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RESEARCH

Cumulus matrix selection leads to isolation of spermatozoa with better motility, morphology, and lower DNA fragmentation

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Abstract

The objective of this study was to compare the motility, morphology, and levels of DNA fragmentation of spermatozoa subjected to conventional swim-up or cumulus matrix (CM) sperm selection. Semen samples were collected from 60 normozoospermic men at a private hospital between December 2021 and March 2022. After liquefaction, semen samples were separated into two portions – one part was subjected to conventional swim-up preparation, and the remaining spermatozoa were subjected to CM selection. The CM was obtained by mechanical isolation from healthy donor oocytes. Semen analysis and evaluation of sperm were performed according to the WHO 6th Edition Laboratory Manual and Kruger's strict criteria, respectively. Sperm DNA fragmentation (SDF, %) of the two preparations was evaluated using the Halosperm G2 detection Kit (Halotech, Madrid, Spain). Wilcoxon rank-sum test was used to compare the characteristics of spermatozoa obtained by the two preparations. Spermatozoa selected by CM showed significantly better rapidly progressive motility (43.5% vs 30.6%, respectively, $P < 0.001$), a higher percentage of morphologically normal forms (14.0% vs 9.0%, respectively, $P < 0.05$), and lower levels of SDF (26.0% vs 45.0%, $P < 0.05$) compared to those prepared by conventional swim-up. Moreover, the incidence of multiple sperm defects was considerably lower in the samples that underwent CM selection compared to those that did not (30.0% vs 49.0%, respectively, $P < 0.05$).The selection by CM significantly increases sperm motility and reduces morphologically abnormal spermatozoa and DNA fragmentation rates compared to the conventional swim-up preparation. The application of this selection technique may increase the chances of successful IVF outcomes.

Lay summary

There are various techniques for selecting high-quality sperm with better shape, mobility, and DNA quality. However, the success of assisted reproduction techniques remains relatively unchanged. In this study, we describe an innovative method that uses the ingredients of a natural coat surrounding the egg (cumulus matrix) to enhance sperm selection procedures. Using this cumulus matrix as a barrier through which sperm cells pass, we mimic natural sperm–egg interactions and are able to select sperm with better characteristics compared to conventional methods. This new sperm selection procedure could lead to increased assisted reproduction success rates.

Keywords: cumulus matrix; DNA fragmentation; sperm morphology; sperm motility; sperm selection

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Introduction

Nowadays, approximately 15–20% of couples worldwide are diagnosed with impaired fecundity (Pathak *et al.* 2020, Gullo *et al.* 2021). About 40—50% of them are associated with male infertility factors (Choy & Eisenberg 2018). The selection of human spermatozoa with better motility, a lower number of morphological abnormalities, and decreased levels of DNA fragmentation has been proven to be useful for improving fertilization, implantation, and ongoing pregnancy (Lundin *et al.* 1997, Kruger & Coetzee 1999, Van Waart *et al.* 2001, Jin *et al.* 2015, Dcunha *et al.* 2022).

One of the most commonly applied techniques for sperm selection is swim-up (Volpes *et al.* 2016). Previous studies have proven that the application of this method selects spermatozoa with better motility (AL-Marayaty *et al.* 2017), morphology (Heidari *et al.* 2018), and lower sperm DNA fragmentation rates compared to native sperm (Younglai *et al.* 2001, Parmegiani *et al.* 2010, Xue *et al.* 2014, Cho & Agarwal 2017).

There have been several advancements in the field of assisted reproductive technology (ART) in the past decades, including the development of innovative sperm selection methods like hyaluronic acid-mediated sperm binding (Huszar *et al.* 2007, West *et al.* 2022) and magnetic-activated cell sorting (Nadalini *et al.* 2014). Another innovative sperm selection technique involving cumulus matrix (CM) penetration in a dish has recently been developed (Gospodinova *et al.* 2019). This selection is based on the fact that relatively few mature and competent spermatozoa could penetrate the CM (Kim *et al.* 2008, Hong *et al.* 2009).

However, the effect of the applied novel technique for selection through the CM membrane on sperm characteristics is still scarcely studied. The present study was designed to assess whether the introduced selection through CM may have a positive influence on sperm motility, morphology, and DNA fragmentation levels.

