibition of this master chaperone leads to the loss of PRL receptors due to proteolytic degradation [23]. The survival of breast cancer cells and normal mammary epithelial cells was found to be promoted by PRL and HSP90 in a cellular context-dependent manner [24]. HSP90 is involved in PRL-induced apoptosis signaling, and the inhibition of PRL receptors promotes spermatogonial apoptosis during spermatogenesis [25]. Thus, in the present study, we assessed seminal PRL levels and sperm *HSP90* transcript content in fertile and infertile men to evaluate the associations of seminal levels of PRL with sperm parameters and *HSP90* transcript content.

Methods

1. Subjects

The present study was approved by the Ethics Committee of the Department of Biology, Shahid Chamran University of Ahvaz (EE/97.24.3.70393/scu.ac.ir). All participants provided written informed consent. Normozoospermic volunteers (n = 18) as controls and infertile patients (n = 18) attending the Narges Medical Genetics and Prenatal Diagnosis Laboratory in Ahvaz, Iran were recruited for semen analysis. The infertile men were individuals with abnormal semen characteristics in at least one parameter according to the 2010 World Health Organization (WHO) guidelines. Smokers, drug users, alcohol consumers, and men with an abnormal body mass index, history of varicocele, cryptorchidism, prostatitis, urinary tract infection, genital trauma, testicular torsion, inguinal or genital surgery, sexually transmitted disease, chronic illness, and serious systemic diseases were excluded from the study.

2. Semen analysis

After 3 days of sexual abstinence, semen samples were collected in sterile containers by masturbation, allowed to liquefy for 30 minutes, and then were analyzed immediately. According to the 2010 WHO guidelines, a concentration of $\geq 15 \times 10^6$/mL, progressive motility of ≥ 32%, normal sperm morphology of ≥ 4% and a leukocyte content of $< 1 \times 10^6$/mL were considered normal.

3. Assessment of seminal PRL levels

Seminal levels of PRL were measured based on the enzyme immunoassay technique using the AccuBind ELISA Microwells Kit (Monobind Inc.) with an Immunoassay Analyzer Cobas e 411 (Roche Diagnostics GmbH). The enzyme immunoassay was carried out according to the manufacturer's instructions, and the absorbance of each microwell was read at 450 nm.

4. Sperm RNA extraction

For the elimination of somatic cells, semen samples were treated with cell lysis buffer (0.1% sodium dodecyl sulfate and 0.5% Triton X in DEPC-treated water) after two washes with phosphate-buffered saline ($Ca^{2+}Mg^{2+}$-free; pH 7.4, 0.1 mM). Then, according to the manufacturer's instructions, TRIzol reagent (Life Technology) was added to each sperm pellet to extract total RNA. After treatment with RNase-free DNase (Qiagen GmbH), the concentration of extracted RNA was determined using a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific).

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5. Real-time reverse-transcription polymerase chain reaction analysis

Total RNA from each sperm pellet was reverse-transcribed into cDNA in a final volume of 10 µL using the PrimeScript RT reagent Kit (TaKaRa) based on the manufacturer's instructions. Real-time quantitative polymerase chain reaction (PCR) analysis for each prepared cDNA was carried out in triplicate using SYBR Green PCR Master mix (TaKaRa) in an ABI Step One Real Time PCR system (Applied Biosystems). β-Actin was applied as a control gene to normalize the *HSP90* mRNA content, and the cycle threshold (ΔCt) value was calculated for each sample.

Oligo primer analysis software version 7.0 (Molecular Biology Insights) was used to design the mRNA primers. The specificity of the primers was verified using the BLAST website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1).

6. Statistical analysis

All statistical analyses were performed in SPSS version 16.0 (SPSS Inc.). The independent-sample *t*-test was used to compare sperm parameters between fertile and infertile men. The non-parametric Mann-Whitney test was performed to assess differences in seminal PRL and ΔCt of sperm *HSP90* mRNA between subjects. Spearman correlation coefficients were used to quantify the relationships between variables. The optimal cutoff value of seminal PRL for diagnosis was determined using receiver operating characteristic (ROC) curve analysis. Differences were considered statistically significant at *p*-values less than 0.05.

Results

The median and interquartile ranges of seminal parameters in infertile patients and normozoospermic controls are presented in Table 2. The mean age of infertile patients (mean ± standard error of the mean [SEM], 34.3 ± 1.50 years [range, 25 to 46]) showed no significant difference ($p = 0.305$) compared to normozoospermic donors (mean ± SEM, 36.8 ± 1.86 years [range, 22 to 56]). The sperm concentration differed significantly ($p = 0.001$) between infertile and fertile subjects. A significantly higher ($p = 0.001$) count of sperm with progressive motility was observed in fertile individuals than in infertile men. Moreover, infertile patients had significantly higher ($p = 0.001$) levels of sperm with abnormal morphology (Table 2).

Infertile patients showed significantly lower ($p = 0.004$) seminal PRL levels than normozoospermic controls (Figure 1A). Seminal PRL showed an optimal diagnostic cutoff value of 15.70 ng/mL with 83.30% sensitivity and 66.7% specificity to distinguish between fertile and infertile subjects (Figure 1B). ROC curve analysis demonstrated that seminal PRL, with an area under the curve (AUC) of 0.776 (95% confidence interval [CI], 0.568 to 0.934; $p = 0.005$), had relatively good diagnostic power for discriminating infertile men. Seminal levels of PRL were correlated significantly with sperm concentration ($r = 0.400$, $p = 0.016$) and progressive motility ($r = 0.422$, $p = 0.010$). No significant correlation was seen between seminal PRL levels and sperm morphology (Table 3).

In infertile patients, the median sperm *HSP90* ΔCt value was significantly lower ($p = 0.040$) than in normozoospermic controls (Table 4). This indicated that there was higher abundance of *HSP90* mRNA in infertile men (Figure 2). A significant correlation was observed between sperm *HSP90* ΔCt values and sperm progressive motility ($r = 0.394$, $p = 0.018$) (Table 3).

Higher sperm *HSP90* transcript abundance was significantly associated with reduced sperm progressive motility. Sperm *HSP90* transcript abundance showed no significant correlations with sperm concentration or morphology. Furthermore, there was a significant correlation between the transcript content of *HSP90* in ejaculated spermatozoa and seminal levels of PRL ($r = 0.351$, $p = 0.036$) (Table 3). Higher levels of seminal PRL were significantly associated with reduced sperm *HSP90* transcript content.

Discussion

Seminal plasma is a biofluid rich in different organic and inorganic compounds that are essential for the maintenance of sperm func-

Table 1. Primers used to assess *HSP90* transcript levels in human sperm

Gene symbol	Primer sequence
HSP90	F: 5'-TGTGCAGTTGCCTACAGGAT-3'
	R: 5'-TGGACTTCGAGCAAGAGATG -3'
β-Actin	F: 5'-GAAGGAAGGCTGGAAGAGT -3'
	R: 5'-CATGAGTCCTTCCACGATACC-3'

HSP90, heat shock protein 90.

Table 2. Semen parameters in fertile controls and infertile patients

Parameter	Fertile (n = 18)	Infertile (n = 18)	*p*-value
Semen volume (mL)	2.5 (2.0–3.5)	2.5 (1.5–3.5)	0.501
Sperm concentration ($\times 10^6$/mL)	36.0 (28.0–47.7)	13.0 (7.0–18.0)	0.001
Progressive motility (%)	42.5 (35.0–50.0)	20.0 (13.7–30.0)	0.001
Normal morphology (%)	11.0 (8.0–13.5)	4.5 (3.0–5.0)	0.001

Values are presented as median (interquartile range). The Mann-Whitney *U* test was done as the test of significance. The level of significance was set at $p<0.05$.

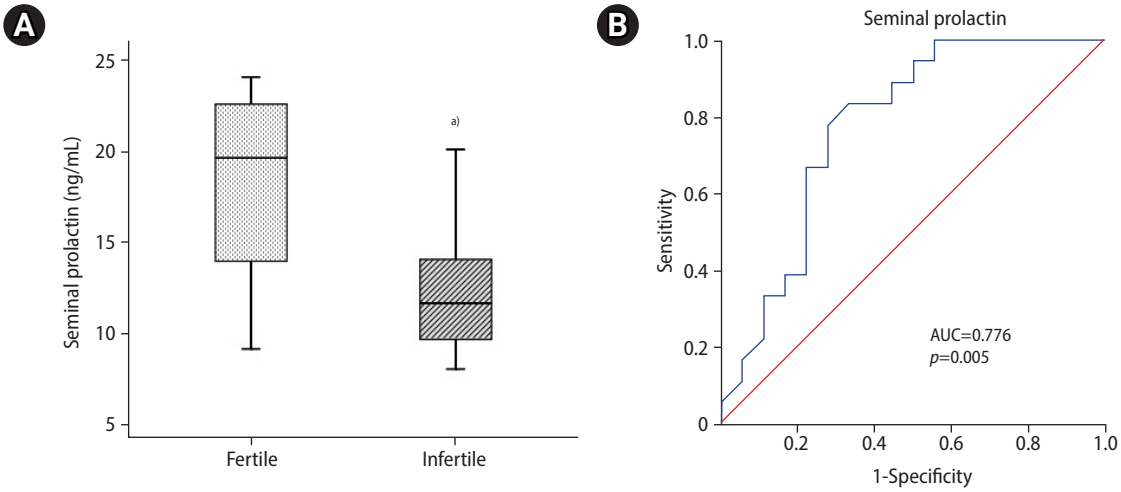

Figure 1. (A) Seminal levels of prolactin (PRL) in infertile patients were significantly lower than in normozoospermic controls (box plot). The Mann-Whitney *U* test was done as the test of significance. (B) Receiver operating characteristic curve analysis revealed that seminal PRL had a fairly good diagnostic value for distinguishing between fertile and unexplained infertile men, with an area under the curve (AUC) of 0.776 (95% confidence interval, 0.618 to 0.934; p=0.005; cutoff value, 15.7 ng/mL; sensitivity, 83.3%; specificity, 66.7%). [a] p=0.004.

Table 3. Spearman correlations between sperm parameters, seminal PRL levels, and sperm *HSP90* ΔCt values

Parameter	Subjects (n=36)			
	Sperm concentration	Progressive motility	Normal morphology	PRL
PRL	r=0.400, p=0.016[a]	r=0.422, p=0.010[a]	r=0.312, p=0.064	1.000
HSP90	r=0.232, p=0.172	r=0.394, p=0.018[a]	r=0.309, p=0.067	r=0.351, p=0.036[a]

PRL, prolactin; *HSP90*, heat shock protein 90; Ct, cycle threshold.
[a] A p-value less than 0.05 indicated statistical significance.

Table 4. Seminal PRL levels and sperm *HSP90* ΔCt values in fertile controls and infertile patients

Parameter	Fertile (n=18)	Infertile (n=18)	p-value
PRL	19.65 (13.32 to 22.83)	11.70 (9.66 to 41.12)	0.004
HSP90	0.063 (–0.757 to 1.079)	–0.861 (–3.15 to 0.417)	0.040

Values are presented as median (interquartile range). The Mann-Whitney *U* test was done as the test of significance. The level of significance was set at p<0.05.
PRL, prolactin; *HSP90*, heat shock protein 90; Ct, cycle threshold.

tion. Reproductive hormones are among the contents of seminal plasma and may play vital roles in the fertilizing ability of spermatozoa. An analysis of seminal hormones enables the identification of underlying molecular pathways associated with the pathology of male infertility and their potential use as non-invasive biomarkers. There have been reports of a role for seminal PRL in male fertility. The present study showed an association of seminal levels of PRL with *HSP90* transcript content in ejaculated spermatozoa. Our findings indicated that the mean concentrations of seminal PRL

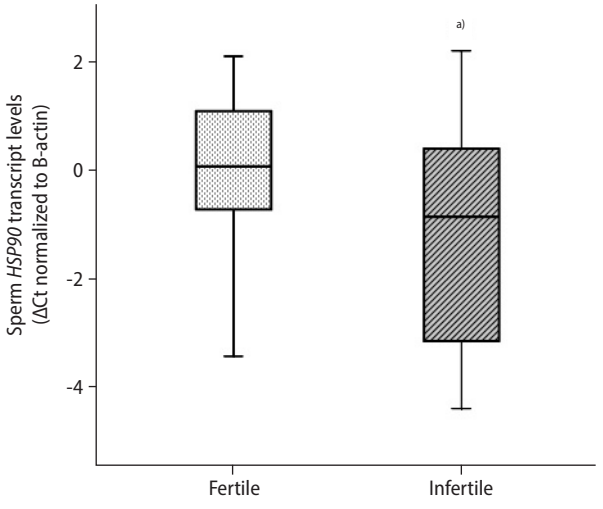

Figure 2. Sperm heat shock protein 90 (*HSP90*) normalized cycle threshold (Ct) values in infertile patients and normozoospermic controls. *HSP90* transcript levels in ejaculated spermatozoa were significantly higher in infertile patients than in normozoospermic men (box plot). Low normalized Ct values indicate high mRNA expression levels. The Mann-Whitney *U* test was done as the test of significance. [a] p=0.040.

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were 18.07 ± 5.3 and 12.69 ± 3.7 ng/mL in fertile and infertile men, respectively. The seminal levels of PRL in normozoospermic controls were significantly higher than that in infertile patients. Higher levels of seminal PRL have been reported in normozoospermic men than in oligozoospermic patients [26-29]. Tang and Chan [30] reported that the seminal levels of PRL in azoospermic patients were significantly lower than in normozoospermic men. Moreover, Chan et al. [11] found that seminal levels of PRL did not meaningfully differ between normozoospermic and oligozoospermic men, but were significantly lower in azoospermic men. They also reported that seminal PRL concentrations were higher in subjects with higher sperm motility. ROC curve analysis demonstrated that seminal PRL with an AUC of 0.776 (95% CI, 0.568 to 0.934; $p = 0.005$) has relatively good diagnostic power to discriminate infertile men. Seminal PRL levels differentiated infertile subjects from fertile individuals at a cutoff value of 15.70 ng/mL with 83.30% sensitivity and 66.7% specificity.

The present study demonstrated significant correlations between seminal levels of PRL and sperm concentration and motility. Significant associations were previously reported between seminal PRL concentrations and sperm count [31], motility [30,31] and viability [32]. Gonzales et al. [33] demonstrated that seminal PRL concentrations had a significant positive correlation with sperm motility and were related negatively to sperm concentration. However, Chan et al. [11] showed that there was no association between seminal levels of PRL and sperm parameters. Our findings also indicated that there was no correlation between seminal PRL levels and sperm morphology. Similarly, Chan et al [11]. found that seminal levels of PRL did not differ between normozoospermic and teratozoospermic men. On the contrary, Wijeratna et al. [34] reported that there was a significant negative relationship between seminal PRL levels and sperm morphology. *In vitro* incubation of human spermatozoa in the presence of PRL increases motility and decreases DNA damage in a dose-dependent manner [13] and stimulates cell metabolism [10]. Ufearo and Orisakwe [35] stated that an increase in endogenous PRL levels or administration of exogenous PRL improves sperm concentration and morphology in hypoprolactinemic infertile men treated with metoclopramide. It has been claimed that PRL increases the efficiency of spermatogenesis in seminiferous tubules by directly affecting steroidogenesis in Leydig cells [8] and improves the viability of sperm cells by activating Akt phosphorylation and reducing caspase activity [13]. Moreover, our findings showed that the abundance of *HSP90* transcripts in ejaculated spermatozoa was significantly higher in infertile patients than in fertile individuals and was significantly related to sperm motility. Reduced sperm progressive motility was found to be significantly associated with higher sperm *HSP90* transcript levels. Higher sperm HSP90 protein levels have been reported in oligozoospermic patients than in normozoospermic men [36]. However, Sagare-Patil et al. [37] found that HSP90 protein levels were lower in oligozoospermic patients than in normozoospermic controls and had a positive correlation with sperm count and motility. Lower levels of HSP90 protein were observed in the spermatozoa of oligoasthenozoospermic patients than in controls [37]. Tian et al. [38] reported that seminal *HSPA2* transcripts differed significantly between normozoospermic men and asthenozoospermic patients and were negatively associated with sperm concentration and motility. Chan et al. [39] demonstrated that sperm HSP70 and HSP90 protein levels were elevated in infertile men with varicocele. Zhang et al. [22] found that *HSP90* transcript abundance decreased in cryopreserved bovine sperm and was associated with sperm plasma membrane and acrosome integrity. Elevated *HSP90* mRNA expression has been observed in the ejaculated sperm of oligozoospermic men [36]. Erata et al. [40] reported that *HSP70* transcript abundance was significantly higher in asthenozoospermic and oligoasthenozoospermic infertile patients than in fertile controls.

HSPs play a major role in the process of spermatogenesis and sperm function [41]. Molecular disorders in the reproductive process can lead to abnormal sperm function. HSP90 interacts with cell division cycle 37 (CDC37) to regulate total protein threonine phosphorylation and SRC phosphorylation during human sperm capacitation [42]. Sun et al. [43] showed that HSP90 regulates human sperm capacitation via the Erk1/2 and p38 mitogen-activated protein kinase (MAPK) signaling pathways. Calle-Guisado et al. [44] reported that HSP90 protects the motility of porcine sperm during prolonged exposure to heat stress. HSP90 protects sperm function after freezing, and the abundance of *HSP90* transcripts correlates with sperm viability [45]. HSP90 localizes in the neck, midpiece, and tail regions of human sperm and might play a crucial role in regulating sperm motility [41,46]. It has been suggested that HSP90 improves motility in spermatozoa by providing sufficient energy via inhibiting adenosine triphosphate (ATP) degradation [45]. Increased expression of *HSP90* during the process of spermatogenesis can be due to its protective role against heat stress. HSP90 could improve sperm quality by activating nitric oxide synthase, which protects sperm from oxidative damage caused by ROS [47].

Furthermore, the present study showed that there was a significant association between seminal levels of PRL and the abundance of *HSP90* transcripts in ejaculated spermatozoa. Higher seminal PRL levels were associated with lower *HSP90* transcript abundance in ejaculated spermatozoa. It has been proposed that PRL exerts antioxidant activity [14,16], which in turn can reduce the adverse

effects of oxygen free radicals on sperm cells. It can be inferred that a decrease in seminal antioxidant capacity causes oxidative stress, which in turn increases *HSP90* expression. Given the proposed antioxidant properties of PRL, it is thought that seminal PRL depletion can lead to oxidative stress and LPO, thereby reducing sperm viability and motility and damaging sperm DNA [48]. Furthermore, it has been reported that HSP90 is involved in PRL-induced apoptosis signaling during spermatogenesis. Inhibition of the PRL receptor, as a client protein of HSP90, promotes spermatogonial apoptosis [25]. However, this study is subject to several limitations. Further studies with a larger sample size and specific methods, as well as experimental studies on the role of seminal PRL, are required.

This study showed that lower seminal PRL levels may be associated with impaired quality of sperm parameters in men. Given the proposed antioxidant properties of PRL and the relationship between seminal PRL levels and sperm *HSP90* transcript abundance, it can be assumed that an imbalance between the seminal antioxidant capacity and the amount of free radicals leads to oxidative stress and reduced sperm quality in infertile patients. Therefore, in addition to the importance of assessing *HSP90* transcript content in ejaculated spermatozoa, the present study suggests that seminal PRL levels can be analyzed as a diagnostic marker to evaluate semen quality and predict male fertility.

