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ORIGINAL RESEARCH

Effects of Cumulus Cell Extracellular Matrix–Assisted Sperm Preparation Protocols on In Vitro Sperm Quality*

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ABSTRACT

This study aimed to determine the effects of adding cumulus cell extracellular matrix (CCECM) to the culture medium used in sperm preparation techniques for assisted reproductive treatments (ART) on routine sperm parameters, fertilization capacity, and sperm DNA fragmentation, and to compare sperm separation protocols.

Ejaculate samples from 30 normozoospermic male patients and cumulus–oocyte complexes collected from their spouses were included. Each semen sample was divided into five equal volumes and allocated to five groups: control, density gradient (DG), density gradient plus cumulus cell extracellular matrix (DG+K), swim-up (SU), and swim-up plus cumulus cell extracellular matrix (SU+K).

The results showed that sperm concentration was markedly lower in all experimental groups than in the control group, with the decrease reaching statistical significance, particularly in the swim-up group. Motility was highest in the SU+K group and was also significantly increased in the DG+K and SU groups. In contrast, the TPMSS value decreased significantly in the SU and SU+K groups. No marked differences were observed among the groups in terms of morphology. Viability increased notably in the CCECM-supplemented groups, and this increase remained significant across all experimental groups compared with the control group. ARIC scores, reflecting acrosomal reaction capacity, were significantly higher in the DG+K and SU+K groups. Moreover, DFI values, indicating DNA damage, were significantly lower in these two groups, suggesting that CCECM supplementation may help preserve sperm DNA integrity.

Adding CCECM to the culture medium used in sperm washing improved sperm quality and efficiency, particularly with the swim-up method. We believe this approach may be a feasible option for treating male infertility within assisted reproductive technologies (ART).

Keywords: Cumulus cell extracellular matrix. Sperm quality. Density gradient. Swim-up, DNA fragmentation. Acrosome reaction.

Kumulus Hücresi Ekstraselüler Matriksi ile Desteklenen Sperm Hazırlama Protokollerinin İn Vitro Sperm Kalitesi Üzerindeki Etkisi

ÖZET

Bu çalışmada, üremeye yardımcı tedavilerde (ÜYTE) uygulanan sperm hazırlama tekniklerinde kültür medyumuna kumulus hücresi ekstraselüler matriksi (CCECM) eklenmesinin; rutin sperm parametreleri, fertilizasyon kapasitesi ve sperm DNA fragmantasyonu üzerindeki etkilerini belirlemek ve sperm ayrıştırma protokollerini karşılaştırmalı olarak analiz etmek amaçlanmıştır.

Çalışmada, normozoospermik 30 farklı erkek hastadan alınan ejakülat örnekleri ile bu hastaların eşlerinden toplanan kumulus-oosit kompleksleri kullanıldı. Her hastadan elde edilen semen örneği beş eşit hacme ayrılarak kontrol, dansite gradiyent (DG), dansite gradiyent + kumulus hücresi ekstraselüler matriksi (DG+K), swim-up (SU), swim-up + kumulus hücresi ekstraselüler matriksi (SU+K) olmak üzere beş grup oluşturuldu.

Elde edilen sonuçlar, tüm deney gruplarında sperm konsantrasyonunun kontrol grubuna kıyasla belirgin olarak azaldığını; bu azalmanın özellikle swim-up uygulanan grupta anlamlı düzeye ulaştığını gösterdi. Motilite oranı en yüksek SU+K grubunda saptanırken, DG+K ve SU gruplarında da motilitenin anlamlı biçimde arttığı belirlendi. TPMSS değeri ise SU ve SU+K gruplarında anlamlı olarak azaldı. Morfoloji açısından gruplar arasında belirgin bir farklılık izlenmezken, canlılık oranının CCECM ilavesi yapılan gruplarda kontrol grubuna göre dikkat çekici şekilde arttığı görüldü. Akrozomal reaksiyon kapasitesini yansıtan ARIC skorları DG+K ve SU+K gruplarında anlamlı derecede yüksek bulundu. Ayrıca, DNA hasarını gösteren DFI değerleri bu iki grupta anlamlı olarak daha düşük saptandı ve CCECM katkısının sperm DNA bütünlüğünü koruyabileceği ortaya kondu.

