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Preimplantation genetic testing for aneuploidy: The management of mosaic embryos

Eun Jeong Yu1, Min Jee Kim2,3, Eun A Park2,4, Inn Soo Kang5

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As the resolution and accuracy of diagnostic techniques for preimplantation genetic testing for aneuploidy (PGT-A) are improving, more mosaic embryos are being identified. Several studies have provided evidence that mosaic embryos have reproductive potential for implantation and healthy live birth. Notably, mosaic embryos with less than 50% aneuploidy have yielded a live birth rate similar to euploid embryos. This concept has led to a major shift in current PGT-A practice, but further evidence and theoretically relevant data are required. Proper guidelines for selecting mosaic embryos suitable for transfer will reduce the number of discarded embryos and increase the chances of successful embryo transfer. We present an updated review of clinical outcomes and practice recommendations for the transfer of mosaic embryos using PGT-A.

Keywords: Embryo transfer; Mosaicism; Preimplantation genetic testing

Introduction

In the field of preimplantation genetic testing for aneuploidy (PGT-A), mosaicism was first identified 25 years ago in a validation study, where it was thought to be caused by an insufficient trophectoderm (TE) sample size [1]. Technological innovations, such as next-generation sequencing (NGS), have significantly improved the identification and quantification of mosaicism. Some authors recently proposed that an “intermediate copy number” of individual chromosomes is a more accurate term than mosaicism [2].

Mosaic embryos have the potential to implant and develop into genetically normal babies [3,4]. Greco et al. [5] first reported in 2015 that 18 women who had mosaic embryo transfers gave birth to six healthy euploid newborns. In a recent prospective study, the authors demonstrated that mosaic embryos had a similar implantation rate (55% vs. 55.8%, \( p = 0.86 \)) and live birth rate (43.4% vs. 42.9%, \( p = 0.82 \)), as well as equivalent developmental potential to that of euploid embryos [6]. In addition, multicenter studies found no significant differences between euploid and mosaic embryo transfers in terms of the preterm delivery rate, birth weight, or risk of congenital malformations [3,7,8].

However, arguments for and against transferring mosaic embryos still exist [9]. The International Do No Harm Group in in vitro fertilization (IVF) argued against the 2019 Preimplantation Genetic Diagnosis International Society (PGDIS) guideline for mosaic embryo transfer [10] on the basis that the interpretation of mosaicism in PGT-A was misleading [9,11]. On the contrary, a recently published prospective non-selection study reported that the risk of clinical error in the diagnosis of uniform aneuploidy by NGS-based PGT-A was exceedingly low (0%–2%), suggesting that PGT-A has high predictive power [12]. Given the variability in the management of mosaic embryos, it is important for clinicians to have informative genetic counseling resources available when informing their patients of PGT-A results and giving recommendations for mosaic embryo transfer.
Therefore, we aimed to provide the latest clinical outcomes following mosaic embryo transfers in PGT-A cycles and a summary of updated practice recommendations.

Definition and types of mosaicism

Mosaicism is the presence of more than one genotypically distinct cell population within a single zygote [13]. Mosaic cellular populations are thought to arise from post-zygotic mitotic errors during post-zygotic cell division [13]. In PGT-A, mosaicism is defined as a mixture of 20% to 80% aneuploid and euploid DNA content; those with less than 20% aneuploid DNA are called euploid, and those with more than 80% aneuploid DNA are called aneuploid. [14]. The incidence of mosaic embryos has been reported to be 5%, but some have found rates of 20%–30% using PGT-A [15]. Mosaicism is more frequently found in cleavage-stage embryos (30%–70%) [16] compared with blastocyst-stage embryos (5%–15%) [17,18].

Mosaicism can be classified into four types based on cell lineage and the timing of mitotic errors in the blastocyst stage [19,20]. An embryo is defined as “total mosaic” when both the inner cell mass (ICM) and TE contain aneuploid and euploid cells. If the mosaic population is exclusively ICM, the embryo is defined as “ICM mosaic,” and if exclusively TE, the embryo is “TE mosaic.” Finally, if all cells in the ICM are aneuploid and all cells in the TE are euploid (or vice versa), the embryo is “ICM/TE mosaic.”

Factors contributing to the diagnosis of mosaicism

Mosaicism may not be associated with maternal age [21]. Some authors suggested a slight increase in mosaicism in younger patients compared to women over 37 years of age [22]. In cases of low-degree mosaicism and segmental aneuploidies, the incidence of mosaicism showed a negative correlation with maternal age [23,24]. Contrary to the effects of maternal age, ovarian response to stimulation was positively related to the occurrence of segmental aneuploidy. In one study, the oocyte vitrification and ovarian response showed no effect on the mosaicism rate [22].

A high proportion of mosaic embryos was found in couples with low sperm concentrations [25,26]. The prevalence of mosaic and chaotic aneuploidy in blastomeres ranges from 35% to 68% in oligozoospermic and azoospermic men [27,28]. There is a higher proportion of mosaic embryos in PGT-A cycles with male infertility compared to patients with normal sperm parameters. The highest mosaicism rates were related to the severity of male infertility [25,26].

Technical laboratory factors may affect the quality of a biopsy and thus may affect the occurrence of mosaicism within the TE. Differences in platform specificity and sensitivity, the protocols for DNA amplification, and the threshold settings established for interpretation can lead to differences in the proportion of mosaicism and the number of euploid embryos to transfer [29]. Other factors associated with the biopsy technique, including the conditions surrounding cell loading and the number of cells biopsied, can also affect the results [30]. The method of fertilization [31] and laboratory conditions, such as oxygen concentration, pH and osmolality in the embryo culture medium, and temperature are related to an increased rate of mosaicism [30].

Management: transfer of mosaic embryos

Multiple factors determine the fate and viability of mosaic embryos, such as the degree of mosaicism in the biopsied sample, the specific type and number of chromosomes involved, and the type of mosaicism.

1. Priority for mosaic embryo transfer

In 2016, the position statement of the PGDIS recommended priorities for mosaic embryo transfers based on the specific chromosome involved and the level of mosaicism [32]. In 2017, the World Congress on Controversies in Preconception, Preimplantation and Prenatal Genetic Diagnosis highlighted the need for PGT-A in IVF practice and updated the PGDIS position statement on recommendations for clinical practice [33]. In 2018, Grati et al. [34] published a study on the chorionic villi samples (CVS) and products of conception (POC) after natural pregnancy to provide a practice guideline whereby mosaic embryos could lead to healthy live births. In 2020, Munne et al. [35] suggested classifying mosaic embryos into high- (> 50%) and low-level (< 50%) groups, with preference for transferring single segmental mosaic embryos over other types of mosaicism. In 2021, Viotti et al. [4] formulated a ranking system using outcome data from one thousand mosaic embryo transfers for the prioritization of mosaic embryos in the clinical setting. They confirmed that combined mosaic embryos have significantly lower implantation and pregnancy rates than euploid embryos. They also found that the type and level of mosaicism had a significant impact on the embryo transfer outcomes. Their study helped to elucidate the problems presented by mosaic transfer and attempted to provide firm conclusions. Relevant medical society practice guidelines and recommendations, including the recent PGDIS 2021 guidelines [36], are summarized in Table 1.

Despite these diverse ranking approaches, attempts to provide clinical recommendations for patients may yet be in early stages. Uncertainty remains regarding related factors affecting the clinical outcome data of mosaic embryo transfer. Some studies have suggested differences in live-birth rates based on the type and level of mosa-
### Table 1. A list of professional medical society guidelines and recommendations regarding mosaic embryo transfer

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>More favorable clinical outcomes in euploid than mosaic embryos</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>More favorable clinical outcomes in low than high levels of mosaicism</td>
<td>Not assessed</td>
<td>Yes (20%–40% vs. 40%–70%)</td>
<td>Not assessed</td>
<td>Yes (&lt; 40% vs. &gt; 40%)</td>
<td>Yes (but controversial)</td>
<td>Yes</td>
</tr>
<tr>
<td>Specific chromosome(s) involved</td>
<td>Lowest priority: chr 13, 18, 21</td>
<td>Lowest priority: chr 13, 18, 21, 22</td>
<td>Lowest priority: chr 13, 18, 21 and 45, X</td>
<td>Embryos mosaic for chromosomes that are associated with potential for uniparental disomy, severe intrauterine growth restriction, or liveborn syndromes may be given lower priority.</td>
<td>Some studies have found risky outcomes depending on the specific chromosome numbers involved; while others have reported that mosaic aneuploidies involving most chromosomes have pregnancies and live births with an abnormal phenotype.</td>
<td>No specific comments</td>
</tr>
<tr>
<td>lesser priority: potential for uniparental disomy (chr 14, 15), intrauterine growth restriction (chr 2, 7, 16)</td>
<td>Lesser priority: lowest priority: potential for uniparental disomy (chr 14, 15), intrauterine growth restriction (chr 2, 7, 16)</td>
<td>Lesser priority: potential for uniparental disomy (chr 14, 15), intrauterine growth restriction (chr 2, 7, 16)</td>
<td>Lesser priority: potential for uniparental disomy (chr 14, 15), intrauterine growth restriction (chr 2, 7, 16)</td>
<td>Lesser priority: potential for uniparental disomy (chr 14, 15), intrauterine growth restriction (chr 2, 7, 16)</td>
<td>Lesser priority: potential for uniparental disomy (chr 14, 15), intrauterine growth restriction (chr 2, 7, 16)</td>
<td>Lesser priority: potential for uniparental disomy (chr 14, 15), intrauterine growth restriction (chr 2, 7, 16)</td>
</tr>
<tr>
<td>More favorable clinical outcomes in monosomies than trisomies</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Different clinical outcomes between mosaic types (segmental vs. whole chromosome vs. complex)</td>
<td>Not assessed</td>
<td>In the case of complex mosaicism, transfer is not recommended</td>
<td>Not assessed</td>
<td>Not assessed</td>
<td>Controversial</td>
<td>Yes</td>
</tr>
<tr>
<td>Recommendation of prenatal test method</td>
<td>Amniocentesis</td>
<td>Amniocentesis</td>
<td>Amniocentesis</td>
<td>Amniocentesis</td>
<td>Amniocentesis</td>
<td>Amniocentesis</td>
</tr>
<tr>
<td>Special considerations</td>
<td>If a decision is made to transfer a non-complex, low-level mosaic embryo, one can prioritize selection based on the specific chromosome involved.</td>
<td>If a decision is made to transfer embryos mosaic for a single chromosome, one can prioritize selection primarily based on the level of mosaicism and then the specific chromosome involved.</td>
<td>Before transfer of mosaic embryos, comprehensive genetic counseling should be provided.</td>
<td>The relative percentage of mosaicism seems to be a better predictor of outcome than the specific chromosomes involved.</td>
<td>No specific comments</td>
<td></td>
</tr>
</tbody>
</table>

PGDIS, Preimplantation Genetic Diagnosis International Society; CoGEN, Congress on Controversies in Preconception, Preimplantation and Prenatal Genetic Diagnosis; ASRM, American Society for Reproductive Medicine; Chr, chromosome.
2. The degree of mosaicism

Chromosomal mosaicism has been defined as low-level mosaicism if abnormal cells are in the 30%–50% range and high-level mosaicism if abnormal cells are in the 50%–70% range using the NGS validation algorithm [41]. Clinical outcome data related to high-versus low-level mosaicism still show conflicting results. Some studies found that low-level mosaicism was related to improvement in ongoing pregnancy rates [38], while others did not find statistically significant results [40,42,43]. Embryos with low-level mosaicism are more likely to develop into healthy babies than high-level mosaic embryos, whereas high-level mosaic embryos increase the risk of miscarriage [35,38,41,44]. A recent prospective study found that embryos with more than 50% mosaicism have a significantly lower implantation rate (24.4% vs. 54.6%; \( p < 0.002 \)), clinical pregnancy rate (15.2% vs. 46.4%; \( p < 0.001 \)), and live birth rate (15.2% vs. 46.6%; \( p < 0.001 \)) compared to euploid embryos in the NGS profile [38]. Both these studies found that embryos with a significant degree of mosaicism had low implantation and clinical pregnancy rates [41,42].

3. Specific chromosomes involved

The clinical outcomes of mosaicism can be highly dependent on the chromosomes involved. Autosomes were ranked in order of their risk of placental insufficiency, intrauterine growth restriction, and uniparental disomy (UPD). The mosaic trisomy 16 chromosome is commonly affected in preimplantation embryos and leads to a high risk of abnormal perinatal outcomes, such as intrauterine growth restriction, preterm birth, and hypertensive disorders [45]. Chromosomes X, 21, and 22 have been reported to be susceptible to whole chromosome trisomies [62,63]. Most monosomic cells are removed at the post-implantation phase [54,55]. Trisomic mosaicism can occur in live births with chromosomal aneuploidy and is associated with cognitive and physical impairments [56]. Although PGDIS recommended the transfer of embryos with mosaic monosomies over those with mosaic trisomies in 2016 [32], this statement was updated and removed in 2019 [10]. In addition, some authors did not find a significant difference in pregnancy rates between monosomic and trisomic mosaic embryos [40].

5. Whole versus segmental aberrations

When duplication or deletion errors occur in a small portion of DNA during mitotic division, the embryo will have a mosaic of the segmental error, allowing some cells to have a normal copy number of chromosomes and others to have segmental deletion or duplication of the chromosomes [57]. In one study, segmental gain or loss was affected in 25% of mosaicism [58]. Some authors suggested that the incidence of segmental mosaicism may be underestimated due to biological and technical errors [59]. Clinical perspectives of embryo mosaicism, with respect to full versus partial aneuploidies, have been inconsistent. Some studies have reported a higher clinical pregnancy rate in partial aneuploid mosaicism [39,42,60], while others have not found a significant difference [40].

Regarding chromosome type, large chromosomes such as chromosomes 1 to 9 are prone to breakage, resulting in segmental mosaicism [62,63], while a significantly lower percentage of copy number errors were observed in small chromosomes and acrocentric chromosomes (e.g., chromosomes 19, 21, 22, and Y) [64,65].
Segmental aneuploidies originate because of mitotic errors during preimplantation development [24]. This is related to blastocyst morphology, not to maternal age or clinical and embryological parameters [66]. A previous multicenter study of 822 mosaic embryo transfers demonstrated that the reproductive potential of mosaic embryos is affected by the number of euploid cells and the complexity in the TE biopsy sample [67]. The embryos with segmental aneuploidy had better clinical outcomes than mosaic embryos with one or two involved chromosomes (implantation rate: $p < 0.001$, ongoing pregnancy rate/birth rate: $p < 0.001$).

6. The number of chromosomes involved (single versus double versus complex aneuploidies)

Several studies found reduced pregnancy capacity in mosaic embryos that had three or more chromosomes involved [40] and in segmental mosaicism that had two or more chromosomes involved [42], whereas other studies did not report clinically significant differences between mosaic embryos involving one or two chromosomes [40,68]. Complex mosaic embryos had the lowest implantation rates among single aneuploid, double aneuploid, and segmental mosaic embryos [40].

Genetic counseling

A recent statement by the American Society for Reproductive Medicine highlighted the importance of patient education prior to PGT-A [37]. Before the transfer of mosaic embryos, counseling should include a discussion of the potential challenges in interpreting mosaic results, the potential risks of mosaic embryo transfers, and the limited neonatal outcome data available. In addition, counseling should provide information regarding the genetic advantages, risks, and limitations of a prenatal diagnosis. Thus far, most prenatal testing results after mosaic embryo transfers have shown normal healthy fetuses with no specific chromosomal abnormalities [3]. However, we found two reports of babies with abnormal karyotypes: a baby with 15q duplication syndrome after transfer of a 57% segmental mosaic embryo [69] and a healthy baby with 2% mosaic monosomy 2 after transfer of a 35% mosaic monosomy 2 embryo [70].

Patients should be informed about the risk of mosaicism in a biopsy specimen, the complexities of the various possible outcomes after transfer of a mosaic embryo, and the need for close prenatal monitoring, including amniocentesis. Until definitive data is available, patients should be advised to go through additional cycles if possible to obtain euploid embryos instead of transferring a mosaic embryo. A schematic prioritization of mosaic embryos according to clinical outcomes is shown in Table 2.

### Prenatal diagnosis after transfer of mosaic embryos

If a pregnancy has been confirmed after mosaic embryo transfer, prenatal diagnosis is recommended to identify fetal chromosomes and other genetic conditions. Although evidence-based guidance for prenatal testing after mosaic embryo transfer is still lacking, most practice statements consistently recommend amniocentesis as the gold standard for prenatal diagnosis [32,33,37,71]. Karyotyping of the amniocytes obtained by amniocentesis is done to diagnose aneuploidy in the fetus [72]. CVS can be useful for patients seeking a diagnosis during the first trimester; however, CVS results represent placental cells derived from the TE. Thus, mosaic findings detected using CVS may indicate placental mosaicism, and follow-up amniocentesis is required to clarify the results. The major advantage of amniocentesis is the ability to analyze fetal cells directly, but it may miss low-level mosaicism. Therefore, amniocentesis best represents the chromosome complement within fetal tissues, but patients should know that some mosaicism may not be detectable. Depending on the PGT-A result, further analysis of prenatal samples should also be considered; chromosomal microarray can be performed if segmental aneuploidy or UPD is involved [37,73]. Cell-free DNA (cfDNA) testing, also known as noninvasive prenatal testing (NIPT), has not been validated to detect mosaicism because NIPT analyzes circulating cfDNA fragments in the maternal plasma derived from both the mother’s and apoptotic trophoblasts, but not from the fetus itself [74].

### Table 2. Schematic prioritization of mosaic embryo classified according to favorable clinical outcomes

<table>
<thead>
<tr>
<th>Priority</th>
<th>Percentage of mosaicism</th>
<th>Monosomy vs. trisomy</th>
<th>Segmental vs. whole chromosome</th>
<th>Specific chromosomes involved</th>
<th>No. of Chr involved (single vs. double vs. complex)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low clinical risk</td>
<td>Low (&lt; 50%)</td>
<td>Monosomy</td>
<td>Segmental</td>
<td>Chr 1, 3, 4, 5, 6, 10, 12, 17, 19, 20, 22, X, Y</td>
<td>Single</td>
</tr>
<tr>
<td>High clinical risk</td>
<td>High (&gt; 50%)</td>
<td>Trisomy</td>
<td>Whole</td>
<td>Chr 13, 18, 21: best-avoided</td>
<td>Complex</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chr 6, 7, 11, 14, 15, 20: UPD risk</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chr 2, 16: IUGR risk</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chr 8, 9: aneuploidy viability</td>
<td></td>
</tr>
</tbody>
</table>

Chr, chromosome; UPD, uniparental disomy; IUGR, intrauterine growth restriction.
Conclusion

Although interest in mosaic embryo transfers is increasing, the debate over whether mosaic embryos can be transferred is ongoing. In practice, the identification of mosaic subgroups that are viable and worthy of transfer is very important, but it is also vital to inform patients that the data on postnatal and neonatal outcomes following mosaic embryo transfers are still limited and that clinical outcomes have been mixed. We emphasize the need for further research on the genetic and clinical outcomes of mosaic embryo transfers. Large-scale multicenter studies would be of particular value in collecting data for the risk evaluation of mosaic embryo transfers and could potentially reduce the disposal of viable embryos for implantation and live births.

Conflict of interest

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

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Author contributions

Conceptualization: ISK. Writing–original draft: EJY. Writing–review & editing: all authors.

References

analysis of inner cell mass and trophectoderm samples of previously array-CGH screened blastocysts shows high accuracy of diagnosis and no major diagnostic impact of mosaicism at the blastocyst stage. Hum Reprod 2013;28:2298–307.


Platelet-rich plasma treatment in patients with refractory thin endometrium and recurrent implantation failure: A comprehensive review

Min Kyoung Kim¹, Haengseok Song², Sang Woo Lyu³, Woo Sik Lee¹

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Refractory thin endometrium and recurrent implantation failure are among the most challenging infertility-related factors hindering successful pregnancy. Several adjuvant therapies have been investigated to increase endometrial thickness and the pregnancy rate, but the treatment effect is still minimal, and for many patients, these treatment methods can be quite costly and difficult to approach. Platelet-rich plasma (PRP) is an autologous concentration of platelets in plasma and has recently been elucidated as a better treatment option for these patients. PRP is rich in cytokines and growth factors, which are suggested to exert a regenerative effect at the level of the injured tissue. Another advantage of PRP is that it is easily obtained from the patient’s own blood. We aimed to review the recent findings of PRP therapy used for patients with refractory thin endometrium and recurrent implantation failure.

Keywords: In vitro fertilization; Platelet-rich plasma; Recurrent implantation failure; Refractory thin endometrium
ant roles of cytokines and GFs in the embryonic implantation process [11], PRP has been a “rising star” in improving endometrial receptivity. Another advantage of PRP is that it is easily obtained directly from the patient’s own blood. We aimed to review the recent findings of PRP therapy used for patients with refractory thin endometrium and RIF.