Conflict of interest

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

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References

1. Ombelet W, Cooke I, Dyer S, Serour G, Devroey P. Infertility and the provision of infertility medical services in developing countries. Hum Reprod Update 2008;14:605–21.
2. Winters BR, Walsh TJ. The epidemiology of male infertility. Urol Clin North Am 2014;41:195–204.
3. Bisht S, Faiq M, Tolahunase M, Dada R. Oxidative stress and male infertility. Nat Rev Urol 2017;14:470–85.
4. Guthrie HD, Welch GR. Effects of reactive oxygen species on sperm function. Theriogenology 2012;78:1700–8.
5. Bromfield JJ. Seminal fluid and reproduction: much more than previously thought. J Assist Reprod Genet 2014;31:627–36.
6. Majumdar A, Mangal NS. Hyperprolactinemia. J Hum Reprod Sci 2013;6:168–75.
7. Karayazi Atici O, Urbanska A, Gopinathan SG, Boutillon F, Goffin V, Shemanko CS. ATM is required for the prolactin-induced HSP90-mediated increase in cellular viability and clonogenic growth after DNA damage. Endocrinology 2018;159:907–30.
8. Hair WM, Gubbay O, Jabbour HN, Lincoln GA. Prolactin receptor expression in human testis and accessory tissues: localization and function. Mol Hum Reprod 2002;8:606–11.
9. Smith ML, Luqman WA. Prolactin in seminal fluid. Arch Androl 1982;9:105–13.
10. Shah GV, Desai RB, Sheth AR. Effect of prolactin on metabolism of human spermatozoa. Fertil Steril 1976;27:1292–4.
11. Chan SY, Tang LC, Chan PH, Tang GW, Ma HK. Relationships of seminal plasma prolactin with spermatozoal characteristics and fertilizing capacity in vitro. Arch Androl 1984;12:17–24.
12. Gonzales GF, Garcia-Hjarles M, Velazquez G, Coyotupa J. Seminal prolactin and its relationship to sperm motility in men. Fertil Steril 1989;51:498–503.
13. Pujianto DA, Curry BJ, Aitken RJ. Prolactin exerts a prosurvival effect on human spermatozoa via mechanisms that involve the stimulation of Akt phosphorylation and suppression of caspase activation and capacitation. Endocrinology 2010;151:1269–79.
14. Rivero-Segura NA, Coronado-Mares MI, Rincon-Heredia R, Perez-Torres I, Montiel T, Pavon N, et al. Prolactin prevents mitochondrial dysfunction induced by glutamate excitotoxicity in hippocampal neurons. Neurosci Lett 2019;701:58–64.
15. Sugino N, Hirosawa-Takamori M, Zhong L, Telleria CM, Shiota K, Gibori G. Hormonal regulation of copper-zinc superoxide dismutase and manganese superoxide dismutase messenger ribonucleic acid in the rat corpus luteum: induction by prolactin and placental lactogens. Biol Reprod 1998;59:599–605.
16. Melendez Garcia R, Arredondo Zamarripa D, Arnold E, Ruiz-Herrera X, Noguez Imm R, Baeza Cruz G, et al. Prolactin protects retinal pigment epithelium by inhibiting sirtuin 2-dependent cell death. EBioMedicine 2016;7:35–49.

https://doi.org/10.5653/cerm.2022.05757

17. Morimoto RI. Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging. Genes Dev 2008; 22:1427–38.
18. Fukuda A, Osawa T, Oda H, Tanaka T, Toyokuni S, Uchida K. Oxidative stress response in iron-induced acute nephrotoxicity: enhanced expression of heat shock protein 90. Biochem Biophys Res Commun 1996;219:76–81.
19. Richter K, Buchner J. Hsp90: chaperoning signal transduction. J Cell Physiol 2001;188:281–90.
20. Kumar K, Deka D, Singh A, Chattopadhyay P, Dada R. Expression pattern of PRM2, HSP90 and WNT5A in male partners of couples experiencing idiopathic recurrent miscarriages. J Genet 2012; 91:363–6.
21. Amaral A, Paiva C, Attardo Parrinello C, Estanyol JM, Ballesca JL, Ramalho-Santos J. Identification of proteins involved in human sperm motility using high-throughput differential proteomics. J Proteome Res 2014;13:5670–84.
22. Zhang XG, Hu S, Han C, Zhu QC, Yan GJ, Hu JH. Association of heat shock protein 90 with motility of post-thawed sperm in bulls. Cryobiology 2015;70:164–9.
23. Taipale M, Jarosz DF, Lindquist S. HSP90 at the hub of protein homeostasis: emerging mechanistic insights. Nat Rev Mol Cell Biol 2010;11:515–28.
24. Perotti C, Liu R, Parusel CT, Bocher N, Schultz J, Bork P, et al. Heat shock protein-90-alpha, a prolactin-STAT5 target gene identified in breast cancer cells, is involved in apoptosis regulation. Breast Cancer Res 2008;10:R94.
25. Saribek B, Jin Y, Saigo M, Eto K, Abe S. HSP90beta is involved in signaling prolactin-induced apoptosis in newt testis. Biochem Biophys Res Commun 2006;349:1190–7.
26. Biswas S, Ferguson KM, Stedronska J, Baffoe G, Mansfield MD, Kosbab MH. Fructose and hormone levels in semen: their correlations with sperm counts and motility. Fertil Steril 1978;30:200–4.
27. Schoenfeld C, Amelar RD, Dubin L, Numeroff M. Prolactin, fructose, and zinc levels found in human seminal plasma. Fertil Steril 1979;32:206–8.
28. Aiman J, McAsey M, Harms L. Serum and seminal plasma prolactin concentrations in men with normospermia, oligospermia, or azoospermia. Fertil Steril 1988;49:133–7.
29. Sueldo CE, Berger T, Kletzky O, Marrs RP. Seminal prolactin concentration and sperm reproductive capacity. Fertil Steril 1985;43: 632–5.
30. Tang LC, Chan SY. Seminal plasma levels of luteinizing hormone, prolactin and testosterone in the evaluation of male infertility. Asia Oceania J Obstet Gynaecol 1986;12:275–83.
31. Smith ML, Luqman WA, Rakoff JS. Correlations between seminal radioimmunoreactive prolactin, sperm count, and sperm motility in prevasectomy and infertility clinic patients. Fertil Steril 1979; 32:312–5.
32. Arowojolu AO, Akinloye O, Shittu OB, Adejuwon CA. Correlations between seminal plasma hormones and sperm biophysical parameters in infertile males in Ibadan. Trop J Obstet Gynaecol 2003;20:7–11.
33. Gonzales GF, Velasquez G, Garcia-Hjarles M. Hypoprolactinemia as related to seminal quality and serum testosterone. Arch Androl 1989;23:259–65.
34. Wijeratna S, Seneviratne HR, Ratnasooriya WD. Reproductive hormones in seminal plasma: it's effect on semen quality. Ceylon J Med Sci 2005;48:53–60.
35. Ufearo CS, Orisakwe OE. Restoration of normal sperm characteristics in hypoprolactinemic infertile men treated with metoclopramide and exogenous human prolactin. Clin Pharmacol Ther 1995;58:354–9.
36. Ferlin A, Speltra E, Patassini C, Pati MA, Garolla A, Caretta N, et al. Heat shock protein and heat shock factor expression in sperm: relation to oligozoospermia and varicocele. J Urol 2010;183:1248–52.
37. Sagare-Patil V, Bhilawadikar R, Galvankar M, Zaveri K, Hinduja I, Modi D. Progesterone requires heat shock protein 90 (HSP90) in human sperm to regulate motility and acrosome reaction. J Assist Reprod Genet 2017;34:495–503.
38. Tian Y, Li L, Zhang F, Xu J. Seminal plasma HSPA2 mRNA content is associated with semen quality. J Assist Reprod Genet 2016; 33:1079–84.
39. Chan CC, Sun GH, Shui HA, Wu GJ. Differential spermatozoal protein expression profiles in men with varicocele compared to control subjects: upregulation of heat shock proteins 70 and 90 in varicocele. Urology 2013;81:1379.
40. Erata GO, Kocak Toker N, Durlanik O, Kadioglu A, Aktan G, Aykac Toker G. The role of heat shock protein 70 (Hsp 70) in male infertility: is it a line of defense against sperm DNA fragmentation? Fertil Steril 2008;90:322–7.
41. Li K, Xue Y, Chen A, Jiang Y, Xie H, Shi Q, et al. Heat shock protein 90 has roles in intracellular calcium homeostasis, protein tyrosine phosphorylation regulation, and progesterone-responsive sperm function in human sperm. PLoS One 2014;9:e115841.
42. Li K, Sun P, Wang Y, Gao T, Zheng D, Liu A, et al. Hsp90 interacts with Cdc37, is phosphorylated by PKA/PKC, and regulates Src phosphorylation in human sperm capacitation. Andrology 2021; 9:185–95.
43. Sun P, Wang Y, Gao T, Li K, Zheng D, Liu A, et al. Hsp90 modulates human sperm capacitation via the Erk1/2 and p38 MAPK signal-

ing pathways. Reprod Biol Endocrinol 2021;19:39.
44. Calle-Guisado V, Bragado MJ, Garcia-Marin LJ, Gonzalez-Fernandez L. HSP90 maintains boar spermatozoa motility and mitochondrial membrane potential during heat stress. Anim Reprod Sci 2017;187:13–9.
45. Wang P, Wang YF, Wang H, Wang CW, Zan LS, Hu JH, et al. HSP90 expression correlation with the freezing resistance of bull sperm. Zygote 2014;22:239–45.
46. Huang SY, Kuo YH, Tsou HL, Lee YP, King YT, Huang HC, et al. The decline of porcine sperm motility by geldanamycin, a specific inhibitor of heat-shock protein 90 (HSP90). Theriogenology 2000;53:1177–84.
47. Flores E, Cifuentes D, Fernandez-Novell JM, Medrano A, Bonet S, Briz MD, et al. Freeze-thawing induces alterations in the protamine-1/DNA overall structure in boar sperm. Theriogenology 2008;69:1083–94.
48. Takeshima T, Usui K, Mori K, Asai T, Yasuda K, Kuroda S, et al. Oxidative stress and male infertility. Reprod Med Biol 2020;20:41–52.

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Measurement of serum anti-Müllerian hormone by revised Gen II or automated assay: Reproducibility under various blood/serum storage conditions

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Objective: We investigated the agreement between anti-Müllerian hormone (AMH) levels measured with revised Gen II (rev-Gen II) and automated AMH (Access) assays and evaluated the reproducibility of each method under various blood/serum storage conditions.
Methods: AMH levels in blood samples from 74 volunteers were measured by rev-Gen II and Access assays under various conditions: immediate serum separation and AMH measurement (fresh control); serum stored at –20 ℃ and AMH measured after 48 hours, 1 week, and 2 years; serum stored at 0 to 4 ℃ and AMH measured after 48 hours and 1 week; and blood kept at room temperature and delayed serum separation after 48 hours and 1 week, with immediate AMH measurement.
Results: In fresh controls, all rev-Gen II-AMH values were higher than comparable Access-AMH values (difference, 8.3% to 19.7%). AMH levels measured with the two methods were strongly correlated for all sample conditions (r=0.977 to 0.995, all p<0.001). For sera stored at –20 ℃ or 0 to 4 ℃ for 48 hours, Access-AMH values were comparable to control measurements, but rev-Gen II-AMH values were significantly lower. AMH levels in sera stored at –20 ℃ or 0 to 4 ℃ for 1 week were significantly lower than in fresh controls, irrespective of method. Across methods, long-term storage at –20 ℃ for 2 years yielded AMH measurements significantly higher than control values. When serum separation was delayed, rev-Gen II-AMH values were significantly lower than control measurements, but Access-AMH values varied.
Conclusion: The rev-Gen II and Access-AMH assays showed varying reproducibility across blood/serum storage conditions, but automated Access yielded superior stability to rev-Gen II.

Keywords: Anti-Müllerian Hormone; Automation; Biological assay; Blood; Reproducibility of results; Serum

Introduction

Serum anti-Müllerian hormone (AMH) is a valuable clinical marker of ovarian reserve and is routinely measured in women receiving infertility treatment. In addition, it is commonly assessed in various areas of women's health and fertility care [1].

To demonstrate the value of serum AMH as an ovarian reserve marker in clinical and research settings, a highly reliable commercial assay is essential [2]. Various AMH immunoassays have been developed and revised, of which the revised Gen II (rev-Gen II) version predominated until recently. However, a fully automated AMH assay was introduced in 2014 and is now the major assay for serum AMH. With the automated AMH assay, several technical problems associated with rev-Gen II (low accuracy, inter-laboratory variation, and relatively long measurement time) have been largely resolved [3].

One concern is whether the AMH values measured by automated assay are interchangeable with those measured by rev-Gen II. Although the manufacturer guidelines indicate that this is true for AMH measurements obtained by the Access automated assay (Beckman-Coulter), the potential for discordance between the two meth-

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ods requires investigation [4].

In immunoassays conducted on patient serum samples, both pre-analytical and analytical variation, as well as intra-individual biological variation, should be considered [5]. To improve the automated AMH assay, several types of variation should be minimized [6].

In the original Gen II assay, factors influencing the pre-analytical variation included blood sample handling, serum separation time, storage conditions, and sample freezing, all of which can produce fluctuations in AMH values. Thus, both pre-analytical variation and reproducibility should be investigated for the automated AMH assay [6].

Few studies have been conducted on the pre-analytical variation associated with the automated AMH assay, which should prove to be reproducible irrespective of storage conditions [7]. In particular, further investigation is required regarding whether long-term serum storage could affect the AMH measurements obtained by automated assay. In clinical settings, AMH values are often measured after long-term storage of serum. In fact, published large-scale AMH data commonly include measurements taken under varying storage conditions or with varying freezing times, up to several years; this hampers the stability and validity of the AMH results [8]. Concern about the long-term stability of serum AMH measurement remains unresolved. However, a few studies have indicated that the long-term storage of serum samples at –20, –70, and 80 ℃ has little impact on serum AMH levels [7,9].

The rev-Gen II assay reportedly exhibits good stability and reliability of AMH values in serum, but studies of its stability in whole blood are scarce [10]. In real clinical situations, some delay between blood collection and serum separation commonly occurs. Few studies have been conducted on the reliability and reproducibility of the AMH measurements obtained by automated assay (Access) across blood or serum storage conditions. Considering the worldwide use of the automated AMH assay, validation of its reliability and reproducibility under specific pre-analytical conditions is urgently required.

The aims of this study were to investigate the concordance of AMH measurements obtained by rev-Gen II and automated (Access) assays across serum or blood sample storage conditions, as well as to evaluate the reliability and reproducibility of each AMH assay according to serum or blood sample storage conditions.

Methods

1. Participants

A prospective study was conducted, and 74 female volunteers were enrolled between October 2015 and November 2015; all were non-pregnant women aged 25 to 45 years with no relevant comorbidities or medications. Eight women had polycystic ovary syndrome. Written informed consent was obtained from all women. The study was approved by the Hamchoon Institutional Review Board (no. 73507-201507-BR-003).

2. Blood sample preparation and serum AMH measurement

Eight blood sample storage conditions were set according to the timing of AMH measurement and serum separation (Figure 1): (1) Immediate serum separation and immediate AMH measurement (fresh control, n = 74); (2) Serum stored at –20 ℃ and AMH measured after 48 hours (n = 23), 1 week (n = 23), and 2 years (n = 74); (3) Serum stored at 0 to 4 ℃ and AMH measured after 48 hours and 1 week (n = 22); (4) Delayed serum separation: blood kept at room temperature and serum separation conducted after 48 hours and 1 week, followed by immediate AMH measurement (n = 24).

Peripheral blood samples were collected in serum separator gel tubes (V-Tube; AB Medical) and allowed to clot at room temperature, then centrifuged at 1,000 × *g* for 10 minutes within 2 hours of collection to separate the sera. In fresh controls, sera were separated and serum AMH values were measured immediately. The remaining sera were distributed into five Eppendorf tubes and stored at –20 ℃ or 0 to 4 ℃ .

For delayed serum separation, portions of the blood samples were distributed into two V-Tubes and left at room temperature. After either 48 hours or 1 week, blood samples were centrifuged at 1,000 × *g* for 10 minutes, and sera were separated. Serum AMH levels were measured immediately.

In all blood/serum samples, AMH values were measured once

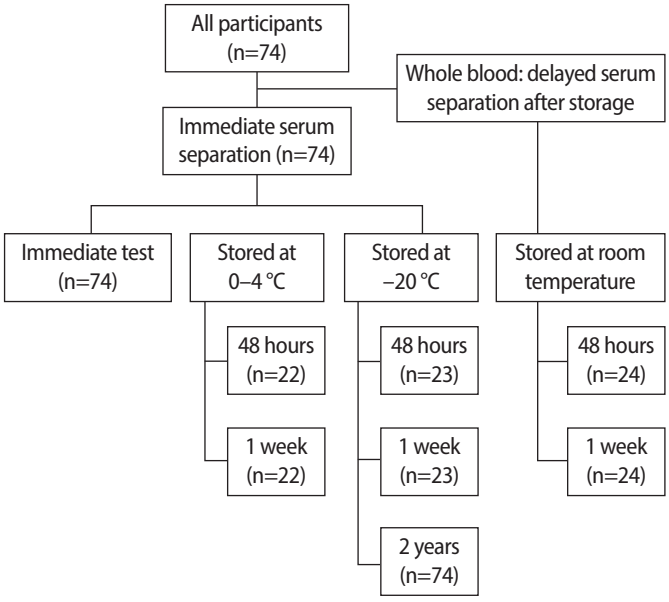

Figure 1. Flowchart of blood/serum sample storage conditions in 74 women.

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each by rev-Gen II and automated (Access) assays. All measurement procedures were performed by one experienced technician according to the manufacturer's protocols.

For the rev-Gen II assay, the Gen II kit (A79765; Beckman-Coulter) was used with revised instructions (IFU-REF A92268D). The revised protocol included an additional assay step that involved pre-mixing all of the calibrators, controls, and samples with the Gen II assay buffer before adding the sample to the Gen II microplate. The measurement range was 0.08 to 22.5 ng/mL. AMH values ≤0.08 were censored at a value of 0.08 ng/mL. The intra- and inter-assay coefficients of variability (CVs) were 5.4% and 5.6%, respectively.

For the automated assay, an Access 2 Immunoassay System (Beckman-Coulter) was used. The AMH level was determined using a 6-point calibration curve, and calibrators were prepared with recombinant human AMH in a synthetic matrix. The limit of quantitation was 0.08 ng/mL, and the measurement range was 0.02 to 24.0 ng/mL. The total imprecision was a CV ≤10.0% at concentrations ≥0.16 ng/mL.

3. Statistical analysis

The correlations between the rev-Gen II-AMH and Access-AMH values were assessed using the Pearson correlation test and the paired Wilcoxon signed-rank test. The intraclass correlation coefficient with absolute agreement (ICC-aa) and 95% confidence intervals were calculated based on a single-rating, absolute agreement, two-way random-effects model. For regression equations, the method described by Passing and Bablok was used. For limits of agreement (LOAs) and likelihood of bias analyses, a Bland-Altman plot was created [11].

To analyze the reproducibility of each assay, AMH levels under seven storage conditions were compared with measurements taken in fresh controls using the Pearson correlation test, ICC-aa values, and the paired Wilcoxon signed-rank test.

All statistical analyses were performed using R for Windows version 3.2.0 (R Foundation for Statistical Computing) and STATA 14 (StataCorp LP). A $p < 0.05$ was considered to indicate statistical significance.

Results

1. Comparison between the rev-Gen II-AMH and Access-AMH values

The rev-Gen II-AMH and Access-AMH values displayed strong correlations for all eight blood/serum sample storage conditions (Table 1). The correlation coefficients ranged from 0.977 to 0.995 ($p < 0.001$ for all). ICC-aa values ranged from 0.93 to 0.97, and all showed good concordance.

Figure 2 includes scatter plot diagrams with regression lines associated with the eight storage conditions. The regression showed no significant deviation from linearity. The representative regression equation for the fresh control was (Access-AMH) = 0.74 × (rev-Gen II-AMH)+0.28. For example, a rev-Gen II-AMH measurement of 1.0 ng/mL corresponded to an Access-AMH value of 1.02 ng/mL, and a rev-Gen II-AMH measurement of 2.0 ng/mL corresponded to an Access-AMH value of 1.76 ng/mL.

Figure 3 shows the associations between the rev-Gen II-AMH and Access-AMH values based on the Bland-Altman plots. Of the values, 5.4% (4/74) were outside the LOAs, but all fell below the lower limit (Figure 3A). Under the assumption of normal distribution, AMH values were naturally log-transformed, then expressed as ln[rev-Gen II-AMH] and ln[Access-AMH]. Natural logarithmic transformation was successful in producing differences unrelated to the mean.

Table 2 summarizes the mean differences and 95% LOAs between ln[rev-Gen II-AMH] and ln[Access-AMH]. We calculated the anti-logarithms for the differences between ln[rev-Gen II-AMH] and ln[Access-AMH] to obtain values representing the ratios of rev-Gen II-AMH

Table 1. Correlation and agreement between AMH values measured by the revised Gen II and automated (Access) assays under eight blood/serum storage conditions

Storage conditions	Pearson correlation coefficient (*p*-value)	Wilcoxon signed-rank test (*p*-value)	ICC-aa (95% CI)
Fresh control[a] (n = 74)	0.992 (< 0.001)	< 0.001	0.97 (0.95–0.98)
Serum stored at –20 °C for 48 hours (n = 23)	0.977 (< 0.001)	0.012	0.97 (0.93–0.99)
Serum stored at –20 °C for 1 week (n = 23)	0.991 (< 0.001)	< 0.001	0.95 (0.89–0.98)
Serum stored at –20 °C for 2 years (n = 74)	0.995 (< 0.001)	< 0.001	0.93 (0.89–0.96)
Serum stored at 0–4 °C for 48 hours (n = 22)	0.978 (< 0.001)	0.0025	0.93 (0.84–0.97)
Serum stored at 0–4 °C for 1 week (n = 22)	0.988 (< 0.001)	< 0.001	0.93 (0.85–0.97)
Delayed serum separation[b] at 48 hours (n = 24)	0.995 (< 0.001)	0.6729	0.97 (0.92–0.99)
Delayed serum separation[b] at 1 week (n = 24)	0.993 (< 0.001)	0.0096	0.96 (0.91–0.98)

AMH, anti-Müllerian hormone; ICC-aa, intraclass correlation coefficient with absolute agreement; CI, confidence interval.
[a]Sera separated within 2 hours and AMH measured within 4 hours; [b]Blood left at room temperature and sera separated after 48 hours and 1 week, then AMH measured immediately.

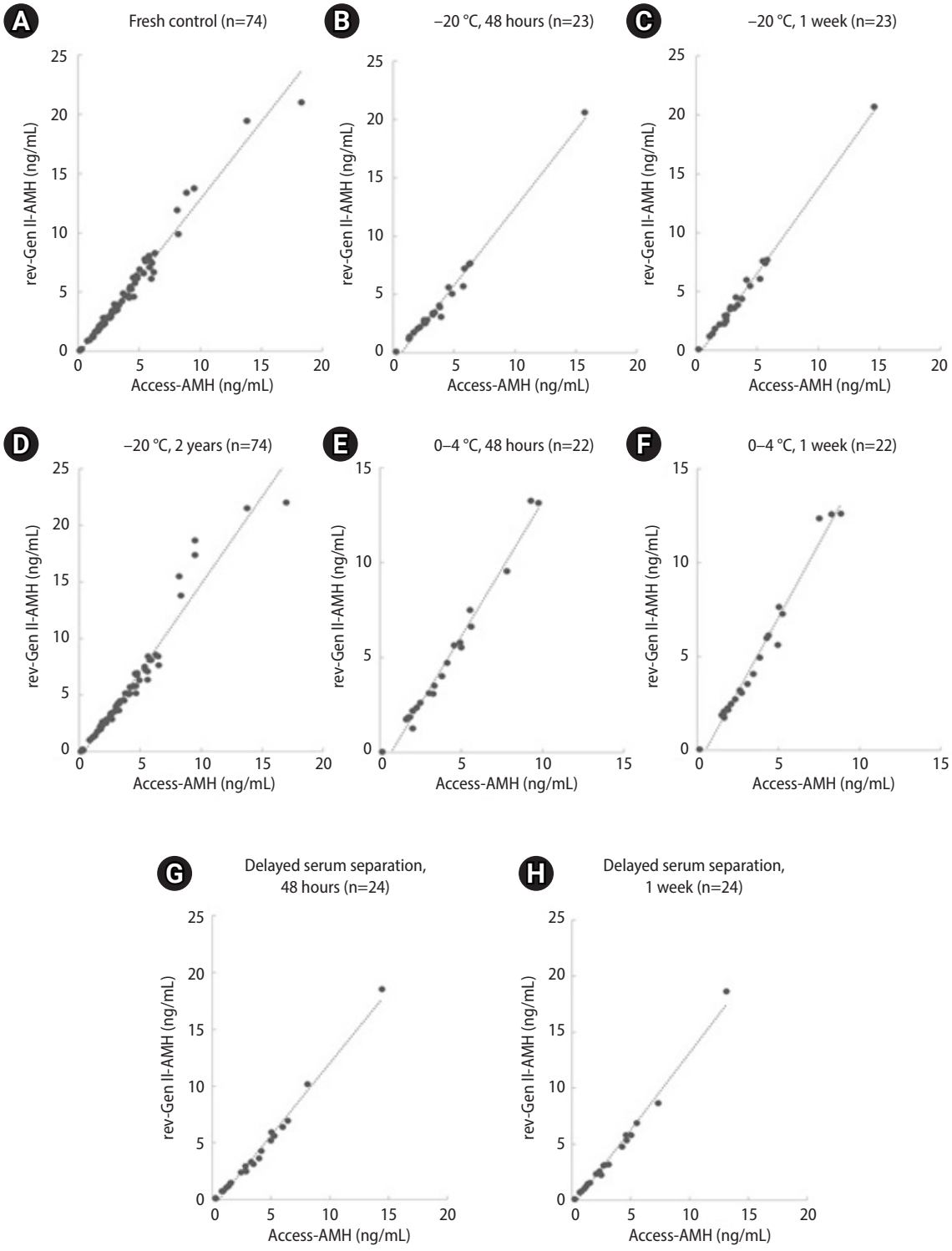

Figure 2. Correlations of serum anti-Müllerian hormone (AMH) values measured by the revised Gen II (rev-Gen II) and automated (Access) assays under eight sample storage conditions (Passing and Bablok regression plots). (A) Fresh control, (B) −20 ℃, 48 hours (n=23), (C) −20 ℃, 1 week (n=23), (D) −20 ℃, 2 years (n=74), (E) 0 to 4 ℃, 48 hours (n=22), (F) 0 to 4 ℃, 1 week (n=22), (G) delayed serum reparation, 48 hours (n=24), and (H) delayed serum reparation, 1 week (n=24).