Materials and methods

Study design and participants

The study was carried out at a private fertility center. In total, 60 normozoospermic men participated in the study between December 2021 and March 2022. Eligibility criteria included progressive sperm motility (class A+B, %) ≥45%, and sperm concentration ≥90 million spermatozoa/mL. Inclusion criteria were based on preliminary unpublished experiments in order to obtain enough spermatozoa able to pass/swim up through the CM for further analysis. Men with genetic disorders, chronic or acute inflammation, severe oligospermia (<1 million spermatozoa/mL), and age above 45 years were excluded from the study. Only fresh semen samples were used for the purposes of the present study. Written informed consent was obtained from all participants. All experimental procedures were approved by the Nadezhda Women's Health Hospital Research Ethics Committee (No. 60/06.12.2021).

Isolation of the cumulus matrix and preparation of cumulus matrix-enriched filters

Donor cumulus–oocyte complexes (COC) were collected during follicular puncture from healthy female patients who had signed informed consent. Separation of the cumulus from COC was performed by mechanical dissection using a 22G needle, followed by centrifugation at 600 ***g*** for 5 min. The supernatant contained the CM but no cells or debris was collected. All supernatant samples were mixed in a pool and stored at −20°C. The Bradford method (Bradford, 1976) was used to determine the protein concentration in the pool.

The working protein concentration of CM (2.5 mg/mL), as determined by preliminary experiments, was prepared after dilution with carbonate buffer (pH 9.3; sodium bicarbonate/sodium carbonate) at room temperature. Then, 28 µL of the prepared solution were placed on a 40 µm filter (pluriSelect, San Diego, CA, USA). The filter surface was allowed to dry for 1 h at room temperature.

Semen handling and analysis

Semen samples were collected by masturbation after 3–5 days of abstinence. After liquefaction (within 30 min from sample collection), all semen samples were examined according to the WHO 6th Edition Laboratory Manual (World Health Organization 2021).

Sperm concentration was assessed using a hemocytometer (Improved Neubauer; Hauser Scientific Inc.) using a bright-field microscope (CKX41, Olympus INC.) and was expressed as million spermatozoa (10^6) per mL.

For each semen sample, at least 200 spermatozoa were assessed in two replicates by two independent researchers blind to preparation groups. In cases with a large difference between the replicate counts, a third count was made by a third researcher.

Evaluation of sperm motility

Sperm motility was evaluated using an eyepiece reticle with grid – Makler chamber according to the WHO 6th edition Laboratory Manual. A four-category grading of motile spermatozoa was determined: grade A – rapidly progressive (25 µm/s); grade B – slowly progressive (5 to <25 µm/s); grade C – non-progressive (<5 µm/s); and grade D – immotile. The percentage of progressively motile spermatozoa (grades A+B) was also evaluated.

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Evaluation of sperm morphology

Sperm morphology in each sample was evaluated according to Kruger's strict criteria (Kruger *et al.* 1986, Kruger *et al.* 1988) using the Diff-Quick staining Kit (Microptic SL, Barcelona, Spain). In total, 23 types of morphological abnormalities of the head, midpiece, and tail were determined. Defective conditions for heads included – small, large, amorphous, elongated, round, pear-shaped, double, acephalic, detached head, presence of small or large acrosomal areas, and spermatozoa without an acrosome. Midpiece defects were defined as thick, thin, bent, asymmetric midpiece, and the presence of cytoplasmic droplets. Tail defects included – short, coiled, and double tails. The presence of acrosomal vacuoles and nuclear vacuoles was also evaluated. Spermatozoa that had more than one morphological abnormality were classified as having multiple defects.

Multiple abnormalities index (MAI%), teratozoospermia index (TZI%), and sperm deformity index (SDI%) were also calculated. The MAI is evaluated as the mean number of anomalies per abnormal spermatozoon, which includes all head, midpiece, and tail defects (World Health Organization 2021). The TZI takes into account only one anomaly of each part of the sperm cell – one head defect, one midpiece defect, and one tail defect, respectively (World Health Organization 2021). The SDI is the number of defects divided by the total number of spermatozoa and includes variations of several sperm head defects but only one for each midpiece and tail anomaly (World Health Organization 2021).

Sperm selection by swim-up and cumulus matrix

Native semen samples were subjected to several sperm analysis procedures (motility, morphology, and DNA fragmentation assessment) (Fig. 1).

The remaining semen sample from each patient was separated into two portions, and one half was processed with the conventional swim-up preparation process according to the WHO 6th edition Laboratory Manual (World Health Organization 2021). The obtained spermatozoa from this method were retained for sperm analysis. The other half of the sample was placed in a small tube at a 45° angle for selection through a CM-enriched filter. The filter was placed above the semen sample, and 500 µL of culture media (ORIGIO Sequential Fert; Origio, Denmark) was overlayed on top of the filter. After 20 min, the media above the filter containing the spermatozoa that managed to pass through the CM were collected and subsequently analyzed (Fig. 1).