Sperm yıkama sırasında kullanılan kültür medyumuna CCECM eklenmesi, özellikle swim-up yöntemiyle birlikte uygulandığında sperm kalitesi ve verimliliğini artırdı. Bu yaklaşımın, ÜYTE kapsamında erkek infertilitesi tedavisinde uygulanabilir bir seçenek olabileceğini düşünüyoruz.

Anahtar Kelimeler: Kumulus hücresi ekstraselüler matriksi. Sperm kalitesi. Dansite-gradiyent. Swim-up. DNA fragmantasyonu. Akrozom reaksiyonu.

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Infertility is a major reproductive health problem worldwide, affecting approximately 15% of couples of reproductive age and male-factor infertility can be identified in about 50% of infertile couples[1]. From an anatomical perspective, male infertility is commonly categorized as pre-testicular, testicular, and post-testicular disorders[1]. According to the World Health Organization (WHO) laboratory manual (6th edition, 2021), standard evaluation of male fertility primarily relies on routine semen parameters, including ejaculate volume and pH, sperm concentration/total sperm number, total and progressive motility, vitality, and morphology[2]. However, routine semen parameters do not always accurately predict male fertility potential, as infertility may still be present despite semen parameters within reference limits[2].

In assisted reproductive technologies (ART), one key laboratory step is the isolation of functionally competent spermatozoa capable of fertilizing the oocyte. Conventional sperm preparation techniques such as swim-up/down, density-gradient centrifugation, and glass wool filtration are widely used in ART laboratories[3]. Nevertheless, these approaches may not consistently isolate sperm populations free of DNA damage, and preparation-related handling/centrifugation steps may adversely affect sperm quality in susceptible samples[3]. In clinical practice, spermatozoa are frequently selected for intracytoplasmic sperm injection (ICSI) based on visually assessed criteria under optical magnification (primarily motility and morphology); however, this approach does not directly inform on the genomic integrity of the selected spermatozoon[4].

The cumulus–oocyte complex (COC) surrounds the oocyte and consists of cumulus cells embedded within an extracellular matrix (ECM). The cumulus ECM is rich in hyaluronan (hyaluronic acid, HA) and plays essential roles in ovulation and subsequent fertilization[5]. Mature human spermatozoa express HA-binding properties that have been associated with cellular maturity, viability, and an unreacted acrosomal status[6]. In line with these biological principles, HA-based selection strategies have been proposed to favor spermatozoa with better nuclear quality and reduced DNA fragmentation[7].

Several studies support the relevance of the COC/ECM microenvironment for physiologic sperm selection. For example, sperm selection using cumulus oophorus complexes has been compared with conventional preparation methods, with reports suggesting differences in sperm quality and DNA fragmentation outcomes[4]. However, the literature specifically addressing the direct integration of cumulus cell–derived ECM into routine sperm preparation media remains limited, and comparative evidence across commonly used protocols (e.g., density-gradient vs. swim-up) is scarce. Therefore, the present study was designed to investigate whether supplementing sperm separation media with cumulus cell extracellular matrix (CCECM) can improve sperm efficiency and in vitro sperm quality. In this context, CCECM was incorporated into the media used during density-gradient and swim-up sperm preparation procedures, and the effects of CCECM-assisted protocols on routine semen parameters were evaluated through comparative analyses.

Sperm DNA fragmentation (SDF) testing provides an additional parameter for assessing male-factor infertility. Sperm DNA integrity has been associated with fertilization, embryogenesis, implantation, and pregnancy outcomes; consequently, SDF testing has been described as a valuable tool in selected clinical scenarios[8]. Accordingly, we investigated whether supplementing the preparation media with CCECM improves in vitro sperm quality across commonly used protocols (density gradient and swim-up), assessing routine semen parameters alongside fertilization capacity, acrosome reaction, and sperm DNA fragmentation[8].