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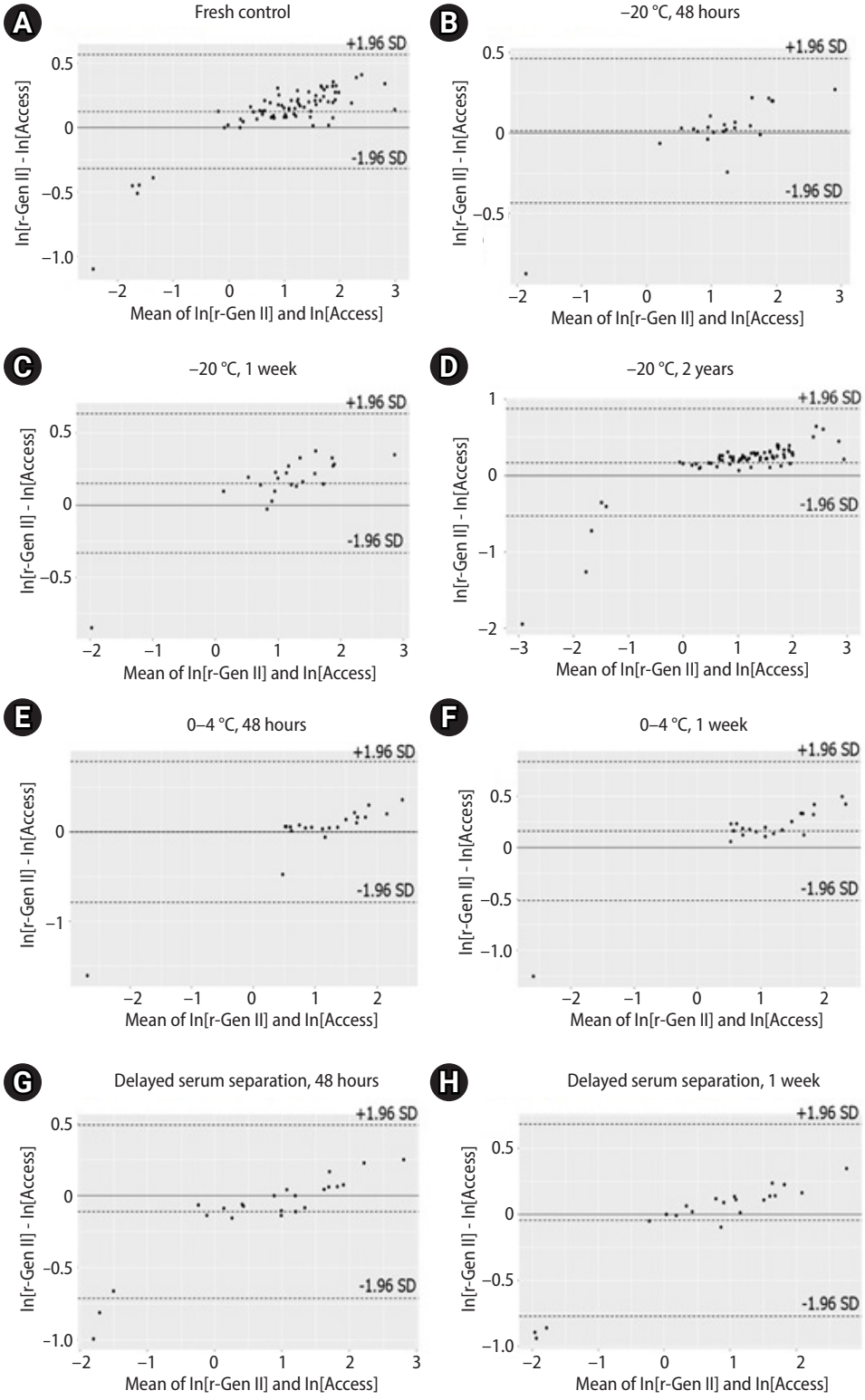

Figure 3. Agreement of serum anti-Müllerian hormone (AMH) values measured by revised Gen II (rev-Gen II) and automated (Access) assays under eight sample storage conditions. Serum AMH values were natural log-transformed and expressed as ln[rev-Gen II-AMH] and ln[Access-AMH] (Bland-Altman plots). (A) Fresh control, (B) −20 °C, 48 hours, (C) −20 °C, 1 week, (D) −20 °C, 2 years, (E) 0 to 4 °C, 48 hours, (F) 0 to 4 °C, 1 week, (G) delayed serum reparation, 48 hours, and (H) delayed serum reparation, 1 week. SD, standard deviation.

Table 2. Mean difference and 95% LOAs between natural log-transformed AMH values measured by the revised Gen II and automated (Access) assays under eight blood/serum storage conditions

Sample storage conditions	Mean difference	95% LOA
Fresh control[a] (n = 74)	0.13	0.08 to 0.18
When rev-Gen II-AMH < 10 ng/mL (n = 69)	0.11	0.06 to 0.17
Serum stored at –20 ℃ for 48 hours (n = 23)	0.01	–0.08 to 0.11
Serum stored at –20 ℃ for 1 week (n = 23)	0.15	0.05 to 0.25
Serum stored at –20 ℃ for 2 years (n = 74)	0.18	0.1 to 0.26
Serum stored at 0–4 ℃ for 48 hours (n = 22)	–0.01	–0.15 to 0.18
Serum stored at 0–4 ℃ for 1 week (n = 22)	0.17	0.03 to 0.31
Delayed serum separation[b] at 48 hours (n = 24)	–0.11	–0.23 to 0.02
Delayed serum separation[b] at 1 week (n = 24)	–0.04	–0.19 to 0.11

LOA, limit of agreement; AMH, anti-Müllerian hormone.
[a]Sera separated within 2 hours and AMH measured within 4 hours; [b]Blood left at room temperature and sera separated after 48 hours and 1 week, then AMH measured immediately.

and Access-AMH (ln[rev-Gen II-AMH]–ln[Access-AMH] = ln[rev-Gen II-AMH/Access-AMH]). We also calculated the anti-logarithms of the 95% LOAs of the differences between ln[rev-Gen II-AMH] and ln[Access-AMH] to obtain 95% LOAs of the ratios of rev-Gen II-AMH and Access-AMH. The geometric mean ratio of rev-Gen II-AMH and Access-AMH values in the fresh control was 1.132 with a 95% LOA of 1.083 to 1.197 (representing the anti-logarithms of the values in Table 2), meaning that the rev-Gen II-AMH exceeded the Access-AMH value by between 8.3% and 19.7% for most measurements.

2. Reproducibility analysis of each assay: comparison of AMH values at seven storage conditions with fresh controls

After storage for 48 hours and 1 week at 0 to 4 ℃, the rev-Gen II-AMH level had decreased by 12.6% ± 10.6% (mean ± standard deviation) and 6.5% ± 4.7%, respectively, compared with fresh controls ($p < 0.001$ for all). After storage for 48 hours and 1 week at –20 ℃, the rev-Gen II-AMH level had decreased by 11.8% ± 6.3% and 7.6% ± 7.0%, respectively, compared with fresh controls ($p < 0.001$ for all). On the contrary, sera stored at –20 ℃ for 2 years yielded significantly higher rev-Gen II-AMH values than fresh controls (10.8% ± 14.0%, $p < 0.01$). The progressive effects of storage-dependent serum AMH values are shown in Figure 4.

The Access-AMH level decreased by 0.2% ± 4.1% ($p = 0.554$) in sera stored for 48 hours at 0 to 4 ℃ and 1.3% ± 7.1% ($p = 0.424$) in sera stored for 48 hours at –20 ℃, relative to fresh controls. The Access-AMH measurement remained stable in sera stored for up to 48 hours at both 0 to 4 ℃ and –20 ℃. The Access-AMH level decreased by 7.7% ± 4.6% and 7.1% ± 6.9% in sera stored for 1 week at 0 to 4 ℃ and –20 ℃, respectively, compared with fresh controls ($p < 0.001$ for all). On the contrary, sera stored at –20 ℃ for 2 years yielded significantly higher Access-AMH values compared with fresh controls (4.5% ± 8.6%, $p < 0.01$).

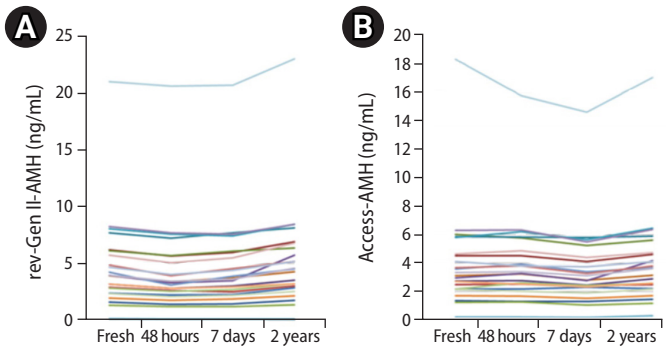

Figure 4. Progressive anti-Müllerian hormone (AMH) change in 23 women from whom three aliquots of serum were frozen for 48 hours, 1 week, and 2 years at –20 ℃. (A) AMH values measured by revised Gen II (rev-Gen II) assay, and (B) AMH values measured by automated assay (Access).

Table 3 summarizes the results of the Wilcoxon signed-rank test and ICC-aa values for a comparison of AMH values measured by each assay under various storage conditions relative to fresh controls. All AMH measurements taken in stored samples had excellent ICCs and Pearson correlations with those in fresh controls, whereas the Wilcoxon signed-rank test indicated differences between AMH values according to storage conditions. Only in sera stored for 48 hours (both at 0 to 4 ℃ and –20 ℃) did the Wilcoxon signed-rank test reveal no significant difference between the Access-AMH values.

With delayed serum separation, the decrease in rev-Gen II-AMH was 13.2% ± 7.7% after 48 hours and 17.6% ± 11.8% after 1 week, relative to fresh controls ($p < 0.001$ for all) (Table 4). The Access-AMH level had increased by 5.5% ± 8.5% after storage for 48 hours ($p < 0.05$) but had decreased by 7.2% ± 6.2% after storage for 1 week, again relative to fresh controls ($p < 0.001$).

Overall, little change was noted in the Access-AMH assay results across storage conditions compared with rev-Gen II-AMH.

Discussion

The goals of this study were to investigate the agreement between the rev-Gen II-AMH and Access-AMH assays and to evaluate the effect of sample storage conditions on the reproducibility of each AMH assay.

The optimal statistical approach to assess the degree of agreement between old and new assays is not obvious, but many studies have described the product-moment correlation coefficient as an indicator of agreement [12]. Comparative studies have shown high

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Table 3. Reproducibility analysis: comparison of the rev-Gen II-AMH and Access-AMH values under seven blood/serum storage conditions (relative to fresh control)

Storage conditions	Pearson correlation coefficient (*p*-value)	Wilcoxon signed-rank test (*p*-value)	ICC-aa (95% CI)
Rev-Gen II-AMH			
Serum stored at –20 °C for 48 hours (n = 23)	0.989 (< 0.001)	< 0.001	0.99 (0.97–1.0)
Serum stored at –20 °C for 1 week (n = 23)	0.990 (< 0.001)	< 0.001	0.99 (0.98–1.0)
Serum stored at –20 °C for 2 years (n = 74)	0.997 (< 0.001)	< 0.01	0.99 (0.99–0.99)
Serum stored at 0–4 °C for 48 hours (n = 22)	0.988 (< 0.001)	< 0.001	0.98 (0.96–0.99)
Serum stored at 0–4 °C for 1 week (n = 22)	1.0 (< 0.001)	< 0.001	1.0 (0.99–1.0)
Delayed serum separation[a] at 48 hours (n = 24)	0.997 (< 0.001)	< 0.001	0.99 (0.98–1.0)
Delayed serum separation[a] at 1 week (n = 24)	0.996 (< 0.001)	< 0.001	0.98 (0.96–0.99)
Access-AMH			
Serum stored at –20 °C for 48 hours (n = 23)	0.984 (< 0.001)	0.424	1.0 (0.99–1.0)
Serum stored at –20 °C for 1 week (n = 23)	0.984 (< 0.001)	< 0.001	0.99 (0.98–1.0)
Serum stored at –20 °C for 2 years (n = 74)	0.993 (< 0.001)	< 0.01	1.0 (0.99–1.0)
Serum stored at –4 °C for 48 hours (n = 22)	0.998 (< 0.001)	0.554	1.0 (1.0–1.0)
Serum stored at –4 °C for 1 week (n = 22)	0.989 (< 0.001)	< 0.001	0.99 (0.98–1.0)
Delayed serum separation[a] at 48 hours (n = 24)	0.994 (< 0.001)	< 0.05	1.0 (0.99–1.0)
Delayed serum separation[a] at 1 week (n = 24)	0.992 (< 0.001)	< 0.001	1.0 (0.99–1.0)

rev-Gen II, revised Gen II; AMH, anti-Müllerian hormone; ICC-aa, intraclass correlation coefficient with absolute agreement; CI, confidence interval.
[a]Blood left at room temperature and sera separated after 48 hours and 1 week, then AMH measured immediately.

Table 4. Rev-Gen II-AMH and Access-AMH levels in fresh control and delayed serum separation samples

	Fresh control[a] (n = 24)		Delayed serum separation at 48 hours[b] (n = 24)		Delayed serum separation at 1 week[c] (n = 24)	
	rev-Gen II-AMH	Access-AMH	rev-Gen II-AMH	Access-AMH	rev-Gen II-AMH	Access-AMH
Mean ± SD (ng/mL)	4.17 ± 4.21	3.36 ± 3.05	3.78 ± 4.03	3.52 ± 3.15	3.61 ± 4.11	3.06 ± 2.97
Median (ng/mL)	3.12	2.68	2.75	2.86	2.45	2.42
IQR (ng/mL)	1.27–5.74	1.22–4.54	1.17–5.30	1.34–4.99	1.07–5.19	1.07–4.50

rev-Gen II, revised Gen II; AMH, anti-Müllerian hormone; SD, standard deviation; IQR, interquartile range.
[a]Sera separated within 2 hours and AMH measured within 4 hours; [b]Blood left at room temperature and sera separated after 48 hours, then AMH measured immediately; [c]Blood left at room temperature and sera separated after 1 week, then AMH measured immediately.

correlations ($r > 0.99$) between the Access and rev-Gen II assays [7,13,14]. Similar results were obtained in our study. However, two serious problems limit the use of correlation coefficients. First, a correlation depends on the range and distribution of the variables. Second, a correlation ignores any systematic bias between the two variables. Furthermore, the true AMH values in a sample are unknown, and we can only estimate relative bias between two AMH assays. Therefore, in the present research, an additional comparison study using the Bland-Altman method was performed. Some discordance between AMH assays is inevitable, and what matters is whether we can accept the degree of disagreement between the two AMH values. The main objective of the Bland-Altman approach is the comparison of experimentally observed deviations with a preset clinical acceptance limit [15].

Clinical users have no choice but to rely on manufacturers' claims and package inserts containing precision information. Data provided by manufacturers often reflect better precision than is achieved in clinical practice. According to the package insert of the Access kit, values obtained with the Access assay are equivalent to those reported with rev-Gen II for the critical range of 0.16 to 10 ng/mL, with 4.0% bias [16]. Previous studies have also revealed good correlations between these values, along with differences that fall within clinically acceptable ranges, indicating that the methods are interchangeable [13,14]. However, discordance of approximately 11% to 22% between values obtained by rev-Gen II and Access has been reported [4]. Furthermore, similar discordance has been demonstrated between the rev-Gen II and Elecsys assay values [17]. Comparisons have been performed between frozen serum samples or between fresh and frozen-thawed serum samples [4,14,18].

We compared samples under the same conditions, and our results also showed considerable discrepancy between the AMH measurements obtained by rev-Gen II and Access (Table 2), which was consistent with earlier studies reporting a difference of 9% to 11% between the two methods [7,13]. As Figure 3 shows, the points on the

Bland-Altman plot were uniformly scattered between the LOAs, which may suggest good agreement between the two measurement methods. However, the relatively wide span of the LOA and the considerable differences observed would not be negligible in clinical settings. Despite manufacturer suggestions that the Access assay offers standardized results consistent with rev-Gen II through the use of identical antibodies and calibration, such discordance raises potential issues concerning Access performance [4,7]. If a consistent bias is present, adjusting for it is simple. However, widely spaced LOAs and discrepancies based on the storage of samples constitute a much more serious problem. Although a conversion factor can be generated by linear regression methods, the conversion from rev-Gen II-AMH into Access-AMH is potentially highly inaccurate [19].

While the cause of such discrepancies is unclear, the systematic nature suggests that a calibration error of one method or the high inter-laboratory variability of the manual AMH assay documented in AMH external ring trial schemes are potential causes of bias [20-22]. Because the lack of universal calibration means that the AMH values are quite different compared with values from manual assays, one should not compare absolute AMH values between clinical studies that use different assays [1,4]. To maximize the clinical utility of AMH measurement, it is also critical to develop an international standard for AMH assessment [1].

Published studies involving the Bland-Altman technique have indicated various acceptable differences, but few have described the rationale for this choice [23]. Because AMH is used with age-specific reference values and several diagnostic cut-off levels, it is difficult to determine the acceptable degree of disagreement. The mean percentage difference has been compared to the acceptable change limit (ACL) according to ISO 5725-6 as an alternative to the acceptable degree of disagreement [24]. The ACL for interpreting a measured difference is based on the analytical imprecision (CV) according to the formula $ACL = 2.77 \times CV$ [25]. Assay precision was evaluated for both intra- and inter-run precision using AMH quality control material consisting of human recombinant AMH (Beckman-Coulter) at three known concentrations [14]. A CV of 4.96% was obtained from in-house routine mean data collection of the quality control value over 6 months, and the ACL was calculated as 13.74%. Considering the ACL of Access, the discrepancy between rev-Gen II-AMH and Access-AMH may constitute a clinically acceptable level.

Imprecision caused by analytical variation has significantly decreased in recent times due to automation. Although analytic variation can be reduced by the judicious choice of methodology and by adherence to strict standard operating procedures, it can never be eliminated entirely, and a growing body of evidence has demonstrated that the quality of laboratory results cannot be assured merely by focusing on purely analytical aspects [26].

Perhaps one of the most important clinical advances in the recent medical literature on AMH is the recognition of the meaningful pre-analytical variability in AMH results, which must be considered for appropriate interpretation in clinical care [6]. In clinical practice, the pre-analytical phase is usually poorly standardized; it is very difficult to control all of the pre-analytical components, such as the conditions of sample transport, storage, and handling. Recent works have established that the original Gen II assay was significantly susceptible to pre-analytical variability, and the rev-Gen II assay yielded more consistent results regardless of storage conditions [27]. Previous studies have shown that two fully automated immunoassays exhibited excellent analytical performance, superior to the current manual assay [13]. However, reproducibility of the automated assay has not yet been adequately confirmed. Our study showed that all AMH measurements by each assay had excellent ICC and Pearson correlations, whereas the Wilcoxon signed-rank test indicated that AMH values across sample storage conditions were less reliable for most measurements. All three statistical methods appeared to support that the Access-AMH level remained unchanged versus baseline only for the first 48 hours at 0 to 4 ℃ and −20 ℃. In contrast, Access-AMH showed a significant decrease after 1 week of storage, which corresponds well with the results of earlier studies [7,18]. The time-dependent stability of the Access-AMH measurements in stored serum at 0 to 4 ℃/−20 ℃ was superior to that of the rev-Gen II-AMH measurements. Antibodies in the automated kit may be unaffected by complement, allowing for stable assay performance over time. Our study also showed that storage at 0 to 4 ℃ is sufficient to maintain a proper assay outcome at −20 ℃ for up to 7 days.

Concerns about the long-term stability of serum AMH remain unresolved. Long-term stability is essential in epidemiological studies involving longitudinal laboratory results, but it is hindered by various challenges, such as changes in analysis methods. We found only two papers on long-term stability, only one of which involved an evaluation of AMH stability using the same AMH method [7,9]. Demirdjian et al. [7] reported that long-term storage of samples at −20 and −70 ℃ for up to 15 months had no significant impact on AMH level measured with Access. In contrast, we found a significant increase in AMH values measured with rev-Gen II and Access after 2 years of storage, but the magnitude of difference for Access-AMH was small (average, 4.5%).

Whole blood stability testing is necessary in clinical laboratory situations. The performance of AMH tests in infertility clinics lacking appropriate laboratory facilities has led to the use of clinical laboratory services utilizing dispatch collected into serum tubes with gel separators [10]. In a practical setting involving blood sampling in

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weekend sessions, AMH analysis may be performed after 48 hours on the Monday after a weekend during which whole blood in a serum separator tube was kept at room temperature. This means that blood samples may be centrifuged 2 to 3 days after collection and not processed according to recommended protocols, especially over weekends. Researchers must understand the impact of this delay on AMH results. We found only one study about Access-AMH changes caused by storage in serum gel tubes at room temperature with delayed centrifugation. That research indicated that the variation between days 0 and 6 was < 5% and that unseparated serum gel tubes can easily be stored at room temperature or couriered to a remote assay service without the need for centrifugation and refrigeration for up to 6 days [7]. Our results indicated that the change in the Access-AMH level over time was smaller than the change in the rev-Gen-II-AMH level, and storage in unseparated serum gel tubes for 1 week at room temperature profoundly influenced the rev-Gen II-AMH measurements (with an average decrease of 17.6%, constituting a clinically significant result) (Table 4). The stability of Access-AMH during storage at room temperature with delayed centrifugation suggests that using the Access assay may decrease enzymatic problems in AMH measurement.