Determination of sperm DNA fragmentation

Sperm DNA fragmentation (SDF, %) was evaluated using the Halosperm G2 detection Kit (Halotech,

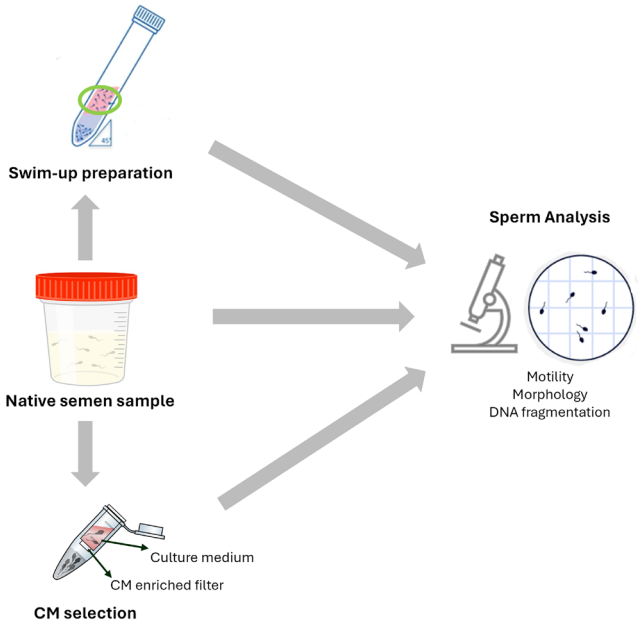

Figure 1

Experimental design.

Madrid, Spain) according to the manufacturer's instructions. A minimum of 200 spermatozoa per sample were scored under 400× magnification of a bright-field microscope (CKX41, Olympus Inc.). Sperm SDF was classified according to their halo formation: fragmented DNA (spermatozoa with small halo, without halo, or degraded ones) or those with non-fragmented DNA (spermatozoa with big and medium halo formation). Results were presented as the percentage spermatozoa with fragmented DNA – Sperm DNA fragmentation (SDF %).

Statistical analysis

None of the data were normally distributed according to the Shapiro–Wilk test. Therefore, the Wilcoxon rank-sum test was used to compare the analyzed variables. Quantitative data are expressed as median and range (minimum–maximum). Statistical significance was set at $P < 0.05$. Statistical analyses were performed with SPSS statistical software for Windows, version 21.0 (SPSS).

Results

Baseline characteristics

Median baseline characteristics, as well as results from sperm motility and morphological assessment of the studied patients, are presented in Table 1. The most common sperm abnormalities were elongated head, acrosome vacuoles, thick midsection, and cytoplasmic droplets. Multiple defects were found in 59.0% (36.0–74.0%) of spermatozoa.

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Table 1 Baseline characteristics of the study population (men, *n* = 60) in terms of standard semen parameters, sperm motility, and morphology characteristics and DNA fragmentation.

Characteristics	**Median**	**Range** (minimum–maximum)
Age, years	36.0	25.0–45.0
Duration of abstinence, days	4.0	3.0–5.0
Semen pH	7.9	7.2–8.6
Semen volume, mL	3.1	1.8–5.8
Sperm count, × 10^6/mL	118.1	94.1–228.7
Motility grading, %		
'A' (rapidly progressive >25 µm/s)	36.2	27.3–50.0
'B' (slowly progressive 5–25 µm/s)	29.0	19.0–42.7
'C' (non-progressive < 5 µm/s)	11.0	5.0–15.0
'D' (immotile)	30.5	22.0–50.0
'A+B' (progressive motility)	58.6	50.0–69.0
Morphology, strict criteria		
Normal forms, %	5.0	2.0–10.0
Morphological abnormalities, %		
Head defects		
Small head	22.0	14.0–46.0
Large head	17.0	8.0–33.0
Amorphous head	13.0	6.0–24.0
Elongated head	26.5	14.0–34.0
Round head	14.0	6.0–30.0
Pear-shaped head	2.0	0.0–6.0
Double head	0.0	0.0–2.0
Acephalic	4.0	0.0–12.0
Detached head	4.0	0.0–9.0
Acrosome vacuoles	35.0	13.0–49.0
Nuclear vacuoles	11.0	4.0–26.0
Small acrosomes	14.0	2.0–34.0
Large acrosomes	2.0	0.0–11.0
Absence of acrosomes	13.5	6.0–25.0
Midpiece defects		
Thick neck	24.0	11.0–41.0
Bent neck	6.5	2.0–12.0
Asymmetric	20.5	7.0–36.0
Thin midsection	4.5	1.0–12.0
Cytoplasmic droplets	28.0	14.0–43.0
Tail defects		
Short tail	2.5	0.0–8.0
Coiled tail	6.0	1.0–21.0
Double tail	1.0	0.0–6.0
Multiple sperm defects, %	59.0	36.0–74.0
Multiple anomalies index, %	2.6	2.2–3.0
Teratozoospermia index, %	1.9	1.6–2.0
Sperm deformity index, %	1.8	1.5–1.9
Sperm DNA fragmentation, %	61.0	30.0–93.0