Materials and Methods

Our study was conducted in the In Vitro Fertilization Center Laboratories and the Histology and Embryology Laboratories of the Faculty of Medicine at Bursa Uludağ University, with approval from the Clinical Research Ethics Committee of the Faculty of Medicine.

Study population and sample collection

This study was conducted using ejaculate samples obtained from 30 normozoospermic cases. Semen samples were collected by masturbation after 2–3 days of sexual abstinence. Only couples in whom oocyte retrieval was performed on the same day as semen collection were included. Inclusion criteria for male participants were normozoospermia, defined as sperm concentration ≥16 million/mL and semen volume ≥3

mL. Inclusion criteria for female partners were retrieval of at least 5 oocytes. Exclusion criteria comprised severe oligospermia (total progressive motile sperm count, TPMSS <5 million), azoospermia, prior surgical sperm retrieval procedures (TESA, TESE, micro-TESE), inability to provide a semen sample, and refusal to participate.

Group allocation and experimental design

For each participant, the semen sample was divided into five aliquots and assigned to the following groups (Figure 1):

- Group 1 (Raw semen control): Semen was analyzed without any processing.
- Group 2 (DGC—standard medium): Sperm were prepared using the routine density-gradient centrifugation (DGC) protocol with a commercially available MOPS-buffered culture medium.
- Group 3 (DGC + CCECM): The routine DGC protocol was performed, except that the culture medium was supplemented with cumulus cell extracellular matrix (CCECM).
- Group 4 (Swim-up—standard medium): Sperm were prepared using the routine swim-up method with a commercially available MOPS-buffered culture medium.
- Group 5 (Swim-up + CCECM): The routine swim-up protocol was performed, except that the culture medium was supplemented with CCECM.

Baseline semen assessment and post-preparation routine semen parameters were evaluated according to the WHO Laboratory Manual[2].

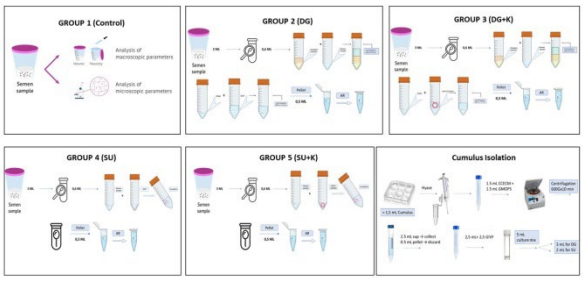

Figure 1.
Schematic overview of the experimental design showing the study groups, sperm preparation protocols applied in each group, and the CCECM isolation procedure.

Standard semen analysis

In all groups, standard semen analysis was performed according to the WHO 2021 criteria[2]. Patients provided a second ejaculate sample 2 h after the first collection, following written informed consent. Samples were collected in sterile plastic containers and incubated at 37°C for 15–30 min to liquefy. After liquefaction, semen was transferred to a conical tube and volume was recorded; macroscopic appearance (color/viscosity) and liquefaction time were noted. Following gentle mixing, 10 µL of semen was loaded into a Makler counting chamber, and sperm concentration and motility were assessed under a phase-contrast microscope. Sperm vitality was evaluated using Eosin-Y staining. Smears were prepared for morphology and sperm DNA fragmentation analyses, air-dried, and stored until processing. After baseline assessment, the remaining ejaculate was divided into five equal aliquots for the washing protocols. Group 1 was analyzed without processing, whereas Groups 2–5 were analyzed after the respective sperm preparation procedures. Residual samples were used for acrosome reaction assessment by fluorescent Pisum sativum agglutinin (PSA) staining[9,10].

Eosin Y test (sperm vitality)

Sperm vitality was assessed using the Eosin Y staining method according to the WHO 2021 criteria[2]. After homogenization, 10 µL of each ejaculate sample was mixed on a microscope slide with an equal volume of 0.5% Eosin Y solution, covered with a 22×22 mm coverslip, and incubated for 30 s. Evaluation was performed under a phase-contrast microscope at ×100 magnification using immersion oil. For each preparation, 200 spermatozoa were randomly selected and scored. Spermatozoa with an unstained head were considered viable, whereas those showing pink/red staining were considered non-viable. Cells showing staining limited to the neck region were also classified as viable, given preserved plasma membrane integrity. The percentage of viable spermatozoa was calculated and recorded after rounding to the nearest whole number[2].