Methods

A literature search was done using PubMed to investigate recently reported PRP studies in the reproductive endocrinology field. The search period was from January 2018 to May 2022 to find the most up-to-date studies regarding PRP use in endometrium-related infertility. The search words included “platelet-rich plasma,” “gynecology,” “infertility,” and “endometrium.” Mostly case reports, pilot studies with small sample sizes, and a few randomized controlled trials (RCTs) were found.

1. PRP preparation

There is still no consensus on a standardized protocol for preparing therapeutically effective PRP. The basis of PRP preparation is mainly the differential centrifugation of the whole blood [12]. Each component of the whole blood is separated into different layers by centrifugation due to differences in specific gravity. Two main methods are known for preparing PRP: the PRP method and the buffy-coat method [12]. In the PRP method, fresh blood is obtained by venipuncture in acid citrate dextrose tubes and centrifuged right away using soft spin. The supernatant plasma containing platelets is separated and centrifuged at a higher speed (hard spin) to obtain a platelet concentrate. The lower third is PRP, and at the bottom platelet pellets are formed. The buffy-coat method uses whole blood stored at 20°C–24°C and centrifuged at a “high” speed. Due to its density, three layers are formed: red blood cells at the bottom, platelets and white blood cells in the middle, and platelet-poor plasma (PPP) on top. The PPP layer is removed, and the buffy-coat layer is transferred to another tube for centrifugation at low speed to separate white blood cells. Alternatively, a leukocyte filter can be used.

Arora and Agnihotri [13] described the importance of anticoagulants in preparing PRP. Anticoagulant citrate dextrose-A is the most commonly used anticoagulant in commercial kits since it maintains an optimal pH for platelets at 7.2. The citrate binds to calcium and prevents the coagulation cascade. They also emphasized the importance of minimizing the PRP’s surface area in contact with the atmosphere (using small diameter tubes with caps) in order to stop CO₂ from diffusing into the plasma and increasing the pH. An increased pH may potentially cause spontaneous aggregation of the platelets, making it difficult to utilize PRP.

Several commercial PRP preparation kits are available internationally, but there is substantial heterogeneity in the concentrations of platelets, leukocytes, and GFs in PRP. No general consensus exists regarding the optimal component concentrations [14]. Future research should focus on finding the most suitable PRP concentration for applications in infertility.

2. Proposed PRP mechanism

The mechanism through which PRP acts on refractory thin endometrium has not yet been definitely established, but it is believed that several GFs play important roles. Platelets are anucleated cytoplasmic fragments of megakaryocytes that contain α-granules with various GFs [15,16]. GFs are known to control angiogenesis, cell proliferation, stem cell migration, and inflammation [17]. For refractory thin endometrium, angiogenesis and cell proliferation may be the key mechanisms that need to be enhanced to stimulate the recovery process. Among the GFs in α-granules, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), fibroblast growth factor, and insulin-like growth factor are considered to be important in the effects of PRP [18]. PDGF has several effects on the endometrium; it exerts a mitogenic effect in endometrial stromal, decidual, and epithelial cells; enhances DNA synthesis in endometrial stromal cells; stimulates the chemotactic migration of endometrial stromal cells; and promotes endometrial stromal cell motility [19]. VEGF stimulates neovascularization through its endothelial chemokine and mitogenic properties [20]. TGF-β has been shown to regulate endometrial decidualization, the uterine immune response, and endometrium repair during menstruation [21]. Fibroblast growth factor initiates angiogenic processes in the endometrium, upregulates VEGF receptor 2, and promotes endothelial proliferation and organization [22]. Insulin-like growth factor induces endometrial proliferation through the protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway and initiates endometrial cell decidualization [23] (Table 1).

Interestingly, the α-granules contained in PRP have both pro- and anti-angiogenic properties. In order for PRP to promote angiogenesis, it is necessary to activate the pro-angiogenic cell surface receptors (VEGF, PDGF, TGF-β1, epidermal GF, serotonin, angiopoietin-1 and -2, matrix metalloproteinase-1 and -2, and interleukin-8) [24]. Some studies have shown that higher concentrations of PRP attenuated the endometrial cell proliferation rate and led to negative results [25,26]. This is speculated to be due to an excess amount of GFs resulting in the activation of an increased amount of anti-angiogenic factors (TGF-β1, plasminogen activator inhibitor, thrombospondin, angiotatin, endostatin, platelet factor 4, CXCL4L, tissue inhibitors of metalloproteases) to hinder cell proliferation. Giusti et al. reported in an in vitro study that 1.5 × 10⁵ platelet/μL was the optimal concen-
Table 1. Growth factors that play important roles in platelet-rich plasma

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast growth factor [22]</td>
<td>Initiates angiogenic processes</td>
</tr>
<tr>
<td></td>
<td>Upregulates VEGFR2</td>
</tr>
<tr>
<td></td>
<td>Promotes endothelial proliferation and organization</td>
</tr>
<tr>
<td>Insulin-like growth factor [23]</td>
<td>Induces endometrial proliferation through the AKT/mTOR pathway</td>
</tr>
<tr>
<td></td>
<td>Initiates endometrial cell decidualization</td>
</tr>
<tr>
<td>Platelet-derived growth factor [19]</td>
<td>Exerts a mitogenic effect in endometrial stromal, decidual, and epithelial cells</td>
</tr>
<tr>
<td></td>
<td>Enhances DNA synthesis in endometrial stromal cells</td>
</tr>
<tr>
<td></td>
<td>Stimulates the chemotactic migration of endometrial stromal cells</td>
</tr>
<tr>
<td></td>
<td>Promotes endometrial stromal cell motility</td>
</tr>
<tr>
<td>Transforming growth factor-β [21]</td>
<td>Regulates endometrial decidualization</td>
</tr>
<tr>
<td></td>
<td>Regulates uterine immune response</td>
</tr>
<tr>
<td></td>
<td>Regulates endometrial repair during menstruation</td>
</tr>
<tr>
<td>VEGF [20]</td>
<td>Stimulates neovascularization by its endothelial chemokine and mitogenic properties</td>
</tr>
</tbody>
</table>

VEGFR2, VEGF receptor 2; AKT, protein kinase B; mTOR, mammalian target of rapamycin; VEGF, vascular endothelial growth factor.

3. PRP use in refractory thin endometrium and Asherman’s syndrome

Molina et al. [27] prospectively evaluated 19 patients with a history of refractory thin endometrium to whom PRP was given by intrauterine injections. In all cases, endometrial thickness reached > 9 mm after the second PRP injection. The pregnancy rate was 73.7%, of which 26.3% yielded live births and 26.3% ongoing pregnancies.

Chang et al. [28] investigated a larger study population of 64 patients with refractory thin endometrium (< 7 mm) and administered intrauterine injections of PRP to 34 patients. The PRP group had significantly thicker endometrium than that of the control group. The implantation and clinical pregnancy rates in the PRP group were significantly higher than in the control group (27.94% vs. 11.67%, p < 0.05; 44.12% vs. 20%, p < 0.05, respectively).

Kim et al. [29] studied 22 patients with a history of two or more failed in vitro fertilization (IVF) cycles and refractory thin endometrium of < 7 mm. Their prospective interventional study compared the study participants’ previous non-treated and later PRP-treated FET cycles. PRP was injected two or three times from menstrual cycle day 10 of the FET cycle, and FET was done 3 days after the final PRP injection. The implantation, clinical pregnancy, and live birth rates were 12.7%, 30%, and 20%, respectively in the PRP-treated cycles, while all previous cycles reported rates of 0%. However, the endometrial thickness showed no significant difference between the PRP and previous non-treated cycles.

A preliminary study using a mouse model of Asherman’s syndrome (AS) was done by Kim et al. [30] to assess the effectiveness of human PRP for endometrial recovery. Three separate experiments were performed. First, the effects of PRP on endometrial regeneration were assessed by evaluating the endometrial histology and expression of fibrosis-related factors. Second, the mice implantation sites and embryo weights were compared between the PRP and control groups. Third, live births were compared. Human PRP improved endometrial morphology, reduced the degree of fibrosis, and downregulated the expression of fibrosis markers. Higher numbers of implantation sites and live births were also noted.

A consecutive study by the same group of authors used a mouse model of AS to discover the molecular mechanisms of PRP that act on damaged endometrium [31]. They showed that the GFs in PRP promoted angiogenesis by increasing proangiogenic factors such as Hif1α, Hif2α, VEGF-α, Ang-1, Hgf, and IGF-1. PRP also promoted the migration of endometrial stromal cells to injured uterine areas, leading to uterine regeneration in pathologic conditions. Furthermore, PRP significantly increased the phosphorylation of STAT3, which is a critical transcription factor for tissue remodeling and regeneration, in both stroma and epithelial compartments in uteri with AS. Additionally, the mice that received PRP treatment had significantly higher mean weights of embryos and their placentas than the control mice, suggesting that PRP treatment considerably alleviates intrauterine growth restriction phenotypes in AS.

de Miguel-Gomez et al. [32] conducted an in vitro composition analysis and murine model of AS to study the effect of PRP from different sources on endometrial damage. The authors [32] tested whether plasma from human umbilical cord blood had stronger effects than adult PRP (aPRP) on endometrial recovery. The in vitro cell proliferation and migration rate after treatment with umbilical cord
### Table 2. Clinical application of PRP in refractory thin endometrium

<table>
<thead>
<tr>
<th>Study</th>
<th>Study design</th>
<th>Transfer type</th>
<th>PRP injection method</th>
<th>Control group (n)</th>
<th>Intervention group (n)</th>
<th>Endometrium pre-PRP (mm)</th>
<th>Endometrium post-PRP (mm)</th>
<th>p-value</th>
<th>Pregnancy in PRP group (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molina et al. (2018)</td>
<td>Prospective interventional study</td>
<td>Frozen</td>
<td>(1) 1 mL of PRP on day 10 of HRT (2) Day 12 of HRT (72 hr after the first injection)</td>
<td>-</td>
<td>19</td>
<td>&lt; 6</td>
<td>&gt; 9</td>
<td>-</td>
<td>73.7</td>
</tr>
</tbody>
</table>
| Chang et al. (2019)    | Prospective cohort study | Frozen       | (1) 0.5–1 mL of PRP on MCD #10 (2) Progesterone starting day 72 hr after the first injection | 30                | 34                    | < 7                      | > 7                       | 0.013   | 27.94 vs. 11.67
| Kim et al. (2019)      | Prospective interventional study | Frozen       | (1) 0.7–1 mL of PRP on MCD #10 (2) Repeated at 3-day intervals 2–3 times until the EMT reached 7 mm | -                 | 22                    | 4–6.8                    | 4.2–9.1                   | 0.07    | 12.7                        |
| Agarwal et al. (2020)  | Cross-sectional study | Frozen       | Hysteroscopic subendometrial injection with 4 mL PRP (1 mL in each of 4 walls) 7–10 days after injecting leuprolide during the previous cycle | -                 | 32                    | < 7                      | > 7                       | -       | 41.66                        |
| Dogra et al. (2022)    | Prospective interventional study | Fresh and frozen | (1) 0.5–1 mL of PRP on day 8 of HRT (2) Repeated 2–3 times every 48 hr until the EMT reached more than 7 mm | -                 | 20                    | < 7                      | > 7                       | 0.001   | 13.8 (fresh) 25 (fresh) 9.1 (frozen) 3.8 (frozen) |

PRP, platelet-rich plasma; HRT, hormone replacement therapy; MCD, menstrual cycle day; EMT, endometrial thickness.

* *p<0.05*

### Table 3. Clinical applications of PRP in patients with recurrent implantation failure

<table>
<thead>
<tr>
<th>Study</th>
<th>Study design</th>
<th>Transfer type</th>
<th>PRP injection method</th>
<th>Control group (n)</th>
<th>Intervention group (n)</th>
<th>Implantation</th>
<th>Clinical</th>
<th>Ongoing</th>
<th>Live birth</th>
<th>Miscarriage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zamaniyan et al. (2021)</td>
<td>RCT</td>
<td>Frozen</td>
<td>0.5 mL of PRP injection 48 hr before ET</td>
<td>43</td>
<td>55</td>
<td>25 vs. 58.3</td>
<td>23.3 vs. 48.3</td>
<td>11.7 vs. 46.7</td>
<td>-</td>
<td>3.3 vs. 1.7</td>
</tr>
<tr>
<td>Nazari et al. (2021)</td>
<td>RCT</td>
<td>Frozen</td>
<td>0.5 mL of PRP injection 48 hr before ET</td>
<td>197</td>
<td>196</td>
<td>-</td>
<td>19.28 vs. 48.97</td>
<td>-</td>
<td>5.58 vs. 39.28</td>
<td>13.7 vs. 9.69</td>
</tr>
<tr>
<td>Bakhsh et al. (2022)</td>
<td>RCT</td>
<td>Frozen</td>
<td>0.5 mL of PRP injection 48 hr before ET</td>
<td>50</td>
<td>50</td>
<td>-</td>
<td>13.33 vs. 20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xu et al. (2022)</td>
<td>Retrospective</td>
<td>Frozen</td>
<td>0.5 mL of PRP injection 48–72 hr before ET</td>
<td>150</td>
<td>138</td>
<td>17.62 vs. 27.18</td>
<td>24.67 vs. 36.23</td>
<td>-</td>
<td>14 vs. 29.71</td>
<td>27.03 vs. 18</td>
</tr>
</tbody>
</table>

PRP, platelet-rich plasma; RCT, randomized controlled trial; ET, embryo transfer; NC, statistical difference not calculated.
plasma was the highest, and aPRP also revealed a significant increment. The mouse model study showed higher expression of Ki67 and Hoxa-10 in the endometrium after applying aPRP, and the proteomic analysis revealed a specific protein expression profile. The damaged uterine tissue showed more pro-regenerative markers after the application of umbilical cord plasma than after other treatments (nonactivated umbilical cord plasma, activated aPRP, and no treatment).

An interesting novel PRP injection method was reported by Agarwal et al. [33] by injecting PRP in the endo-myometrial junction hysteroscopically. Thirty-two patients with a refractory thin endometrium received hysteroscopic PRP injections, and 24 of them (75%) had improved endometrial thickness (> 7 mm). They underwent FET and among them, 10 had clinical pregnancies with positive fetal heartbeat and two had biochemical pregnancies.

The most recent prospective interventional study, reported in January 2022, found that PRP optimized endometrial thickness in both fresh and FET cycles [34]. Twenty women with refractory thin endometrium (< 7 mm), regardless of hormone replacement therapy, underwent 26 PRP cycles during fresh embryo transfer and FET. PRP infusions were repeated every 48 hours if needed, and the maximum number of PRP infusions was limited to 3. The mean endometrial thickness increased significantly after PRP infusion (p < 0.001) with average increases of 1.07 mm and 0.83 mm after the first PRP treatment (p < 0.001) during fresh IVF and FET, respectively. The clinical pregnancy rates, implantation rates, and live birth rates were not significantly different between fresh embryo transfer and FET cycles (p > 0.05) (Table 2).

4. PRP use in RIF

Zamanian et al. [35] investigated 98 RIF patients (who failed to become pregnant after three or more transfers of good-quality embryos) in a RCT. Fifty-five patients were given intrauterine PRP infusions 48 hours before embryo transfer in FET cycles. The other 43 patients comprised the control group, and these two groups showed significant differences in clinical (52.7% vs. 23.3%, p = 0.003) and ongoing pregnancy rates (50.9% vs. 16.3%, p < 0.001). Interestingly, although the study participants already had normal endometrial thickness, the PRP-treated group showed significantly increased endometrial thickness compared to the control group (13.15 ± 1.42 mm vs. 10.00 ± 0.93 mm, p < 0.001).

The largest RCT was reported in 2021 by Nazari et al. [36], including 418 women with a history of RIF (failure to achieve pregnancy after three or more embryo transfers with high-quality embryos) undergoing FET. Patients were randomly assigned to PRP and control groups. The PRP group received 0.5 mL of PRP by intrauterine injection 48 hours before FET. Among the 418 candidates, 393 participants completed the study (PRP: n = 196; control: n = 197) and higher chemical pregnancy, clinical pregnancy, and live birth rates were observed in the PRP group (p < 0.0001, p < 0.0001, p < 0.0001, respectively). There were no significant differences in the rates of multiple pregnancies and pregnancy complications. Only the spontaneous abortion rate was lower in the PRP group than in the control group.

The most recent RCT study, published in January 2022 by Bakhsh et al. [37], found that 100 women with an unexplained RIF history (previously failed to conceive after three or more transfers of high-quality embryos) had positive pregnancy outcomes after using PRP. These patients, undergoing FET, were divided randomly into PRP and control groups. The pregnancy rate was 20% in the PRP group and 13.33% in the control group, but this difference did not reach statistical significance (p = 0.62). The authors still concluded that PRP may play a role in improving the fertility status of RIF patients and that larger RCT studies are needed.

Xu et al. [38] retrospectively evaluated 288 women with a RIF history (three or more consecutive failed embryo implantations with good-quality embryos, defined as at least six cleavage-stage embryos or three blastocysts). In total, 138 patients with PRP treatment and 150 patients who did not receive treatment were compared and the implantation, clinical pregnancy, and live birth rates were higher in the PRP group. Except for the implantation and miscarriage rates, the other results were statistically significant (Table 3).

Conclusions

Autologous PRP injections have shown substantial benefits as a feasible method to treat refractory thin endometrium and RIF. Recent studies are gathering evidence in support of the hypothesis that the GFs in PRP increase endometrial receptivity. However, larger-scale, high-quality RCTs will be needed to address some of the issues and determine the proper PRP preparation and dosage necessary to effectively treat endometrium-related infertility.

Conflict of interest

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

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Conceptualization: HS, SWL, WSL. Data curation: MKK. Formal analysis: MKK. Methodology: MKK, SWL. Project administration: WSL. Visualization: HS, SWL. Writing–original draft: MKK. Writing–review & editing: MKK, SWL.

References


34. Dogra Y, Singh N, Vanamal P. Autologous platelet-rich plasma optimizes endometrial thickness and pregnancy outcomes in women with refractory thin endometrium of varied aetiology during fresh and frozen-thawed embryo transfer cycles. JBRA Assist Reprod 2022;26:13–21.


Effect of endometrial cell-conditioned medium and platelet-rich plasma on the developmental competence of mouse preantral follicles: An *in vitro* study

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**Objective:** The aim of this study was to evaluate the impacts of platelet-rich plasma (PRP) and conditioned medium (CM) derived from endometrial stromal cells on mouse preantral follicle culture in a two-dimensional system to produce competent mature oocytes for fertilization.

**Methods:** In total, 240 preantral follicles were isolated from female mouse ovarian tissue and divided into four groups. The preantral follicles were isolated three times for each group and then cultured, respectively, in the presence of alpha minimum essential medium (control), PRP, CM, and PRP+CM. The *in vitro* growth, *in vitro* maturation, and cleavage percentage of the preantral follicles were investigated. Immunocytochemistry (IHC) was also conducted to monitor the meiotic progression of the oocytes. Additionally, the mRNA expression levels of the two folliculogenesis-related genes (*Gdf9* and *Bmp15*) and two apoptosis-related genes (*Bcl2* and *Bax*) were investigated using real-time polymerase chain reaction.

**Results:** In the PRP, CM, and PRP+CM groups, the preantral follicle maturation (evaluated by identifying polar bodies) were greater than the control group. The cleavage rate in the CM, and PRP+CM groups were also greater than the control group. IHC analysis demonstrated that in each treatment group, meiotic spindle was normal. In the PRP+CM group, the gene expression levels of *Bmp15*, *Gdf9*, and *Bcl2* were greater than in the other groups. The *Bax* gene was more strongly expressed in the PRP and control groups than in the other groups.

**Conclusion:** Overall, the present study suggests that the combination of CM and PRP can effectively increase the growth and cleavage rate of mouse preantral follicles *in vitro*.