Comparison between swim-up and CM-selected spermatozoa

Sperm motility

The median percentage of fast progressive motile spermatozoa 'grade A' selected by CM was significantly higher compared to the spermatozoa prepared by swim-up (43.5% (20.0–81.1%) vs 30.6% (20.3–71.5%), respectively, $P < 0.001$), as shown in Fig. 2A and Table 2.

Sperm morphology

CM selection significantly increased the percentage of spermatozoa with morphologically normal forms compared to swim-up (14.0% (5.0–23.0%) vs 9.0% (4.0–13.0%), $P = 0.001$) (Fig. 2B). Furthermore, selection by

CM significantly reduced the presence of all types of morphological abnormalities – head, midpiece, and tail defects (Table 2). In addition, the percentage of multiple sperm defects was also considerably lower in samples that underwent CM selection than in those prepared by swim-up (30.0% (18.0–44.0%) vs 49.0% (36.0–60.0%), respectively, P = 0.001) as shown in Fig. 2C. Finally, significantly lower levels of MAI% (2.0% (1.7–2.4%) vs 2.4% (2.0–3.0%), P = 0.001), TZI% (1.5% (1.4–1.8%) vs 1.7% (1.5–2.0%), P = 0.001), and SDI% (1.3% (1.0–1.6%) vs 1.6% (1.4–1.9%), P = 0.001) were found in the CM-selected spermatozoa in comparison to the swim-up prepared ones.

Sperm SDF

The median SDF% in the CM-selected spermatozoa was significantly lower than that in the conventionally prepared swim-up spermatozoa (26.0% (7.0–85.0%) vs 45.0% (16.0–67.0%), respectively, P = 0.03) (Fig. 2D).

Discussion

It has been hypothesized that CM selection may significantly improve sperm characteristics. The present study aimed to examine the efficacy of sperm selection by CM in terms of motility, morphology, and DNA fragmentation and to compare it with the use of conventional swim-up.

Adequate sperm motility is an established factor associated with the success of intra-cytoplasmic sperm injection procedures (Van Der Westerlaken *et al.* 2006, Zheng *et al.* 2016). Unlike its previous version, the latest WHO 6th Edition Laboratory Manual (World Health Organization 2021) distinguishes between slow and fast progressively motile spermatozoa, suggesting that these separate categories may have clinical utility. Some authors have discussed the importance of differentiating between the two groups of progressive sperm (grade A and grade B) in terms of speed and linearity of movement as a qualitative and standardizable measure of progressive motility (Eliasson, 2010, Boitrelle *et al.* 2021). Several studies have reported that specifically fast progressive motility (grade A) has prognostic value for assisted reproduction outcome – in particular, that the complete lack of or limited quantity of such spermatozoa could predict fertilization failure (Verheyen *et al.* 1999, Sifer *et al.* 2005) and is associated with lower pregnancy rates in both intrauterine insemination and IVF cycles (Bollendorf *et al.* 1996). One of the initial findings of this investigation was the increased percentage of fast progressively motile spermatozoa after CM selection, compared to those subjected to swim-up. This indicates that the CM technique allows for the selection of spermatozoa with improved motility. A possible explanation for the observed result could be due to the protein and lipid components prostaglandin E1 (PGE1), prostaglandin E2 (PGE2), prostaglandin F2 (PGF2), pituitary adenylate cyclase-activating polypeptide (ADCYAP1), and pentraxin-3 (PTX3) contained within the cumulus extracellular matrix, which is known to exert a beneficial effect on sperm motility (Turathum *et al.* 2021).