This study has limitations due to its small sample size and use of only a single measurement for each assay system. Additionally, the limits of maximum acceptable differences (expected LOAs) could not be defined *a priori*, based on clinical necessity.

This study confirmed that the sample storage condition is a major pre-analytical variable. This suggests that samples should be tested with the same storage conditions when comparing AMH values between patients. Compared with rev-Gen II, the automated assay is superior given its pre-analytical stability. Repeated future studies are required to minimize variabilities in AMH measurement.

Conflict of interest

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

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References

1. Dewailly D, Andersen CY, Balen A, Broekmans F, Dilaver N, Fanchin R, et al. The physiology and clinical utility of anti-Mullerian hormone in women. Hum Reprod Update 2014;20:370–85.
2. Kumar A, Kalra B, Patel A, McDavid L, Roudebush WE. Development of a second generation anti-Müllerian hormone (AMH) ELISA. J Immunol Methods 2010;362:51–9.
3. Schiffner J, Roos J, Broomhead D, Helden JV, Godehardt E, Fehr D, et al. Relationship between anti-Müllerian hormone and antral follicle count across the menstrual cycle using the Beckman Coulter Access assay in comparison with Gen II manual assay. Clin Chem Lab Med 2017;55:1025–33.
4. Nelson SM, Pastuszek E, Kloss G, Malinowska I, Liss J, Lukaszuk A, et al. Two new automated, compared with two enzyme-linked immunosorbent, antimullerian hormone assays. Fertil Steril 2015;104:1016–21.
5. Rynning M, Wentzel-Larsen T, Bolann BJ. A model for an uncertainty budget for preanalytical variables in clinical chemistry analyses. Clin Chem 2007;53:1343–8.
6. Rustamov O, Smith A, Roberts SA, Yates AP, Fitzgerald C, Krishnan M, et al. The measurement of anti-Mullerian hormone: a critical appraisal. J Clin Endocrinol Metab 2014;99:723–32.
7. Demirdjian G, Bord S, Lejeune C, Masica R, Riviere D, Nicouleau L, et al. Performance characteristics of the Access AMH assay for the quantitative determination of anti-Müllerian hormone (AMH) levels on the Access family of automated immunoassay systems. Clin Biochem 2016;49:1267–73.
8. Dehghan A, Dupuis J, Barbalic M, Bis JC, Eiriksdottir G, Lu C, et al. Meta-analysis of genome-wide association studies in > 80 000 subjects identifies multiple loci for C-reactive protein levels. Circulation 2011;123:731–8.
9. Morse H, Ora I, Turkiewicz A, Andersen CY, Becker C, Isaksson A, et al. Reliability of AMH in serum after long-term storage at -80°C and an extended thawing episode. Ann Clin Lab Res 2016;4:61.
10. Fleming R, Fairbairn C, Blaney C, Lucas D, Gaudoin M. Stability of AMH measurement in blood and avoidance of proteolytic changes. Reprod Biomed Online 2013;26:130–2.
11. Bland JM, Altman DG. Measuring agreement in method comparison studies. Stat Methods Med Res 1999;8:135–60.

12. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Int J Nurs Stud 2010;47:931–6.
13. van Helden J, Weiskirchen R. Performance of the two new fully automated anti-Mullerian hormone immunoassays compared with the clinical standard assay. Hum Reprod 2015;30:1918–26.
14. Pearson K, Long M, Prasad J, Wu YY, Bonifacio M. Assessment of the Access AMH assay as an automated, high-performance replacement for the AMH Generation II manual ELISA. Reprod Biol Endocrinol 2016;14:8.
15. Dewitte K, Fierens C, Stockl D, Thienpont LM. Application of the Bland-Altman plot for interpretation of method-comparison studies: a critical investigation of its practice. Clin Chem 2002;48:799–801.
16. Beckman-Coulter. Access AMH: diagnostic confidence with automated Access AMH testing [Internet]. Beckman-Coulter; 2014 [cited 2023 Mar 6]. Available from: http://www.beckmancoulter.cz/Media/Default/Dx/amh/DS-19401A%20Access%20AMH%20Assay%20Final.pdf
17. Anckaert E, Oktem M, Thies A, Cohen-Bacrie M, Daan NM, Schiettecatte J, et al. Multicenter analytical performance evaluation of a fully automated anti-Mullerian hormone assay and reference interval determination. Clin Biochem 2016;49:260–7.
18. Li HW, Wong BP, Ip WK, Yeung WS, Ho PC, Ng EH. Comparative evaluation of three new commercial immunoassays for anti-Mullerian hormone measurement. Hum Reprod 2016;31:2796–802.
19. Su HI, Sammel MD, Homer MV, Bui K, Haunschild C, Stanczyk FZ. Comparability of antimullerian hormone levels among commercially available immunoassays. Fertil Steril 2014;101:1766–72.
20. Giavarina D. Understanding Bland Altman analysis. Biochem Med (Zagreb) 2015;25:141–51.
21. Zuvela E, Walls M, Matson P. Within-laboratory and between-laboratory variability in the measurement of anti-mullerian hormone determined within an external quality assurance scheme. Reprod Biol 2013;13:255–7.
22. Clark CA, Laskin CA, Cadesky K. Anti-Mullerian hormone: reality check. Hum Reprod 2014;29:184–5.
23. Shermock KM, Connor JT, Lavallee DC, Streiff MB. Clinical decision-making as the basis for assessing agreement between measures of the international normalized ratio. J Thromb Haemost 2009;7:87–93.
24. International Organization for Standardization. ISO 5725-6:1994. Accuracy (trueness and precision) of measurement methods and results. Part 6: Use in practice of accuracy values [Internet]. ISO; 1994 [cited 2023 Mar 6]. Available from: https://www.iso.org/standard/11837.html
25. Oddoze C, Lombard E, Portugal H. Stability study of 81 analytes in human whole blood, in serum and in plasma. Clin Biochem 2012;45:464–9.
26. Fraser CG. Test result variation and the quality of evidence-based clinical guidelines. Clin Chim Acta 2004;346:19–24.
27. Han X, McShane M, Sahertian R, White C, Ledger W. Pre-mixing serum samples with assay buffer is a prerequisite for reproducible anti-Mullerian hormone measurement using the Beckman Coulter Gen II assay. Hum Reprod 2014;29:1042–8.

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Association between polycystic ovarian morphology and insulin resistance in women with polycystic ovary syndrome

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Objective: The aim of the present study was to determine whether polycystic ovarian morphology (PCOM) is related to insulin resistance in women with polycystic ovary syndrome (PCOS).
Methods: A total of 147 Korean women aged 18 to 35 years and diagnosed with PCOS were included in this study. Fasting blood tests and standard 2-hour 75-g oral glucose tolerance tests were performed for all participants. PCOM-related parameters including total antral follicle count (TFC) and total ovarian volume (TOV) were assessed using transvaginal or transrectal ultrasonography. Correlation analysis was conducted to assess the relationships of TFC and TOV with insulin resistance-related clinical and biochemical parameters using Spearman rank correlation coefficients and linear regression analysis, with partial correlations used to control for the effects of confounding covariates.
Results: Fasting insulin levels, low-density lipoprotein levels, and insulin sensitivity assessment indices (ISAIs) were significantly correlated with TFC, but neither postprandial blood glucose levels nor insulin levels were significantly associated with TFC. No insulin resistance-related parameter was significantly correlated with TOV. These results did not change after adjustments for other anthropometric covariates. Fasting insulin and some ISAIs differed significantly between groups categorized by the median TFC value (TFC ≤54 and TFC >54).
Conclusion: TFC, but not TOV, was found to be related to fasting insulin resistance-related parameters in women with PCOS.

Keywords: Insulin resistance; Polycystic ovarian morphology; Polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in reproductive-age women and can cause chronic ovulatory dysfunction or irregular menstruation [1]. PCOS is an important issue for women's health, as it can lead to not only reproductive problems, but also psychological and metabolic disturbances throughout the lifespan [2].

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In patients with PCOS, multiple hormonal factors inhibit the selection of the dominant follicle and induce follicular arrest [3,4], producing a characteristic ultrasound pattern in which small preantral follicles are gathered around the edge of the ovary. This feature resembles that seen in the immature ovaries in adolescence. According to the revised diagnostic criteria [5], polycystic ovarian morphology (PCOM) is considered to be present in the early follicular phase when the number of 2- to 9-mm antral follicles is over 20 or increased ovarian volume (≥ 10 mL) is observed in either ovary on ultrasonography.

PCOS is intricately associated with diverse phenotypes of metabolic abnormalities. Glucose intolerance and type 2 diabetes mellitus, atherosclerotic dyslipidemia, and coronary heart disease are common clinical features of PCOS along with hormonal abnormalities, and these metabolic diseases are generally known to be associated with abnormal insulin sensitivity [4]. Although the pathophysiology

of PCOS is complex and has not been fully established, insulin resistance is the major component affecting various metabolic features and clinical phenotypes.

As interest in the role of insulin resistance in PCOS has increased, many studies have been conducted on this issue. However, research on the relationship between insulin resistance and ultrasonographic morphologic features of PCOS is still scarce and inconclusive [6-8]. Therefore, the purpose of this study was to determine whether the PCOM characteristics of increased antral follicle count and ovarian volume are related to insulin resistance parameters in women with PCOS.

Methods

1. Participants

Korean women between the ages of 18 and 35 years who were newly diagnosed with PCOS at Inje University Haeundae Paik Hospital between January 2010 and December 2013 were recruited for this study. Among all patients diagnosed with PCOS based on the previous 2003 Rotterdam criteria, those who met the recently revised diagnostic criteria in the international consensus guidelines for PCOS were enrolled in this study with the exclusion of other etiologies (including congenital adrenal hyperplasia, androgen-secreting tumor, and Cushing syndrome) [5,9]. Clinical hyperandrogenism was defined by the presence of hirsutism (modified Ferriman-Gallwey score >6) [5], and biochemical hyperandrogenism was defined as a serum androgen level above the 95% confidence limits defined in controls in the study by Chae et al. [10] (total testosterone >0.68 ng/mL and/or free testosterone >1.72 pg/mL). The exclusion criteria were as follows [11-13]: diagnosis of diabetes, thyroid disease, or hyperprolactinemia; history of ovarian surgery; or history of taking medications known to affect the level of any sex hormone or gonadotropin (oral contraceptives, ovulation induction agents, glucocorticoids, or anti-androgens) or anti-diabetic drugs, including insulin sensitizers, in the 6 months prior to enrollment. This retrospective study was approved by the Institutional Review Board (IRB) of Inje University Haeundae Paik Hospital (IRB No. 129792-2014-035), which waived the requirement for informed consent from patients. Ultimately, a total of 147 patients with PCOS were enrolled in the present study.

2. Measurement of anthropometric parameters and ultrasound examination

Clinical variables including age, parity, height, body weight, body mass index, waist circumference, hip circumference, and waist-to-hip ratio were evaluated for all study patients when they first visited the outpatient department. Pelvic ultrasound examinations (transvaginal or transrectal) were conducted in the early follicular phase using a Voluson LOGIQ S7 device (GE Ultrasound Korea Ltd.) equipped with a microconvex intracavitary probe with an approximate frequency range of 3.6 to 9.0 MHz. PCOM was defined as the presence of over 20 follicles (of 2 to 9 mm) and/or an ovarian volume ≥ 10 cm^3 [5,9,14]. All ultrasound examinations were performed by the same expert in reproductive endocrinology based on the international consensus on ultrasound assessment of PCOS [15].

3. Biochemical measurements and assessment of insulin resistance

Blood samples for biochemical analyses were taken from all participants in the early follicular phase after overnight fasting according to the guidelines of the Declaration of Helsinki. Glucose levels were measured 60 and 120 minutes after glucose ingestion during a 2-hour 75-g oral glucose tolerance test, and in some study participants ($n=60$), postprandial insulin levels at 60 and 120 minutes were measured simultaneously with glucose levels during the 2-hour oral glucose tolerance test. Serum glucose and insulin levels were analyzed using an L-Type Glul device (Wako) and an Elecsys Insulin assay (Roche), respectively. Cholesterol and triglyceride levels were measured using Pureauto S (Sekisui), and serum high-density lipoprotein and low-density lipoprotein (LDL) levels were measured using Cholestest (Sekisui). Both intra- and inter-assay coefficients of variation were below 8% for all assays.

Insulin sensitivity assessment indices (ISAIs) were calculated for all study participants. Established fasting ISAIs derived from a combination of fasting insulin and glucose levels were calculated as follows [11-13]: the homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as glucose level (mg/dL) $\times$ insulin level (μU/mL)/405, the glucose-to-insulin ratio (GIR) was calculated by dividing the glucose level (mg/dL) by the insulin level (μU/mL), and the quantitative insulin sensitivity check index (QUICKI) was calculated as 1/[log(insulin level [μU/mL])+log(glucose level [mg/dL])].

4. Statistical analysis

Values are expressed as mean $\pm$ standard deviation or median (range). Correlation analysis was conducted to assess the relationships between PCOM-related parameters and insulin resistance-related clinical and biochemical parameters using Spearman rank correlation coefficients and linear regression analysis, with partial correlations used to control for the effects of confounding covariates. The unpaired *t*-test or the Mann-Whitney *U* test was used to compare continuous parameters between the two groups, which were defined by the median total antral follicle count (TFC) (TFC ≤ 54 and TFC >54). All statistical analyses were conducted using SPSS version

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25.0 (IBM Corp.), with *p*-values < 0.05 considered to indicate statistical significance.

Results

Table 1 shows the baseline clinical anthropometric and ultrasonographic characteristics and laboratory biochemical parameters of the study participants. The median values for parity and TFC were 0 and 54, respectively.

No anthropometric parameter was significantly correlated with any PCOM-related parameter (Table 2). Among the metabolic parameters related to insulin resistance, fasting insulin levels, HOMA-IR, GIR, QUICKI, and LDL levels were significantly related to TFC (Table 3, Figure 1). Neither postprandial blood glucose levels nor insulin levels were significantly associated with TFC (Table 3). None of the insulin resistance-related parameters were significantly correlated with total ovarian volume (TOV), either fasting or postprandial. These results did not change after adjustments for other anthropometric covariates (Table 3).

All patients were divided into two groups based on the median TFC (TFC ≤ 54 and TFC > 54). As shown in Table 4, fasting insulin levels, HOMA-IR, and QUICKI differed significantly between the two groups. The mean LDL level was higher in the TFC > 54 group than in the TFC ≤ 54 group, but this difference was not statistically significant ($p = 0.058$).

Table 1. Baseline clinical and laboratory characteristics of study participants

Characteristic	Participants (n = 147)
Age (yr)	26.27 ± 5.67
Parity	0.19 ± 0.45
Height (cm)	162.34 ± 5.45
Body weight (kg)	58.82 ± 14.85
Body mass index (kg/m^2)	22.27 ± 5.30
Waist-to-hip ratio	0.80 ± 0.07
Total follicle count	65.77 ± 33.34
Total ovarian volume (cm^3)	22.66 ± 9.77
Fasting insulin (μIU/mL)	6.85 (1.70–134.50)
Fasting glucose (mg/dL)	92.24 ± 14.98
INS_2 (μIU/mL)[a]	51.85 (11.30–208.10)
PG_2 (mg/dL)	112.20 ± 42.44
HOMA-IR (fasting)	1.51 (0.33–36.63)
GIR (fasting)	15.77 ± 10.90
QUICKI (fasting)	0.36 ± 0.05
Cholesterol (mg/dL)	175.55 ± 30.37
Triglyceride (mg/dL)	93.60 ± 81.86
HDL (mg/dL)	59.97 ± 14.95
LDL (mg/dL)	95.95 ± 26.59

Values are presented as mean±standard deviation or median (range).
INS_2, postprandial insulin at 2 hours; PG_2, postprandial glucose at 2 hours; HOMA-IR, homeostasis model assessment of insulin resistance; GIR, glucose-to-insulin ratio; QUICKI, quantitative insulin sensitivity check index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
[a]Sixty participants.

Table 2. Correlations between ultrasonographic parameters related to polycystic ovarian morphology and clinical and anthropometric parameters

Variable	Total follicle count		Total ovarian volume (cm^3)	
	r	*p*-value	*r*	*p*-value
Age (yr)	−0.094	0.260	−0.039	0.640
Height (cm)	−0.012	0.881	0.029	0.732
Body weight (kg)	0.137	0.099	0.018	0.826
Body mass index (kg/m^2)	0.105	0.209	−0.009	0.912
Waist-to-hip ratio	0.140	0.130	−0.026	0.777

r, Spearman rank correlation coefficient.

Discussion

PCOS is a heterogeneous disorder characterized by metabolic and reproductive phenotypes, along with hormonal imbalances. Type 2 diabetes mellitus, nonalcoholic liver dysfunction, and dyslipidemia are metabolic disorders common in women with PCOS, and insulin resistance is considered the cardinal mechanism that induces these metabolic disorders [4,16]. Accordingly, several studies on the correlation between hormonal or clinical features and insulin resistance have been published [17-21], but research on the relationship between ultrasonographic morphologic features and insulin resistance in PCOS is still lacking. In this study, we investigated whether the ultrasound findings characteristic of PCOS are related to insulin resis-

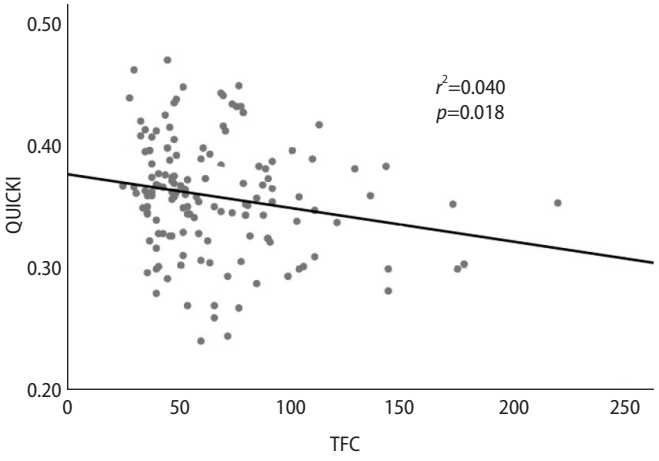

Figure 1. Correlation between total antral follicle count (TFC) and quantitative insulin sensitivity check index (QUICKI) in women with polycystic ovary syndrome. A r^2 was determined by linear regression analysis.

Table 3. Correlations between ultrasonographic parameters related to polycystic ovarian morphology and biochemical metabolic parameters

Variable	Total follicle count				Total ovarian volume (cm^3)			
	r	*p*-value	*r*[a]	*p*-value	*r*	*p*-value	*r*[a]	*p*-value
Fasting insulin (μIU/mL)	0.203	0.016[b]	0.216	0.020[b]	0.099	0.243	0.137	0.143
Fasting glucose (mg/dL)	0.080	0.348	0.054	0.568	0.019	0.823	0.040	0.673
INS_2 (μIU/mL)[c]	0.071	0.592	−0.022	0.873	0.119	0.366	0.041	0.761
PG_2 (mg/dL)	0.120	0.160	0.060	0.525	0.031	0.721	0.079	0.404
HOMA-IR (fasting)	0.213	0.011[b]	0.193	0.038[b]	0.098	0.250	0.134	0.153
GIR (fasting)	−0.193	0.022[b]	−0.259	0.005[b]	−0.105	0.216	−0.120	0.201
QUICKI (fasting)	−0.213	0.011[b]	−0.266	0.004[b]	−0.098	0.246	−0.143	0.126
Cholesterol (mg/dL)	0.129	0.138	0.128	0.177	0.004	0.963	0.062	0.517
Triglyceride (mg/dL)	0.055	0.533	−0.075	0.431	−0.020	0.824	−0.051	0.589
HDL (mg/dL)	−0.053	0.548	0.135	0.153	0.093	0.291	0.066	0.484
LDL (mg/dL)	0.182	0.038[b]	0.190	0.045[b]	0.014	0.874	0.071	0.456

r, Spearman rank correlation coefficient; INS_2, postprandial insulin at 2 hours; PG_2, postprandial glucose at 2 hours; HOMA-IR, homeostasis model assessment of insulin resistance; GIR, glucose-to-insulin ratio; QUICKI, quantitative insulin sensitivity check index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
[a]*r*, partial correlation coefficient adjusted for age, body mass index, and waist-to-hip ratio; [b]$p<0.05$; [c]Sixty participants.

Table 4. Comparison of insulin resistance-related parameters between groups according to total follicle count in women with polycystic ovary syndrome

Variable	Group 1 (n = 74)[a]	Group 2 (n = 73)[b]	*p*-value
Fasting insulin (μIU/mL)	6.10 (1.70–42.50)	7.90 (1.90–134.50)	0.022[c]
Fasting glucose (mg/dL)	90.49 ± 10.66	94.00 ± 18.27	0.166
INS_2 (μIU/mL)[d]	44.50 (15.10–142.60)	56.40 (11.30–208.10)	0.483[e]
PG_2 (mg/dL)	107.52 ± 37.54	117.00 ± 46.90	0.192
HOMA-IR (fasting)	1.33 (0.33–30.55)	1.73 (0.42–36.63)	0.025[c]
GIR (fasting)	17.20 ± 10.90	14.29 ± 10.83	0.114
QUICKI (fasting)	0.37 ± 0.04	0.35 ± 0.05	0.033[c]
Cholesterol (mg/dL)	173.11 ± 32.62	182.12 ± 27.70	0.088
Triglyceride (mg/dL)	93.43 ± 78.66	94.21 ± 85.96	0.957
HDL (mg/dL)	59.12 ± 15.25	60.73 ± 14.82	0.540
LDL (mg/dL)	94.71 ± 25.88	103.54 ± 26.80	0.058

Values are presented as mean ± standard deviation or median (range). *p*-values are unpaired *t*-test.
INS_2, postprandial insulin at 2 hours; PG_2, postprandial glucose at 2 hours; HOMA-IR, homeostasis model assessment of insulin resistance; GIR, glucose to insulin ratio; QUICKI, quantitative insulin sensitivity check index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
[a]Group 1, total follicle count ≤ 54; [b]Group 2, total follicle count > 54; [c]$p < 0.05$; [d]Sixty participants; [e]Mann-Whitney *U* test.

tance parameters, and we found that TFC, but not TOV, was associated with fasting (but not postprandial) insulin resistance-related parameters in women with PCOS. Hong et al. [6] previously demonstrated that TFC could be a major indicator of insulin resistance and metabolic disturbance in women with PCOS, which supports our results. Another study compared hormonal and metabolic features between women with PCOM and those with normal ovaries, revealing that the fasting insulin level and calculated index of insulin resistance were significantly correlated with PCOM, but the fasting glucose level was not significantly different between the two groups [7]. This was also highly consistent with our results.