**Keywords:** Conditioned medium; Culture; Endometrial cells; Platelet-rich plasma; Preantral follicles

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**Introduction**

Fertility preservation strategies, which involve cryopreservation of the oocyte and embryo, can help achieve successful pregnancy; however, some obstacles exist. For example, these methods require time to stimulate ovulation, delaying the therapeutic process for patients with cancer [1]. Furthermore, substantial concerns exist regarding cancer recurrence after ovarian tissue cryopreservation and autotransplantation due to the possibility of malignant cell contamination [2-4]. In this context, *in vitro* follicle culture may be superior to the mentioned methods because it does not increase the risk of can-
Advanced technologies to facilitate oocyte growth and development in culture enable experimental research on the mechanisms regulating oocyte development, in addition to clinical applications [8]. Due to ethical and practical limitations, the application of human research to evaluate oocyte maturation has been limited and requires the use of animal studies. Some of these issues can be addressed by utilizing murine follicles, which are easily accessible [9, 10]. In the last two decades, preantral follicle cultures have provided many oocytes for embryo production [11]. A two-step culture method was discovered to create competent oocytes for maturation, fertilization, and development into live offspring from newborn mouse primordial follicles [12, 13]. Follicle culture must be optimized to improve oocyte maturation and quality regarding genetic components such as meiotic spindles [5]. Furthermore, coculture with uterine epithelial cells by promoting oocyte maturation to meiosis II can increase the maturation of germinal vesicle oocytes [14]. Compared to the co-culture of somatic cells, using conditioned medium (CM) is superior as it avoids the close contact of certain somatic cells, which can result in physiological impairment and/or the reinitiation of latency in follicles. Furthermore, the CM can be retained for later use [15]. Some studies have indicated greater viability and larger diameter of follicles cultured with CM than control medium, indicating that CM could improve follicle culture [16]. Sources of CM include endometrial cells, which secrete various factors involved in cell proliferation and migration [17-19]. CM derived from human endometrial cell lines produces several cytokines and growth factors, including interleukin 6, platelet-derived growth factor, and interleukin 10 [17-19]. Interestingly, these factors play an important role in the modulation of ovarian function, including follicular growth, maturation, and theca cell proliferation [20-22]. Platelet-rich plasma (PRP), an accumulation of platelets suspended in plasma, is another source that can promote the viability and growth of various cell lines, likely due to the presence of growth factors [23-26]. Some of these growth factors, such as epidermal growth factor (EGF), connective tissue growth factor, and transforming growth factor beta (TGF-β), are involved in follicular development and oocyte maturation [27, 28]. Several studies have shown that PRP factors benefit follicle growth and the survival of primordial follicles in in vivo and in vitro three-dimensional culture [27, 29]. Thus, PRP and CM can affect the growth, maturation, and viability of preantral follicles.

In ovarian tissue culture, preantral follicles typically do not progress to meiosis II [11]. In contrast, the in vitro culture of preantral follicles is promising for the evaluation of oocyte maturation and comparative analyses of factors such as culture medium in in vitro follicular culture [30]. Therefore, this investigation was carried out to assess the influences of endometrial stromal cell-derived PRP and CM on in vitro growth, in vitro maturation (IVM), cell viability, and the cleavage rate of mouse preantral follicle culture in a two-dimensional system.

Methods

1. Animals

In this experimental animal study, female National Medical Research Institute (BALB/c) mice were purchased from the Royan Institute in Iran for the isolation of ovarian tissue. Additionally, 20 adult male BALB/c mice were purchased for sperm collection. The mice were maintained in the animal house under light- and temperature-controlled conditions (12 hours of light and 12 hours of darkness; 21°–24°C and 30%–60% humidity), and fresh food and water were provided. The animals were kept and used according to the standards of the Animal Ethics Committee. The experimental procedure of this study was approved by the Ethics Committee of Shiraz University of Medical Sciences (reference No. IR. Sums.REC.1398.688).

2. Ovarian tissue isolation

The female mice were sacrificed with carbon dioxide (CO₂), and the ovaries were transferred to a HEPES (N-2-hydroxyethyl-piperazine-N’-2-ethanesulfonic acid)-based potassium simplex optimization medium (KSOM). The preantral follicles were isolated from the ovarian tissue and were cultured in four treatment groups: alpha minimum essential medium FBS (control), CM, PRP, and PRP+CM.

3. Preantral follicle isolation

From the fresh ovarian tissues, 240 preantral follicles were mechanically isolated. These preantral follicles were isolated three times for each group. To accomplish this, preantral follicles were separated in the KSOM medium (at 37°C) under a stereomicroscope using a 26-gauge needle. The isolated preantral follicles were selected if they (1) were normal preantral follicles with two or more compact granulosa cell layers and some adjoining theca cells and (2) had a clear, round oocyte in the center of the preantral follicles. The isolated preantral follicles were cultured in FBS (control), CM, PRP, or PRP+CM media.

4. PRP collection

Blood samples from female BALB/c mice were obtained via cardiac puncture and poured into a tube containing an anticoagulant agent (3.2% sodium citrate). The blood samples were then centrifuged at 250 × g for 10 minutes at 20°C. The supernatant was transferred to another tube and then centrifuged at 2,000 × g for 15 minutes. The two upper layers were removed, and the residual plasma, including precipitated platelets, was considered the PRP. Finally, the obtained
clots were centrifuged at 3,000 × g for 5 minutes at 4°C; the platelet fragments were separated and kept at −20°C for further experiments [27,29]. To count the platelets in the obtained PRP, the Sysmex XT-1600i system (Sysmex, Kobe, Japan) was utilized and revealed an average of 2,380 × 10³ platelets/mL.

5. Isolation and culture of endometrial stromal cells

In this investigation, female BALB/c mice were purchased from the Royan Institute in Iran. The animals in the estrus cycle were euthanized using narcosis induced by CO₂. Using sterile surgical instruments, the abdominal region was opened, and the intestines were pushed aside to observe both uterine horns. The uterine horns were isolated at the most distal portion of the Fallopian tube. Then, the uterine horns were dissected from the Fallopian tube, and surrounding connective and adipose tissues were removed. Afterward, the endometrial samples were obtained and washed in PBS [30-32].

Next, the samples were minced and incubated at 37°C with 0.2% HBSS with 15 mM HEPES, gentamicin (10 µg/mL), 2% (v/v) fetal calf serum, and 1 × antibiotic-antimycotic solution (fungizone [2.5 µg/mL], streptomycin [100 µg/mL], and penicillin [100 IU/mL]) for 30 minutes with shaking (50 rpm). Next, the samples were isolated according to Clercq De protocol [10].

1) Endometrial stromal cell-derived CM

When cells reached approximately 80% confluence, daily changes of medium were carried out for 24 hours; the mixture used included serum-free DMEM:F12 (1:1) medium added to insulin (10 µg/mL), selenium (6.7 ng/mL), transferrin (5.5 µg/mL), hydrocortisone (5 µg/mL), penicillin (100 IU/mL), fungizone (2.5 µg/mL), streptomycin (100 µg/mL), and gentamicin (10 µg/mL) [32].

6. 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

The survival rate of the preantral follicles was analyzed to determine the effective dose of PRP and endometrial cell-derived CM using the MTT assay. For this purpose, 60 preantral follicles were collected and randomly divided into six groups, then seeded in a 96-well plate. Each group contained 10 preantral follicles in an α-MEM culture medium, comprising 1% insulin-transferrin-selenium (ITS), ascorbic acid (50 µg/mL), penicillin-streptomycin (50 µg/mL), follicle-stimulating hormone (FSH; 100 mIU/mL), and luteinizing hormone (LH; 10 mIU/mL). After 24 hours, fresh media (200 µL) containing concentrations of either 5% PRP, 10% PRP, 5% CM, or 10% CM was added to four of the groups; the remaining two groups were not treated. The plates were incubated for 48 hours. A fresh medium (200 µL) containing an MTT agent (5 mg/mL in medium) was substituted and the plates incubated at 37°C for 4 hours. Then, dimethyl sulfoxide (50 µL) was substituted, and the plates were incubated at 37°C for 30 minutes. Subsequently, the optical densities of all wells were evaluated using an enzyme-linked immunoassay reader at a wavelength of 570 nm [29-33].

7. In vitro growth

Penicillin-streptomycin (50 µg/mL), 1% ITS, FSH (100 mIU/mL), LH (10 mIU/mL), and ascorbic acid (50 µg/mL). Moreover, each group was complemented with either 10% FBS (control), 10% PRP 10% CM, and 10% PRP+10% CM, then incubated for 8 days at 37°C. The medium was substituted once every 2 days [6,11,34].

8. In vitro maturation

After 8 days of culture, the follicles in the four groups were transferred to the culture medium supplemented with 1.5 IU/ml HCG and then incubated at 37°C. After 24 hours, the existence of polar bodies and the expansion of cumulus cells were evaluated. Mature oocytes were identified based on the identification of polar bodies via stereomicroscopy. Next, the mature follicles were assessed regarding the presence of the meiotic spindle (through fluorescence microscopy), relevant gene expression, and in vitro fertilization (IVF) [6].

9. In vitro fertilization

The oocytes accompanied by polar bodies in meiosis II were incubated with capacitated spermatozoa isolated from male BALB/c mice. Then, the oocytes were separated and transferred to a global medium (20 µL) under mineral oil at 37°C and 5% CO₂, and then the IVF rate in the cleavage stage was assessed [35].

10. Immunocytochemistry

The obtained meiosis II oocytes were fixed with a microtubule-stabilizing buffer containing Triton X-100 (0.1%), formaldehyde (3.7%), dithiothreitol (1 mM), Taxol (1 mM), deuterium oxide (50%), and aprotinin (0.01%). The oocytes were added to a phosphate-buffered saline blocking solution consisting of normal goat serum (2%), bovine serum albumin (1%), sodium azide (0.2%), powdered milk (0.2%), Triton X-100 (0.01%), and glycine (0.1 M) and were stored at 4°C. Chromatin was detected using the triple staining method. To do this, the oocytes were incubated for 4 hours in a mixture of mouse monoclonal alpha and beta anti-tubulins (Thermo Fisher Scientific, Waltham, MA, USA) at 1:500 final dilution and then were washed with a phosphate-buffered saline blocking solution and incubated in a 1:400 dilution of Alexa 488 goat anti-rabbit IgG (1:500) (Thermo Fisher Scientific) and rhodamine phalloidin (1:200) (Sigma, St. Louis, MS, USA) for 3 hours at 37°C. The oocytes were transferred to a glycerol-based medium with propidium iodide (1 ng/mL) on posts of Vaseline (1 mm) to stop the oocyte compression. A Labomed device (Labomed Inc., Culver City, CA, USA) was utilized for fluorescent mi-
croscopy, and photos were taken with a water immersion objective with stimulation lines at 400 nm (an argon laser for anti-tubulin) [11].

11. Real-time quantitative polymerase chain reaction

The extraction of total RNA from the preantral follicle after 9 days was performed using an RNA isolation kit according to the manufacturer’s instructions. Next, the synthesis of complementary DNA (cDNA) was conducted via reverse transcription. The mRNA expression levels of the BAX, BCL2, BMP15, and GDF9 genes were investigated using real-time quantitative PCR (RT-qPCR). The primer sequences used are presented in Table 1. The RT-qPCR (Applied Biosystems, Waltham, MA, USA) was carried out in a 10-μL volume including PCR pre-Mix (5 μL RR820L; Takara Bio Inc., Kusatsu, Shiga, Japan), forward/reverse primers (5 μM), and cDNA (1 μL) as follows: initial denaturation (1 minute at 94°C), denaturation (10 seconds at 94°C), annealing (30 seconds at 59°C), and extension (20 seconds at 72°C). The mRNA levels of the β-actin gene were evaluated as the endogenous control. Calculations were performed using the 2−ΔΔCt (Livak) formula [36].

12. Data analysis

Statistical analyses were carried out using GraphPad Prism statistical software (GraphPad Software, San Diego, CA, USA). The differences between groups were measured using the Tukey test, and statistical software (GraphPad Software, San Diego, CA, USA). The differences between groups were measured using the Tukey test, and statistical significance between more than two groups was investigated using the analysis of variance test. All obtained data were reported as mean ± standard deviation. A p-value of < 0.05 was considered to indicate statistical significance.

Results

1. Oocyte maturation

Preantral follicles with two or more compact granulosa cell layers were isolated three times for each group (Figure 1). The oocyte maturation rates in the four groups are presented in Figure 2. The maturation rate (based on the observation of polar bodies) of the group treated with CM was significantly greater than that of the control group (p < 0.01). Oocyte maturation was also dramatically greater in the PRP group than in the control group (p < 0.05). Finally, the oocyte maturation rate was significantly elevated in the PRP+CM group relative to the control group (p < 0.01).

2. The oocyte cleavage rate

The oocyte cleavage rate for each group is shown in Table 2. Our findings indicated that the cleavage percentage in the CM group was %31.25, in the PRP group was %20, and finally in the treated group with both CM and PRP was %41.17.

3. Preantral follicle viability

The viability of preantral follicles in the groups, analyzed to determine the effective dosage of PRP and CM, is exhibited in Figure 3. Our findings demonstrate that the viability of preantral follicles treated with 10% PRP was significantly greater than the viability in the groups treated with other concentrations of PRP (10% PRP vs. 5% PRP, p < 0.05; 10% PRP vs. 0% PRP, p < 0.05). Furthermore, the viability of the group exposed to 10% CM was significantly greater than the viability of those exposed to other CM concentrations (10% CM vs. 5% CM, p < 0.05; 10% CM vs. 0% CM, p < 0.01).

4. Meiotic spindle positioning

Immunocytochemistry analysis indicated the presence of meiotic spindle, meaning that these oocytes had proceeded in meiosis and attained meiotic competence. We found that the spindles were oriented parallel to the oocyte surface in the treatment groups (PRP, CM, and PRP+CM) (Figures 4 and 5).

5. Gene expression

The expression levels of the BAX, BCL2, BMP15, and GDF9 genes of the preantral follicles were cultured in the four groups are shown in Figure 6. The findings indicated that the expression levels of Bmp15, Gdf9, and Bcl2 were significantly upregulated in the preantral follicles of the PRP+CM group compared with the other groups. In contrast, the Bax gene was expressed most strongly in the control and PRP groups.

Discussion

In vitro, preantral follicle culture is a key method of fertility preservation for either women of reproductive age or prepubertal girls who lack hormonal stimulation or are at risk of cancer. Therefore, the

---

**Table 1.** The sequences and characteristics of the primers used

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDF9</td>
<td>F: AGGTGAGAAGCTGGAAATGG</td>
<td>160</td>
</tr>
<tr>
<td>BCL2</td>
<td>R: GTTGGAGAGTGGAGTGG</td>
<td>176</td>
</tr>
<tr>
<td>BAX</td>
<td>F: TTTGCTTCAGGGTTTCATCC</td>
<td>246</td>
</tr>
<tr>
<td>BMP15</td>
<td>R: CAGTTGAAGTTGCCGTCAGA</td>
<td>170</td>
</tr>
<tr>
<td>ACTB</td>
<td>F: ACCACCTTCAACTCATCATG</td>
<td>238</td>
</tr>
</tbody>
</table>

F, forward; R, reverse; bp, base pair.
cytes is still challenging [5]. Hence, in this work, the development of mouse preantral follicles using CM derived from endometrial cells and PRP was investigated for the first time. Because CM and PRP contain different cytokine and growth factors, they can impact ovarian follicle growth [18-19,27,29]. Thus, in this project, the effects of CM, PRP, and PRP+CM were evaluated on in vitro oocyte development, fertilization potential, quality of the meiotic spindle, and expression of related genes (BMP15, GDF9, BAX, and BCL-2). Our study revealed that the maturation rate of oocytes in all treatment groups (PRP, CM, and PRP+CM) was significantly greater than in the control group.

The impacts of PRP and CM on preantral follicle growth have been posited to be due to different growth factors, such as fibroblast growth factor, supporting the early stage of preantral follicle development [37]. In addition, previous studies have reported the stimula-

**Table 2. Cleavage rate**

<table>
<thead>
<tr>
<th>Group</th>
<th>Cleavage/MII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.10%/20</td>
</tr>
<tr>
<td>PRP</td>
<td>2.10%/20</td>
</tr>
<tr>
<td>CM</td>
<td>5.16%/31.25</td>
</tr>
<tr>
<td>PRP+CM</td>
<td>7.17%/41.17</td>
</tr>
</tbody>
</table>

MII, mature oocytes; PRP, platelet-rich plasma; CM, conditioned medium.


Figure 2. The maturation rate of oocytes (based on the observation of polar bodies) after in vitro growth (7 days) and in vitro maturation (24 hours). The level of oocyte maturation rate were significantly greater in all treatment groups than in the control group. PRP, platelet-rich plasma; CM, conditioned medium. Treatment group vs. control group,

a) $p<0.05$; b) $p<0.01$.

Enhancement of preantral follicle culture medium may help obtain a better embryo and improve the odds of successful pregnancy [11]. Numerous approaches to in vitro culture have been introduced for female gamete growth; however, the production of competent oo-
Figure 3. The survival rate of the preantral follicles was analyzed to determine the effective dose of platelet-rich plasma (PRP, A) and endometrial cell-derived conditioned medium (CM, B) using the MTT assay after 48 hours. The samples contained different concentrations of PRP and CM: 0% PRP, 5% PRP, and 10% PRP and 0% CM, 5% CM, and 10% CM. The preantral follicle survival rate was obtained three times for each group. a) $p<0.05$; b) $p<0.01$.

Figure 4. Fluorescent images of preantral follicle culture, including the meiotic spindle positioning in the mouse oocyte. The condensed cumulus is detached from the meiosis II oocytes. The meiosis II oocyte is shown with first polar body disjunction following in vitro growth and in vitro maturation, as well as a meiotic spindle (green) and chromosomes (red) after immunofluorescence staining. The spindles with spired poles are located in the oocyte cortex. An extra meiosis II with more common wide spindle poles and chromosome adjustment is depicted. Original magnification, ×400; scale bar, 100 μm. The meiotic spindle was oriented parallel to the oocyte surface in the treatment groups (platelet-rich plasma [PRP], conditioned medium [CM], and PRP+CM).
**Figure 5.** Meiotic spindle migration. The spindles are located in the oocyte cortex. The migration of the meiotic spindles from the center of the oocytes to the cortex occurred. The outer circle indicates the zona pellucida; microtubules are shown in green and chromosomes in red. PRP, platelet-rich plasma; CM, conditioned medium.

**Figure 6.** mRNA expression levels of the Bmp15 (A), Gdf9 (B), Bcl2 (C), and Bax (D) genes in the meiosis II oocytes in the four groups. The mRNA expression level in the preantral follicles was obtained three times for each group. PRP, platelet-rich plasma; CM, conditioned medium. PRP+CM group vs. control group: \(^{a}p<0.05, ^{b}p<0.01\); PRP+CM group vs. PRP group: \(^{c}p<0.05, ^{d}p<0.01\).
The investigation of Hosseini and colleagues indicated that PRP can improve the growth and viability of human preantral follicles in vitro [29]. Similarly, Adib et al. [35] declared that CM originating from human cumulus cells could enhance oocyte growth and maturation in vitro. However, in the present study, the maturation and cleavage rate in the PRP group were lower than in the CM and PRP+CM groups.

Another result was that meiotic spindles of the oocytes had proceeded to meiosis II in the treatment groups. Along these lines, researchers have posited that some growth factors, such as fibroblast growth factor, EGF, and TGF-β, play a substantial role in the resumption of meiosis in oocytes [38-40]. The spindles were oriented parallel to the oocyte surface in the CM, PRP, and PRP+CM groups, like the orientation of meiosis II spindles parallel to the oocyte cortex surface in the mice studied by McNally [41].

Molecular findings revealed that the expression levels of the BMP15, GDF9, and BCL2 genes were upregulated in the preantral follicles of the PRP+CM group relative to the other groups. In contrast, the BAX gene was less strongly expressed in this group than in the others, whereas BAX was most strongly expressed in the presence of PRP. BCL-2 and BAX are among the most important genes involved in apoptosis. The BCL-2 gene-encoded product inhibits apoptosis and promotes increased cell survival. In contrast, the BAX gene-encoded product induces apoptosis and causes increased cell death [42]. Increased expression of the BCL-2 gene can potentiate the viability and growth of preantral follicles in the presence of PRP+CM.

Likewise, downregulation of the BAX gene in the cultured preantral follicles exposed to PRP+CM can decrease the apoptosis rate. In the present study, we found greater expression of BAX in the PRP group than in the CM and PRP+CM groups. PRP has been reported to be involved in the formation of reactive oxygen species, leading to increased BAX expression [43,44]. In addition, the BMP15 and GDF9 genes, secreted by oocytes, can regulate granulosa cell proliferation and differentiation and promote preantral follicle growth [45]. Previous studies have reported that BMP15 present in the follicular fluid of mature follicles, which may be synthesized by the oocytes of these follicles, regulates cumulus cell expansion. Moreover, evidence suggests that GDF9 is found in mature oocytes [46]. The strong expression of BMP15 and GDF9 can explain the relatively high developmental competence of the preantral follicles in the PRP+CM group. Due to the different effects of PRP reported in our work and others on ovarian follicle culture, which may lead to different impacts on the viability of ovarian follicles, further investigation is warranted on reactive oxygen species in PRP and culture media.

This study suggests that the combination of PRP and CM can increase the growth and cleavage rate of mouse early preantral follicles, as well as the expression of related genes. Additionally, the expression of the BAX gene was increased in the PRP group relative to other groups. However, additional, large in vitro experimental investigations, particularly oxidative assessments of PRP in follicle culture, are required to validate these findings.

Conflict of interest

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

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Author contributions

Data curation: NT. Formal analysis: NT, FD, GM. Funding acquisition: FD, FA. Methodology: NZF. Writing–original draft: NT. Writing–review & editing: FD.