Morphological analysis

Thin smear slides were prepared from ejaculate samples obtained from all groups before and after washing and air-dried at room temperature. The dried smears were stained using the three-step Diff-Quik protocol. Briefly, slides were dipped 10 times in the fixative solution, then 8 times in the Xanthene solution, and 8 times in the Thiazine solution. After staining, slides were rinsed with sterile distilled water for 1 min to remove excess stain and then dried at room temperature. For each smear, 200 spermatozoa were randomly selected and evaluated. Morphological assessment was performed according to Kruger's strict criteria[11], considering abnormalities of the sperm head, midpiece, and tail. The percentage of morphologically normal spermatozoa was calculated, and morphology outcomes were analyzed separately

for each group to determine the distribution of morphological features.

Cumulus cell extracellular matrix (CCECM) isolation

Cumulus granulosa cells are important components of the oocyte microenvironment and have been investigated in previous isolation studies[12]. CCECM was obtained from cumulus cells collected during mechanical/enzymatic oocyte denudation approximately 2 h after OPU in women from whom five oocytes were retrieved. Cumulus material (1.5 mL) was transferred to a conical tube, mixed with an equal volume of MOPS-buffered culture medium (G-MOPS, Vitrolife), and centrifuged at 600×g for 10 min. The supernatant was discarded, and the remaining 0.5 mL pellet was retained. A 2.5 mL portion of the collected supernatant was then mixed 1:1 with sperm buffer, divided into two aliquots (DG+K: 3.0 mL; SU+K: 1.2 mL), and incubated until sperm washing (Figure 1).

Density-gradient centrifugation (DGC) sperm preparation

After liquefaction, semen was divided into five equal aliquots. Group 2 was processed by density-gradient centrifugation according to the WHO 2021 criteria[2]. A two-layer gradient (1 mL 95% over 1 mL 45%) was prepared in a 14-mL conical tube, and 0.5 mL liquefied semen was layered on top and centrifuged at 500 ×g for 15 min. The supernatant was discarded; the pellet was washed with 3 mL sperm buffer, centrifuged at 300 × g for 10 min, and resuspended to a final volume of 0.6 mL. Post-wash concentration and motility were assessed using a Makler chamber. 10-µL smears were prepared for morphology and sperm DNA fragmentation analyses, and the remaining pellet was used for acrosome reaction assessment.

Density-gradient centrifugation with CCECM (DGC + CCECM) sperm preparation

After liquefaction, semen was divided into five equal aliquots. For Group 3 (DGC + CCECM), a two-layer gradient (1 mL 95% over 1 mL 45%) was prepared in a 14-mL conical tube, overlaid with 0.5 mL homogenized semen, and centrifuged at 500 ×g for 15 min. After removing the supernatant, the pellet was washed with 3 mL of medium containing 1.5 mL sperm buffer mixed 1:1 with 1.5 mL of previously isolated CCECM, centrifuged at 300 × g for 10 min, and resuspended to a final volume of 0.6 mL. Sperm concentration and motility were assessed using a Makler chamber; 10-µL smears were prepared for morphology and sperm DNA fragmentation analyses, and the remaining pellet was used for acrosome reaction assessment.

Swim-up sperm preparation

After liquefaction, semen samples were divided into five equal aliquots. Group 4 was processed using the swim-up method according to the WHO 2021 criteria[2]. Briefly, 0.5–0.6 mL of homogenized semen was placed beneath 1.2 mL sperm buffer in a tube and incubated at 37°C for 1 h at a 45° angle. Following incubation, the tube was returned to the upright position, and the upper 1 mL fraction was carefully aspirated. The recovered fraction was resuspended in 1.5 mL sperm buffer and centrifuged at 300–500 ×g for 5 min; the supernatant was discarded, and the pellet was resuspended in 0.5 mL medium for subsequent analyses. Makler chamber analysis (10 µL) was used to assess post-wash sperm concentration and motility; 10-µL smears were air-dried for morphology and SDF, and the remaining pellet was reserved for acrosome reaction analysis.