Despite the differences in population, PCOM with regular ovulatory cycles may represent the mildest form of hyperandrogenism and is associated with greater insulin resistance than normal morphologic features [6,7]. PCOM is related to hyperinsulinemia and low sex hormone-binding globulin levels [7], even in the absence of other metabolic abnormalities or imbalanced gonadotropin secretion. Similarly, Norman et al. [22] suggested that in the presence of PCOM, a more severe disturbance in insulin signaling may constitute a predisposition to the menstrual irregularity characteristic of PCOS.

We observed no significant relationship between ovarian volume and insulin resistance. Several studies have noted that ovarian volume or ovarian blood flow is associated with insulin resistance

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[6,8,23,24], and the discrepancy between these studies and ours may stem from the use of different study designs, diagnostic criteria, and ethnicities of study populations [25]. Some studies have reported that while ovarian volume may be a good surrogate marker for PCOS, the follicle count is more sensitive and specific to PCOM features and could be a more powerful predictor than TOV of insulin resistance in PCOS [6,26]. These suggestions are partially consistent with our findings.

In the present study, serum LDL levels were significantly associated with TFC. A previous study also reported that higher lipid levels, including LDL levels, were observed in patients with PCOS with greater follicle counts despite similar body mass indices [27]. To our knowledge, ours is the first study to conduct a comparison of insulin resistance-related parameters between groups categorized by TFC, and we found that fasting insulin and some ISAIs were significantly different between groups.

Our study has several limitations, including a retrospective design and a relatively small sample size. Based on a previous study [7], the sample size was calculated as 89 people per group (178 people in total). Notably, only 60 participants received a blood test measuring their postprandial insulin levels, which was an insufficient sample size to demonstrate the reliability of the study.

In conclusion, the morphologic features of PCOS, especially an elevated follicle count, are significantly related to insulin resistance in women with PCOS. Further large-scale prospective trials that include sufficient data on both fasting and postprandial blood glucose and insulin are needed to clarify and corroborate our results.

Conflict of interest

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

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References

1. March WA, Moore VM, Willson KJ, Phillips DI, Norman RJ, Davies MJ. The prevalence of polycystic ovary syndrome in a community sample assessed under contrasting diagnostic criteria. Hum Reprod 2010;25:544–51.
2. Rodriguez-Paris D, Remlinger-Molenda A, Kurzawa R, Glowinska A, Spaczynski R, Rybakowski F, et al. Psychiatric disorders in women with polycystic ovary syndrome. Psychiatr Pol 2019;53:955–66.
3. Qiao J, Feng HL. Extra- and intra-ovarian factors in polycystic ovary syndrome: impact on oocyte maturation and embryo developmental competence. Hum Reprod Update 2011;17:17–33.
4. Azziz R, Carmina E, Chen Z, Dunaif A, Laven JS, Legro RS, et al. Polycystic ovary syndrome. Nat Rev Dis Primers 2016;2:16057.
5. Teede HJ, Misso ML, Costello MF, Dokras A, Laven J, Moran L, et al. Recommendations from the international evidence-based guideline for the assessment and management of polycystic ovary syndrome. Hum Reprod 2018;33:1602–18.
6. Hong SH, Sung YA, Hong YS, Jeong K, Chung H, Lee H. Polycystic ovary morphology is associated with insulin resistance in women with polycystic ovary syndrome. Clin Endocrinol (Oxf) 2017;87:375–80.
7. Adams JM, Taylor AE, Crowley WF, Hall JE. Polycystic ovarian morphology with regular ovulatory cycles: insights into the pathophysiology of polycystic ovarian syndrome. J Clin Endocrinol Metab 2004;89:4343–50.
8. Diamanti-Kandarakis E, Dunaif A. Insulin resistance and the polycystic ovary syndrome revisited: an update on mechanisms and implications. Endocr Rev 2012;33:981–1030.
9. Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil Steril 2004;81:19–25.
10. Chae SJ, Kim JJ, Choi YM, Hwang KR, Jee BC, Ku SY, et al. Clinical and biochemical characteristics of polycystic ovary syndrome in Korean women. Hum Reprod 2008;23:1924–31.
11. Chun S. 1-h Postprandial glucose level is related to the serum anti-Mullerian hormone level in women with polycystic ovary syndrome. Gynecol Endocrinol 2015;31:815–8.
12. Park CH, Chun S. Association between serum gonadotropin level and insulin resistance-related parameters in Korean women with polycystic ovary syndrome. Obstet Gynecol Sci 2016;59:498–505.
13. Kim N, Chun S. Association between the serum estrone-to-estradiol ratio and parameters related to glucose metabolism and insulin resistance in women with polycystic ovary syndrome. Clin Exp Reprod Med 2021;48:374–9.
14. The Korean Society of Gynecologic Endocrinology. The Korean Society of Gynecologic Endocrinology clinical guideline 2016. The Korean Society of Gynecologic Endocrinology; 2016. p. 27.

15. Balen AH, Laven JS, Tan SL, Dewailly D. Ultrasound assessment of the polycystic ovary: international consensus definitions. Hum Reprod Update 2003;9:505–14.
16. Carmina E, Lobo RA. Polycystic ovary syndrome (PCOS): arguably the most common endocrinopathy is associated with significant morbidity in women. J Clin Endocrinol Metab 1999;84:1897–9.
17. De Leo V, Musacchio MC, Cappelli V, Massaro MG, Morgante G, Petraglia F. Genetic, hormonal and metabolic aspects of PCOS: an update. Reprod Biol Endocrinol 2016;14:38.
18. Ehrmann DA. Polycystic ovary syndrome. N Engl J Med 2005; 352:1223–36.
19. Shroff R, Syrop CH, Davis W, Van Voorhis BJ, Dokras A. Risk of metabolic complications in the new PCOS phenotypes based on the Rotterdam criteria. Fertil Steril 2007;88:1389–95.
20. Shi Y, Gao X, Sun X, Zhang P, Chen Z. Clinical and metabolic characteristics of polycystic ovary syndrome without polycystic ovary: a pilot study on Chinese women. Fertil Steril 2008;90:1139–43.
21. Christ JP, Vanden Brink H, Brooks ED, Pierson RA, Chizen DR, Lujan ME. Ultrasound features of polycystic ovaries relate to degree of reproductive and metabolic disturbance in polycystic ovary syndrome. Fertil Steril 2015;103:787–94.
22. Norman RJ, Hague WM, Masters SC, Wang XJ. Subjects with polycystic ovaries without hyperandrogenaemia exhibit similar disturbances in insulin and lipid profiles as those with polycystic ovary syndrome. Hum Reprod 1995;10:2258–61.
23. Chen MJ, Yang WS, Chen CL, Wu MY, Yang YS, Ho HN. The relationship between anti-Mullerian hormone, androgen and insulin resistance on the number of antral follicles in women with polycystic ovary syndrome. Hum Reprod 2008;23:952–7.
24. Carmina E, Orio F, Palomba S, Longo RA, Lombardi G, Lobo RA. Ovarian size and blood flow in women with polycystic ovary syndrome and their correlations with endocrine parameters. Fertil Steril 2005;84:413–9.
25. Kim JJ, Choi YM. Phenotype and genotype of polycystic ovary syndrome in Asia: ethnic differences. J Obstet Gynaecol Res 2019;45:2330–7.
26. Jonard S, Robert Y, Cortet-Rudelli C, Pigny P, Decanter C, Dewailly D. Ultrasound examination of polycystic ovaries: is it worth counting the follicles? Hum Reprod 2003;18:598–603.
27. Kim JJ, Hwang KR, Chae SJ, Yoon SH, Choi YM. Impact of the newly recommended antral follicle count cutoff for polycystic ovary in adult women with polycystic ovary syndrome. Hum Reprod 2020; 35:652–9.

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CERM

Physiological intracytoplasmic sperm injection does not improve the quality of embryos: A cross-sectional investigation on sibling oocytes

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Objective: This study aimed to compare the efficacy of physiological intracytoplasmic sperm injection (PICSI) and intracytoplasmic sperm injection (ICSI) in terms of the fertilization rate and embryo quality using sibling oocyte cycles.
Methods: This prospective, cross-sectional study collected data from 76 couples who underwent their first cycle at the Hue Center for Reproductive Endocrinology and Infertility, Vietnam, between May 2019 and November 2021. The inclusion criteria were cycles with at least eight oocytes and a sperm concentration of 5×10^6/mL. Sperm parameters, sperm DNA fragmentation (SDF), fertilization, and the quality of cleavage-stage embryos on day 2 and blastocysts on day 5 were examined.
Results: From 76 ICSI cycles, 1,196 metaphase II (MII) oocytes were retrieved, half of which were randomly allocated to either the PICSI (n=592) or ICSI (n=604) treatment group. The results showed no significant difference between the two groups in terms of fertilization (72.80% vs. 75.33%, p=0.32), day 2 cleavage rate (95.13% vs. 96.04%, p=0.51), blastulation rate (52.68% vs. 57.89%), and high-quality blastocyst rate (26.10% vs. 31.13%, p=0.13). However, in cases where SDF was low, 59 cycles consisting of 913 MII oocytes produced a considerably higher blastulation rate with PICSI than with ICSI (50.49% vs. 35.65%, p=0.00). There were no significant differences between the pregnancy outcomes of the PICSI and ICSI embryo groups following embryo transfer.
Conclusion: Using variable sperm quality provided no benefit for PICSI versus ICSI in terms of embryo outcomes. When SDF is low, PICSI appears to be able to produce more blastocysts.

Keywords: DNA fragmentation; Embryonic structures; Fertilization *in vitro*; Hyaluronic acid; Sperm injections, intracytoplasmic

Introduction

The presence of a hyaluronic acid (HA) receptor in the plasma membrane of the sperm head indicates that the sperm has matured and is capable of binding HA in the extracellular matrix of cumulus cells encircling the oocyte [1]. Sperm with HA receptors can reach the cytoplasm of the oocyte during natural fertilization [2]. Two sperm selection methods have been developed based on the interaction between spermatozoa and HA: (1) recovering spermatozoa attached to HA-coated Petri dishes [3] and (2) selecting spermatozoa that swim slowly in a HA medium [4].

The capacity of mature spermatozoa selected with HA to fertilize is equivalent to that of mature spermatozoa selected with the hemi-zona binding assay. Moreover, the proportion of sperm with normal morphology was found to be greater in the HA-binding group than in the group of fresh sperm [5]. HA is believed to play a role in the selection of mature spermatozoa [6] can be used to select sperm without causing DNA damage [7-10]. An inverse correlation exists between

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the rate of HA-binding and protamine deficiency, as well as between DNA fragmentation and poor sperm morphology [11]. Furthermore, Parmegiani et al. [8] and Huang et al. [12] demonstrated reduced DNA fragmentation in spermatozoa selected in HA solution as opposed to swim-up and in HA-coated dishes as opposed to density gradient centrifugation, respectively. Razavi et al. [13] found that spermatozoa recovered from HA-coated dishes had the same level of DNA fragmentation as spermatozoa recovered from unselected dishes.

In nature, sperm migration through the female reproductive system is a highly selective mechanism that selects mature sperm with a high level of DNA integrity. Intracytoplasmic sperm injection (ICSI), in contrast, overcomes most barriers in natural sperm selection. Using external morphology to select sperm, it is not possible to identify sperm with DNA fragmentation, which may have impacts on embryological and clinical outcomes [14-16]. Several advanced methods of sperm selection are currently being investigated to imitate the dynamics of natural selection. In this context, sperm selection based on the maturation of the cell membrane (physiological intracytoplasmic sperm injection [PICSI]) is often employed [17]. PICSI selects sperm based on an oocyte's ability to bind HA, which improves the success rate of ICSI. Analyses have been conducted on mature spermatozoa with no or minimum DNA damage and no aneuploidy. Previous trials compared the effectiveness of PICSI to that of conventional ICSI. Parmegiani et al. [8] demonstrated that when HA was employed, the embryo development rate and proportion of high-quality embryos were significantly greater than when ICSI was employed. Mokanszki et al. [18] evaluated the efficacy of PICSI using an HA-binding assay threshold (HBA score) and reported significantly higher fertilization rates in the PICSI group with HBA $>60\%$, implantation rates in the PICSI group with HBA $\leq 60\%$, and clinical pregnancy rates in each PICSI group than in the ICSI group.

However, several other studies have reported that PICSI does not improve the success of *in vitro* fertilization (IVF), even in patients with unexplained infertility [19-21]. It was reported that the effectiveness of PICSI in improving treatment outcomes for unexplained infertility was uncertain [22]. Thus, it is unclear whether HA should be employed to select sperm for enhancing ICSI performance or whether physiological sperm selection is effective in certain situations. The goal of this study was to assess the fertilization rate and embryo quality of PICSI and ICSI cycles utilizing sibling oocytes.

Methods

1. Study design

This prospective, cross-sectional study collected data from 76 couples who underwent their initial IVF cycle at the Center for Reproductive Endocrinology and Infertility, Hue University of Medicine and Pharmacy, Vietnam, between May 2019 and November 2021. The inclusion criteria were cycles that had at least eight oocytes to ensure the separation of two sibling groups and a minimum sperm concentration of 5×10^6/mL (to perform an accurate Halosperm test). Sperm parameters, the sperm DNA fragmentation index (DFI), fertilization, and the quality of cleavage-stage embryos on day 2 and blastocysts on day 5 were evaluated. The embryo cleavage rate was defined as the proportion of embryos with at least two blastomeres to those with two pronuclei 44 to 48 hours following ICSI. A blastocyst was recorded based on the presence of an embryonic cavity. At 116 to 118 hours after ICSI, a high-quality blastocyst possessed the following characteristics: a cavity that filled the blastocyst's volume, a densely packed inner cell mass, and a trophectoderm consisting of many cells that formed a cohesive epithelium. The blastulation rate was defined as the number of blastocysts to cleavage-stage embryos, while the quality blastulation rate referred to the proportion of healthy blastocysts to cleavage-stage embryos. This study compared the percentage of embryo development between PICSI and ICSI using different sperm subgroup characteristics, including morphology, motility, and DNA fragmentation. Inability to ejaculate, sperm collected by cryopreservation or surgery, individuals with extremely low sperm counts (less than 5×10^6/mL), severe varicocele, or azoospermia were among the exclusion criteria. This study also excluded IVF cycles involving gamete donors and women with severe endometriosis (grades 3 and 4).

2. Semen analysis

Sperm samples were collected after 3 to 5 days of. Using the 2010 World Health Organization guidelines, semen samples were tested for concentration, sperm motility, vitality, and morphology following 30 minutes of liquefaction at 37 °C.

3. Sperm DNA fragmentation test

All semen samples were examined with Halosperm HT-HS10 (Halotech DNA S.L.) for fragmented DNA. The sperm sample was combined with an agarose microgel and smeared onto a microscope slide before being refrigerated at 40 °C. After agarose was dried at room temperature, the slide was submerged in a denaturation solution for 7 minutes. Following this, the sample was incubated in a lysis solution for 25 minutes. The slide was cleaned with distilled water, dehydrated in successive 70% and 100% ethanol washes for 2 minutes each, and then air-dried and stained with Giemsa. Using a Carl Zeiss Primo Starlight microscope at $\times1,000$ magnification, 500 sperm were counted on each slide. The DFI was computed as the proportion of spermatozoa with DNA fragmentation per 500 spermatozoa; those with DNA fragmentation were detected with small halos, without halos, or degraded.

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4. Semen preparation

Sperm samples were produced using a two-layer density gradient centrifugation procedure with 300 to 400 ×g for 15 minutes and 45% and 90% Sil-Select Plus (Fertipro). Before ICSI, sperm samples were twice cleaned in 3 mL of Spermrinse medium (Vitrolife). During each step of washing with Spermrinse medium, sperm samples were centrifuged at 300 to 400 ×g for 10 minutes, and the supernatants were discarded. In preparation for the sperm selection procedure, 0.3 mL of residual sperm medium was retained.

5. Controlled ovarian hyperstimulation

Utilizing a gonadotropin-releasing hormone antagonist protocol, women who underwent IVF cycles were treated with controlled ovarian hyperstimulation. The starting dosage of recombinant follicle-stimulating hormone (follitropin alfa) was based on the antral follicle count and anti-Müllerian hormone level. Then, 35 to 36 hours after an intramuscular injection of 10,000 IU of human chorionic gonadotropin (hCG) (Pregnyl; Merck Sharp & Dohme Limited), follicles were aspirated with an ultrasound-guided single-lumen needle (Vitrolife).

6. Physiological intracytoplasmic sperm injection

PICSI dishes (Origio) were prepared by soaking hyaluronan microdots in 10 µL of culture medium droplets GMOPS PLUS (Vitrolife). To immobilize the sperm, drops of 10% polyvinylpyrrolidone (PVP) (Vitrolife) were applied to the disk. Then, 2 µL of purified sperm suspension was added to the hyaluronan microdot-containing droplets. To optimize sperm binding, the dishes were covered with 3 to 4 mL of Ovoil (Vitrolife) and incubated at 37 °C for 15 minutes.

7. Intracytoplasmic sperm injection and embryo culture

After using 80 IU of HYASE (Vitrolife) to denude the oocyte cumulus complex, mature oocytes were identified. Three hours after retrieval, ICSI was performed using a mature oocyte and sperm that had been previously prepared. The injected oocyte was cultivated in a single drop of 20 µL of G-TL (Vitrolife) covered by 3 mL of Ovoil (Vitrolife) under conditions of 6% CO_2 and 5% O_2. Next, 16 to 18 hours after the injection, fertilized oocytes were detected by the presence of two pronuclei. On days 2 and 5, embryos were evaluated according to the Istanbul consensus. A high-quality blastocyst was defined as possessing a densely packed inner cell mass and trophectoderm composed of many cells forming a cohesive epithelium [23].

8. Embryo vitrification and thawing

Day 5 blastocysts were chosen for vitrification. Utilizing the Cryotop device and commercially available medium (Kitazato), vitrification was performed according to the manufacturer's instructions. The blastocysts were stored in liquid nitrogen for storage.

The embryos were thawed using the warming solution (Kitazato) per the manufacturer's instructions. Before transfer, embryos were cultured for 2 hours in 20 µL of G-TL (Vitrolife) prepared the previous night at 6.0% CO_2 and 5.0% O_2.

9. Embryo transfer

To prepare the endometrium, 4 mg of oral estradiol (Progynova; Bayer) was administered twice daily beginning on day 2 of the subsequent cycle. Progesterone (Crinone Gel 8%; Merck KGaA) was administered vaginally at a dosage of 90 mg twice daily to induce secretory transformation. Embryos were transferred on days when the endometrial thickness was at least 7 mm. Embryos were immersed for 15 to 30 minutes in 1 mL of Embryoglue (Vitrolife) before being put into the Kitazato catheter and then transferred to the uterus under transvaginal ultrasound monitoring.

10. Clinical follow-up

On the 14th day after embryo transfer, serum β subunit of human chorionic gonadotropin (β-hCG) levels were measured, and a value of more than 50 mIU/mL was considered β-hCG-positive. Two weeks later, transvaginal ultrasound was performed. Four weeks after embryo transfer, the appearance of a gestational sac and fetal cardiac activity were considered to indicate clinical pregnancy. Miscarriage or pregnancy loss was confirmed by ultrasound.

11. Statistical analysis

Statistical analysis was conducted using SPSS version 22.0 (IBM Corp.). Numeric data were presented as mean standard deviation, and frequencies were expressed as a percentage when comparing results between the PICSI and ICSI groups. Samples were classified into subgroups based on their morphology, motility, and sperm DNA fragmentation (SDF) as follows: normal morphology ≥ 4% and normal morphology < 4%; progressive ≥ 30% and progressive < 32%; DFI < 30% (low SDF) and DFI ≥ 30% (high SDF). The chi-square or Fisher exact test, as well as the independent-samples *t*-test, were used to analyze categorical variables. A *p*-value < 0.05 was considered statistically significant in all tests.

12. Ethical statement

The present study was approved by the Ethics Committee of Hue University of Medicine and Pharmacy, with approval number H2020/030. All patients agreed to participate in this study and signed an informed consent form.

13. Availability of data and material

The dataset used and/or analyzed during the current study is available from the corresponding author upon reasonable request.

Results

The clinical and cycle characteristics of the couples are shown in Table 1. From May 2019 to November 2021, 76 couples were selected to undergo either PICSI or ICSI. In addition to typical semen analysis parameters, the average sperm DFI was calculated to be 23.48%±15.10% (range, 4.4% to 76.4%). The majority of cases were due to female-factor infertility, with polycystic ovary syndrome being the most common cause. The mean number of retrieved oocytes was 20.04±7.27 (range, 8 to 43), of which 15.75±5.31 (range, 7 to 31) were metaphase II (MII) oocytes.

The outcomes of fertilization and embryo development using two techniques are summarized in Table 2. In each cycle, half of the oocytes were randomly assigned to one of two treatment groups: PICSI (n=592) and ICSI (n=604), based on inclusion and exclusion criteria. The fertilization rate (72.80% vs. 75.33%, $p=0.32$), cleavage rate on day 2 (95.13% vs. 96.04%, $p=0.51$), blastulation rate (52.68% vs. 57.89%, $p=0.13$), and good-quality blastocyst rate (26.10% vs. 31.58%, $p=0.08$) did not show statistically significant differences between the PICSI and ICSI groups.

Table 3 presents the comparison between embryo culture outcomes from using PICSI and ICSI in different subgroups of spermatozoa concerning morphology and motility. No significant variation was found between PICSI and ICSI in the subgroups with normal and defective sperm morphology and motility.