References

6. De Roo C, Tilleman K. In vitro maturation of oocytes retrieved from ovarian tissue: outcomes from current approaches and fu-
33. Sengupta J, Given RL, Carey JB, Wiltlauf HM. Primary culture of mouse endometrium on floating collagen gels: a potential in vi-


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Sperm DNA fragmentation negatively influences the cumulative live birth rate in the intracytoplasmic sperm injection cycles of couples with unexplained infertility

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Objective: This study aimed to determine the effect of sperm DNA fragmentation (SDF) on the cumulative live birth rate (CLBR) in intracytoplasmic sperm injection (ICSI) cycles in couples with unexplained infertility.

Methods: We conducted a prospective study of 145 couples who underwent ICSI cycles for unexplained infertility. Based on the SDF rate, patients were categorized into a low SDF group (SDF ≤30%, n=97) and a high SDF group (SDF >30%, n=48). SDF was assessed using the acridine orange test on density gradient centrifugation prepared samples. The CLBR was calculated as the first live birth event per woman per egg collection over 2 years.

Results: The high SDF group (SDF >30%) showed a significantly lower CLBR (p<0.05) and a significantly higher miscarriage rate (p<0.05) than the low SDF group (SDF ≤30%). No significant difference was observed in the implantation and cumulative pregnancy rates between the two SDF groups. The total number of embryo transfers was stratified further into fresh and frozen embryo transfers. In the fresh embryo transfers, there were significant differences in the implantation rates, clinical pregnancy rates, and live birth rates (p<0.05) between the low SDF and high SDF groups. However, in the frozen embryo transfers, there were no significant differences in clinical outcomes between the two groups. In the multivariable logistic regression analysis, SDF was a predictor of CLBR (p<0.05) when adjusted for possible confounding factors.

Conclusion: High SDF was associated with a lower CLBR and a higher miscarriage rate in the ICSI cycles of couples with unexplained infertility.

Keywords: Infertility; Intracytoplasmic sperm injection; Live birth rate; Sperm DNA fragmentation

Introduction

Infertility affects approximately 15% of couples of reproductive age [1]. Unexplained infertility refers to couples who fail to conceive despite having a female partner with healthy ovulatory function and patent fallopian tubes and a male partner with standard semen analysis results [2]. Although the underlying reasons for unexplained infertility have not been fully identified, increasing evidence suggests that sperm DNA fragmentation (SDF) should be considered [3-5].

Approximately 25%–80% of couples with unexplained infertility have elevated SDF values [4-6]. Reduced pregnancy and live birth rates with increased miscarriage rates were observed in couples with idiopathic infertility and > 25% SDF after in vitro fertilization (IVF) cycles [4]. Non-male factor infertility couples with SDF ≥ 30% have shown lower rates of normal cleavage speed, high-quality embryos at day 3, blastocyst development, blastocyst quality, and implantation...
tion in intracytoplasmic sperm injection (ICSI) cycles [7].

Previously, intrauterine insemination (IUI) and IVF were the first-line treatments for couples with unexplained infertility [5]. A meta-analysis concluded that, in cases of well-defined unexplained infertility, the use of ICSI was favored over IVF to increase fertilization rates and reduce the risk of total fertilization failure (TFF) [8]. Approximately 5%–25% of IVF cycles lead to TFF in couples with unexplained infertility, whereas ICSI has significant benefits and results in higher cumulative pregnancy rates [8,9].

Two types of assays measure the levels of SDF: one that directly measures the extent of DNA fragmentation using probes and dyes and another that measures the susceptibility of DNA to denaturation, which is higher in fragmented DNA [7]. The acridine orange test (AOT) exemplifies the second type of assay and differentiates sperm with normal double-stranded DNA (green fluorescence) and abnormal denatured or single-stranded DNA (orange-red fluorescence) with the help of the metachromatic shift properties of the stain [10,11]. AOT is a simple and affordable test for the assessment of DNA integrity in infertile men [11,12]. Clinical assessments of SDF by AOT must be performed on the total motile fraction of sperm rather than on raw ejaculate sperm, as raw semen contains a significant number of degenerated and dead sperm with damaged DNA [13].

A study showed that couples with unexplained infertility had elevated SDF but did not explore clinical correlations with the outcomes of the ICSI cycles [4]. In previous studies, SDF values were evaluated prior to assisted reproductive treatment (ART) and, to improve outcomes, the patients were allocated to IVF or ICSI based on their SDF values [5,6,14]. It has been reported that the negative effects of SDF on clinical outcomes are attenuated in young women with high-quality oocytes [15,16]. Only fresh transfers were considered in most of the studies; thus, the cumulative live birth rate (CLBR) was not measured [4,5]. The CLBR, which includes both fresh embryo transfer (ET) and frozen embryo transfer (FET) cycles, measures the success of ART cycles [17]. This study aimed to determine the effect of SDF on the CLBR in ICSI cycles in couples with unexplained infertility.

Methods

1. Study population

We conducted a prospective study of 145 couples with unexplained infertility (median age, 30.25 ± 4.33 years) who were undergoing their first ICSI cycles at the tertiary care center attached to our reproductive medicine unit at a medical college. This study was approved by the ethics committee of our institution. Written consent was obtained from all participating couples. A total of 145 ICSI cycles (one ICSI cycle per couple) were divided into two groups based on SDF rates: a low DNA fragmentation group (SDF ≤ 30%, n = 97) and a high DNA fragmentation group (SDF > 30%, n = 48) [7,18-21]. Clinical and laboratory outcomes were correlated between the two groups.

2. Inclusion and exclusion criteria

Couples undergoing their first ICSI cycles for unexplained infertility were included in this study. The diagnosis of unexplained infertility was based on the following criteria: (1) normal ovarian reserve with an antral follicle count ≥ 8 and anti-Müllerian hormone levels ≥ 1.5 ng/mL, (2) normal tubal patency and uterine function evaluated by diagnostic laparoscopy and hysteroscopy, and (3) normal semen parameters for the male partner according to World Health Organization (WHO) 2010 criteria [22]. None of the female partners were ≥ 41 years of age in this study population. Female partners with < 5 mature metaphase II oocytes and male partners with normal semen parameters (WHO 2010 criteria) altered on the day of transvaginal oocyte recovery (TVOR) or egg collection were excluded. Participants with life-threatening diseases such as cancer or chronic kidney disease were also excluded from the study.

3. Semen analysis and preparation

Patients collected semen samples in sterile, non-toxic containers by masturbation after sexual abstinence of 2–3 days. After 30 minutes of liquefaction, samples were evaluated for count, motility, and morphology according to the WHO 2010 criteria [22]. Semen samples were prepared using two-layer density gradient centrifugation (DGC; V-GRAD 80% and 40%, Vitromed, Jena, Germany) for ICSI. SDF was evaluated on the DGC-prepared semen samples.

4. Acridine orange test

The assessment of SDF was done using AOT [10]. Smears with 10 µL of post-wash samples were prepared and air-dried. Carnoy’s solution (methanol: glacial acetic acid, 3:1 vol/vol) was used to fix the slides overnight. The staining solution was prepared daily from a stock solution of acridine orange (1g/L in distilled water, stored in the dark at 4°C) at a ratio of 10 mL of stock solution to 40 mL of 0.1 M citric acid and 2.5 mL of 0.3 M sodium phosphate dibasic heptahydrate (Na2HPO4·7H2O), and the pH was adjusted to 2.5. The slides were stained with the above stain for 5 minutes, rinsed in distilled water, and covered with coverslips.

The slides were examined for SDF using a fluorescence microscope (Olympus CX31, Tokyo, Japan) under oil at × 1,000 with an excitation of 450–490 nm. Green fluorescence represented normal intact sperm, whereas red indicated fragmented and denatured sperm. Sperm with orange or yellow heads, as well as those display-
ing green and red colors simultaneously, were also considered fragmented [10,23]. At least 400 sperm were assessed in each slide to calculate the average SDF. Slides were fixed on the same day as semen preparation and examined the next day for SDF by AOT. A single highly skilled and trained andrologist evaluated all slides for consistency and to prevent interpersonal variability. Each stained slide was read immediately after staining to reduce variation in fluorescence intensity.

5. Ovarian stimulation

Controlled ovarian stimulation was started from day 3 of the menstrual cycle using recombinant follicle-stimulating hormone (Recagon, MSD; Gonal-F, Merck, Kenilworth, NJ, USA). A gonadotropin-releasing hormone antagonist (Cetrorelix Acetate, Emcure, Pune, India) was administered to suppress the pituitary function when a minimum of one follicle ≥ 14 mm was seen. Recombinant human chorionic gonadotropin (Ovidrel, Merck) was administered when three or more follicles reached a diameter of ≥ 17 mm and appropriate serum estradiol values were detected. TVOR was performed 35 hours after triggering with human chorionic gonadotropin.

6. ICSI procedure

The recovered oocytes were incubated in culture medium (One-step; Vitromed) for 1–2 hours at 37°C in an atmosphere of 6% CO₂, 5% O₂, and the remainder N₂. The oocytes were denuded by hyaluronidase (Hyadase 80 IU; Vitromed) at 37°C. The ICSI procedure, as described by Palermo et al. [24], was performed by a highly skilled embryologist. A morphologically normal and motile sperm was selected and immobilized in polyvinylpyrrolidone (PVP 7%; Vitromed). The immobilized sperm was aspirated tail-first into the injection pipette and injected into the oocyte. At 16–18 hours after ICSI, the oocytes that presented with two pronuclei and a second polar body were counted as fertilized. The fertilized zygotes were cultured until day 3 or day 5 of ICSI for ET or cryopreservation.

7. Embryo grading

According to the Istanbul consensus, day-3 embryos were graded as A, B, and C based on the blastomere number, fragmentation percentage, and multinucleation [25]. Grade A indicated a good embryo with stage-specific 6–8 blastomeres, < 10% fragmentation, and no multinucleation; grade B indicated a fair embryo with stage-specific 6–8 blastomeres, 10%–25% fragmentation, and no multinucleation; and grade C indicated a poor embryo with non-stage-specific blastomeres, severe fragmentation (> 25%), and the presence of multinucleation.

Day-5 blastocysts were graded according to Gardner and Schoolcraft [26]. Expansion of the blastocysts was graded 3 to 6, and trophoectoderm (TE) and inner cell mass (ICM) were graded as A, B, or C. Expansion of the blastocyst was graded as follows: 3, full; 4, expanded; 5, hatching; and 6, hatched. The TE was categorized as: grade A, a TE with many cells forming a cohesive epithelium; grade B, a TE with few cells forming a loose epithelium; and grade C, a TE with very few cells. Similarly, for ICM, the following grading was applied: grade A, a tightly packed ICM with many cells; grade B, a loosely grouped ICM with many cells; and grade C, an ICM with very few cells.

8. Embryo vitrification and warming

The surplus embryos were vitrified on either day 3 or day 5 by the Kitazato vitrification protocol (Kitazato, Japan) [27]. Briefly, the embryos were placed in an equilibration solution for 10–15 minutes at room temperature (RT), then transferred to a vitrification solution (VS1, VS2) for 1 minute, and later the embryos were loaded with minimum media onto the top of a vitrification device (Cryolock; Fujifilm/Irvine Scientific, Santa Ana, CA, USA). The device was plunged immediately into liquid nitrogen (LN₂), capped inside the LN₂, and then stored for future use.

Similarly, warming of the day-3 or day-5 embryos was done using the Kitazato thawing protocol (Kitazato) [27]. Briefly, the uncapped vitrification device from the LN₂ was placed directly in a thawing solution pre-warmed to 37°C and the embryos were allowed to float. After 1 minute, the embryos were transferred to a diluent solution at RT for 3 minutes and then transferred to a washing solution (WS1, WS2) for 5 minutes and 1 minute consecutively. The embryos were finally moved to a culture dish and incubated at 37°C in an atmosphere of 6% CO₂, 5% O₂, and the remainder N₂ until the ET.

9. Endometrium preparation

After oocyte retrieval in patients undergoing ET cycles, daily micronized progesterone was administered vaginally (Crinone 8% gel, Merck, Kenilworth, NJ, USA) and on alternate days intramuscularly (Hald 100 mg, Intas, Ahmedabad, India) until the pregnancy test was confirmed negative, or continued for an additional 3 months if the pregnancy test was positive.

In FET cycle patients, oral estradiol valerate (Evadiol, Intas, Ahmedabad, India) was used in a stepwise increasing dose pattern for preparation of the endometrium. The endometrial lining and thickness were observed regularly prior to the ET. Progesterone was administered in a method like that described in the ET cycles. For a day-3 or day-5 ET, 4 or 6 days of progesterone was administered, respectively.

10. Embryo transfer

ET was performed under abdominal guided ultrasound (a maximum of 3 embryos) on either day 3 or day 5, depending on the quality of the embryos and the age of the patient. The embryos were
transferred using a soft catheter (Cook, Brisbane, Australia). The serum β-hCG level was obtained 14 days after the transfer to confirm a positive pregnancy. Embryo utilization was calculated as the ratio of the number of embryos transferred and the number of embryos frozen to the total number of embryos formed. The high-quality embryo (grade A) rate at day 3 was calculated as the ratio of grade A embryos at day 3 to the total number of embryos cleaved. An intrauterine sac with the presence of a fetal heartbeat was considered a clinical pregnancy. The implantation rate was calculated as the proportion of gestational sacs determined by ultrasound to the total number of embryos transferred. Miscarriage was defined as a pregnancy loss after detection of an intrauterine pregnancy by ultrasound before 20 weeks. The CLBR was calculated as the first live birth event per woman per egg collection over 2 years.

11. Statistical analysis

Data were shown as mean ± standard deviation for continuous variables and analyzed using the unpaired Student t-test. The categorical variables were presented as proportions between two groups and analyzed using the chi-square test. A stratified analysis for potentially biasing factors such as day of transfer (day 3 and day 5) and type of transfer (fresh and frozen) on the CLBR was conducted using the chi-square test. The effect of SDF on the CLBR and the modifying effects of the biasing factors were assessed using logistic regression analysis. Multivariable logistic regression was used to analyze the effect of SDF on the CLBR and miscarriage rate while adjusting for possible confounders between the positive live birth group and the negative live birth group. Sample size calculation was done using G*Power version 3.1.9.7 (Franz Faul, University of Kiel, Germany), which indicated that 138 cycles would be adequate to demonstrate a 20% proportion difference with 80% power and a 5% significance level considering the miscarriage rate as the primary outcome. A p-value of < 0.05 was used to indicate statistical significance. The statistical analysis was executed using IBM SPSS ver. 21.0 (IBM Corp., Armonk, NY, USA).

Results

1. Demographic and embryological characteristics of couples with unexplained infertility in ICSI cycles

When the demographic and embryological characteristics of couples with unexplained infertility were compared between the two SDF groups (low SDF ≤ 30% and high SDF > 30%), similar findings were observed for the ages of the female and male partners, years of infertility, number of previous failed IUI cycles, number of oocytes retrieved, number of metaphase II oocytes, fertilization rates, cleavage rates, embryo utilization rates, number of transferred embryos, and grade A embryo rates at day 3. The only meaningful difference was observed in the number of ET cycles per ICSI. A higher number of ET cycles per ICSI (p = 0.018) was seen in the high SDF group compared to the low SDF group (Table 1).

2. Comparative analysis of semen parameters according to SDF group

Semen parameters such as sperm count, total sperm count, motility, progressive motility, and morphology were similar between the

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>SDF ≤ 30%</th>
<th>SDF &gt; 30%</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>97</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Female age (yr)</td>
<td>30.15 ± 4.27</td>
<td>30.44 ± 4.48</td>
<td>0.705</td>
</tr>
<tr>
<td>Male age (yr)</td>
<td>34.40 ± 4.64</td>
<td>34.85 ± 4.03</td>
<td>0.567</td>
</tr>
<tr>
<td>Year of infertility</td>
<td>2.98 ± 1.52</td>
<td>3.06 ± 1.47</td>
<td>0.785</td>
</tr>
<tr>
<td>No. of previous failed IUI cycles</td>
<td>1.94 ± 0.65</td>
<td>2.06 ± 0.69</td>
<td>0.334</td>
</tr>
<tr>
<td>No. of oocytes retrieved</td>
<td>14.83 ± 5.60</td>
<td>13.66 ± 4.41</td>
<td>0.209</td>
</tr>
<tr>
<td>No. of MII oocytes</td>
<td>12.60 ± 5.38</td>
<td>11.35 ± 4.26</td>
<td>0.162</td>
</tr>
<tr>
<td>Fertilization rate</td>
<td>84.08 ± 14.62</td>
<td>87.47 ± 14.30</td>
<td>0.188</td>
</tr>
<tr>
<td>Cleavage rate</td>
<td>82.19 ± 15.69</td>
<td>84.77 ± 16.74</td>
<td>0.364</td>
</tr>
<tr>
<td>Embryo utilization rate</td>
<td>65.82 ± 22.33</td>
<td>70.84 ± 22.42</td>
<td>0.206</td>
</tr>
<tr>
<td>Good quality embryo rate at day 3</td>
<td>41.18 ± 20.87</td>
<td>43.15 ± 23.66</td>
<td>0.610</td>
</tr>
<tr>
<td>No. of embryos transferred</td>
<td>2.11 ± 0.59</td>
<td>2.09 ± 0.56</td>
<td>0.818</td>
</tr>
<tr>
<td>No. of embryo transfer cycles</td>
<td>1.25 ± 0.48</td>
<td>1.46 ± 0.54</td>
<td>0.018</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviation.
ICSI, intracytoplasmic sperm injection; SDF, sperm DNA fragmentation; IUI, intrauterine insemination; MII, metaphase II.

*p<0.05.
two SDF groups, whereas a significant difference was observed in the SDF rates ($p < 0.001$) (Table 2).

### 3. Clinical outcomes in patients with unexplained infertility in ICSI cycles

A total of 145 patients underwent 191 ET cycles (both ET and FET). In the low SDF group, 97 patients underwent 97 ICSI cycles and 121 ET cycles, while 48 patients in the high SDF group underwent 48 ICSI cycles and 70 ET cycles. When the clinical outcomes between the two groups were compared, the high SDF group had a significantly lower CLBR ($p = 0.029$) and a significantly higher miscarriage rate ($p = 0.045$) than the low SDF group (Table 3). No significant differences in the implantation rates and cumulative pregnancy rates were observed between the two groups (Table 3). The cycles were further stratified according to the type of transfer (i.e., ET or FET). In the ET cycles ($n = 96$), 66 were in the low SDF group and 30 were in the high SDF group. The high SDF group had a significantly lower implantation rate ($p = 0.031$), clinical pregnancy rate ($p = 0.005$), and live birth rate ($p = 0.004$) than the low SDF group, although there was no significant difference in the miscarriage rate. In the FET cycles ($n = 95$), 55 were in the low SDF group and 40 were in the high SDF group, and no significant differences were found in the clinical outcomes between the groups (Table 3).

In the low SDF group, out of 121 ET cycles, 66 (54.54%) were ET cycles and 55 (45.45%) were FET cycles, whereas in the high SDF group, out of 70 ET cycles, 30 (42.85%) were ET cycles and 40 (57.14%) were FET cycles. There was no notable difference in the ET and FET cycles when the two groups were compared ($p = 0.119$) (Figure 1). In addition, ET cycles on day 3 and day 5 were also compared between the two groups. Couples in the low SDF group underwent 50 (41.32%) day-3 and 71 (57.85%) day-5 ET cycles, and couples in the high SDF group underwent 34 (48.57%) day-3 and 36 (51.42%) day-5 ET cy-

### Table 2. Comparative analysis of semen parameters according to SDF group

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SDF ≤ 30%</th>
<th>SDF &gt; 30%</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm count ($\times 10^6$/mL)</td>
<td>39.06 ± 11.92</td>
<td>35.66 ± 12.78</td>
<td>0.117</td>
</tr>
<tr>
<td>Total sperm count ($\times 10^6$)</td>
<td>94.85 ± 37.49</td>
<td>86.91 ± 36.39</td>
<td>0.228</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>58.79 ± 8.52</td>
<td>58.47 ± 8.24</td>
<td>0.833</td>
</tr>
<tr>
<td>Progressive motility (%)</td>
<td>45.64 ± 7.10</td>
<td>46.37 ± 7.23</td>
<td>0.228</td>
</tr>
<tr>
<td>Morphology (%)</td>
<td>5.18 ± 0.93</td>
<td>5.20 ± 0.89</td>
<td>0.889</td>
</tr>
<tr>
<td>SDF rate</td>
<td>14.19 ± 8.02</td>
<td>53.81 ± 16.28</td>
<td>$&lt; 0.001^a$</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation. SDF, sperm DNA fragmentation.

$^a p<0.001$.