Swim-up with CCECM (Swim-up + CCECM) sperm preparation

After liquefaction, semen samples were divided into five equal aliquots. Group 5 was processed using the swim-up method with cumulus cell extracellular matrix (CCECM). Briefly, 0.5–0.6 mL of homogenized semen was placed beneath 1.2 mL of medium containing 0.6 mL sperm buffer mixed 1:1 with 0.6 mL previously isolated CCECM. The tube was incubated at 37°C for 1 h at a 45° angle, then returned to the upright position, and the upper 1 mL fraction was carefully aspirated. The recovered fraction was resuspended in 1.5 mL sperm buffer and centrifuged at 300–500 ×g for 5 min; the supernatant was discarded, and the pellet was resuspended in 0.5 mL medium for subsequent analyses.

Acrosome reaction assessment (FITC-PSA)

After sperm washing, 500 µL aliquots were prepared for control and ionophore-stimulated conditions. Pellets were resuspended in 500 µL G-MOPS containing 3% albumin and capacitated at 37°C for 3 h; the stimulated group received 10 µL of the calcium ionophore (HY-136460) and was incubated for 30 min. Samples were centrifuged (1600 rpm, 7 min), and pellets were incubated with 100 µL PBS + 100 µL Hoechst 33342 (CAS: 23491-52-3) for 10 min in the dark, washed, and resuspended in PBS. Smears (10 µL) were prepared on cold slides, fixed in 95% ethanol (30 min), air-dried, stained with 10 µL FITC-PSA (15 min, dark), and washed 10–15 times with PBS. Fluorescence microscopy was used for evaluation[9,10,13]. Among viable sperm, an equatorial FITC-PSA band indicated acrosome reaction (+), whereas diffuse head staining indicated acrosome-intact (−); 200 viable spermatozoa were scored per slide from two fields.

TUNEL assay (sperm DNA fragmentation)

After standard semen analysis and sperm washing, all samples were smeared onto slides, air-dried at room temperature, and stored at −20 °C until staining. Sperm DNA fragmentation was assessed using the TUNEL In Situ (HRP-DAB) Cell Apoptosis Detection Kit (Cat. No: abx092376) according to the manufacturer's instructions. Slides were examined using a Zeiss LSM 900 Airyscan2 confocal microscope with a ×63 oil-immersion objective. Spermatozoa showing brown nuclear staining were classified as TUNEL-positive, whereas those without nuclear staining were classified as TUNEL-negative. For the TUNEL analysis, 200 spermatozoa were evaluated per sample. All evaluations and counts were performed by the same investigator in duplicate. DNA fragmentation was expressed as a percentage, calculated as the number of TUNEL-positive spermatozoa divided by the total number of spermatozoa evaluated.

Statistical analysis

At the end of the study, sperm concentration, motility, vitality, normal morphology, acrosome reaction, and sperm DNA fragmentation were compared across the five groups based on the media used during sperm preparation. The Shapiro-Wilk test was used to assess the normality of the variables. All comparisons were performed using non-parametric methods. The Friedman test was used for dependent samples comparisons to determine overall differences among samples. For variables showing a significant Friedman test result, pairwise comparisons were performed using the Bonferroni correction. A two-sided $p<0.05$ was considered statistically significant. Statistical analyses were conducted using IBM SPSS Statistics (v29.0).

Results

In this study, ejaculate samples obtained after 2–3 days of sexual abstinence were analyzed from 30 normozoospermic men with normal semen parameters whose partners underwent oocyte retrieval on the same day. Each sample was divided into five aliquots to compare sperm washing protocols: Group 1, unprocessed semen; Group 2, density-gradient centrifugation (DGC) with a commercial MOPS-buffered medium; Group 3, DGC with MOPS medium supplemented with CCECM; Group 4, swim-up with a commercial MOPS-buffered medium; and Group 5, swim-up with MOPS medium supplemented with CCECM. After processing, routine semen parameters, morphology, vitality, sperm DNA fragmentation, and acrosome reaction were evaluated across groups, and outcomes were statistically compared (Table I).