The association between the degree of SDF and the outcomes of PICSI versus ICSI was also evaluated. As shown in Table 4, in the low SDF subgroup (DFI <30%), 913 MII oocytes subjected to 59 cycles had a significantly higher blastulation rate with PICSI as compared to ICSI (50.49% vs. 35.65%, $p=0.00$). However, in the high SDF group, no significant difference was observed between PICSI and ICSI. The relationship of the fertilization rate with these four factors—sperm morphology, motility, DNA fragmentation, and blastocyst quality—are presented in Figure 1, respectively.

Table 5 shows the results of embryo transfer for 31 cycles with embryos following PICSI and 63 cycles with embryos following ICSI. Although the percentage of hCG-positive patients in the PICSI group was somewhat higher than in the ICSI group (61.19% vs. 57.14%), this difference was not statistically significant ($p=0.53$). Additionally, the frequencies of clinical pregnancy and stillbirth between the two groups did not demonstrate a statistically significant difference.

Table 1. General characteristics of study participants in infertile couples with IVF

Characteristic	Results (n=76)
Male age (yr)	35.71±5.07 (28–51)
Female age (yr)	31.93±4.17 (25–44)
Infertility duration (yr)	4.20±2.19
Primary infertility	46 (60.53)
Secondary infertility	30 (39.47)
Male BMI (kg/m^2)	23.75±2.78 (17.19–33.70)
Female BMI (kg/m^2)	21.32±2.57 (15.19–32.89)
Semen analysis	
Concentration (10^6/mL)	36.09±14.18 (5–86)
PR (%)	32.32±10.75 (5–58)
Viability (%)	82.34±8.66 (23–92)
Normal morphology (%)	3.89±1.92 (1–14)
Abnormal head (%)	88.67±5.59 (78–97)
Abnormal neck: tail (%)	52.07±11.43 (30–92)
DFI (%)	23.48±15.10 (4.4–76.4)
Indication for IVF	
With male factor	11 (14.47)
With PCOS	39 (51.32)
With tubal factor	18 (23.68)
With endometriosis	6 (7.89)
With low ovarian reserve	4 (5.26)
With ≥2 factors	15 (19.74)
Main findings of IVF cycles	
AMH (ng/mL)	4.82±3.02 (1.40–21.23)
FSH day 2 (mIU/mL)	6.33±1.44 (4.06–10.50)
Total no. of retrieved oocytes	20.04±7.27 (8–43)
No. of obtained MII oocytes	15.75±5.31 (7–31)

Values are presented as mean±standard deviation (range) or number (%).
IVF, *in vitro* fertilization; BMI, body mass index; PR, progressive; DFI, DNA fragmentation index; PCOS, polycystic ovary syndrome; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; MII, metaphase II.

Discussion

The usefulness of utilizing HA in sperm selection has been demonstrated in a number of previous studies [8,18,24]. Parmegiani et al. [8] found that the total rate of good-quality embryos was significantly

Table 2. Comparison of the outcomes of embryo culture following PICSI versus ICSI

	PICSI	ICSI	*p*-value[a]
No. of MII oocytes	592	604	
Fertilization rate	431/592 (72.80)	455/604 (75.33)	0.32
Cleavage rate	410/431 (95.13)	437/455 (96.04)	0.51
Blastulation rate	216/410 (52.68)	253/437 (57.89)	0.13
Good-quality blastocyst rate	107/410 (26.10)	138/437 (31.58)	0.08

Values are presented as number (%).
PICSI, physiological intracytoplasmic sperm injection; ICSI, intracytoplasmic sperm injection; MII, metaphase II.
[a]Statistical test using chi-square test.

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Table 3. Comparison of the result of embryo culture following PICSI and ICSI in terms of sperm morphology and motility subgroups

Outcome	Sperm morphology						Sperm motility					
	Normal morphology ≥ 4% (n = 40)			Normal morphology < 4% (n = 36)			PR ≥ 32% (n = 42)			PR < 32% (n = 34)		
	PICSI (317 MII oocytes)	ICSI (325 MII oocytes)	*p*-value[a]	PICSI (275 MII oocytes)	ICSI (279 MII oocytes)	*p*-value[a]	PICSI (337 MII oocytes)	ICSI (347 MII oocytes)	*p*-value[a]	PICSI (255 MII oocytes)	ICSI (257 MII oocytes)	*p*-value[a]
Fertilization rate	231/317 (72.87)	252/325 (77.54)	0.17	200/275 (72.73)	204/279 (73.12)	0.92	245/337 (72.70)	273/347 (78.67)	0.07	186/255 (72.94)	183/257 (71.21)	0.66
Cleavage rate	218/231 (94.37)	241/252 (95.63)	0.52	192/200 (96.00)	197/204 (96.57)	0.76	233/245 (95.10)	263/273 (96.34)	0.49	177/186 (95.16)	175/183 (95.63)	0.83
Blastulation rate	138/218 (63.30)	159/241 (65.97)	0.55	78/192 (41.67)	94/197 (47.71)	0.16	128/233 (54.94)	155/263 (58.94)	0.37	88/177 (49.72)	98/175 (56.00)	0.24
Good-quality blastocyst rate	71/218 (32.57)	89/241 (36.91)	0.33	36/192 (18.75)	49/197 (24.87)	0.14	64/233 (27.47)	88/263 (33.46)	0.15	45/177 (25.42)	50/175 (28.57)	0.51

Values are presented as number (%).
PICSI, physiological intracytoplasmic sperm injection; ICSI, kintracytoplasmic sperm injection; PR, progressive; MII, metaphase II.
[a]Statistical test using chi-square test.

higher in the PICSI group (95.0% ± 0.8%) compared to the ICSI group (84.0% ± 1.1%, $p < 0.001$). The proportion of top-grade embryos was also significantly greater in the PICSI group than in the ICSI group (35.8% vs. 24.1%, $p = 0.046$). While there was no statistical significance, there were tendencies toward increased rates of fertilization, implantation, and pregnancy in the PICSI group [8]. Furthermore, when deciding whether to use PICSI or ICSI during cycles, it is important to measure the HBA score of sperm in fresh semen. PICSI is considered a new and effective procedure that can significantly improve clinical outcomes in patients with a low HBA score [18].

Contrary to our expectations, PICSI did not provide any additional benefits in terms of fertilization and subsequent embryo cleavage compared to ICSI cycles. The difference in blastocyst development between PICSI and conventional ICSI was not statistically significant. This finding was also documented in a study conducted by Majumdar and Majumdar [22] in 2013. Two previous studies had also used HA-containing media to select spermatozoa and had similarly found that this did not significantly increase the fertilization rate and the proportion of high-quality embryos [19,20]. There are numerous possible explanations for this finding. One possible theory is that the mechanical act of removing adhering sperm from the PICSI plate may damage the sperm, which could be exacerbated by the toxicity of PVP [21]. As another possibility, we discovered that the technique of sperm selection in the PICSI dish followed by sperm injection into the oocytes, took significantly longer than conventional ICSI. When oocytes spend an extended period of time outside, their quality can decrease. As Liu et al. [20] reported, a longer injection time was seen in the medium containing HA (Sperm Slow) group than in the ICSI group. The authors suggest that a possible approach would be to limit the number of oocytes that are injected into each dish, thereby minimizing the amount of time that oocytes are exposed to conditions outside of a controlled incubator [20].

The effect of HA-based selection of normal sperm was also presented by Prinosilova et al. [5]. Their results showed that when mature spermatozoa are chosen with HA, their capacity to fertilize normally was similar to that observed when the hemi-zona assay was used. Moreover, the percentage of sperm meeting strict normal shape criteria was higher in the HA-binding group than in the initial semen sample [5]. Erberelli et al. [25] examined PICSI and ICSI cycles in couples with the moderate to severe male factor fertility. The researchers concluded that teratozoospermia cases could benefit from the PICSI technique and suggested using PICSI in all cases of abnormal morphology spermatozoa [25]. Kim et al. [24] also found support for the advantage of PICSI when a medium containing HA is used in cases of severe teratozoospermia cases (≤ 1% of sperm with normal morphology). The fertilization rate and ratio of good-quality embryos were significantly higher in the PICSI group than in the ICSI group

Table 4. The relationship between embryo culture results and the sperm DNA fragmentation following PICSI and ICSI groups

Outcome	DFI < 30% (n = 59)			DFI ≥ 30% (n = 17)		
	PICSI (450 MII oocytes)	ICSI (463 MII oocytes)	*p*-value[a]	PICSI (142 MII oocytes)	ICSI (141 MII oocytes)	*p*-value[a]
Fertilization rate	320/450 (71.11)	346/463 (74.73)	0.22	111/142 (78.17)	109/141 (77.30)	0.86
Cleavage rate	305/320 (95.31)	331/346 (95.66)	0.83	105/111 (94.59)	106/109 (97.25)	0.32
Blastulation rate	154/305 (50.49)	118/331 (35.65)	0.00	62/105 (59.05)	(65/106) (61.32)	0.74
Good-quality blastocyst rate	72/305 (23.61)	94/331 (28.40)	0.17	35/105 (33.33)	44/106 (41.51)	0.22

Values are presented as number (%).
PICSI, physiological intracytoplasmic sperm injection; ICSI, intracytoplasmic sperm injection; DFI, DNA fragmentation index; MII, metaphase II.
[a]Statistical test using chi-square test.

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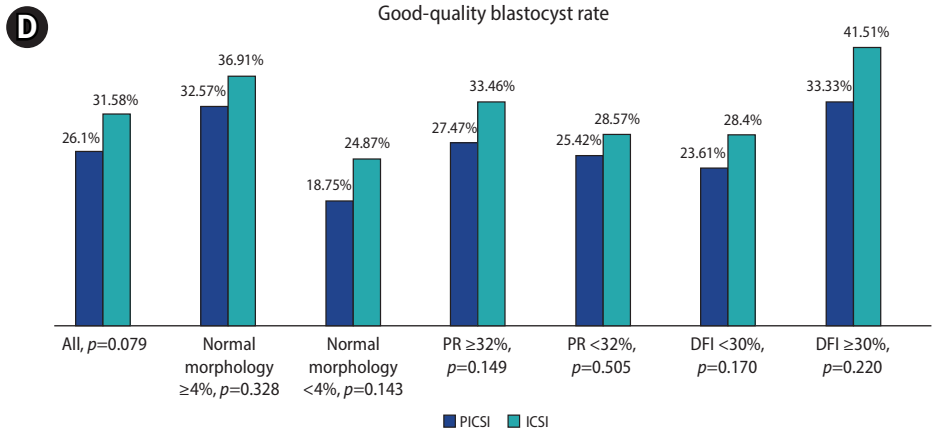

Figure 1. Embryo outcomes of physiological intracytoplasmic sperm injection (PICSI) versus intracytoplasmic sperm injection (ICSI) in all cases and in each subgroup in terms of sperm morphology, motility, and DNA fragmentation. (A) Fertilization rate, (B) cleavage rate, (C) blastulation rate, and (D) good-quality blastocyst rate. PR, progressive; DFI, DNA fragmentation index. Statistical significance is defined at $p<0.05$.

Table 5. Comparison of transfer embryo results following PICSI and ICSI groups

Outcome	PICSI	ICSI	*p*-value
No. of transfer embryos cycles	31	63	
No. of embryos transferred per cycle	1.97±0.31	1.90±0.49	0.52[a]
Endometrium thickness (mm)	9.78±1.76	9.56±1.47	0.83[a]
β-hCG positive	19 (61.19)	36 (57.14)	0.53[b]
Clinical pregnancy	16 (51.61)	34 (53.97)	0.42[b]
Miscarriage	4 (12.90)	11 (17.46)	0.91[b]

Values are presented as mean±standard deviation or number (%).
PICSI, physiological intracytoplasmic sperm injection; ICSI, intracytoplasmic sperm injection; β-hCG, β subunit of human chorionic gonadotropin.
[a]Statistical test using independent-samples *t*-test; [b]Statistical test using chi-square test.

(82.7% vs. 71.7%, $p<0.001$; and 52.8% vs. 34.0%, $p<0.001$, respectively). Furthermore, the PICSI group had a lower ratio of poor-quality embryos on day 3. In cases with severe teratozoospermia, PICSI appears to be superior to ICSI in terms of fertilization rate and embryo quality [24]. We also distinguished between normal and abnormal spermatozoa morphology groups, but found no benefit of PICSI in the subgroups with teratozoospermia (normal morphology of sperm <4%) or normal morphology (normal morphology of sperm ≥4%), and motility. This finding may be explained by the embryologist's ability to select high-quality sperm during traditional ICSI by observing and picking them before insemination.

HA has been shown to be capable of selecting spermatozoa with greater DNA integrity and normal morphology [8,9,11,26]. Sperm DNA damage has been linked to the inability to conceive, spontaneous abortion, and assisted reproductive failure [27,28]. Although SDF did not have a negative effect on fertilization in ICSI cycles, it was associated with the formation of cleavage-stage embryos and blastocysts [29-31]. DNA fragmentation was substantially lower in spermatozoa bound to HA than in spermatozoa after being washed and collected in PVP or spermatozoa from a fresh semen sample [8]. Kirkman-Brown et al. [21] found a correlation between the hyaluronan-based (HAB) score and sperm motility, concentration, fertilization rate, and DNA fragmentation. Although sperm DNA compaction is a weak predictor of clinical pregnancy rates, neither the HAB score nor SDF was indicative of any clinical outcomes [21]. We discovered that when using the PICSI procedure on sperm samples with low DNA fragmentation (DFI <30%), a higher percentage of blastocysts developed. Therefore, PICSI should be considered in cases of low-level SDF to maximize the number of blastocysts. However, the good-quality blastocyst rates were not significantly different between the two procedures. Furthermore, no statistically significant difference was found between sperm samples with a high degree of DNA fragmentation (DFI ≥30%) according to whether PICSI or ICSI was used.

The use of sibling oocytes to evaluate embryos in PICSI and ICSI promotes stability in oocyte quality throughout each cycle. However, because the number of embryo transfer cycles was not large, we assessed the outcomes following embryo transfer using the total number of embryo transfer cycles for each procedure. There was no discernible difference in the hCG-positivity rate, clinical pregnancy rate, or miscarriage rate across the groups. Although PICSI has superiority in fertilization and high-quality embryo rates, a recent study with a small sample size analyzed sibling oocyte cycles and showed no improvement in embryo transfer cycles [32]. A study on male factor infertility demonstrated no difference in biochemical or clinical pregnancy between PICSI and ICSI, although the sample size was also

fairly small [25]. The rates of clinical pregnancy, live birth, and preterm birth did not differ significantly between the PICSI and ICSI groups in a major blinded randomized controlled trial study by Kirkman-Brown et al. [21], with the exception of the stillbirth rate, which was higher in the PICSI group. When Scaruffi et al. [33] evaluated the efficiency of PICSI in cases that had failed in the prior ICSI cycle, they found that HA-ICSI had considerably higher pregnancy and implantation rates than ICSI cycles ($p = 0.001$ and $p = 0.0001$, respectively). In comparison to ICSI, PICSI recorded statistically significant lower rates of miscarriage (4.3% for PICSI vs. 7.0% for ICSI; odds ratio, 0.61; 95% confidence interval, 0.43 to 0.84; $p = 0.003$) [21]. Thus, the effectiveness of PICSI in improving clinical outcomes remains unclear. To evaluate the role of PICSI, more research with larger sample sizes on other types of patients is needed.

In conclusion, a group of sibling oocytes was randomly divided and subjected to both PICSI and ICSI procedures to ensure consistent quality during each cycle. Our findings indicate that although PICSI does not demonstrate any advantages in terms of overall grading and evaluation of specific sperm characteristics during fertilization and embryo development, it appears to have a greater ability to generate blastocysts with minimal SDF. Further studies are required to assess the effectiveness of PICSI by analyzing clinical pregnancy rates, miscarriage rates, and live birth rates in comparison to conventional ICSI.

Conflict of interest

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

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References

1. Dandekar P, Aggeler J, Talbot P. Structure, distribution and composition of the extracellular matrix of human oocytes and cumulus masses. Hum Reprod 1992;7:391–8.
2. Myles DG, Primakoff P. Why did the sperm cross the cumulus?: to get to the oocyte: functions of the sperm surface proteins PH-20 and fertilin in arriving at, and fusing with, the egg. Biol Reprod 1997;56:320–7.
3. Huszar G, Ozkavukcu S, Jakab A, Celik-Ozenci C, Sati GL, Cayli S. Hyaluronic acid binding ability of human sperm reflects cellular maturity and fertilizing potential: selection of sperm for intracytoplasmic sperm injection. Curr Opin Obstet Gynecol 2006;18:260–7.
4. Barak Y, Menezo Y, Veiga A, Elder K. A physiological replacement for polyvinylpyrrolidone (PVP) in assisted reproductive technology. Hum Fertil (Camb) 2001;4:99–103.
5. Prinosilova P, Kruger T, Sati L, Ozkavukcu S, Vigue L, Kovanci E, et al. Selectivity of hyaluronic acid binding for spermatozoa with normal Tygerberg strict morphology. Reprod Biomed Online 2009;18:177–83.
6. Huszar G, Ozenci CC, Cayli S, Zavaczki Z, Hansch E, Vigue L. Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status. Fertil Steril 2003;79 Suppl 3:1616–24.
7. Huszar G, Jakab A, Sakkas D, Ozenci CC, Cayli S, Delpiano E, et al. Fertility testing and ICSI sperm selection by hyaluronic acid binding: clinical and genetic aspects. Reprod Biomed Online 2007;14:650–63.
8. Parmegiani L, Cognigni GE, Bernardi S, Troilo E, Ciampaglia W, Filicori M. "Physiologic ICSI": hyaluronic acid (HA) favors selection of spermatozoa without DNA fragmentation and with normal nucleus, resulting in improvement of embryo quality. Fertil Steril 2010;93:598–604.
9. Yagci A, Murk W, Stronk J, Huszar G. Spermatozoa bound to solid state hyaluronic acid show chromatin structure with high DNA chain integrity: an acridine orange fluorescence study. J Androl 2010;31:566–72.
10. Mongkolchaipak S, Vutyavanich T. No difference in high-magnification morphology and hyaluronic acid binding in the selection of euploid spermatozoa with intact DNA. Asian J Androl 2013;15:421–4.
11. Nasr-Esfahani MH, Razavi S, Vahdati AA, Fathi F, Tavalaee M. Evaluation of sperm selection procedure based on hyaluronic acid binding ability on ICSI outcome. J Assist Reprod Genet 2008;25:197–203.
12. Huang MT, Kuo-Kuang Lee R, Lu CH, Chen YJ, Li SH, Hwu YM. The

https://doi.org/10.5653/cerm.2023.05918

efficiency of conventional microscopic selection is comparable to the hyaluronic acid binding method in selecting spermatozoa for male infertility patients. Taiwan J Obstet Gynecol 2015;54:48–53.

13. Razavi SH, Nasr-Esfahani MH, Deemeh MR, Shayesteh M, Tavalaee M. Evaluation of zeta and HA-binding methods for selection of spermatozoa with normal morphology, protamine content and DNA integrity. Andrologia 2010;42:13–9.
14. Celik-Ozenci C, Jakab A, Kovacs T, Catalanotti J, Demir R, Bray-Ward P, et al. Sperm selection for ICSI: shape properties do not predict the absence or presence of numerical chromosomal aberrations. Hum Reprod 2004;19:2052–9.
15. Esbert M, Pacheco A, Soares SR, Amoros D, Florensa M, Ballesteros A, et al. High sperm DNA fragmentation delays human embryo kinetics when oocytes from young and healthy donors are microinjected. Andrology 2018;6:697–706.
16. Jerre E, Bungum M, Evenson D, Giwercman A. Sperm chromatin structure assay high DNA stainability sperm as a marker of early miscarriage after intracytoplasmic sperm injection. Fertil Steril 2019;112:46–53.
17. Hasanen E, Elqusi K, ElTanbouly S, Hussin AE, AlKhadr H, Zaki H, et al. PICSI vs. MACS for abnormal sperm DNA fragmentation ICSI cases: a prospective randomized trial. J Assist Reprod Genet 2020;37:2605–13.
18. Mokanszki A, Tothne EV, Bodnar B, Tandor Z, Molnar Z, Jakab A, et al. Is sperm hyaluronic acid binding ability predictive for clinical success of intracytoplasmic sperm injection: PICSI vs. ICSI? Syst Biol Reprod Med 2014;60:348–54.
19. Choe SA, Tae JC, Shin MY, Kim HJ, Kim CH, Lee JY, et al. Application of sperm selection using hyaluronic acid binding in intracytoplasmic sperm injection cycles: a sibling oocyte study. J Korean Med Sci 2012;27:1569–73.
20. Liu Y, Feenan K, Chapple V, Roberts P, Matson P. Intracytoplasmic sperm injection using hyaluronic acid or polyvinylpyrrolidone: a time-lapse sibling oocyte study. Hum Fertil (Camb) 2019;22:39–45.
21. Kirkman-Brown J, Pavitt S, Khalaf Y, Lewis S, Hooper R, Bhattacharya S, et al. Efficacy mechanism evaluation 61: sperm selection for assisted reproduction by prior hyaluronan binding: the HABSelect RCT. NIHR Journals Library; 2019.
22. Majumdar G, Majumdar A. A prospective randomized study to evaluate the effect of hyaluronic acid sperm selection on the intracytoplasmic sperm injection outcome of patients with unexplained infertility having normal semen parameters. J Assist Reprod Genet 2013;30:1471–5.
23. ALPHA Scientists In Reproductive Medicine; ESHRE Special Interest Group Embryology. Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. Reprod Biomed Online 2011;22:632–46.
24. Kim SJ, Kim H, Kim TH, Jeong J, Lee WS, Lyu SW. Effect of sperm selection using hyaluronan on fertilization and quality of cleavage-stage embryos in intracytoplasmic sperm injection (ICSI) cycles of couples with severe teratozoospermia. Gynecol Endocrinol 2020;36:456–9.
25. Erberelli RF, Salgado RM, Pereira DH, Wolff P. Hyaluronan-binding system for sperm selection enhances pregnancy rates in ICSI cycles associated with male factor infertility. JBRA Assist Reprod 2017;21:2–6.
26. Tarozzi N, Nadalini M, Bizzaro D, Serrao L, Fava L, Scaravelli G, et al. Sperm-hyaluronan-binding assay: clinical value in conventional IVF under Italian law. Reprod Biomed Online 2009;19 Suppl 3:35–43.
27. Zini A, Boman JM, Belzile E, Ciampi A. Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: systematic review and meta-analysis. Hum Reprod 2008;23:2663–8.
28. Zhao J, Zhang Q, Wang Y, Li Y. Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: a systematic review and meta-analysis. Fertil Steril 2014;102:998–1005.
29. Sivanarayana T, Ravi Krishna C, Jaya Prakash G, Krishna KM, Madan K, Sudhakar G, et al. Sperm DNA fragmentation assay by sperm chromatin dispersion (SCD): correlation between DNA fragmentation and outcome of intracytoplasmic sperm injection. Reprod Med Biol 2013;13:87–94.
30. Kim SM, Kim SK, Jee BC, Kim SH. Effect of sperm DNA fragmentation on embryo quality in normal responder women in in vitro fertilization and intracytoplasmic sperm injection. Yonsei Med J 2019;60:461–6.
31. Borges E Jr, Zanetti BF, Setti AS, Braga DP, Provenza RR, Iaconelli A Jr. Sperm DNA fragmentation is correlated with poor embryo development, lower implantation rate, and higher miscarriage rate in reproductive cycles of non-male factor infertility. Fertil Steril 2019;112:483–90.
32. Novoselsky Persky M, Hershko-Klement A, Solnica A, Bdolah Y, Hurwitz A, Ketzin El Gilad M, et al. Conventional ICSI vs. physiological selection of spermatozoa for ICSI (picsi) in sibling oocytes. Andrology 2021;9:873–7.
33. Scaruffi P, Bovis F, Casciano I, Maccarini E, De Leo C, Gazzo I, et al. Hyaluronic acid-sperm selection significantly improves the clinical outcome of couples with previous ICSI cycles failure. Andrology 2022;10:677–85.