### Table 3. Clinical outcomes of patients with unexplained infertility who underwent ICSI cycles

<table>
<thead>
<tr>
<th>Clinical outcome</th>
<th>SDF ≤ 30%</th>
<th>SDF &gt; 30%</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total embryo transfer cycles ($n = 191$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Implantation rate</td>
<td>38.61 (95/246)</td>
<td>29.10 (39/134)</td>
<td>0.063</td>
</tr>
<tr>
<td>Cumulative pregnancy rate</td>
<td>75.25 (73/97)</td>
<td>66.66 (32/48)</td>
<td>0.276</td>
</tr>
<tr>
<td>Cumulative live birth rate</td>
<td>60.82 (59/97)</td>
<td>41.66 (20/48)</td>
<td>0.029$^a$</td>
</tr>
<tr>
<td>Miscarriage rate</td>
<td>19.17 (14/73)</td>
<td>37.5 (12/32)</td>
<td>0.045$^a$</td>
</tr>
<tr>
<td>Fresh embryo transfers ($n = 96$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Implantation rate</td>
<td>34.72 (50/144)</td>
<td>20.00 (13/65)</td>
<td>0.031$^a$</td>
</tr>
<tr>
<td>Clinical pregnancy rate</td>
<td>66.66 (44/66)</td>
<td>36.66 (11/30)</td>
<td>0.005$^a$</td>
</tr>
<tr>
<td>Live birth rate</td>
<td>54.54 (36/66)</td>
<td>23.33 (7/30)</td>
<td>0.004$^a$</td>
</tr>
<tr>
<td>Miscarriage rate</td>
<td>18.18 (8/44)</td>
<td>36.36 (4/11)</td>
<td>0.191</td>
</tr>
<tr>
<td>Frozen embryo transfers ($n = 95$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Implantation rate</td>
<td>44.11 (45/102)</td>
<td>37.68 (26/69)</td>
<td>0.402</td>
</tr>
<tr>
<td>Clinical pregnancy rate</td>
<td>52.72 (29/55)</td>
<td>52.50 (21/40)</td>
<td>0.982</td>
</tr>
<tr>
<td>Live birth rate</td>
<td>41.81 (23/55)</td>
<td>32.50 (13/40)</td>
<td>0.355</td>
</tr>
<tr>
<td>Miscarriage rate</td>
<td>20.68 (6/29)</td>
<td>38.09 (8/21)</td>
<td>0.176</td>
</tr>
</tbody>
</table>

Values are presented as percent (positive number/total number). ICSI, intracytoplasmic sperm injection; SDF, sperm DNA fragmentation.

$^a p<0.05$.  

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There were no significant differences in the transfers \( (p = 0.330) \) among these groups (Figure 1). A minimum of one and a maximum of three ET cycles were done per ICSI.

4. Stratification of biasing factors and their effect on the CLBR

The day of transfer (day3/day5) and type of transfer (fresh/frozen) were considered as biasing factors. There was no significant difference in the live birth rate of the day-3 or day-5 transfers \( (p = 0.145) \). Similarly, there was no significant difference in the live birth rate between the fresh and frozen transfer cycles \( (p = 0.494) \) (Table 4). The biasing factors did not modify the effect of SDF as an independent predictor of cumulative live birth \( (\text{odds ratio [OR], } 0.986; 95\% \text{ confidence interval [CI], } 0.971–1.001; p = 0.071) \) when evaluated using logistic regression analysis. The day of transfer did not modify the effect of SDF on the probability of cumulative live birth \( (\text{OR}, 0.986; 95\% \text{ CI}, 0.971–1.002; p = 0.083) \) (Table 4).

5. Demographic and embryological characteristics of couples with unexplained infertility in live birth groups

The couples with unexplained infertility were divided into two groups based on live birth outcomes: (1) the positive live birth group and (2) the negative live birth group. These two groups showed significant differences in the ages of both male \( (p = 0.020) \) and female partners \( (p = 0.034) \), the embryo utilization rate \( (p = 0.023) \), and grade A embryos \( (p = 0.045) \). No remarkable difference was noted in the SDF rates, number of mature oocytes, fertilization rates, cleavage rates, number of embryos transferred, and number of ET cycles per ICSI between the two groups (Table 4).

6. SDF as a predictor of cumulative live birth and miscarriage in the ICSI cycles of couples with unexplained infertility

When adjusted for the possible confounders between the positive live birth and negative live birth groups, multivariate logistic regression analysis showed that SDF was a predictor of cumulative live birth \( (\text{OR}, 0.986; 95\% \text{ CI}, 0.971–1.002; p = 0.083) \) (Table 4).

---

**Table 4. Stratification of the biasing factors between positive live birth and negative live birth groups**

<table>
<thead>
<tr>
<th>Biasing factor</th>
<th>Positive live birth group</th>
<th>Negative live birth group</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day of transfer (day 3/day 5)</td>
<td>79 (30/49)</td>
<td>66 (33/33)</td>
<td>0.145</td>
</tr>
<tr>
<td>Type of transfer (fresh/frozen)</td>
<td>79 (38/41)</td>
<td>66 (28/38)</td>
<td>0.494</td>
</tr>
</tbody>
</table>

**Table 5. Demographic and embryological characteristics of couples with unexplained infertility subdivided into the live birth groups**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Positive live birth group</th>
<th>Negative live birth group</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>79</td>
<td>66</td>
<td>-</td>
</tr>
<tr>
<td>Female partner’s age (yr)</td>
<td>29.56 ± 3.77</td>
<td>31.08 ± 4.81</td>
<td>0.034 *</td>
</tr>
<tr>
<td>Male partner’s age (yr)</td>
<td>33.77 ± 3.92</td>
<td>35.48 ± 4.85</td>
<td>0.020 *</td>
</tr>
<tr>
<td>SDF rate</td>
<td>24.28 ± 19.07</td>
<td>30.92 ± 24.53</td>
<td>0.153</td>
</tr>
<tr>
<td>No. of MII oocytes</td>
<td>11.90 ± 5.14</td>
<td>11.73 ± 5.36</td>
<td>0.068</td>
</tr>
<tr>
<td>Fertilization rate</td>
<td>86.78 ± 12.67</td>
<td>83.31 ± 16.43</td>
<td>0.088</td>
</tr>
<tr>
<td>Cleavage rate</td>
<td>84.99 ± 14.16</td>
<td>80.71 ± 17.86</td>
<td>0.110</td>
</tr>
<tr>
<td>Embryo utilization rate</td>
<td>72.43 ± 22.66</td>
<td>63.96 ± 21.75</td>
<td>0.023 *</td>
</tr>
<tr>
<td>High-quality embryo rate</td>
<td>45.13 ± 21.16</td>
<td>37.88 ± 21.99</td>
<td>0.045 *</td>
</tr>
<tr>
<td>No. of embryos transferred</td>
<td>2.09 ± 0.59</td>
<td>2.24 ± 0.56</td>
<td>0.088</td>
</tr>
<tr>
<td>No. of embryo transfer cycles</td>
<td>1.28 ± 0.50</td>
<td>1.36 ± 0.52</td>
<td>0.347</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation.

SDF, sperm DNA fragmentation; MII, metaphase II.

*\( p<0.05 \).
Table 6. SDF as a predictor of cumulative live birth and miscarriage in ICSI cycles of unexplained infertility couples

<table>
<thead>
<tr>
<th>Clinical outcome</th>
<th>Adjusted OR</th>
<th>95% CI</th>
<th>p-value</th>
<th>Confounder adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumulative live birth</td>
<td>0.984</td>
<td>0.968–1.000</td>
<td>0.047</td>
<td>Female partner’s age, embryo utilization rate, high-quality embryo rate</td>
</tr>
<tr>
<td>Miscarriage</td>
<td>1.005</td>
<td>0.985–1.025</td>
<td>0.621</td>
<td>Female partner’s age, embryo utilization rate, high-quality embryo rate</td>
</tr>
</tbody>
</table>

SDF, sperm DNA fragmentation; ICSI, intracytoplasmic sperm injection; OR, odds ratio; CI, confidence interval.

Discussion

Routine semen analysis plays a salient role in the infertility evaluation of men. However, its role is minor for couples with unexplained infertility since sperm abnormalities at the DNA level cannot be identified by routine methods. SDF, rather than normal semen analysis, has good diagnostic and prognostic capabilities for men with idiopathic infertility based on routine semen parameters [28-30].

SDF can occur pre- or post-ejaculation due to various mechanisms, as described by Sakkas and Alvarez [31] and others [32]. The integrity of sperm DNA is necessary for proper fertilization and embryo development. A study suggested that early paternal effects, before embryonic genome activation, were not related to SDF, but that SDF was related to late paternal effects and could increase the risk of miscarriage [33]. Other studies also determined that the effect of SDF on pregnancy rates was modest in IVF cycles and had slight to no effect in ICSI cycles [4,7,34-37]. In contrast, some studies reported the negative effect of SDF on pregnancy rates in ICSI cycles [21,38,39]. In most studies, the correlation of SDF with clinical outcomes was limited to pregnancy rates only. According to a recent meta-analysis, very few studies correlated SDF with live birth rates in cycles of ICSI and even fewer correlated SDF with CLBR. This is the first study to correlate SDF with CLBR in couples with unexplained infertility undergoing ICSI cycles. The data on CLBRs provided by this study are particularly significant because both fresh and frozen ET outcomes were included in the analysis. It is challenging to report the CLBR, as the definition of this rate is inconsistent. In our study, the CLBR was defined as the first live birth event achieved from one TVOR/egg collection cycle over a period of 2 years [40]. All patients in the present study underwent at least one ET cycle after TVOR.

The main outcome measure of the present study was the CLBR in correlation with SDF in couples with unexplained infertility in ICSI cycles. The high SDF group had a 1.5-fold lower CLBR (p = 0.029) (Table 3) and a 2.0-fold higher miscarriage rate (p = 0.045) (Table 3) than the low SDF group. In this study, SDF was not correlated with fertilization, cumulative pregnancy, and implantation rates, but there was a trend for high SDF to be associated with a lower implantation rate (p = 0.063) (Table 3), which was also observed in other studies [7,41]. In the stratification of transfer cycles, ET cycles had significant differences in the clinical outcomes between the high and low SDF groups (Table 3), whereas in FET cycles, clinical outcomes were similar between the high and low SDF groups, probably because some patients underwent more than one FET cycle and tended to opt for the maximum number of embryos to be transferred (i.e., 3) due to previous failed cycles. Nonetheless, the analysis of all cycles showed significant associations between SDF and the live birth rate and miscarriage rates. Other studies have also found lower rates of implantation, clinical pregnancy, and live birth in ET cycles [4,7,41]. As reported in other studies, we also found no remarkable differences in the grade A embryo rate at day 3 in both SDF groups after ICSI cycles [34,35,37]. In contrast, some studies contradicted these results and reported poor quality embryo outcomes in the high SDF group [7,36]. In most studies, the embryo utilization rate was not mentioned because only ET cycles were considered [4,5,7].

ICSI has been the most favored method for treating couples with well-defined idiopathic infertility [8]. It was evident from a previous study that couples can achieve a higher take-home baby rate with ICSI cycles rather than with conventional IVF cycles [42]. Therefore, in this study, all couples underwent ICSI irrespective of the SDF percentage. In most studies, SDF was evaluated prior to the ART cycles and IVF or ICSI cycles were chosen based on the SDF values, or samples were frozen and/or evaluated when needed [5,41,43,44]. In this study, the SDF was evaluated in the actual sperm to be used for the ICSI cycles and clinically correlated in an unbiased manner to improve the outcome.

The negative correlation of SDF with the live birth rate in IVF cycles was established in a recent meta-analysis [14] where the pooled data of six studies identified a negative correlation between SDF and live birth rates in ICSI cycles. However, the detrimental effect was nullified in a sub-group analysis that only included studies with female factors (age and ovarian reserve). Further studies on this issue are needed [14].

One study reported a significant difference in the live birth rate in IVF cycles with a high SDF and, to a lesser degree, in ICSI cycles; the weaker findings in ICSI cycles can be explained by the fact that there were many fewer patients in the low SDF group (<25%). The same study showed an approximately 12% lower live birth rate in ICSI patients with SDF of 25%–50% compared to those with SDF >50% [4]. Similarly, other studies stated that couples in the high SDF groups had lower rates of ongoing pregnancy in ICSI cycles, which was corroborated by the present study [41,43]. In contrast, other studies reported no significant correlation between the live birth rate and SDF.
SDF was positively correlated with the miscarriage rate in this study at a threshold of 30%. Spontaneous abortion rates were higher in ICSI cycles with SDF > 30%, as reported by Zini et al. [36]. In a meta-analysis by Robinson et al. [45], a review of 16 studies and other recent studies corroborated that SDF was positively correlated with spontaneous miscarriage [7,46]. Even with optimizations such as semen sample preparation by DGC, morphologically good sperm selection through ICSI, and the selection of high-quality embryos for transfer, the miscarriage rates were significant when correlated with SDF in this study. As mentioned earlier, this may be attributed to the late paternal effect of male gene expression [33].

To some extent, the effect of SDF on the clinical outcome depends on the quality of the oocyte [15]. Sperm depends on the oocyte for post-fertilization DNA repair, and high-quality oocytes can help mitigate the effect of SDF on pregnancy outcomes [15]. The female partners were significantly younger in the positive live birth group (29.56 years) than in the negative live birth group (31.08 years, \( p = 0.034 \) (Table 5). Growing evidence suggests that high-quality oocytes from younger women can overcome the effect of SDF on pregnancy outcomes [15,16], as corroborated by the present study.

The SDF, fertilization, and cleavage rates showed no notable differences between the live birth groups. In the positive live birth group, the grade A embryo rate was higher \( (p = 0.045) \) (Table 5), which led to a higher embryo utilization rate \( (p = 0.023) \) (Table 5). This may be attributed to the young female partners with high-quality oocytes in the positive live birth group as compared to the negative live birth group, whose female partners were comparatively older. High-quality oocytes have the capacity to repair damaged sperm DNA even despite SDF. Similar conclusions have been proposed in other studies [15]. Since female age, embryo utilization rate, and the grade A embryo rate showed statistically significant differences between the live birth groups, they were considered as confounding factors. When the effect of SDF on the cumulative live birth was adjusted for these confounding factors, SDF was a significant predictor of cumulative live birth \( (p = 0.047) \) (Table 6) in the ICSI cycles of couples with unexplained infertility. The effect of SDF on the CLBR was not significant \( (p > 0.05) \) (Table 4) when modified by these biasing factors; therefore, they were not considered as confounding factors for the CLBR in couples with unexplained infertility.

AOT is an established method for assessing the integrity of sperm DNA in infertile men [11,12]. Using AOT, an unfavorable effect of SDF on pregnancy and implantation rates was found in the high SDF group in ICSI cycles [21], and this finding has clinical significance for patients with repeated early pregnancy loss [46]. The miscarriage rate was directly correlated with SDF in this study. Of the 16 studies included in a meta-analysis on SDF and miscarriage, eight used AOT, six used the TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end (TUNEL) assay, and two used the comet assay [45].

The AOT method is simple, inexpensive, and convenient to use routinely in-house. The principle of AOT is similar to that of the sperm chromatin structural assay (SCSA) except for the number of sperm counted. In this study, a trained and technically skilled in-house embryologist evaluated the slides for SDF. We have been using the AOT method to assess SDF since 2012 for various research projects [47]. Although the AOT is not as robust as the SCSA, the cells can be differentiated easily, and the SDF rate can be evaluated technically. However, there is a lack of consistency across studies regarding the threshold value for AOT, which is set at 30%–50% for clinical correlations [12,21,48]. In this study, at a threshold value of 30%, the SDF was inversely correlated with the CLBR and directly correlated with the miscarriage rate of ICSI cycles in couples with unexplained infertility.

The percentage of couples with high SDF in this study was approximately 33% of all couples with unexplained infertility. The low percentage compared to other studies may have been due to the use of prepared sperm samples to evaluate SDF rather than raw semen samples [4,5]. Although ICSI was performed in order to optimize outcomes in all couples, the couples with high SDF needed to undergo a significantly higher number of ET cycles \( (p = 0.018) \) (Table 1) than the low SDF group, which is both financially and emotionally burdensome to couples. Furthermore, even with more ET cycles, the fertility rate was significantly lower in the high SDF group than in the low SDF group. After a negative result, many couples did not return for another transfer even if they have embryos frozen. Therefore, treatment interventions to reduce the SDF such as antioxidant therapy, lifestyle modifications, and dietary supplements [49-52], or the use of techniques (e.g., microfluidics and magnetic-activated cell sorters) to select sperm with low or barely detectable levels of SDF without further damaging the sperm cells can be used to improve the clinical outcomes [53,54].

Despite the valuable results obtained in the study, the authors recognize its limitations. The sample size was small because only couples with unexplained infertility who underwent ICSI cycles were included. The AOT method may not be as robust as the gold-standard SCSA method but, as already mentioned, the AOT method is simple, inexpensive, and comparable to the SCSA method. We were unable to calculate the blastulation rate as some patients underwent both day-3 and day-5 ET cycles. Finally, SDF is a contributing factor along with other confounders, not an independent predictor of CLBR in the ICSI cycles of couples with unexplained infertility.

In conclusion, SDF negatively influenced the CLBR, and a high SDF
was associated with a higher miscarriage rate in the ICSI cycles of couples with unexplained infertility. These findings suggest that there is a need to evaluate SDF prior to ART cycles in couples with unexplained infertility to enable better counseling.

Conflict of interest

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

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Author contributions

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

References


47. Repalle D, Chittawar PB, Bhandari S, Joshi G, Pananjape M, Joshi C. Does centrifugation and semen processing with swim up at 37°C yield sperm with better DNA integrity compared to centrifugation and processing at room temperature? J Hum Reprod Sci 2013;6:23–6.


Sperm DNA fragmentation in consecutive ejaculates from patients with cancer for sperm cryopreservation

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Objective: This prospective consecutive study investigated the variation in sperm DNA fragmentation (SDF) in multiple semen samples from patients with cancer.

Methods: Eighty-one patients with various cancers underwent multiple semen collections on 3 consecutive days for sperm cryopreservation prior to cancer treatment. A commercial Halosperm kit was used to measure SDF. Within- and between-subject coefficients of variation were estimated via random-effects analysis of variance to assess the consistency of semen parameters and SDF. Intraclass correlation coefficients (ICCs) were calculated to assess the magnitude of the between-subject component of variance relative to the total variance.

Results: The volume of semen in the day-2 and day-3 samples was significantly lower compared with the day-1 sample. Most parameters showed high ICC values, suggesting that within-subject fluctuations were small relative to the between-subject variability. The highest ICC values were identified for the SDF (ICC, 0.68; 95% confidence interval [CI], 0.45–0.84) and semen volume (ICC, 0.67; 95% CI, 0.45–0.84).

Conclusion: Our findings showed that repeated ejaculates from patients with cancer had stable SDF levels.

Keywords: Cryopreservation; DNA fragmentation; Fertility preservation; Semen analysis; Sperm

Introduction

The number of male cancer survivors of reproductive age has been steadily increasing, and concerns for the quality of life of patients with cancer, including fertility preservation, have received widespread attention. Sperm cryopreservation has been strongly recommended before cancer treatment since sperm quality may decrease posttreatment [1,2]. To obtain the desired number of sperm samples for cryopreservation, male patients are required to ejaculate multiple times within several days.

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The World Health Organization (WHO) recommends that semen be collected after abstinence for 3–7 days [3]. To collect enough samples, the American Society of Clinical Oncology guideline recommends that sperm banking be performed quickly, at 24-hour intervals [4]. To the best of our knowledge, only one study has evaluated the quality of semen in male patients with cancer who underwent several sessions of ejaculation within a short period [5]. When analyzing the consistency of conventional semen parameters, repeated ejaculates did not show significant variation in semen quality over a maximum of 5 consecutive days. However, we cannot conclude that the sperm is completely normal because conventional semen parameters do not include all functions of sperm.

In current clinical practice, the evaluation of male fertility is largely dependent on conventional semen analysis. However, conventional semen analysis can be unreliable for predicting in vitro fertilization (IVF) outcomes. To overcome these limitations, the use of sperm DNA fragmentation (SDF) analysis has gained increasing popularity. Recent studies [6-8] have demonstrated that SDF levels have a signifi-
cant association with IVF outcomes. In IVF/intracytoplasmic sperm injection (ICSI) cycles, a high SDF level was shown to be associated with low embryo formation rates [6]. A high SDF level was also associated with a high miscarriage rate [7]. A meta-analysis including 13 prospective studies showed that male partners with a history of recurrent pregnancy loss have significantly higher levels of SDF compared with fertile control participants [8].

There are various techniques for measuring SDF, including the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling assay, sperm chromatin structure assay, Comet assay, and sperm chromatin dispersion (SCD) assay. The SCD assay is widely used because it is simple, quick, and highly reproducible [9]. Several studies [10-12] have reported on the consistency of semen parameters using within-subject coefficients of variation (CVw). However, variation in the semen quality of repeated ejaculates related to SDF has never been investigated, especially in patients with cancer. In the present study, we used the SCD assay to analyze the variation of conventional semen parameters as well as SDF in patients with cancer who visited our sperm bank clinic before cancer treatment.