Table I. Descriptive statistics of variables by group.

	Control	DGC	DGC + CCECM	SU	SU+ CCECM	p
Sperm concentration (×10⁶/mL)						<0.001
Median (min-max)	29(10-45)[a]	15(5-39)[b]	13(6-40)[b]	8(0.5-20)[c]	8(0.4-20)[c]	
Mean± SD	29±9.9	17±9.8	16±9.5	8±4.8	9±5.3	
Motility (%)						<0.001
Median (min-max)	60(30-77)[a]	72(40-95)[b]	84(59-100)[b]	85(60-100)[c]	100(89-100)[c]	
Mean± SD	58±13.3	73±13.7	82±11.1	84±8.0	98±3.5	
TPMSS (total progressive motile sperm count)						<0.001
Median (min-max)	9(1.5-17)[a]	6(0.8-17)[b]	6(1.6-17)[b]	4(0.16-8.5)[c]	4(0.16-10)[c]	
Mean± SD	9±3.9	6±3.8	6±3.5	4±2.1	4±2.4	
Normal morphology (%)						<0.001
Median (min-max)	3(1-6)[a]	3(1-7)[b]	3(1-7)[b]	4(0-8)[c]	3(1-7)[c]	
Mean± SD	2.9±1.4	3.0±1.5	3.3±1.7	3.4±1.8	3.4±1.6	
Vitality (%)						<0.001
Median (min-max)	57(30-75)[a]	75(60-90)[b]	85(70-95)[b]	80(60-95)[c]	87(70-96)[c]	
Mean± SD	56±10	74±8	83±8	80±8	87±6	
ARIC score (%)						<0.001
Median (min-max)	2.5(1.6-4.7)[a]	2.8(1.7-6.9)[b]	5.7(2.7-14)[b]	3.7(2.5-6.9)[c]	5,7(3.2-18)[c]	
Mean± SD	2.5±0.8	3.5±1.8	6.8±3.4	4.1±1.3	6.7±4.3	
DFI (%) (DNA fragmentation index)						<0.001
Median (min-max)	35(18-43)[a]	17(12-32)[b]	9(3-15)[b]	16(9-20)[c]	2(1-4)[c]	
Mean± SD	32±7.6	19±6.1	8±3.3	15±4.2	2±0.9	

SD: standard deviation, Min: minimum, Max: maximum.
[abc] indicates the results of pairwise comparisons among groups. Values sharing the same letter are not significantly different, whereas values with different letters differ significantly ($p<0.05$).

Semen parameter

Sperm concentration, motility, and TPMSS were compared across the five groups using the Friedman test, followed by Bonferroni-corrected pairwise comparisons. Descriptive statistics for sperm concentration are presented in Table I. The median sperm concentration in the control group was 29 (10–45), compared with 15 (5–39) in DGC, 13 (6–40) in DGC+CCECM, 8 (0.5–20) in SU, and 8 (0.4–20) in SU+CCECM (Table I). Relative to the control group, all processed groups showed significantly lower concentrations. CCECM supplementation did not affect concentration within either technique. In contrast, concentration differed between DGC- and SU-based methods.

Motility differed across groups. The median motility in the control group was 60 (30–77), compared with 72 (40–95) in DGC, 84 (59–100) in DGC+CCECM, 85 (60–100) in SU, and 100 (89–100) in SU+CCECM (Table I). Compared with the control group, motility was significantly higher in DGC+CCECM, SU, and SU+CCECM, whereas the increase in DGC did not reach statistical significance. CCECM supplementation was not associated with a significant change in motility within the density-gradient protocol; however, motility was higher in SU+CCECM than in SU. No significant difference was observed between DGC and SU, while DGC+CCECM and SU+CCECM differed significantly.

For TPMSS, the median value in the control group was 8.7 (1.5–17), compared with 5.5 (0.8–17) in DGC, 5.5 (1.6–17) in DGC+CCECM, 3.5 (0.16–8.5) in SU, and 4.0 (0.16–10) in SU+CCECM (Table I). Compared with the control group, TPMSS was significantly lower in SU and SU+CCECM and decreased in DGC, whereas no difference was observed between the control group and DGC+CCECM. CCECM supplementation did not affect TPMSS within either preparation technique. In contrast, TPMSS differed between DGC- and SU-based methods.