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CERM

Effects of controlled ovarian stimulation regimens on top-quality blastocyst development and perinatal outcomes with the freeze-all strategy: A retrospective comparative study

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Objective: This study aimed to determine the effect of ovarian stimulation regimens on the top-quality blastocyst development rate and perinatal outcomes with the freeze-all strategy.
Methods: A retrospective comparative cohort analysis of 149 *in vitro* fertilization (IVF) cycles using the freeze-all strategy was conducted. The IVF cycles were stimulated with either a gonadotropin-releasing hormone antagonist or clomiphene citrate along with gonadotropin based on the patient's serum anti-Müllerian hormone level. Oocyte retrieval, fertilization, and embryo culture were performed following standard procedures. All good-quality blastocysts were cryopreserved and used for frozen-thawed embryo transfer (FET) in subsequent cycles. The fertilization, blastulation, and top-quality blastocyst development rates were calculated. The perinatal outcomes of FET cycles, gestational period, and birth weight were assessed.
Results: The main outcome of this study was the top-quality blastocyst development rate, and the secondary outcomes were perinatal parameters (e.g., gestational period and birth weight) between the stimulation regimens. Despite the higher number of usable-quality embryos in the antagonist group, the blastocyst development rate remained comparable (p=0.105). Similarly, perinatal outcomes were comparable in subsequent FET cycles (p=0.538).
Conclusion: These findings suggest that the choice between antagonist and clomiphene citrate with gonadotropin as stimulation in controlled ovarian stimulation regimens may not affect the top-quality blastocyst development rate. The IVF outcomes (e.g., clinical pregnancy, miscarriage, and live birth rates) remained unaffected in subsequent FET cycles. Unlike fresh embryo transfer, the birth weight and gestational length were not associated with prior controlled ovarian stimulation regimens when the freeze-all strategy was used.

Keywords: Abortion, spontaneous; Antagonists; Birth weight; Blastocyst; Clomiphene; Embryo transfer; Fertilization in vitro; Live birth; Ovulation induction; Pregnancy outcome

Introduction

Assisted reproductive technologies are widely used for the treatment of infertility/subfertility. The first live birth using *in vitro* fertilization (IVF) was achieved through natural-cycle IVF. Later, to increase the number of recruited follicles per cycle and to prevent the spontaneous surge of luteinizing hormone (LH), gonadotropin-releasing hormone (GnRH) analogs were introduced into controlled ovarian stimulation (COS). The use of these agents in COS led to increases in the number of oocytes retrieved and fertilized embryos per cycle. The availability of multiple embryos provides the ability to select the best-quality embryo for transfer, leading to an increased success rate. Moreover, surplus good-quality embryos can be cryopreserved and used for future transfer [1]. COS is aimed at the recruitment of multiple follicles and the inhibition of spontaneous ovulation simultane-

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ously. To increase the oocyte yield per cycle, gonadotropins have been used [2], and GnRH analogs (agonists and antagonists) are used to prevent the LH surge [3]. The estimated pregnancy rate per started cycle with COS is approximately 20% to 30% [4]; however, when using minimal ovarian stimulation, only about a 10% pregnancy rate per stimulated cycle is observed [5]. This difference in pregnancy rates highlights the importance of COS for the success of assisted reproductive technologies. To retrieve multiple mature oocytes in a single cycle, high doses of exogenous gonadotropins are administered that stimulate the development of multiple oocytes, leading to their maturity. Ovarian stimulation is a major component of the IVF procedure; however, aggressive ovarian stimulation using higher doses has negative effects on oogenesis, embryo quality, endometrium receptivity, and possibly the perinatal outcomes of IVF [6]. Therefore, it is necessary to develop an ideal IVF protocol that aims at providing good-quality multiple embryos, a high chance of good-quality embryo transfer with a low cycle cancellation rate, a high pregnancy success rate, fewer side effects, lower costs, and fewer required hospital visits.

Clomiphene citrate (CC) is an ovarian stimulation agent approved by the U.S. Food and Drug Administration in 1961 that is commonly used in minimal stimulation in patients with a poor ovarian response along with gonadotropins and GnRH antagonists (GnRHa) [7]. This drug exerts an antiestrogen effect on the pituitary gland, primarily by binding to the estrogen receptors in the hypothalamus, thereby releasing follicle-stimulating hormone (FSH) to produce more follicles and complementing the activity of externally administered gonadotropins. Simultaneously, it inhibits the release of LH, thereby preventing a premature LH surge, which is responsible for premature ovulation [8]. CC is orally administered, inexpensive, and easily available. Co-administration of CC and human menopausal gonadotropin (hMG) (CC+hMG) reduces the gonadotropin dose requirement, particularly for patients who prefer fewer injections. The extended use of CC until the day of ovulation triggering has been advised recently, as CC exerts antiestrogenic activity to prevent a spontaneous LH surge, enabling it to replace GnRHa or GnRH agonists at a lower cost [8,9]. However, due to its antiestrogen effects on reproductive organs, its prolonged use poses serious concerns, since it may affect endometrial receptivity in fresh embryo transfer cycles. Some additional disadvantages are associated with this stimulation protocol, such as a lower number of oocytes retrieved and, sometimes, cycle cancellation.

GnRHa has recently been applied in clinical practice for COS in IVF. GnRHa-based stimulation protocols offer several advantages over GnRH agonist use. These advantages include a shorter duration of treatment, a shorter duration of FSH administration, and a lower risk of ovarian hypersensitivity syndrome (OHSS) [10]. In addition, the GnRHa protocol overcomes some of the disadvantages associated with the GnRH agonist protocol, such as lower oocyte yield and serum estradiol (E_2) levels on the ovulation trigger day [11]. However, the results of the previous study have highlighted the effectiveness of the GnRHa protocol in COS in terms of higher fertilization rates, mean numbers of transferrable-quality embryos, and successful pregnancy rates, as well as a lower incidence of OHSS [11].

Previous studies have suggested that ovarian stimulation protocols are associated with top-quality blastocyst development and an increased risk of adverse perinatal outcomes, such as lower birth weight and preterm delivery, in fresh embryo transfer cycles [12]. However, it remains unknown whether the top-quality blastocyst development after COS differs between the distinct stimulation regimens. Whether an increased risk of adverse perinatal outcomes still exists even after using frozen-thawed embryo transfer (FET) in subsequent cycles is an intriguing question. The present study was undertaken to evaluate the effects of COS with CC+hMG and GnRHa regimens on oocyte and embryo quality and subsequent FET outcomes. The main outcome of this study was the top-quality blastocyst development rate between the COS regimens. The secondary outcomes were fertilization rate, clinical pregnancy, miscarriage, and live birth rates between these two groups.

Methods

1. Patient selection

This is a retrospective cohort analysis of a total of 118 patients (149 IVF cycles) 30 to 39 years of age with a diagnosis of infertility treated at Kinoshita Ladies Clinic, between June 2017 and December 2018. The inclusion criteria were an indication of IVF or intracytoplasmic sperm injection (ICSI) and COS with a GnRHa or CC+hMG protocol (Figure 1). All the patients provided written informed consent for their' anonymized medical records to be used for clinical research purposes, and the study was approved by the Institutional Ethics Committee of Kinoshita Ladies Clinic (approval number: 006).

2. COS protocols

At the start of the cycle, serum anti-Müllerian hormone (AMH) levels were measured. Patients with serum AMH levels >1.0 ng/mL were stimulated with GnRHa, whereas patients with serum AMH levels ≤ 1.0 ng/mL were stimulated with a mild stimulation protocol using CC+hMG.

1) GnRHa protocol

Dose adjustments during treatments were chosen on a case-by-case basis according to patients' characteristics. A dose of 0.25 mg of GnRHa (Cetrotide, Merck BioPharma Co. Ltd.) every other day and

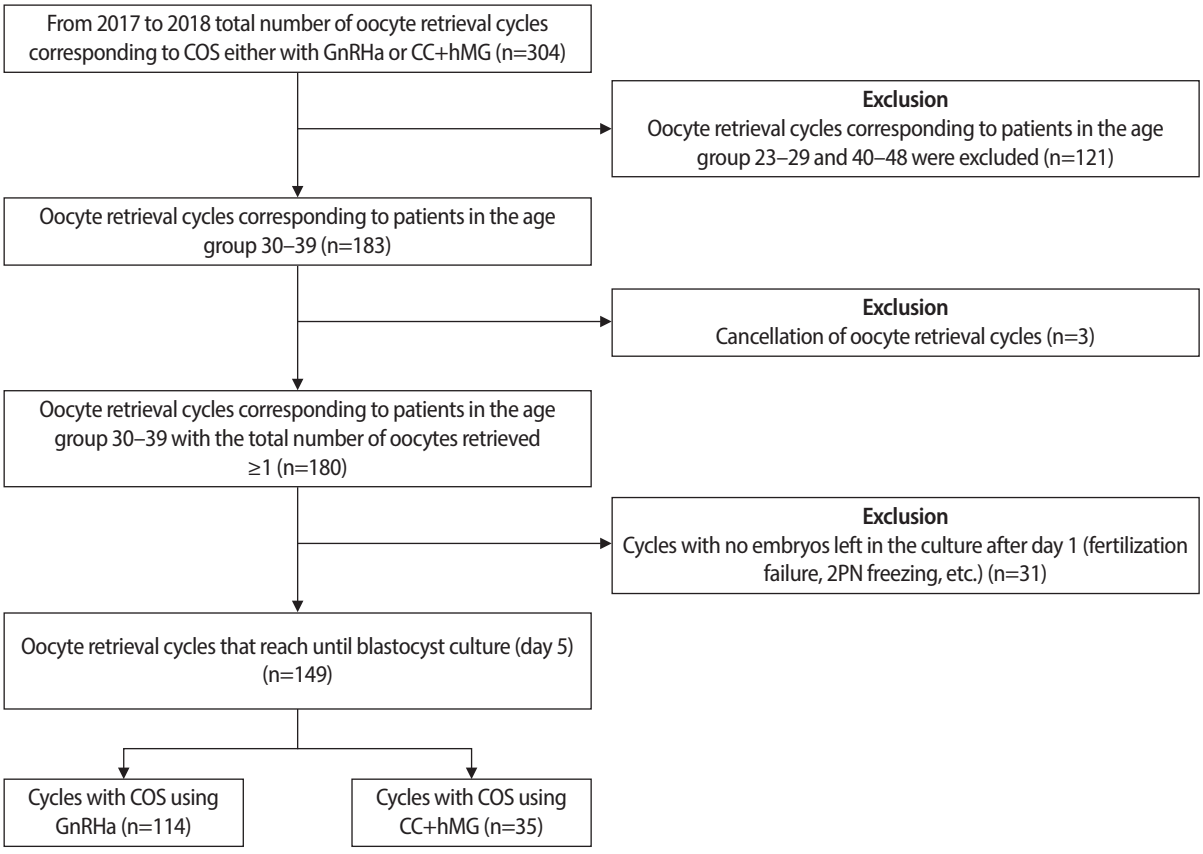

Figure 1. A flow chart of the included and excluded cycles in the present study. COS, controlled ovarian stimulation; GnRHa, gonadotropin-releasing hormone antagonist; CC, clomiphene citrate; hMG, human menopausal gonadotropin; 2PN, two pronuclei.

150 to 450 IU/day of hMG (HMG Ferring, Ferring Pharmaceuticals Co. Ltd.) was started on day 3 to 5 of the menstrual cycle. When a leading follicle reached a diameter of ≥ 18 mm, ovulation was triggered with a GnRH agonist nasal spray (Buserecur 1.25 mg, Fuji Pharma Co. Ltd.). In patients at high risk for OHSS, ovulation was triggered with 1.25 mg GnRH agonist and 5,000 IU (intramuscular) of human chorionic gonadotropin (hCG) (Ovidrel, Merck Biopharma Co. Ltd.). Transvaginal ultrasound (TVS)-guided oocyte retrieval was performed 34 to 38 hours after the trigger, following the standard procedure.

2) CC+hMG protocol

For COS with the CC+hMG protocol, on day 3 of the menstrual cycle, serum basal FSH, LH, E_2, and progesterone (P_4) levels were assayed. From menstrual cycle days 3 to 5 onwards, patients in this group were given 50 to 100 mg/day of CC (Clomid, Fuji Pharma Co. Ltd.) and 150 IU/day of hMG for about 10 days. Cycle monitoring was started on days 7 to 8 of the menstrual cycle, and then TVS was performed every 2 to 4 days to adjust the hMG dose according to follicle development. During each monitoring, the number(s) and sizes (mm) of follicles were recorded by TVS, along with measurements of serum FSH, LH, E_2, and P_4 levels on the same days. The final stage of oocyte maturation was induced by an intramuscular injection of a 10,000-IU hCG trigger (HCG Mochida, Mochida Pharmaceutical Co. Ltd.), once at least one follicle reached 18 mm or greater in diameter. TVS-guided oocyte retrieval was performed 34 to 38 hours after the trigger. Attempts were made to retrieve all follicles more than 10 mm in diameter.

3. Oocyte fertilization, embryo culture, and transfer

Based on semen parameters, the previous history of failed IVF, the total number of oocytes retrieved, and other relevant factors, either ICSI or a split protocol (conventional-IVF+ICSI) was used for fertilization. For ICSI, oocytes were denuded using hyaluronidase (80 IU/mL) (Fujifilm, Irvine Scientific Inc.) to inspect the extrusion of the first polar body, and metaphase II (MII) oocytes were subjected to sperm injection, as described elsewhere [13]. The fertilization of oocytes was determined by the presence of two pronuclei and polar bodies 18 to 20 hours after insemination. After fertilization, zygotes were cultured in 25 μL of one-step medium (Naka Medical Corp.) under mineral oil (Fuso Pharmaceutical Industries Ltd.) at 37 ºC and 6% CO_2, 5% O_2,

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and 89% N_2. Additionally, an examination for the number and regularity of blastomere and embryonic fragmentation was performed on day 3 embryos, and scoring of cleavage stage embryos was performed according to Veeck's classification [14]. On the morning of days 5 and 6, the development of blastocysts was reviewed and recorded using the Gardner criteria by trained embryologists at the Kinoshita Ladies Clinic [15]. Day 5 and 6 blastocysts were rated based on: (1) the degree of expansion and hatching status (1 = early blastocyst, less than half volume of the embryo is occupied by the blastocoel; 2 = blastocyst, more than half of the volume is occupied by the blastocoel; 3 = full blastocyst, the entire volume of the embryo is occupied by the blastocoel, 4 = fully expanded blastocyst, the blastocoel volume is larger than the previous stage embryo and thinning of the zona pellucida has started; 5 = hatching blastocyst, herniation of the trophectoderm [TE] has started through the zona layer; 6 = hatched blastocyst, the blastocyst has completely escaped from the zona). For blastocysts graded as 3 to 6 (that is, from full blastocyst onwards), the further assessment was based on (2) the inner cell mass (ICM) score or quality, defined in a range from A to C (A: good—prominent, easily noticeable, composed of many cells that form compact and tightly bound structure; B: fair—easily noticeable, many numbers of the cell but are loosely held together; C: poor—difficult to notice, comprising few cells), and (3) the TE score or quality, ranging from A to C (A: good—many cells collectively forming a cohesive, tightly knit epithelium layer; B: fair—few cells, therefore, forming a loose epithelium layer, and C: poor—very few numbers of cells). Based on these three parameters, a standard alphanumeric rating was assigned. Accordingly, a "top-quality" blastocyst was defined as an expanded or hatched blastocyst (score 3 or 4), with both the ICM and TE having at least a fair score (BB). Therefore, all blastocysts of grade ≥ 3BB were considered top-quality. The blastocysts were cryopreserved using a vitrification protocol (Cryotech, Repro Life Co. Ltd.). The top-quality blastocysts were subjected to FET using a Warming Kit 102 as per the manufacturer's instructions (Cryotech, Repro Life Co. Ltd.). Embryo quality was scored after thawing and before embryo transfer.

4. Outcomes

The main outcome of this study was a comparison of the usable blastocyst development rate between the COS regimens with GnRHa and CC+hMG. Only usable blastocysts, defined as 3BB or better on day 5 or 6 of culture, were included in the calculation and selected for cryopreservation. Other parameters, such as the duration of stimulation, the total dose of gonadotropins, and the number of oocytes retrieved, were also compared. The secondary outcomes of oocyte and embryo quality between the two groups were the fertilization, clinical pregnancy, miscarriage, and live birth rates. A clinical pregnancy was defined as the detection of a gestational sac on ultrasound. A clinical miscarriage was a case with a documented loss of fetal cardiac activity in an intrauterine pregnancy, loss of a gestational sac, or lack of development of an embryo after at least 7 days. A live birth was defined as a viable infant born after 24 weeks of gestation.

5. Statistical analysis

Outcome measures between the groups were compared using Mann-Whitney *U* test, Kruskal-Wallis test, and Fisher exact test as appropriate, using the Easy R (EZR) statistical analysis software [16].

Results

1. Study population

AMH is a useful endocrine marker for assessing ovarian reserve. Therefore, based on serum AMH levels all the patients enrolled in this study were grouped into two COS regimens. In total, 95 patients (80.5%; 118 cycles) with serum AMH levels > 1.0 ng/mL underwent COS with GnRHa, whereas 23 patients (19.5%; 35 cycles) with serum AMH levels ≤ 1.0 ng/mL were stimulated with the CC+hMG protocol. There was a 4.1-fold higher number of patients recruited for COS with GnRHa than for COS with CC+hMG.

2. Baseline characteristics

The baseline characteristics of the sample population are depicted in Table 1. A significant difference in the serum AMH levels between the two groups (3.58 ± 3.05 ng/mL vs. 1.29 ± 1.73 ng/mL, $p < 0.001$) was noted (Table 1). The indications for IVF/ ICSI–embryo transfer were a tubal factor (5.1%), male factor (8.5%), polycystic ovary syndrome (6.8%), and antisperm antibody (0.8%). The majority of the women undergoing infertility treatment (n = 76) were diagnosed with unexplained infertility. Out of the 149 IVF cycles, 87 (58.4%) were performed in patients diagnosed with unexplained infertility. Sixteen women (13.5%) diagnosed with endometriosis underwent 11 and 13 IVF cycles of the GnRHa and CC+hMG regimens, respectively. The body mass index of patients in both groups was comparable (21.6 ± 3.2 kg/m^2 vs. 22.2 ± 2.8 kg/m^2), without a statistically significant difference ($p = 0.172$). The mean age of patients in the CC+hMG group (34.6 ± 3.3 years) was comparable to that of patients in the GnRHa group (34.3 ± 2.7 years), and there was no statistically significant difference in the mean maternal age ($p < 0.622$) between the groups (Table 1).

3. COS parameters

Both COS regimens used gonadotropins, and the average gonadotropin dosage utilized by patients in the GnRHa group was

more than 2-fold that of the CC+hMG group (4,664.0±1,377.4 IU vs. 2,159.6±1,182.7 IU, $p<0.001$). Similarly, there was a statistically significant difference in the duration of gonadotropin injections (14.8±2.8 days vs. 10.8±3.8 days) between the groups ($p<0.001$) (Table 2). At the time of the hCG trigger, serum P_4 and E_2 levels were measured. The mean serum P_4 level on the day of the hCG trigger was higher in the GnRHa group (2.54±1.56 ng/mL) than in the CC+hMG group (0.75±0.38 ng/ mL); however, these results did not reach statistical significance ($p=0.041$). Similarly, an approximately four-fold higher serum E_2 level on the hCG trigger day was noted in the GnRHa group patients than in the CC+hMG group (12,114.56±6,700.17 pg/mL vs. 2,756.98±2,897.21 pg/mL); this difference was statistically significant ($p<0.001$). A significant difference was also found in endometrial thickness on the hCG triggering day between the groups (11.1±2.7 mm vs. 8.6±2.7 mm, $p<0.001$) (Table 2).

Table 1. Baseline characters of the sample population[a)]

Characteristic	GnRHa	CC+hMG	*p*-value
No. of patients	95	23	
No. of cycles	114	35	0.744
Age (yr)	34.3±2.7	34.6±3.3	0.622
AMH (ng/mL)	3.58±3.05	1.29±1.73	<0.001
Basal FSH (pg/mL)	7.60±2.26	6.24±0.88	0.268
Basal LH (pg/mL)	6.25±2.69	3.58±3.14	<0.001
BMI (kg/m^2)	21.59±3.24	22.18±2.78	0.172
Infertility diagnosis			
Endometriosis	11 (9.6)	13 (37.1)	<0.001
PCOS	8 (7.0)	0	-
Antisperm antibody	2 (1.4)	0	-
Male factor	9 (7.9)	6 (17.1)	<0.001
Tubal factor	6 (5.3)	2 (5.7)	0.015
Unexplained	74 (64.9)	13 (37.1)	<0.001
No data	4 (3.5)	1 (2.8)	0.134

Values are presented as mean±standard deviation or number (%). The *p*-values were assessed using the Mann-Whitney *U* test, Kruskal-Wallis test (chi-square test), or Fisher exact test, as appropriate.
GnRHa, gonadotropin-releasing hormone antagonist; CC, clomiphene citrate; hMG, human menopausal gonadotropin; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; BMI, body mass index; PCOS, polycystic ovary syndrome.
[a)]Baseline characteristics of the sample population stratified by ovarian stimulation protocols (n=149).