Methods

1. Subjects

Eighty-one patients with various cancers underwent one or multiple semen collections for sperm cryopreservation between 2016 and 2017 at the Seoul National University Bundang Hospital. Germ cell tumors (16 patients) and lymphomas (16 patients) were the two most common cancers, followed by gastrointestinal cancer (15 patients) and leukemia (7 patients). The mean age of the patients at the time of semen collection was 27.5 ± 7.5 years (range, 14–42 years) and most were not married. None of the patients had received chemotherapy before semen collection. The Institutional Review Board of the Seoul National University Bundang Hospital approved the use of individual data from patients’ medical records (No. B-1403-242-102). All participants provided written informed consent.

2. Laboratory analysis

Semen collections were repeated one to five times (mean, 2.6 ± 0.8) for each patient within a maximum 5 days. All semen samples were obtained in sterile containers by masturbation. After liquefaction for 30 minutes at room temperature (RT), routine sperm quality was assessed via a computer-assisted semen analysis system (SAIS-PLUS 10.1; Medical Supply, Seoul, Korea) within 1 hour of collection. The evaluated semen parameters were semen volume (mL), sperm concentration (×10^6/mL), total sperm count (semen volume × sperm concentration), progressive motility (%), and total motile count (TMC) (semen volume × sperm concentration × progressive motility/100). To ensure accuracy of the results, a manual assessment was also performed.

For the SCD assay, a Halosperm kit (Halotech DNA, Madrid, Spain) was used, as described previously [13]. The semen samples (25 µL) were mixed with pre-warmed agarose gel and dropped onto slides. The slides were covered with a glass coverslip and kept in a refrigerator for 5 minutes at 4°C to create a microgel with the implanted sperm. The coverslip was then removed and the slides were immersed in a prepared acid solution (80 µL of hydrogen chloride in 10 mL of distilled water) for 7 minutes at RT. The slides were then transferred to the tray with a lysis solution and incubated for 25 minutes at RT. The slides were rinsed with distilled water for 5 minutes, followed by dehydration in increasing concentrations of ethanol (70%, 90%, and 100%, for 2 minutes each). After drying, the slides were stained with Diff-Quik (Baxter Diagnostics Inc., McGaw Park, IL, USA), rinsed under tap water, and air-dried at RT.

Each slide was examined under a light microscope at ×400 magnification, and at least 200 sperms were assessed for halo patterns. Each sperm was categorized as having a large halo, medium halo, small halo, no halo, or degraded. Sperms with a small halo or no halo and degraded sperms were classified as sperms with fragmented DNA. The SDF level was the percentage of sperms with fragmented DNA per total sperms.

3. Data analysis

We initially obtained 172 semen analysis results from 97 male patients. The semen samples collected on the first day were regarded as day-1 (D1) samples. There were 81 D1 samples, 51 day-2 (D2) samples, 20 day-3 (D3) samples, 11 day-4 (D4) samples, and 9 day-5 (D5) samples. Because the D4 and D5 samples were too small, we only analyzed the D1, D2, and D3 samples (81 men, 152 samples). The semen analysis results of the D2 and D3 samples were compared with the results of the D1 samples using the Wilcoxon signed-rank test. Based on our previous study [5], we selected the parameters (volume, concentration, motility, and SDF) that we thought were the most meaningful. The relationship between SDF levels and the other semen parameters was evaluated using linear regression analysis.

The coefficient of variation (CV) was calculated as the square root of the variance component estimate divided by the overall mean and expressed as a percentage (CV = [standard deviation/mean] × 100). Correlations between the within-subject standard deviation and individual means were analyzed using the Spearman correlation test. To compare the size of the between-subject coefficient of variance (CVb) to the total (between- and within-subject) component of variance, intraclass correlation coefficients (ICCs) in a two-way random effects model were used. In this setting, the ICC could estimate how
strongly repeated measures in the same individual were correlated,
thereby providing a measure of the within-subject consistency (sta-
bility) of the semen parameters. A high ICC value indicates that with-
in-subject fluctuations were small relative to the between-subject
variability. In the present study, the following scale was used to inter-
pret reliability: excellent, > 0.75; good, 0.60–0.74; fair, 0.40–0.59; and
poor, < 0.4.

Results

Semen volume, sperm concentration, motility, and SDF levels from
the D1 to D3 samples are depicted in Figure 1 as box and whisker
plots. As shown in Table 1, semen volume and sperm concentration
were significantly reduced in the D2 and D3 samples when compared
with the D1 samples. Total sperm count, motility, and TMC were not
changed significantly in D1 through D3 samples. The SDF level was
significantly reduced in D2 samples only, when compared with D1
samples. Correlations between SDF levels and other semen parame-
ters are shown in Table 2. There was no association between SDF lev-
els and semen volume, sperm concentration, or total sperm count in
the D1, D2 and D3 samples. The SDF level had a significant negative
relationship with motility in the D1 samples only ($r = -0.273$,
$p = 0.014$).

The CVw, CVb, and ICC values for various semen parameters are
presented in Table 3. All analyzed parameters showed higher be-
tween-subject variability than within-subject variability. Semen vol-
ume and motility demonstrated the smallest degree of variation,
both within and between subjects. Semen volume and motility
showed the lowest CVw (26.4 and 27.4, respectively), whereas total
sperm count and TMC showed the highest CVw (68.3 and 58.0, re-
spectively).

Semen volume and SDF showed an ICC value with good reliability
(between 0.60 and 0.74). The ICC value of SDF was highest (0.68;
95% confidence interval [CI], 0.45–0.84), followed by semen volume
(0.67; 95% CI, 0.45–0.84). The ICC values of total sperm count and
sperm concentration were less than 0.40.

Figure 1. Box whisker plots showing consecutive changes in semen volume (A), sperm concentration (B), sperm motility (C), and sperm DNA
fragmentation (SDF; D) levels from day 1 to day 3 semen samples in 81 patients with cancer (number of samples: day 1, 81; day 2, 51; day 3, 20).
Discussion

All semen parameters of the D1, D2, and D3 samples were maintained within the normal range according to the 2010 WHO guidelines [3]. This suggested that the presence of various cancers did not significantly affect semen quality in the study group. Although there have been conflicting results, previous studies have shown normal semen analysis of patients with various cancers (except testicular cancer) [14,15]. This study confirmed that repeated ejaculates from patients with a variety of cancers maintained good sperm quality, as we also reported in a previous study [5]. In our data, the median SDF level was 17.0% in D1 samples, 15.7% in D2 samples, and 17.8% in D3 samples. All values were less than 30% (a cutoff point suggested by previous literature [16-18]), which supports the finding that repetitive ejaculates from patients with a variety of cancers maintained relatively stable DNA integrity during the 3 days of sperm collection.

The conventional semen parameters of repetitive ejaculates in the same individual are known to have a wide CV due to high biological variation [12,19]. Therefore, at least two semen samples should be examined after 3-7 days of ejaculatory abstinence to assess the fertility of male partners [3]. In the present study, the CVw ranged from 26.4%–68.3% and the CVb ranged from 41.2%–181.0%. Similar results were observed in our previous study (CVw, 17.2%–51.5%; CVb, 29.6%–146.8%) [5], as well as another study on healthy men [12]. The present results showed that the CVb in all parameters was higher than the CVw, and that sperm concentration showed the highest

Table 1. The median values of semen parameters from the first day sample to the third day samples

<table>
<thead>
<tr>
<th>Variable</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>81</td>
<td>51</td>
<td>20</td>
</tr>
<tr>
<td>Semen volume (mL)</td>
<td>2.5 (0.6–7.5)</td>
<td>2.0 (0.5–5.0)*</td>
<td>2.0 (0.5–4.5)*</td>
</tr>
<tr>
<td>Total sperm count (×10⁹)</td>
<td>181.0 (9.4–1710)</td>
<td>115.8 (6.0–7470)</td>
<td>105.8 (40.5–473)</td>
</tr>
<tr>
<td>Sperm concentration (×10⁹/mL)</td>
<td>98.0 (9.0–261.0)</td>
<td>50.5 (4.0–442)*</td>
<td>56.0 (13.0–133.1)*</td>
</tr>
<tr>
<td>Sperm motility (%)</td>
<td>44.8 (8.9–84.1)</td>
<td>43.7 (3.5–88.6)</td>
<td>42.9 (20.8–96.1)</td>
</tr>
<tr>
<td>Total motile sperm count (×10⁶)</td>
<td>70.4 (2.4–1049.6)</td>
<td>52.4 (0–2888.6)</td>
<td>64.7 (12.2–379.4)</td>
</tr>
<tr>
<td>Sperm DNA fragmentation (%)</td>
<td>17.0 (5.0–78.3)</td>
<td>15.7 (2.0–71.0)*</td>
<td>17.8 (2.7–51.7)</td>
</tr>
</tbody>
</table>

Values are presented as median (range).
*p<0.05 when compared with day 1 sample (Wilcoxon signed-rank test).

Table 2. Correlation coefficients between sperm DNA fragmentation level and other semen parameters from the first day sample to the third day samples

<table>
<thead>
<tr>
<th>Variable</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p-value</td>
<td>r</td>
</tr>
<tr>
<td>Semen volume (mL)</td>
<td>0.189</td>
<td>0.090</td>
<td>0.235</td>
</tr>
<tr>
<td>Total sperm count (×10⁹)</td>
<td>0.166</td>
<td>0.138</td>
<td>0.069</td>
</tr>
<tr>
<td>Sperm concentration (×10⁹/mL)</td>
<td>0.138</td>
<td>0.220</td>
<td>−0.055</td>
</tr>
<tr>
<td>Sperm motility (%)</td>
<td>−0.273</td>
<td>0.014</td>
<td>0.220</td>
</tr>
<tr>
<td>Total motile sperm count (×10⁶)</td>
<td>0.036</td>
<td>0.751</td>
<td>−0.041</td>
</tr>
</tbody>
</table>

Number of samples: day 1, 81; day 2, 51; day 3, 20.

Table 3. Within-subject coefficients of variation, between-subject coefficients of variation, and intraclass correlation coefficients of semen parameters in three-times ejaculates from 81 men

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CVw (%)</th>
<th>CVb (%)</th>
<th>ICC</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semen volume (mL)</td>
<td>26.4</td>
<td>50.5</td>
<td>0.67</td>
<td>0.45 to 0.84</td>
</tr>
<tr>
<td>Sperm count (×10⁹)</td>
<td>58.0</td>
<td>155.1</td>
<td>0.36</td>
<td>0.09 to 0.64</td>
</tr>
<tr>
<td>Sperm concentration (×10⁹/mL)</td>
<td>47.4</td>
<td>75.0</td>
<td>0.19</td>
<td>−0.05 to 0.49</td>
</tr>
<tr>
<td>Sperm motility (%)</td>
<td>27.4</td>
<td>41.2</td>
<td>0.43</td>
<td>0.15 to 0.69</td>
</tr>
<tr>
<td>Total motile sperm count (×10⁶)</td>
<td>68.3</td>
<td>181.0</td>
<td>0.42</td>
<td>0.15 to 0.68</td>
</tr>
<tr>
<td>Sperm DNA fragmentation (%)</td>
<td>33.3</td>
<td>72.6</td>
<td>0.68</td>
<td>0.45 to 0.84</td>
</tr>
</tbody>
</table>

CVw, within-subject coefficients of variation; CVb, between-subject coefficients of variation; ICC, intraclass correlation coefficients; CI, confidence interval.
variation, as reported in previous studies [5, 10].

To the best of our knowledge, this is the first report to demonstrate the consistency of SDF during repetitive semen collections. The SDF levels in the present study were much higher in the CVb than the CVw, which suggests that SDF is highly individual. Because SDF levels showed relatively low CVw and high ICC values, the SDF level was a highly reliable parameter among several semen parameters.

There have been several studies analyzing the association between SDF and semen parameters [16, 20-22]. Nevertheless, the conclusions are still unclear and controversial. In the present study, SDF levels in the D1 samples had a significantly negative relationship with motility, which is consistent with previous reports [16, 20, 21]. The non-association between SDF levels and motility in the D2 and D3 samples might be attributed to the small number of samples. The association between SDF level and sperm concentration has shown conflicting results [16, 22]. In our study, the SDF level showed no relationship with sperm concentration. One reason to consider is that the patients included in this study were relatively younger than in other studies.

In conclusion, we demonstrated that repeated ejaculates from patients with a variety of cancers did not show a substantial variation in SDF levels. Further large-scale studies are required to investigate the sperm quality of repeated ejaculates, including D4 and D5 samples.

Conflict of interest

Byung Chul Jee is an Editor-in-Chief and Seul Ki Kim is an Associate Editor of the journal, but they were not involved in the peer reviewer selection, evaluation, or decision process of this article. No other potential conflicts of interest relevant to this article were reported.

Acknowledgments

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References

13. Kim SW, Nho EJ, Lee JY, Jee BC. Specific tail swelling pattern in hy-


Correlation of oocyte number with serum anti-Müllerian hormone levels measured by either Access or Elecsys in fresh in vitro fertilization cycles

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Objective: The aim of this study was to assess the correlation of oocyte number with serum anti-Müllerian hormone (AMH) levels measured by two automated methods (Access or Elecsys) in fresh stimulated in vitro fertilization (IVF) cycles.

Methods: In this retrospective study at a university hospital, data were collected from 243 fresh stimulated IVF cycles performed from August 2016 to December 2020. The serum AMH level was measured by Access in 120 cycles and by Elecsys in 123 cycles. The cut-off of serum AMH for prediction of poor responders (three or fewer oocytes) or high responders (15 or more oocytes) was calculated by the receiver operating characteristic curve analysis.

Results: For the two automated methods, the following equations were derived: total oocyte number = 2.378 + 1.418×(Access-AMH) (r = 0.645, p < 0.001) and total oocyte number = 2.417 + 2.163×(Elecsys-AMH) (r = 0.686, p < 0.001). The following combined equation could be derived: (Access-AMH) = 0.028 + 1.525×(Elecsys-AMH). To predict poor responders, the cut-off of Access-AMH was 1.215 ng/mL (area under the curve [AUC], 0.807; 95% confidence interval [CI], 0.730–0.884; p < 0.001), and the cut-off of Elecsys-AMH was 1.095 ng/mL (AUC, 0.848; 95% CI, 0.773–0.923; p < 0.001). To predict high responders, the cut-off of Access-AMH was 3.450 ng/mL (AUC, 0.922; 95% CI, 0.862–0.981; p < 0.001), and the cut-off of Elecsys-AMH was 2.500 ng/mL (AUC, 0.884; 95% CI, 0.778–0.991; p < 0.001).

Conclusion: Both automated methods for serum AMH measurement showed a good correlation with oocyte number and good performance for predicting poor and high responders in fresh stimulated IVF cycles. The Access method usually yielded higher measured serum AMH levels than the Elecsys method.

Keywords: Anti-Müllerian hormone; In vitro fertilization; Infertility; Oocyte

Introduction

Anti-Müllerian hormone (AMH) is a dimeric glycoprotein that is a member of the transforming growth factor β family. It is produced in the Sertoli cells of testes and plays a role in male sexual differentiation [1]. AMH is also produced in the granulosa cells of pre-antral and small antral follicles in women [2,3]. The serum AMH level is widely used to assess ovarian reserve and predict the ovarian response to an exogenous gonadotropin in in vitro fertilization (IVF) cycles [1,4-9]. It has been shown that the serum AMH level has similar or better performance than the antral follicle count (AFC) for predicting the oocyte yield in stimulated IVF cycles [10]. Measurement of serum AMH by enzyme-linked immunosorbent assays was first reported in the 1990s; this so-called first-generation
AMH assay was developed and produced both by Diagnostic Systems Lab (DSL) and Immunotech (IOT). Each company’s assay used different primary antibodies against AMH and different calibrators, resulting in different values when the same sample was analyzed [11]. The DSL antibody and IOT standard calibrators were later combined in 2010, and the second-generation (Gen II, original) assay was developed by Immunotech Beckman Coulter [12,13]. Shortly thereafter, the revised Gen II assay was introduced by adding a pre-mix step with new AMH reference ranges in 2013 [3]. The revised Gen II-AMH level was usually somewhat higher than the original Gen II-AMH level [3].

In 2015, fully automated AMH assays were released by Beckman Coulter (Access) and by Roche (Elecsys) [14,15]. The automated assay uses recombinant AMH as a calibrator, thereby reducing the test time and improving sample instability or variability. Ultimately, the reproducibility was quite substantially improved compared to the previous manual methods [16-19]. Tadros et al. [19] reported that, on average, Access-AMH levels were 16% lower and Elecsys-AMH levels were 20% lower than the levels reported using the revised Gen II assay in patients with reduced AFC. Therefore, the AMH levels measured by the automated assays are considered to be similar to those obtained using the original Gen II assay [18]. However, Access-AMH showed a better correlation with oocyte number than the revised Gen II assay [15,18]. Theoretically, both Access-AMH and Elecsys-AMH levels in a single person would be expected to be similar because both methods use the same antibody. Nonetheless, the possibility of a difference in these measured values in a single person still exists, since Access-AMH uses five approximate calibration points (0.16, 0.6, 4, 10, and 24 ng/mL), but Elecsys-AMH uses three points.

In previous studies, the Access-AMH levels and Elecsys-AMH levels in the same patient showed a significant correlation, and there was a tendency for higher levels to be measured using Access-AMH than using Elecsys-AMH [14,20,21]. In the present study, we evaluated the association of oocyte number with Access-AMH levels or Elecsys-AMH levels in different cohorts of patients undergoing stimulated IVF cycles, and determined the cut-off of Access-AMH or Elecsys-AMH to predict poor responders (3 or fewer oocytes) or high responders (15 or more oocytes).

Methods

1. Study subjects and AMH measurements

We selected 243 fresh IVF cycles performed between August 2016 and December 2020 at Seoul National University Bundang Hospital. The initial indication of IVF was unexplained infertility in 48 couples, diminished ovarian reserve in 46 couples, tubal factor infertility in 38 couples, endometriosis in 24 couples, male factor infertility in 22 couples, and mixed-cause infertility in 65 couples. The Institutional Review Board of the Seoul National University Bundang Hospital approved the use of patients’ medical records and IVF laboratory data (No. B-2110-714-101). As this study was a retrospective study, only the data on the procedure already performed were used, so patient consent was omitted.

In all cycles, full stimulation with recombinant follicle-stimulating hormone (FSH) with or without purified human menopausal gonadotropin (hMG) (excluding mild stimulation or natural cycle) was used, and the serum AMH level was measured within 1 year before ovarian stimulation by Access (Beckman Coulter, Brea, CA, USA) in 120 cycles and by Elecsys (Roche Diagnostics, Basel, Switzerland) in 123 cycles. The AMH measurement method was assigned at random or at the physician's preference.

The Access-AMH and Elecsys-AMH assays are automated immunoassays that utilize chemiluminescence for detection. They are not susceptible to interference by serum complement [22]. The total duration of assay is 39 minutes for Access-AMH and 18 minutes for Elecsys-AMH. The measurement range of Access-AMH is 0.02–24.00 ng/mL, and the intra- and inter-assay coefficients of variation are ≤ 1.7% and ≤ 2.8% according to the manufacturer’s instructions. For Elecsys-AMH, the measurement range is 0.01–23.00 ng/mL, and the intra- and inter-assay coefficients of variation are ≤ 2.6% and ≤ 3.9%, respectively [22].

2. Ovarian stimulation protocols

Ovarian stimulation was performed with recombinant FSH (Gonal-F; Merck Serono, Darmstadt, Germany) (142 cycles), recombinant FSH and purified hMG (Menopur; Ferring Pharmaceuticals, Kiel, Germany) (7 cycles), or recombinant FSH and recombinant luteinizing hormone (Pergoveris, Merck Serono) (17 cycles). A flexible gonadotropin-releasing hormone (GnRH) antagonist was used for pituitary suppression in all IVF cycles. Briefly, gonadotropins (according to the serum AMH level and individual ovarian response of previous cycles) were started on menstrual day 2–4 and the doses were adjusted. When the leading follicle reached a diameter of 14 mm, cetrorelix (Cetrotide, 0.25 mg/day; Merck Serono) was started and when the leading follicle reached a diameter of 18–19 mm, 250 μg or 500 μg of recombinant human chorionic gonadotropin (hCG; Ovidrel, Merck-Serono) (171 cycles), 5,000 IU of urinary hCG (IVF-C; LG Chemical, Seoul, Korea) (2 cycles), a GnRH agonist (Decapeptyl [0.2 mg], Ferring) (3 cycles), or 250 μg of recombinant hCG with a GnRH agonist (Decapeptyl [0.2 mg]) (67 cycles) was administered for final triggering. Oocytes were retrieved 35–36 hours later. The total oocyte number and the mature oocyte number were recorded. In most cases, oocyte maturity could be easily evaluated under stereomicroscopy on the basis of the cumulus pattern. In situations where the maturity
was unclear due to dark cumulus cells or blood clots, the oocytes were denuded using 85 IU/mL hyaluronidase (Cook, Bloomington, IN, USA) and mechanical pipetting. Mature oocytes were defined according to the presence of the first polar body and absence of a germinal vesicle.