Many studies have reported that spermatozoa which are able to pass through COC have better morphology and higher zona pellucida binding capacity (Zhuo *et al.* 2001, Tanghe *et al.* 2002). The significantly improved morphology of the spermatozoa obtained through the CM method, in contrast to swim-up, could be due to the fact that morphologically normal spermatozoa have markedly better maturity and function compared to those with morphological defects (Gergely *et al.* 1999, Celik-Ozenci *et al.* 2003, Celik-Ozenci *et al.* 2004, Prinosilova *et al.* 2009). In addition, the present study demonstrated a notable reduction in the prevalence of multiple defects among the spermatozoa selected via CM vs those subjected to swim-up. As demonstrated by a previous study, an increased percentage of spermatozoa with multiple defects is associated with an increase in the incidence of spontaneous abortions (Georgieva *et al.* 2017). Furthermore, other authors have reported a link between the presence of sperm with multiple defects in the ejaculate and subsequent unsuccessful fertilization (Menkveld *et al.* 1990, Liu & Baker 1992).

Another result from this study was the significantly lower levels of DNA fragmentation in spermatozoa obtained after CM selection compared to those subjected to conventional swim-up. Multiple sources have demonstrated that spermatozoa with higher DNA quality are associated with improved embryo development as well as a success following embryo transfer (Zheng *et al.* 2018, Parikh *et al.* 2019, Ganeva *et al.* 2021). Therefore, the sperm selection method through CM may improve the outcome after assisted reproduction.

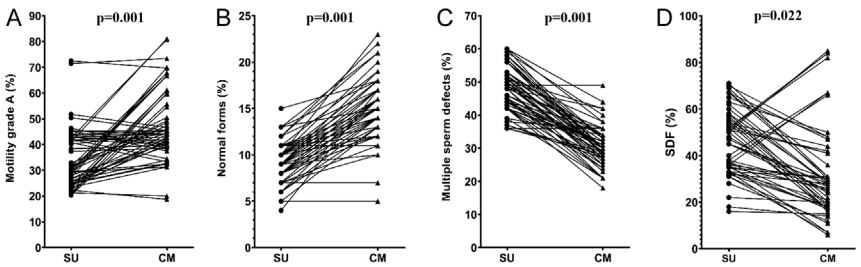

Figure 2

Comparison between sperm subjected to conventional swim-up method and cumulus matrix (CM) selection in terms of percentage: (A) sperm motility grade 'A'; (B) morphologically normal forms; (C) multiple sperm defects; and (D) sperm DNA fragmentation (SDF). Each line represents an individual subject.

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Table 2 Paired comparison of motility and morphology characteristics of the spermatozoa after selection by cumulus matrix (CM) and swim-up procedures. Data are presented as median values.

Characteristics	Selection by swim-up	Selection by CM	*P*
Motility, grading, %			
'A' (rapidly progressive >25 µm/s)	30.6	43.5	0.001
'B' (slowly progressive 5–25 µm/s)	32.2	17.5	0.001
'C' (non-progressive < 5 µm/s)	13.3	16.2	NS
'D' (immotile)	13.8	14.7	NS
'A+B' (progressive motility)	72.2	67.4	NS
Morphology, strict criteria			
Normal forms, %	9.0	14.0	0.001
Morphological abnormalities, %			
Head defects			
Small head	22.0	19.0	0.004
Large head	15.5	14.5	0.014
Amorphous head	10.0	6.0	0.001
Elongated head	22.5	18.0	0.001
Round head	14.0	10.5	0.008
Pear-shaped head	1.0	0.0	0.002
Double head	0.0	0.0	NS
Acephalic	1.5	0.0	NS
Detached head	1.0	0.0	0.020
Acrosome vacuoles	33.0	27.5	0.001
Nuclear vacuoles	11.0	8.0	0.001
Small acrosomes	11.0	7.5	0.002
Large acrosomes	2.0	1.0	NS
Absence of acrosomes	9.5	6.5	0.050
Midpiece defects			
Thick neck	22.0	19.5	0.050
Bent neck	4.0	3.0	0.040
Asymmetric	20.0	16.0	0.001
Thin midsection	2.0	2.0	NS
Cytoplasmic droplets	23.5	16.0	0.001
Tail defects			
Short tail	1.5	1.0	0.024
Coiled tail	3.0	1.5	0.008
Double tail	1.0	1.0	NS
Multiple sperm defects, %	49.0	30.0	0.001
Multiple anomalies index, %	2.4	2.0	0.001
Teratozoospermia index, %	1.7	1.5	0.001
Sperm deformity index, %	1.6	1.3	0.001
Sperm DNA fragmentation, %	45.0	26.0	0.022

NS, not significant.