4. Laboratory outcomes

As the GnRHa protocol is beneficial for obtaining more oocytes in a single IVF cycle, in our study the GnRHa group showed a statistically significant difference in the total number of oocytes retrieved per cycle as compared to the CC+hMG group (18.9±8.0 vs. 6.8±4.8, $p<0.001$). Similarly, a statistically significant difference was observed in the number of mature oocytes (MII) between the groups (15.6±7.7 vs. 5.3±4.4, $p<0.001$). However, the oocyte maturation rate among the groups (82.4%±12.6% vs. 83.8%±18.9%) was comparable and these results did not reach statistical significance ($p=0.131$). The GnRHa group exhibited more fertilized embryos (12.7±7.2) 18 hours post-insemination than the CC+hMG group (4.1±4.2). A significant difference was also found between the two

Table 2. Stimulation parameters and the effect of COS protocols on different laboratory outcomes[a)]

Characteristic	GnRHa (114 cycles)	CC+hMG (35 cycles)	*p*-value
Total gonadotropins dosage (IU)	4,664.0±1,377.4	2,159.6±1,182.7	<0.001
Duration of gonadotropin injections (day)	14.8±2.8	10.8±3.8	<0.001
Serum P_4 level on the day of trigger (ng/mL)	2.54±1.56	0.75±0.38	0.041
Serum E_2 level at the trigger (pg/mL)	12,114.56±6,700.17	2,756.98±2,897.21	<0.001
Serum LH level at the trigger (pg/mL)	1.45±1.59	11.05±9.26	<0.001
Endometrium thickness on hCG triggering day (mm)	11.1±2.7	8.6±2.7	<0.001
No. of oocytes retrieved	18.9±8.3	6.4±4.8	<0.001
No. of mature oocytes (MII)	15.6±7.7	5.3±4.4	<0.001
Oocyte maturation rate (%)	82.4±12.6	83.8±18.9	0.131
No. of fertilized embryos (2PN)	12.7±7.2	4.1±4.2	<0.001
Fertilization rate (%)	81.2±14.7	62.7±37.3	0.103
No. of total blastocysts	6.4±5.0	2.8±3.5	<0.001
Blastocyst development rate (%)	60.3±25.9	50.4±45.0	0.334
No. of top-quality blastocysts	4.4±4.1	2.0±2.7	<0.001
Top-quality blastocyst development rate (%)	42.7±24.5	36.4±39.1	0.105

Values are presented as mean±standard deviation. The *p*-values were assessed using the Mann-Whitney *U* test, Kruskal-Wallis test (χ^2 test), or Fisher exact test, as appropriate.
COS, controlled ovarian stimulation; GnRHa, gonadotropin-releasing hormone antagonist; CC, clomiphene citrate; hMG, human menopausal gonadotropin; P_4, progesterone; E_2, estradiol; LH, luteinizing hormone; hCG, human chorionic gonadotropin; MII, metaphase II; 2PN, two pronuclei.
[a)]Stimulation parameters and laboratory outcomes of the sample population stratified by ovarian stimulation protocols (n=149).

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groups in the total number of fertilized embryos ($p < 0.001$). Although the fertilization rate in the GnRHa group was seemingly higher (81.2% ± 14.7%) than that in the CC+hMG group (62.7% ± 37.3%), this difference did not reach statistical significance ($p < 0.103$) (Table 2). The total number of blastocysts that developed until day 6 of culture in the GnRHa group was significantly higher than in the CC+hMG group (6.4 ± 5.0 vs. 2.8 ± 3.5, $p < 0.001$). However, the blastocyst between the COS regimens (60.3% ± 25.9% vs. 50.4% ± 45.0%), and the difference did not reach statistical significance ($p = 0.334$). Similarly, we calculated the number of top-quality blastocysts (days 5 and 6) in both groups. The average number of top-quality blastocysts was significantly higher in the GnRHa group than in the CC+hMG group (4.4 ± 4.1 vs. 2.0 ± 2.7, $p < 0.001$). However, the main outcome of this study, the top-quality blastocyst development rate (42.7% ± 24.5% vs. 36.4% ± 39.1%, $p = 0.105$) remained comparable (Table 2).

5. Clinical and neonatal outcomes

In total, 105 patients (154 cycles) underwent FET. Out of the 154 FET cycles, 126 (81.8 %) used embryos derived from previous GnRHa-based COS cycles, whereas 28 (18.2%) cycles had embryos from previous cycles that used CC+hMG stimulation (Table 3). No significant difference was found between the cleavage and blastocyst stage FET cycles between both groups ($p = 0.131$ and $p = 0.130$, respectively). As secondary outcomes of the study, the patients in the CC+hMG group had lower clinical pregnancy (51.7% vs. 41.7%, $p = 0.402$), miscarriage (21.0% vs. 6.7%, $p = 0.214$), and live birth rates (40.8% vs. 38.9%, $p = 0.494$) than those in the GnRHa group (Table 3). These differences, however, were not statistically significant. In addition to IVF outcomes, in the present study, we assessed the effect of COS on neonatal outcomes, such as gestational period and birth weight. No statistically significant differences were observed between the groups in the gestational period (38.8 ± 3.2 weeks vs. 37.9 ± 2.9 weeks, $p = 0.036$) and birth weight (3,032.3 ± 446.4 g vs. 2,858.9 ± 469.6 g, $p = 0.036$) (Table 3).

Discussion

The results of our study using data from 149 IVF cycles suggest that COS protocols (GnRHa and CC along with hMG) might be associated with blastulation and top-quality blastocyst development rates, although the observed trends did not reach statistical significance. Both COS protocols were comparable in terms of clinical outcomes (clinical pregnancy rate, miscarriage rate, and live birth rate) and neonatal outcomes (gestational period and birth weight) after FET. CC along with gonadotropins has been used in a minimal stimulation protocol, especially in high responders and patients with immature ovarian insufficiency, advanced reproductive age, and low serum AMH levels. However, a previous study reported that the administration of higher doses of gonadotropins for COS in patients with a decreased ovarian reserve and advanced maternal age had no beneficial effect on IVF success, because only a few primordial follicles would be stimulated in each menstrual cycle [17]. Therefore, a lower dosage of gonadotropins was used in the CC+hMG group for COS than in the GnRHa group. One may object that the higher doses of gonadotropins in the GnRHa protocol may negatively affect the implantation potential of the embryo; however, this possibility was ruled out by the use of FET. Similarly, Kol et al. [18] showed that high doses of GnRHa during ovarian stimulation did not influence the implantation potential of embryos in FET cycles.

In each IVF cycle, serum P_4 and E_2 levels were measured on the day of the ovulation trigger. In our study, higher serum P_4 levels were ob-

Table 3. The effect of COS protocols on clinical and neonatal outcomes after FET[a)]

Characteristic	GnRHa	CC+hMG	*p*-value
No. of patients	87	18	
No. of cycles	126	28	0.079
Stage of embryo transfer			
Cleavage stage	26 (17.7)	12 (33.3)	0.131
Blastocyst stage	121 (82.3)	24 (66.6)	0.130
Clinical pregnancy	76/147 (51.7)	15/36 (41.7)	0.402
Miscarriage	16/76 (21.0)	1/15 (6.7)	0.214
Live birth per embryo transferred	60/147 (40.8)	14/36 (38.9)	0.494
Gestational period (wk)	39.1 ± 3.3	38.0 ± 3.4	0.036
Birth weight (g)	3032.3 ± 446.4	2858.9 ± 469.6	0.538

Values are presented as number (%) or mean±standard deviation. The *p*-values were assessed using the Mann-Whitney *U* test, Kruskal-Wallis test (chi-square test), or Fisher exact test, as appropriate.

COS, controlled ovarian stimulation; FET, frozen embryo transfer; GnRHa, gonadotropin-releasing hormone antagonist; CC, clomiphene citrate; hMG, human menopausal gonadotropin.

[a)]The effect of COS protocols on in vitro fertilization and neonatal outcomes of the sample population stratified by COS protocols after FET (n=154).

served on the day of ovulation trigger in the GnRHa group patients than in the CC+hMG group. However, there were no significant differences between the groups. There is some evidence suggesting that elevated P_4 levels have (1) a detrimental effect on endometrial receptivity, which could be typically managed with a freeze-all strategy rather than fresh embryo transfer [19], and (2) reduce top-quality blastocyst development [20,21]. Similarly, in our study, the higher P_4 levels noted in GnRHa group patients on the day of ovulation trigger resulted in lower blastulation and top-quality blastocyst development rates than in the CC+hMG group. However, this result again did not reach statistical significance. Some contradictory findings have reported comparable top-quality blastocyst development rates between cycles with or without premature P_4 elevation [22,23]. In our study, E_2 levels were also significantly higher in the GnRHa group than in the CC+hMG group. Several studies have assessed the effect of higher levels of E_2 on hCG trigger day on blastulation and top-quality blastocyst development rates. The results of these studies are heterogeneous, and most of the studies included cleavage stage (day 3) embryos. A prospective study with data from 207 IVF cycles found that higher E_2 levels (< 2,446 pg/mL) had a beneficial effect on embryo quality. However, extremely high levels (> 2,446 pg/mL) exhibited adverse effects [24].

In the present study, despite the higher number of retrieved and mature oocytes in the GnRHa group, blastocyst development rates were comparable between both COS regimens. These results again highlight the previously established notion that a higher oocyte yield is not associated with an improved blastocyst development rate [25]. Aggressive ovarian stimulation poses a serious risk of developing OHSS. However, previous studies have confirmed that using GnRHa significantly reduces the risk of developing severe OHSS [26,27]. The incidence of OHSS further significantly decreased when using CC-based mild stimulation regimens [28]. Interestingly, we observed no cases of severe OHSS in either group of patients. Hence, the IVF doctors at our clinic have addressed this key issue by creating an OHSS-free IVF program, which is the objective of many IVF units worldwide. CC-based mild stimulation protocols are associated with a higher rate of cycle cancellation. However, in a cohort of 149 IVF cycles, only three cycles were canceled and excluded at the earliest step of cycle selection. It is a well-recognized fact that the abnormal maternal hormonal milieu produced by COS including CC may negatively affect endometrium receptivity and thereby pregnancy outcomes during fresh autologous IVF cycles; therefore, in GnRHa- and CC+hMG-based ovarian stimulation cycles, FET is recommended [29,30]. The pregnancy outcomes (clinical pregnancy, miscarriage, and live birth rates) among the study groups were comparable. Previous studies have suggested that ovarian stimulation protocols are associated with adverse neonatal outcomes such as low birth weight and risk of preterm delivery in fresh embryo transfer cycles [12]. However, in our study using FET, the neonatal outcomes (gestational period and birth weight) were comparable among both COS regimens. Only three pregnancies in the CC+hMG group resulted in preterm (< 37 weeks) deliveries. Interestingly, none of the newborns in either group reported very low birth weight (< 1,500 g).

Comparative analyses of the present study suggest that COS regimens with GnRHa and CC along with gonadotropins may not affect the top-quality blastocyst development rate. Following FET in subsequent cycles, the IVF outcomes, such as the clinical pregnancy, miscarriage, and live birth rates, remained unaffected. Previous studies have suggested that as compared to natural cycles, COS using CC along with hMG in cycles with fresh embryo transfers were associated with the highest proportion of small for gestational age in the entire cohort. That protocol also showed the highest adjusted odds ratio (AOR) for low for birth weight (< 2,500 g) (AOR, 1.67; 95% confidence interval [CI], 1.45 to 1.73), very low for birth weight (< 1,500 g) (AOR, 2.38; 95% CI, 1.52 to 3.72), and small for gestational age (AOR, 1.71; 95% CI, 1.47 to 1.98). Moreover, the protocol was significantly associated with a higher incidence of cesarean section deliveries [12]. In the present study, unlike fresh embryo transfer cycles, perinatal outcomes such as birth weight and gestational length were not associated with the prior COS regimen. Furthermore, large population studies, including randomized controlled trials, are needed to investigate and the effect on perinatal outcomes. However, the interpretation of the findings of this study is limited by the overall sample size, the distribution of sample sizes between the groups, and the possibility of selection and confounding biases. In the present study, we did not attempt to investigate the association of infertility diagnoses with blastocyst development and IVF or neonatal outcomes. The effect of different infertility diagnoses on pre-implantation embryo development, clinical, and perinatal outcomes after IVF is a new area of future research.

Conflict of interest

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

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References

1. Macklon NS, Stouffer RL, Giudice LC, Fauser BC. The science behind 25 years of ovarian stimulation for in vitro fertilization. Endocr Rev 2006;27:170–207.
2. Segawa T, Kato K, Miyauchi O, Kawachiya S, Takehara Y, Kato O. Evaluation of minimal stimulation IVF with clomiphene citrate and hMG. Fertil Steril 2007;88(Suppl 1):S286.
3. Bhandari S, Ganguly I, Chittawar PB, Agarwal P, Singh A, Gupta N. Use of clomiphene to prevent premature luteinizing hormone surge during controlled ovarian hyper stimulation. Int J Reprod Contracept Obstet Gynecol 2016;5:1944–8.
4. Gnoth C, Maxrath B, Skonieczny T, Friol K, Godehardt E, Tigges J. Final ART success rates: a 10 years survey. Hum Reprod 2011;26:2239–46.
5. Pelinck MJ, Vogel NE, Hoek A, Simons AH, Arts EG, Mochtar MH, et al. Cumulative pregnancy rates after three cycles of minimal stimulation IVF and results according to subfertility diagnosis: a multicentre cohort study. Hum Reprod 2006;21:2375–83.
6. Santos MA, Kuijk EW, Macklon NS. The impact of ovarian stimulation for IVF on the developing embryo. Reproduction 2010;139:23–34.
7. Verberg MF, Eijkemans MJ, Macklon NS, Heijnen EM, Baart EB, Hohmann FP, et al. The clinical significance of the retrieval of a low number of oocytes following mild ovarian stimulation for IVF: a meta-analysis. Hum Reprod Update 2009;15:5–12.
8. Engel JB, Ludwig M, Felberbaum R, Albano C, Devroey P, Diedrich K. Use of cetrorelix in combination with clomiphene citrate and gonadotrophins: a suitable approach to 'friendly IVF'? Hum Reprod 2002;17:2022–6.
9. Singh A, Bhandari S, Agrawal P, Gupta N, Munaganuru N. Use of clomiphene-based stimulation protocol in oocyte donors: a comparative study. J Hum Reprod Sci 2016;9:159–63.
10. Weiss JM, Ludwig M, Ortmann O, Diedrich K. GnRH antagonists in the treatment of infertility. Ann Med 2003;35:512–22.
11. Olivennes F, Belaisch-Allart J, Emperaire JC, Dechaud H, Alvarez S, Moreau L, et al. Prospective, randomized, controlled study of in vitro fertilization-embryo transfer with a single dose of a luteinizing hormone-releasing hormone (LH-RH) antagonist (cetrorelix) or a depot formula of an LH-RH agonist (triptorelin). Fertil Steril 2000;73:314–20.
12. Jwa SC, Nakashima A, Kuwahara A, Saito K, Irahara M, Sakumoto T, et al. Neonatal outcomes following different ovarian stimulation protocols in fresh single embryo transfer. Sci Rep 2019;9:3076.
13. Van Steirteghem AC, Nagy Z, Joris H, Liu J, Staessen C, Smitz J, et al. High fertilization and implantation rates after intracytoplasmic sperm injection. Hum Reprod 1993;8:1061–6.
14. Veeck LL. Oocyte assessment and biological performance. Ann N Y Acad Sci 1988;541:259–74.
15. Gardner DK, Surrey E, Minjarez D, Leitz A, Stevens J, Schoolcraft WB. Single blastocyst transfer: a prospective randomized trial. Fertil Steril 2004;81:551–5.
16. Kanda Y. Statistical analysis using freely-available "EZR (Easy R)" software. Rinsho Ketsueki 2015;56:2258–66.
17. Check JH. Mild ovarian stimulation. J Assist Reprod Genet 2007;24:621–7.
18. Kol S, Lightman A, Hillensjo T, Devroey P, Fauser B, Tarlatzis B, et al. High doses of gonadotrophin-releasing hormone antagonist in in-vitro fertilization cycles do not adversely affect the outcome of subsequent freeze-thaw cycles. Hum Reprod 1999;14:2242–4.
19. Hernandez-Nieto C, Lee JA, Alkon-Meadows T, Luna-Rojas M, Mukherjee T, Copperman AB, et al. Late follicular phase progesterone elevation during ovarian stimulation is not associated with decreased implantation of chromosomally screened embryos in thaw cycles. Hum Reprod 2020;35:1889–99.
20. Vanni VS, Somigliana E, Reschini M, Pagliardini L, Marotta E, Faulisi S, et al. Top quality blastocyst formation rates in relation to progesterone levels on the day of oocyte maturation in GnRH antagonist IVF/ICSI cycles. PLoS One 2017;12:e0176482.
21. Huang B, Ren X, Wu L, Zhu L, Xu B, Li Y, et al. Elevated progesterone levels on the day of oocyte maturation may affect top quality embryo IVF cycles. PLoS One 2016;11:e0145895.
22. Baldini D, Savoia MV, Sciancalepore AG, Malvasi A, Vizziello D, Beck R, et al. High progesterone levels on the day of HCG administration do not affect the embryo quality and the reproductive outcomes of frozen embryo transfers. Clin Ter 2018;169:e91–5.
23. Pardinas ML, Nohales M, Labarta E, De Los Santos JM, Mercader A, Remohi J, et al. Elevated serum progesterone does not impact euploidy rates in PGT-A patients. J Assist Reprod Genet 2021;38:1819–26.
24. Kyrou D, Popovic-Todorovic B, Fatemi HM, Bourgain C, Haentjens P, Van Landuyt L, et al. Does the estradiol level on the day of human chorionic gonadotrophin administration have an impact on preg-

nancy rates in patients treated with rec-FSH/GnRH antagonist? Hum Reprod 2009;24:2902–9.
25. Jones CA, Acharya KS, Acharya CR, Raburn D, Muasher SJ. Patient and in vitro fertilization (IVF) cycle characteristics associated with variable blastulation rates: a retrospective study from the Duke Fertility Center (2013–2017). Middle East Fertil Soc J 2020;24:4.
26. Kolibianakis EM, Collins J, Tarlatzis BC, Devroey P, Diedrich K, Griesinger G. Among patients treated for IVF with gonadotrophins and GnRH analogues, is the probability of live birth dependent on the type of analogue used?: a systematic review and meta-analysis. Hum Reprod Update 2006;12:651–71.
27. Al-Inany HG, Abou-Setta AM, Aboulghar M. Gonadotrophin-releasing hormone antagonists for assisted conception. Cochrane Database Syst Rev 2006;3:CD001750.
28. Karimzadeh MA, Ahmadi S, Oskouian H, Rahmani E. Comparison of mild stimulation and conventional stimulation in ART outcome. Arch Gynecol Obstet 2010;281:741–6.
29. Kato K, Ezoe K, Yabuuchi A, Fukuda J, Kuroda T, Ueno S, et al. Comparison of pregnancy outcomes following fresh and electively frozen single blastocyst transfer in natural cycle and clomiphene-stimulated IVF cycles. Hum Reprod Open 2018;2018:hoy006.
30. Bourgain C, Devroey P. The endometrium in stimulated cycles for IVF. Hum Reprod Update 2003;9:515–22.

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INSTRUCTIONS TO AUTHORS

CERM

Enacted: December 31, 1974
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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.

3. Registration of clinical trial research

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

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

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

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3. Archiving policy

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4. Open data policy

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

5. Clinical data sharing policy

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

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

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

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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.
- Submit each figure as individual files separate from the manuscript. Do not insert figures into the text document. Figures should be in tiff, tif, jpg, jpeg files. Do not submit your manuscript or figures as pdf files.
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- Drug and chemical names should be stated in standard chemical or generic nomenclature.
- Description of genes or related structures in a manuscript should include the names and official symbols provided by the US National Center for Biotechnology Information (NCBI) or the HUGO Gene Nomenclature Committee.
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2. Original article

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

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

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

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

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

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

5) **Results:** The results should be presented in logical sequence in the text, tables, and illustrations. Do not repeat in the text all data in the tables or figures, but describe important points and trends.

6) **Discussion:** Observations pertaining to the results of research and other related materials should be interpreted for your readers. Emphasize new and important observations; do not merely repeat the contents in the Introduction or Results. Explain the meaning of the observed opinion along with its limits, and within the limits of the research results connect the conclusion to the purpose of the research.

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9) **Author contributions:** What authors have done for the study should be described in this section. To qualify for authorship, all contributors must meet at least one of the seven core contributions by CRediT (conceptualization, methodology, software, validation, formal analysis, investigation, data curation), as well as at least one of the writing contributions (original draft preparation, review and editing). Authors may also satisfy the other remaining contributions; however, these alone will not qualify them for authorship. Contributions will be published with the final article, and they should accurately reflect contributions to the work. The submitting author is responsible for completing this information at submission, and it is expected that all authors will have reviewed, discussed, and agreed to their individual contributions ahead of this time. The information concerning sources of author contributions should be included in this section at submitting the final version of manuscript (at the first submission, this information should be included in title page).

Examples of authors' contributions are as follows:
Conceptualization: BCJ. Data curation: DL. Formal analysis: YIA. Funding acquisition: JHA. Methodology: BCJ. Project administration: MYP. Visualization: MHC. Writing – original draft: DL. Writing – review & editing: BCJ.

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

Examples of references

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

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

(3) **Book**

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

(4) **In press**

Yang XL, Chen F, Yang XY, Du GH, Xu Y. Low molecular weight heparin does not reduce miscarriages in non-thrombophilic IVF/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.

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

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

3. Review article

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

4. Case report

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

5. Brief communication

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

6. Letter to the editor

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

VII. AUTHOR'S MANUSCRIPT CHECKLIST

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

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

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

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

IX. MANUSCRIPT ACCEPTED FOR PUBLICATION

1. Final version

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