3. Data analysis

Statistical analysis was performed using IBM SPSS ver. 25.0 (IBM Corp., Armonk, NY, USA). All variables were presented as mean ± standard deviation. Correlations between pairs of numeric parameters (such as serum AMH level, serum estradiol level at triggering day, the number of total or mature oocytes, and ovarian sensitivity (OS)) were assessed by the Spearman rank test. The OS was calculated in two ways; The OS-TO was defined as the total oocyte number per 500 IU of total gonadotropins, and the OS-MO was defined as the mature oocyte number per 500 IU of total gonadotropins. Equations were derived for the relationships between pairs of numeric parameters through linear regression analysis.

Results

The basal characteristics of two cohorts are shown in Table 1. The mean total gonadotropin dose was significantly higher in the Elecsys-AMH cohort than in the Access-AMH cohort. The correlation coefficients between serum AMH levels and five stimulation outcomes are presented in Table 2. Serum estradiol level at triggering day, the number of total or mature oocytes, OS-TO, and OS-MO were all positively associated with the Access-AMH level or Elecsys-AMH level, with statistical significance.

Figure 1 shows the linear regression lines between the Access-AMH level or Elecsys-AMH level and the total oocyte number. Linear regression analysis derived four equations to show the relationships between four stimulation outcomes and AMH levels (Table 3). For each stimulation outcome, when two equations (from Access-AMH and Elecsys-AMH) were combined, a total of four equations to show the correlations between the Access-AMH level and the Elecsys-AMH level were derived.

Table 1. Basal clinical characteristics of Access-AMH cohort and Elecsys-AMH cohort and their stimulation outcomes

<table>
<thead>
<tr>
<th>Variable</th>
<th>Access-AMH cohort (120 cycles)</th>
<th>Elecsys-AMH cohort (123 cycles)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female age (yr)</td>
<td>36.8 ± 4.9</td>
<td>37.1 ± 4.5</td>
<td>0.620</td>
</tr>
<tr>
<td>Male age (yr)</td>
<td>39.9 ± 5.4</td>
<td>39.1 ± 5.1</td>
<td>0.689</td>
</tr>
<tr>
<td>Cause of infertility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male factor</td>
<td>12 (10.0)</td>
<td>10 (8.1)</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Female factor</td>
<td>94 (78.3)</td>
<td>70 (56.9)</td>
<td></td>
</tr>
<tr>
<td>Combined</td>
<td>5 (4.2)</td>
<td>4 (3.3)</td>
<td></td>
</tr>
<tr>
<td>Unexplained</td>
<td>9 (7.5)</td>
<td>39 (31.7)</td>
<td></td>
</tr>
<tr>
<td>Serum AMH level (ng/mL)</td>
<td>2.19 ± 2.54</td>
<td>1.82 ± 1.48</td>
<td>0.253</td>
</tr>
<tr>
<td>Duration between measurement of serum AMH and oocyte pick-up (day)</td>
<td>87.2 ± 93.9</td>
<td>100.1 ± 96.7</td>
<td>0.466</td>
</tr>
<tr>
<td>Total dose of gonadotropin (IU)</td>
<td>2,279 ± 658</td>
<td>2,535 ± 632</td>
<td>0.004</td>
</tr>
<tr>
<td>Serum E level at triggering day</td>
<td>1,214 ± 1,036</td>
<td>1,481 ± 1,077</td>
<td>0.079</td>
</tr>
<tr>
<td>No. of total oocyte (TO)</td>
<td>5.5 ± 5.4</td>
<td>6.4 ± 5.2</td>
<td>0.146</td>
</tr>
<tr>
<td>No. of mature oocyte (MO)</td>
<td>3.3 ± 3.4</td>
<td>3.8 ± 3.5</td>
<td>0.140</td>
</tr>
<tr>
<td>Ovarian sensitivity-TO</td>
<td>1.49 ± 1.97</td>
<td>1.42 ± 1.38</td>
<td>0.877</td>
</tr>
<tr>
<td>Ovarian sensitivity-MO</td>
<td>0.88 ± 1.13</td>
<td>0.84 ± 0.89</td>
<td>0.948</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviation or number (%). AMH, anti-Müllerian hormone.

Table 2. Correlations between serum AMH level and five stimulation outcomes

<table>
<thead>
<tr>
<th>Variable</th>
<th>Access-AMH</th>
<th>Elecsys-AMH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum E level at triggering day</td>
<td>0.608</td>
<td>0.684</td>
</tr>
<tr>
<td>Total oocyte (TO)</td>
<td>0.645</td>
<td>0.686</td>
</tr>
<tr>
<td>Mature oocyte (MO)</td>
<td>0.534</td>
<td>0.578</td>
</tr>
<tr>
<td>Ovarian sensitivity-TO</td>
<td>0.657</td>
<td>0.702</td>
</tr>
<tr>
<td>Ovarian sensitivity-MO</td>
<td>0.57</td>
<td>0.608</td>
</tr>
</tbody>
</table>

*p-value < 0.05 was considered to indicate statistical significance.

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level could be derived. In the calculated four equations, the Access-AMH level was usually higher than the Elecsys-AMH level. For example, using the equations for the total oocyte number, the Access-AMH level was 1.553 ng/mL when the Elecsys-AMH level was 1.0 ng/mL. When using the equation for the mature oocyte number, an Access-AMH level of 1.269 ng/mL corresponded to an Elecsys-AMH level of 1.0 ng/mL.

For the prediction of poor responders, the cut-off of Access-AMH was 1.215 ng/mL (area under the curve [AUC], 0.807; 95% confidence interval [CI], 0.730–0.884; p < 0.001), and the cut-off of Elecsys-AMH was 1.095 ng/mL (AUC, 0.848; 95% CI, 0.773–0.923; p < 0.001) (Table 4, Figure 2). For the prediction of high responders, the cut-off of Access-AMH was 3.450 ng/mL (AUC, 0.922; 95% CI, 0.862–0.981; p < 0.001), and the cut-off of Elecsys-AMH was 2.500 ng/mL (AUC, 0.884; 95% CI, 0.778–0.991; p < 0.001) (Table 5, Figure 3).

**Discussion**

In the present study, we demonstrated that two fully automated AMH measurements could well predict the oocyte number in infertile women who underwent stimulated IVF cycles. No previous study has investigated whether there is a difference in serum AMH levels between these two methods for fully automated measurements of AMH. Although the data are from different cohorts, a correlation be-
between Access-AMH and Elecsys-AMH was identified, assuming the same number of oocytes. We believe that our results will be very useful in interpreting AMH levels measured by other methods at centers that usually use only one method.

The Access-AMH level was usually higher than the Elecsys-AMH level. However, considering the OS-E and OS-MO, it is thought that interoperability would be difficult because the relationship between the two was not consistent at low AMH values.

In addition, the trend for Access-AMH levels to be higher than Elecsys-AMH levels could be related to the higher total gonadotropin dose in the Elecsys-AMH group than in the Access-AMH group. According to European Society of Human Reproduction and Embryology (ESHRE) guidelines on ovarian stimulation for IVF and intracytoplasmic sperm injection, AMH and AFC could predict the ovarian response well during ovarian stimulation. They recommended establishing the FSH starting dose considering AMH and AFC [10]. Due to the retrospective nature of this study and differences in physicians' preferences across groups, it is not possible to draw a firm conclusion.

Table 4. Results of receiver operating characteristic curves of serum AMH level for prediction of poor ovarian response (total oocytes ≤3)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Access-AMH</th>
<th>Elecsys-AMH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cut-off (ng/mL)</td>
<td>1.215</td>
<td>1.095</td>
</tr>
<tr>
<td>AUC (95% CI)</td>
<td>0.807 (0.730–0.884)</td>
<td>0.848 (0.773–0.923)</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>77.2</td>
<td>88.2</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>69.8</td>
<td>74.5</td>
</tr>
<tr>
<td>PPV</td>
<td>69.814</td>
<td>84.833</td>
</tr>
<tr>
<td>NPV</td>
<td>77.188</td>
<td>79.609</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

AMH, anti-Müllerian hormone; AUC, area under the curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

Table 5. Results of receiver operating characteristic curves of serum AMH level for prediction of high ovarian response (total oocytes ≥15)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Access-AMH</th>
<th>Elecsys-AMH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cut-off (ng/mL)</td>
<td>3.450</td>
<td>2.500</td>
</tr>
<tr>
<td>AUC (95% CI)</td>
<td>0.922 (0.862–0.981)</td>
<td>0.884 (0.778–0.991)</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>100</td>
<td>85.7</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>83.5</td>
<td>81.0</td>
</tr>
<tr>
<td>PPV</td>
<td>20.869</td>
<td>21.392</td>
</tr>
<tr>
<td>NPV</td>
<td>100.000</td>
<td>98.946</td>
</tr>
<tr>
<td>p-value</td>
<td>0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

AMH, anti-Müllerian hormone; AUC, area under the curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.
that the total gonadotropin dose fully explains the difference in AMH; therefore, additional research is needed to address this question in the future.

La Marca et al. [20] reported that the Access-AMH level was usually higher than the Elecsys-AMH level. When calculating the dose of follicitropin alfa, Access-AMH value was used instead of the Elecsys-AMH value, and a ≥ 15% difference in the starting dose occurred in only 2 of 113 patients. When calculating the dose of follicitropin delta, the Access-AMH value was used instead of the Elecsys-AMH value, and a ≥ 15% difference in the starting dose occurred in 21 of 113 patients. In general, when using follicitropin delta, the Elecsys-AMH level is considered the gold standard. Considering the results of this paper, the choice between using Elecsys-AMH or Access-AMH values appears to have little effect on the determination of the correct FSH dose used for ovarian stimulation. The authors suggest that the two most widely used automated AMH assays, Elecsys and Access, have modest differences in values, and the clinical significance of this study's results lies in the reliability of the interchangeable use of AMH values obtained from both assays.

In the present study, the correlation coefficient of Access-AMH with the total oocyte number was 0.645, and that of Elecsys-AMH with the total oocyte number was 0.686. Asada et al. [15] reported that the correlation coefficient of Access-AMH with the total oocyte number was 0.655, which is very similar to our result. Homburg et al. [22] reported a correlation coefficient of 0.48 between Access-AMH and the total oocyte number.

Our study also showed that both automated methods for serum AMH measurement had good performance in predicting poor and high responders in fresh stimulated IVF cycles. Based on our observations, the cut-off of Access-AMH was 1.215 ng/mL and the cut-off of Elecsys-AMH was 1.095 ng/mL for predicting poor responders. The cut-off of Access-AMH was 3.450 ng/mL and the cut-off of Elecsys-AMH was 2.500 ng/mL for predicting high responders.

In the Bologna criteria defining poor responders, a serum AMH level > 0.5–1.1 ng/mL was presented as one of the criteria [23]. Broer et al. [24] also presented the cut-off of AMH for predicting poor or high responders in their meta-analysis as 2.0 ng/mL (95% CI, 0.1–5.7 ng/mL) and 4.8 ng/mL (95% CI, 1.3–10.2 ng/mL; p < 0.001), respectively. In their study, AMH was all measured by IOT. We also previously reported that the cut-offs of AMH levels measured using IOT were 1.08 ng/mL and 3.57 ng/mL, respectively, for predicting poor and high responders (≥ 20 oocytes) [25].

After the introduction of automated methods, the cut-off of Access-AMH or Elecsys-AMH for prediction of poor or high responders should be reset. Bosch et al. [10] suggested that the FSH dose and the drug and dose for triggering should be different for ovarian stimulation in poor and high responders, considering the AMH level and AFC. However, they also reported that there was no consistent definition of poor and high responders.

Baker et al. [26] studied whether Access-AMH could be used to predict poor ovarian responders. The mean value of Access-AMH among patients with poor ovarian response to ovarian stimulation, defined as 4 or fewer oocytes retrieved, was 0.74 ng/mL, whereas the cut-off was 3.20 ng/mL for normal to high responders. The cut-off for predicting poor ovarian response at 90% specificity was 0.93 ng/mL (sensitivity, 74.1%; specificity, 90%). There was no AMH cut-off value for high responders, and the AMH cut-off value for an AFC > 15 was 1.75 ng/mL (sensitivity, 90%; specificity, 59.1%). Homburg et al. [22] reported that the cut-offs of Access-AMH were 0.77 ng/mL for poor responders and 2.184 ng/mL for high responders (> 15 oocytes). In that report, the serum AMH levels of 1,787 and 1,258 patients at two different sites were measured by the Access-AMH method. The cut-off level of Access-AMH for predicting poor ovarian response and high ovarian response in our study was somewhat higher than that of Baker et al. [26] and Homburg et al. [22]. This may have been because the average age of the Access group in our study was 39.94 years, which is higher than in previous studies.

Iliodoromiti et al. [27] systematically searched and analyzed the literature measured by two automated measures in the same patient cohort. They found that Access-AMH values were higher than those obtained using Elecsys, and the correlation was linear (Access = −0.05 +1.10 × Elecsys). Access-AMH showed a higher value on average by about 10% compared to Elecsys-AMH, and when using the AMH value measured by Access-AMH, it was reported that attention should be paid because the patients would receive a lower dose of follicitropin delta based on the Access-AMH levels.

Tan et al. [28] prospectively measured and analyzed both Access-AMH and Elecsys-AMH in 43 infertile women aged 21 to 45 years. They reported that the cut-off of AMH for predicting poor ovarian response was 2.23 ng/mL for Access-AMH and 2.02 ng/mL for Elecsys-AMH. Furthermore, the cut-off of AMH for predicting high ovarian response was reported to be 5.19 ng/mL for Access-AMH and 4.60 ng/mL for Elecsys-AMH. The cut-off values in Tan’s study were all higher than in our study, which is probably due to the small sample size. However, the results are consistent with previous papers, which reported that Access-AMH showed slightly higher values than Elecsys-AMH.

It has been reported that very low values of AMH (1.5 pmol/L, which is equivalent to 0.21 ng/mL [15]) for Elecsys-AMH can predict cycle cancellation, and the cut-off value of Elecsys-AMH for a low oocyte yield (defined as ≤ 3 oocytes) was 0.56 ng/mL [29]. That result also confirmed that the cut-off value of Elecsys-AMH was somewhat higher than that of our study. It is presumed that these results were caused by differences in patient groups and stimulation protocols.
The limitations of our study are related to its retrospective nature. The AMH measurement method was determined by the physician's preference, and the interval between AMH measurement and oocyte pick-up was wide, although within 1 year. However, as in previous studies, Access-AMH showed slightly higher values than Elecsys-AMH. The finding that both AMH measurement methods predicted poor and high ovarian response is also consistent with previous studies.

In conclusion, although there is a slight difference between the two methods, both automated AMH measurement methods show good correlations with the number of retrieved oocytes and predict poor and high ovarian response relatively well. In the future, a large-scale prospective study is needed to clarify the differences between the two test methods.

Conflict of interest

Byung Chul Jee is an Editor-in-Chief and Seul Ki Kim is an Associate Editor of the journal, but they were not involved in the peer reviewer selection, evaluation, or decision process of this article. No other potential conflicts of interest relevant to this article were reported.

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References

Intraovarian platelet-rich plasma administration could improve blastocyst euploidy rates in women undergoing in vitro fertilization

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Objective: Platelet-rich plasma (PRP) therapy has received considerable attention as an adjunct to fertility treatments, especially in women with very low ovarian reserve and premature ovarian insufficiency. Although recent studies have demonstrated that PRP led to improvements in folliculogenesis and biomarkers of ovarian reserve, the effect of intraovarian PRP administration on embryo genetics has not been studied.

Methods: We report a pilot study of patients who had preimplantation genetic testing for aneuploidy (PGT-A) before and then within 3 months following PRP administration. Twelve infertile women with at least one prior failed in vitro fertilization (IVF) cycle underwent ovarian stimulation (cycle 1) with a gentle stimulation protocol and PGT-A performed at the blastocyst stage. Following cycle 1, autologous intraovarian PRP administration was performed. Within 3 months following PRP administration, the patients underwent cycle 2 and produced blastocysts for PGT-A. The percentage of euploid embryos between both cycles was compared.

Results: The mean age of all participants was 40.08±1.46 years, and their mean body mass index was 26.18±1.18 kg/m². The number of good-quality embryos formed at the blastocyst stage was similar between cycle 1 and cycle 2 (3.08±0.88 vs. 2.17±0.49, respectively; p=0.11). Among all patients in cycle 1, 3 of 37 embryos were euploid (8.11%) while in cycle 2, 11 out of 28 embryos were euploid (39.28%, p=0.002). Three clinical pregnancies were noted among this patient group.

Conclusion: This novel study is the first to present an improvement in the embryo euploidy rate following intraovarian PRP application in infertile women with prior failed IVF cycles. The growth factors present in PRP may exhibit a local paracrine effect that could improve meiotic aberrations in human oocytes and thus improve euploidy rates. Whether PRP improves live birth rates and lowers miscarriage rates remains to be determined in large trials.

Keywords: In vitro fertilization; Ovary; Platelet-rich plasma; Preimplantation genetic testing

Introduction

Ovarian aging leads to a decline in both the quantity and quality of oocytes, negatively impacting the formation of genetically normal (euploid) embryos during in vitro fertilization (IVF) treatment and increasing the frequency of miscarriage [1]. The aging process leads to cellular and molecular events such as disturbances in mitochondrial dynamics and mRNA storage, translation, and degradation within the oocytes, all of which contribute to meiotic aberrations [1].

Platelet-rich plasma (PRP) is derived from whole blood, which contains plasma (55%), red blood cells (41%), platelets and white blood cells (4%), by centrifugation and separation of its different components [2]. The centrifugation and separation process leads to the removal of red blood cells and the production of plasma with 5–10 times higher concentrations of growth factors. The platelets present in PRP contain alpha granules that, when activated, release many...
factors that contribute to growth, cell proliferation, and angiogenesis [3]. The growth factors present in PRP have been shown to play an important role in enhancing collagen synthesis, proliferation of bone cells, fibroblast chemotaxis, macrophage activation, angiogenesis, chemotaxis of immune cells, migration and mitosis of endothelial cells, differentiation of epithelial cells, and cytokine secretion by mesenchymal and epithelial cells [2].

Autologous PRP therapy uses injections of the patient’s own concentrated platelets and plasma following a venous blood draw. The theory behind using this modality for treatment stemmed from the natural healing process being the body’s initial response to tissue injury by delivering activated platelets and releasing growth factors. The clinical use of PRP has considerably increased over the last decade, and now includes treatments for musculoskeletal injuries [4-6], arthritis [7], periorbital rejuvenation [8], pancreatic problems [9], dentistry [10], wound healing [11], alopecia [12], and infertility [13]. PRP treatment has recently been used as an adjunct in assisted reproduction technology, in particular, as an intraovarian injection in conjunction with IVF for women who have poor ovarian reserve, premature ovarian insufficiency, and even menopause [14-18]. Recent data have shown that intraovarian PRP treatment led to improvement in markers of ovarian reserve such as serum anti-Müllerian hormone (AMH), a marker of ovarian reserve, and increased oocyte yield with IVF [19]. Although recent studies have demonstrated that PRP led to improvements in folliculogenesis and biomarkers of ovarian reserve, the effect of intraovarian PRP administration on embryo genetics has not been studied, except for one case report that demonstrated qualitative improvement in embryo genetics after intraovarian injection of autologous PRP [20]. We report a pilot study of patients who had preimplantation genetic testing for aneuploidy (PGT-A) before and then within 3 months following intraovarian PRP administration.

Methods

1. Participants

The participants underwent infertility treatment at Rejuvenating Fertility Center. Infertility was defined as an inability to conceive with unprotected intercourse after 1 year for women aged < 35 years, and after 6 months for women aged > 35 years. Women with any medical condition that interfered with fertility treatment were excluded from the study. The inclusion criteria were women with at least one previous failed IVF cycle and women who produced fully developed embryos (blastocysts) before and after intraovarian PRP administration (n = 12). Each participant underwent two IVF cycles: the first (cycle 1) was followed by intraovarian PRP administration, after which a second IVF cycle (cycle 2) took place within 3 months following the PRP administration. Informed consent was obtained from all patients and the study was approved by the New England Institutional Review Board (NEIRB; No. 120180241).

2. IVF protocols

The IVF cycles 1 and 2 performed in the same participants used similar ovarian stimulation protocols. In brief, in each cycle, after oral contraceptive pill pre-treatment for approximately 2–3 weeks and adequate suppression, minimal/mild ovarian stimulation was started with an extended regimen (from cycle day 3 until the day before triggering) of clomiphene citrate (50 mg/day orally) in conjunction with letrozole (2.5 mg/day orally) with low-dose gonadotropin (75 IU daily) injections (Follistim, Merck, White House Station, NJ, USA; or Gonal F, EMD Serono, Rockland, MA, USA).