Sperm selection methods based on native female reproductive tract components, particularly the COC, already exist. One such approach involves the use of hyaluronic acid (HA). Previous studies have shown that multiple HA components contained within the CM have a beneficial effect on sperm DNA integrity (Parmegiani *et al.* 2010, Naknam *et al.* 2019). HA binding-based selection has also shown to yield spermatozoa with lower DNA fragmentation compared to conventional methods like swim-up and density gradient centrifugation (Dandekar *et al.* 1992, Parmegiani *et al.* 2012, Huang *et al.* 2015, Oseguera-López *et al.* 2019). Moreover, the spermatozoa obtained via this selection method exhibit higher progressive motility and improved morphological integrity (Rashki Ghaleno *et al.* 2016). This could be explained by the ability of mature spermatozoa to find and bind to the cumulus extracellular matrix (Rashki Ghaleno *et al.* 2016). Nonetheless, the only utilized component in this selection technique is synthetic and chemically modified and therefore lacks factors crucial for the spermatozoa such as TNF-stimulated gene-6 (TNFAIP6), PTX3, and heavy chains of serum-derived inter-α-inhibitor proteins (Carrette *et al.* 2001, Ploutarchou et al 2015). In contrast, in the present study, native structural components making up the CM, which reflect the

physiological surrounding of the egg, were used. These components are known to act as chemoattractants to spermatozoa, to be involved in the process of capacitation, initiate the acrosomal reaction, and improve sperm motility and DNA integrity (Eisenbach & Tur-Kaspa 1999, Russell & Salustri 2006, Parmegiani *et al.* 2010, Naknam *et al.* 2019, Van Soom *et al.* 2002).

Applying whole COC-containing cells in sperm selection has already been described previously, showing improvement of motility, morphology, and DNA fragmentation, subsequently resulting in higher quality embryos and increased clinical pregnancy rates (Sabet *et al.* 2021). However, the presence of donor cumulus cells in our experimental design was avoided because they may interfere with the spermatozoa behavior by producing metabolites and oxidative species, which may adversely affect sperm vitality.

An additional strength of the present study was the fact that each semen sample was divided in two portions, with one portion used for the novel CM selection technique and the other for the conventional swim-up, significantly reducing possible confounding factors and selection bias. Nonetheless, the described technique has several practical limitations associated with the technical time for obtaining the COC as well as the subsequent mechanical separation of the CM. Future studies should focus on optimizing the CM extraction method, as well as expanding the study population to include patients with various pathological diagnoses such as oligozoospermia and teratozoospermia.

In terms of clinical relevance, the herein-described sperm selection method could be tested in the future with regard to IVF outcomes. Despite the research setting of this study, all possible measures were taken to ensure safety and avoid contamination. These included testing of the donor oocytes for infectious diseases, the use of a sterile laminar flow box, sterile tubes, filters, and containers for the preparation and storage of the CM pool.

While it is possible to prepare a CM matrix filter from the partner's COC on the same day for a personalized approach, the applicability of this strategy could be limited by the small number of oocytes, insufficient quantity of cumulus cells obtained, or advanced age compromising the cumulus quality. Conversely, utilizing a pool of donor oocytes arguably provides a more rigorous quality check of the starting material, as donor women are typically younger and undergo testing for infectious diseases. In addition, using pre-prepared ready-to-use CM matrix filters would ease the integration of this method in routine practice, provided that the shelf life and storage conditions of these filters are determined on a larger scale.

Conclusion

The present study demonstrates that CM sperm selection yields a significantly higher percentage of rapid and linear progressive motile spermatozoa, increased numbers of morphologically normal spermatozoa, reduced incidence of multiple sperm defects, and decreased DNA fragmentation rates when compared to the swim-up preparation technique. The results from this study show that sperm preparation by CM could optimize the selection of high-quality spermatozoa, potentially leading to increased ART success rates.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the study reported.

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Author contribution statement
All authors qualify for authorship by contributing substantially to this article. MH, RG, DP, and GS developed the original concept and design of the study. MH, RG, DP, MR, VG, DV, and RG collected the data, MH performed the statistical analysis, and MH, RG, DP, MR, and GS provided their inputs as regards data interpretation. All authors have contributed to critical discussion and reviewed the final version of the manuscript and approved it for publication.

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