Hypothalamic-pituitary suppression using a gonadotropin-releasing hormone (GnRH) antagonist was conducted to prevent ovulation. The final maturation of oocytes was induced by a GnRH agonist or by human chorionic gonadotropin trigger when the lead follicle was > 18 mm. The retrieved oocytes were fertilized by intracytoplasmic sperm injection as clinically indicated. All embryos were cultured until the blastocyst stage followed by trophectoderm biopsies for PGT-A and then vitrified to be transferred in a subsequent frozen embryo transfer cycle.

3. Intraovarian PRP administration

PRP was prepared as we previously described [21,22]. Approximately 32 mL of blood was collected from the patient by peripheral venipuncture. The blood sample was placed in a room-temperature centrifuge set to 1,500 × g for 5 minutes. After centrifugation, the upper layer, corresponding to relatively platelet-poor plasma, was aspirated and discarded, after which the PRP layer was aspirated and placed in a separate tube for a second round of centrifugation, and the lower level corresponding to red blood cells was discarded. The process was repeated a second time. A total of 8 mL of PRP was collected from the tubes, and no activators were used. Under intravenous sedation and transvaginal ultrasound guidance, intraovarian injection of approximately 4 mL of PRP per ovary was performed. The injection was performed in multifocal spots, and diffusion of the PRP in the subcortical layers was achieved by applying 5–7 punctures per ovary transvaginally using a 22-gauge needle and guide. The patients tolerated the procedure well and were discharged home.

4. Statistical analysis

Because the data were normally distributed, we used the paired t-test to compare continuous clinical data between cycles 1 and 2. The chi-square test was used to compare the proportion of euploid embryos between cycles 1 and 2. The statistical analysis was con-
ducted using GraphPad Prism statistical software (GraphPad Software, San Diego, CA, USA), and a \( p \)-value of \(< 0.05\) was considered statistically significant.

## Results

The mean age of all participants was 40.08 ± 1.46 years, and their mean body mass index was 26.18 ± 1.18 kg/m\(^2\). When comparing clinical data between cycle 1 and cycle 2, statistically significant differences were not found for serum follicle-stimulating hormone levels (7.98 ± 1.01 mIU/mL vs. 8.45 ± 1.55 mIU/mL, respectively; \( p = 0.66\)), the antral follicle count calculated by transvaginal ultrasound (11.09 ± 1.82 vs. 12.36 ± 2.36, respectively; \( p = 0.25\)), the number of oocytes collected (6.18 ± 1.61 vs. 7.27 ± 1.68, respectively; \( p = 0.21\)), and the number of good-quality embryos formed at the blastocyst stage (3.08 ± 0.88 vs. 2.17 ± 0.49, respectively; \( p = 0.11\)) (Table 1). Because we used exactly the same protocol for gonadotropins before and after PRP, there was no significant difference in the dose of medications used between cycle 1 and cycle 2 (\( p > 0.05\)). Among all participants, 3 out of 37 embryos were euploid in cycle 1 (8.11%), while 11 out of 28 embryos were euploid in cycle 2 (39.28%, \( p = 0.002\)). Table 2 shows the individual results for each participant. Three clinical pregnancies were noted among the outcomes of this patient group. The remainder of the patients are either still banking more euploid embryos or in the process of preparing for embryo transfer.

## Discussion

For many older infertile women with low ovarian reserve, the production of an euploid embryo is a major challenge along their journey [23]. Here, we present, to our knowledge, the first case series comparing IVF euploidy rates pre-PRP and post-PRP (within 3 months following the PRP procedure). The autologous PRP is known to contain cytokines, chemokines, and growth factors including platelet-derived growth factor, stromal cell derived factor 1, and hematocyte growth factor [24]. These molecular signals are known to initiate the recruitment, proliferation, and activation of fibroblasts.

### Table 1. Clinical data comparison between cycle 1 (before PRP) and cycle 2 (after PRP)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cycle 1</th>
<th>Cycle 2</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (mIU/mL)</td>
<td>7.98 ± 1.01</td>
<td>8.45 ± 1.55</td>
<td>0.66</td>
</tr>
<tr>
<td>Antral follicle count</td>
<td>11.09 ± 1.82</td>
<td>12.36 ± 2.36</td>
<td>0.25</td>
</tr>
<tr>
<td>Number of oocytes retrieved</td>
<td>6.18 ± 1.61</td>
<td>7.27 ± 1.68</td>
<td>0.21</td>
</tr>
<tr>
<td>Number of good-quality embryos formed at the blastocyst stage</td>
<td>3.08 ± 0.88</td>
<td>2.17 ± 0.49</td>
<td>0.11</td>
</tr>
<tr>
<td>Percentage of euploid embryos (%)</td>
<td>8.11</td>
<td>39.28</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard error of the mean. PRP, platelet-rich plasma; FSH, follicle-stimulating hormone.

### Table 2. The age of the patients, the PGT-A results between cycles 1 and 2, and the clinical outcomes

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Cycle 1</th>
<th>Cycle 2</th>
<th>Clinical outcome</th>
<th>Percent of euploid embryos before cycle 1 ( \rightarrow ) after PRP cycle 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>CP</td>
<td>0 → 100</td>
</tr>
<tr>
<td>2</td>
<td>33</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0 → 100</td>
</tr>
<tr>
<td>3</td>
<td>39</td>
<td>0</td>
<td>2</td>
<td>4 (1 Mosaic)</td>
<td>0 → 0</td>
</tr>
<tr>
<td>4</td>
<td>39</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>25 → 66</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>1</td>
<td>3</td>
<td>14</td>
<td>0 → 50</td>
</tr>
<tr>
<td>6</td>
<td>41</td>
<td>1</td>
<td>0</td>
<td>78</td>
<td>26 → 75</td>
</tr>
<tr>
<td>7</td>
<td>41</td>
<td>0</td>
<td>12</td>
<td>8</td>
<td>8 → 0</td>
</tr>
<tr>
<td>8</td>
<td>42</td>
<td>0</td>
<td>3</td>
<td>90</td>
<td>1 → 0</td>
</tr>
<tr>
<td>9</td>
<td>44</td>
<td>1</td>
<td>0</td>
<td>61</td>
<td>100 → 0</td>
</tr>
<tr>
<td>10</td>
<td>44</td>
<td>2</td>
<td>0</td>
<td>75</td>
<td>1 → 0</td>
</tr>
<tr>
<td>11</td>
<td>45</td>
<td>1</td>
<td>0</td>
<td>33</td>
<td>1 → 0</td>
</tr>
<tr>
<td>12</td>
<td>45</td>
<td>0</td>
<td>1</td>
<td>70</td>
<td>0 → 100</td>
</tr>
</tbody>
</table>

PGT-A, preimplantation genetic testing for aneuploidy; PRP, platelet-rich plasma; CP, clinical pregnancy; NA, not applicable; ET, embryo transfer.
neutrophils, monocytes, which are expected to regulate angiogenesis and tissue perfusion which might be an independent way to achieve ooplasm improvement within the adult human ovary [19].

Placing autologous cytokines within ovarian tissue may facilitate the production of higher AMH levels by granulosa cells and improve blastocyst ploidy. One possible mechanism is that any new follicles recruited and good quality oocytes obtained after the intraovarian injection of these growth factors have always resided in the ovaries, but are then stimulated by the PRP administration [25]. Another mechanism could be that the platelet growth factors present in the PRP activate, by supplying molecular signals, the existing ovarian stem cells to differentiate into de novo oocytes [2]. In vitro studies demonstrating the effect of PRP on the growth and survival of isolated early human follicles tend to support such theories, as the development and survival rates of preantral follicles in PRP-supplemented culture media have been found to be significantly higher than in media without PRP supplementation, as demonstrated in a dose-dependent manner with both fresh and vitrified ovarian samples [25]. Finally, the oocytes of older women have aberrant meiotic events and impaired fertilization, resulting in poor embryonic development, partly due to altered mitochondrial number and function [26]. Studies have shown that aneuploid embryos have relatively high mitochondrial DNA copy numbers [27]. It is plausible that PRP improves ooplasm quality by altering the mitochondria, leading to improvements in meiosis and thus resulting in ploidy rescue of the embryos.

The limitations of this case series include a small sample size and the lack of a control group, such a group would have been women who underwent ovarian puncture without the injection of PRP, since mechanical puncture of the ovaries could have an effect on ovarian function. Even though all the patients in this reported a history of prior failed IVF, other limitations include the wide range in the ages of patients (from 28 to 45 years old) and the lack of complete pregnancy outcomes in all participants because many of them are still trying to bank more euploid embryos.

In summary, there is a clear need for well-designed studies pertaining to the effect of the commonly used intraovarian PRP administration in women who struggle to form euploid embryos and who ultimately resort to the use of donor oocytes. Some investigators have explored the ovarian germline stem cell niche and its probable regulatory mechanisms with the hope of yielding valuable insights for the treatment of ovarian aging [28]. Investigations related to the effect of PRP on ovarian stem cells are likely to clarify the signaling pathways involved in de novo oocyte replenishment and follicular development, potentially helping older women with abnormal embryo genetics [29].

Conflict of interest

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

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References

9. Zarin M, Karbalaei N, Keshtgar S, Nemati M. Platelet-rich plasma improves impaired glucose hemostasis, disrupted insulin secre-


A case of congenital cloacal extrophy/omphalocele-exstrophy-imperforate anus-spinal defects syndrome and a successful pregnancy

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Herein, we report an exceptionally rare case of a 25-year-old woman with cloacal extrophy/omphalocele-exstrophy-imperforate anus-spinal defects (OEIS) syndrome achieving a viable pregnancy despite many gastrointestinal and genitourinary malformations and multiple respective corrective operations. The patient was born with two vaginas, two uteruses, four ovaries, an imperforate anus, a large omphalocele including bowel and bladder extrophy, and diaphysis of the pubic rami. This patient is the only documented OEIS patient not to have tethered spinal cord as an anomaly, perhaps contributing to her successful pregnancy. After experiencing preeclampsia with severe features at 35 weeks, the baby was born via cesarean section.

Keywords: Cloacal extrophy; Exstrophy epispadias complex; Exstrophy epispadias sequence; Exstrophy of cloaca; Imperforate anus and spinal defects; Omphalocele

Introduction

Cloacal extrophy, a condition on the spectrum of omphalocele-exstrophy-imperforate anus-spinal defects (OEIS) syndrome, is regarded as being the most severe manifestation of exstrophy epispadias sequence (EES). While OEIS, exstrophy epispadias complex (EEC), EES, and cloacal extrophy are often used interchangeably, the OEIS acronym specifically refers to omphalocele, exstrophy of the cloaca, imperforate anus, and spinal defects. OEIS is a very rare congenital defect resulting in an array of pathological phenotypes, with the most common being bladder and colon extrophy, leading to a myriad of anomalies affecting multiple organ systems (Figure 1) [1,2]. Duplication of multiple sex organs is an additional phenotypic variant sometimes seen in OEIS patients. OEIS is estimated to affect 1 in 200,000 to 400,000 live births [1,3]. EEC refers to epispadias, classic bladder extrophy, and cloacal extrophy complex, with cloacal extrophy being the most severe of the three abnormalities.

Generally, early surgical intervention is preferred due to better outcomes relating to urinary continence, infection control, physical appearance, self-esteem, sexual function, and fertility [1]. Despite these better outcomes, most OEIS patients are rarely able to achieve a viable pregnancy because of their severe malformations. Their Müllerian anomalies lead to an increased risk of obstetric and urologic complications, but birth is possible. However, if they do achieve pregnancy, many risks are imposed [4]. The relatively fragile, reconstructed sexual organs typically cannot withstand the birthing process, and these patients also usually require additional postpartum operations [1]. These factors compel extensive obstetric and surgical evaluations throughout pregnancy, often warranting caution when an OEIS patient is considering conception. This paper presents the exceptionally rare case of a woman with OEIS syndrome who achieved viable childbirth and elaborates on various aspects of EES-OEIS, including prognosis and interventions. The Institutional Review Board approval was waived and informed consent was obtained.
Case report

A 25-year-old G1P0 woman born with OEIS syndrome presented to the office following a positive pregnancy test. Consistent with OEIS, this patient had many congenital malformations, including two vaginas, two uteruses, four ovaries, an imperforate anus, a large omphalocele including bowel and bladder exstrophy, and diaphysis of the pubic rami. This patient is the only documented OEIS patient not to have tethered spinal cord as an anomaly. The patient was given a colostomy bag until she turned 2 years old, when she underwent a colon pull-through procedure with continued regular enemas for bowel management. By age 4, she underwent a total hysterectomy and bilateral salpingo-oophorectomy, leaving her with a single uterus and cervix, bilateral fallopian tubes and two ovaries. Her second vagina with the vaginal cuff was closed off as well, leaving her with a single vaginal opening, which restored normal gynecologic anatomy. The patient had her bladder neck closed with a Mitrofanoff operation, as represented in the image below (Figure 2), urinating through a catheter through a stroma in the abdomen that she continues to use now. The patient began menstruating at 12 years old; she experienced irregular menstrual cycles on a sporadic basis, but was told this may happen IRB waived and informed consent was given.

Despite her substantial surgical history, in June 2020, the patient learned she was pregnant after having several missed menstrual cycles. The patient had been counseled throughout most of her life by several specialists stating that her chances of pregnancy were substantially low. Because of this, the patient did not utilize any form of contraception. Once pregnant, additional extensive counseling took place regarding the high-risk nature of her pregnancy. Nonetheless, she decided to proceed with very close obstetric follow-up. The pregnancy was further complicated by a preexisting right-sided ovarian mass, the diagnostic impression of which was a benign ovarian cyst. The cyst was followed with serial sonography, which demonstrated progressive enlargement, quite possibly due to pregnancy-induced hormonal changes. At 35 weeks, the patient developed preeclampsia with proteinuria, necessitating delivery via primary cesarean section with ovarian cystectomy and/or possible salpingo-oophorectomy. Given her extensive adhesions from past corrective procedures and because of the large ovarian cyst requiring excision, cesarean section was performed via a midline vertical abdominal incision with a classical uterine incision [1,6].

At the time of delivery, the ovarian cyst measured approximately 30 cm (Figure 3). A viable male infant was delivered, weighing 1,842 g and measuring 41.91 cm. The hysterotomy was closed and attention was then turned to the right ovary. The mass had obliterated all normal ovarian architecture and a decision was made to proceed with unilateral salpingo-oophorectomy. The patient recovered well postoperatively and was discharged home on postoperative day 5. The pathology of the ovarian mass showed a benign ovarian cyst. The infant was admitted to the neonatal intensive care unit for 16 days and is reportedly doing well nearly 1 year after birth. The pa-
Patient’s future fertility was preserved with a unilateral ovary and fallopian tube; however, the patient was extensively counseled on the high risk of complications for any future pregnancies. She was not placed on any birth control.

Discussion

A “typical” OEIS patient is difficult to define, as a spectrum of malformations can be found. The most common findings of OEIS, however, are omphalocele, imperforate anus, shortened hindgut, an open cecal plate flanked by open hemi-bladders on either side, and spinal cord and ureteral deformities [7]. Based on histopathologic studies in human embryos, current data attribute cloacal exstrophy to an early defect in the closure of the ventral body [8,9]. Cloacal exstrophy patients typically have normal ovaries (unlike our patient), but their Müllerian ducts do not fuse, resulting in uterine and vaginal duplication [7]. Some variants of OEIS result in the uterus remaining inside the body; however, patients often develop complications like uterine prolapse and intrauterine hemorrhage [9].

Few OEIS patients reproduce, limiting data on their offspring [1]. The current data do not demonstrate an association between cloacal exstrophy and aneuploidy, but also do not definitively exclude the possibility [8]. The exact underlying cause of cloacal exstrophy/OEIS is unknown and not currently attributed to any medications [10]. Many familial cases have been reported, suggesting an inherited component, but exact genetic relationships have not been found [11]. Interestingly, as with many other types of neural tube defects, it is highly associated with female monozygotic twins along with a risk of recurrence in subsequent pregnancies [8].

The OEIS complex comprises a wide spectrum of phenotypes, preventing a protocol-based standard of care. Each patient undergoes reconstructive operations pertaining to their own individualized malformations. Even after reconstruction, OEIS patients often experience life-long complications. As cloacal exstrophy is one of the most severe forms of an abdominal wall defect, always presenting with omphalocele, patients are frequently destined to suffer from bowel and urinary issues throughout their life [12,13]. One article studied the long-term functionality of patients born with OEIS syndrome and showed high rates of urinary and bowel incontinence in addition to constipation, with 42% of the patients requiring intermittent catheterization and 22% requiring urinary diversion [8]. Only 35% of female patients reported normal menstruation in this study [8]. Our patient reported that many doctors throughout her life stated that she would never get pregnant because of her malformations and history of multiple operations. The patient in this case report had cloacal exstrophy, as opposed to just bladder exstrophy, which is more commonly seen in anomalies with OEIS. A small study showed that out of 22 pregnancies in EEC patients, only 64% resulted in live births, none of which occurred in patients in the cloacal exstrophy group (i.e., live births only occurred in the bladder exstrophy patients) (Table 1) [14]. The glaring weakness of that study, however, is that it only had two cloacal exstrophy patients, an inevitable consequence of exceedingly rare conditions.

![Figure 3](imageurl) Ovarian cyst removed during cesarean section.

Table 1. Outcomes of 22 pregnancies in 12 EEC patients

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Total pregnancy (n = 22)</th>
<th>Bladder exstrophy pregnancy (n = 20)</th>
<th>Cloacal exstrophy pregnancy (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total pregnancy</td>
<td>22 (100)</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Spontaneous abortion &lt; 24 wk</td>
<td>4 (18)</td>
<td>3 (15)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Termination</td>
<td>4 (18)</td>
<td>3 (15)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Live birth</td>
<td>14 (64)</td>
<td>14 (70)</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are presented as number (%). Previous findings [14] from a study on 22 pregnancies in exstrophy patients, in which 0% of cloacal exstrophy patients had viable live births (as our patient did).

EEC, exstrophy epispadias complex.
Only a few case reports exist of OEIS patients achieving pregnancy, substantially limiting the formulation of management guidelines [1]. Additionally, each case of OEIS syndrome varies greatly, further limiting standardization. The patient described herein is one of only a few in whom a live birth was reported. Fewer than 10% of women with cloacal and bladder exstrophy have been reported to have pregnancies, and these pregnancies were usually complicated by new-onset or worsening of recurrent urinary tract infections, uterine prolapse, and urinary incontinence [1].

In general, females born with OEIS can expect to face considerable challenges in life, not only relating to extensive operations and high-risk pregnancies, but also to urinary incontinence, poor body image, uterine prolapse, and fertility issues [6,13,15]. Documenting the rare success story of an OEIS pregnancy is vital for learning more about how to care for future similar cases. More data are not only needed regarding OEIS pregnancies, but also on the health and outcomes of the offspring. Additionally, further investigation is needed to elucidate some of the unique features of our patient that may have contributed to her successful pregnancy. Being the only known OEIS patient to not have a tethered cord could perhaps be one of those factors. Notwithstanding these patients’ many hurdles, pregnancy should not necessarily be ruled out early on in their life, as evidenced by the patient featured in this report.

Conflict of interest

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

Author contributions

Conceptualization: MS. Project administration: MS. Visualization: MS. Writing—original draft: MS. Writing—review & editing: MS, GG, MB, AH.

References

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

6. Letter to the editor

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

VII. AUTHOR’S MANUSCRIPT CHECKLIST

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

VIII. PEER REVIEW PROCESS

All manuscripts will be evaluated by two peer reviewers who are selected by the editors. The acceptance criteria for all papers are based on the quality and originality of the research and its clinical and scientific significance. An initial decision will normally be made within 4 weeks of receipt of a manuscript, and the reviewers’ comments are sent to the cor-
responding authors. Revised manuscripts must be submitted online by the corresponding author. The corresponding author must indicate the alterations that have been made in response to the referees' comments item by item in response note. Failure to resubmit the revised manuscript within 8 weeks of the editorial decision is regarded as a withdrawal. Please notify the editorial office if additional time is needed or if you choose not to submit a revision. Authors can track the progress of a manuscript on the journal’s web-site. Articles that are accepted for publication are listed in the “Articles in Press” section of the journal's website. The manuscript, when published, will become the property of the journal. All published papers become the permanent property of the Korean Society for Reproductive Medicine, and must not be published elsewhere without written permission.

Any appeal against the editorial decision to publish a text must be made within 2 weeks of the date of the decision letter. Authors who wish to appeal a decision should contact the Editor-in-Chief, explaining in detail their reasons for the appeal. All appeals will be discussed with at least one other associate editor. If the associate editor(s) does 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

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

2. Manuscript corrections

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

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

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XI. FEEDBACK AFTER PUBLICATION

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