Morphological analysis

For morphological assessment, Diff-Quik–stained sperm smears were evaluated by examining the head, neck/midpiece, cytoplasmic droplet, detached head, and tail regions for morphological abnormalities (Figure 2). Based on these findings, the percentage of morphologically normal spermatozoa was calculated for each sample, and differences between groups were subsequently analyzed. For normal sperm morphology, the median value in the control group was 3 (1–6), compared with 3 (1–7) in DGC, 3 (1–7) in DGC+CCECM, 4 (0–8) in SU, and 3 (1–7) in SU+CCECM (Table I). Bonferroni-corrected pairwise comparisons showed no significant differences between the control group and any processed group. Likewise, no differences were observed between processed groups (DGC vs DGC+CCECM; DGC vs SU; SU vs SU+CCECM; DGC+CCECM vs SU+CCECM). Overall, these findings indicate that the applied sperm preparation protocols did not have a statistically significant effect on normal morphology.

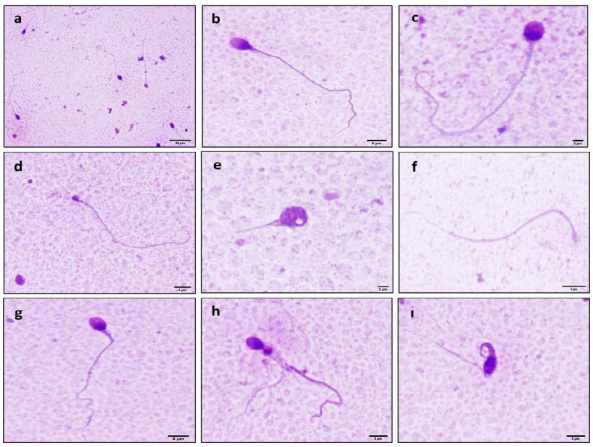

Figure 2.
Sperm morphology assessment on Diff-Quik–stained smears at ×100 magnification. (a) Representative field containing multiple spermatozoa; (b) normal morphology; (c) globozoospermia (round-headed sperm); (d) microcephaly (small head); (e) macrocephaly (large head); (f) pin-head (tapered head); (g) neck/midpiece abnormality; (h) cytoplasmic droplet; and (i) tail abnormality.

Vitality

Sperm vitality assessed by the Eosin Y test differed across groups (Figure 3). The median vitality in the control group was 57 (30–75), compared with 75 (60–90) in DGC, 85 (70–95) in DGC+CCECM, 80 (60–95) in SU, and 87 (70–96) in SU+CCECM (Table I). Compared with the control group, vitality was significantly higher in all processed groups. In addition, CCECM supplementation was associated with higher vitality within both techniques (DGC vs DGC+CCECM; SU vs SU+CCECM). No significant differences were observed between DGC and SU or between DGC+CCECM and SU+CCECM.

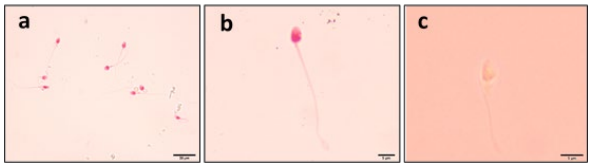

Figure 3.
Sperm vitality assessment using Eosin Y staining at ×100 magnification. (a) Representative field containing multiple spermatozoa; (b) non-viable (dead) spermatozoa; and (c) viable (live) spermatozoa.

Acrosome reaction

In this study, the acrosome reaction–inducing capacity (ARIC) was calculated to quantify the extent of acrosome responsiveness in viable sperm, as identified by Hoechst staining. Specifically, ARIC was defined as the ionophore-induced acrosome reaction rate minus the spontaneous acrosome reaction rate within the same group. Acrosome status was assessed using a lectin-based acrosomal marker, FITC-conjugated Pisum sativum agglutinin (FITC-PSA), according to established protocols[13,14]. The analysis was performed in the unprocessed control group and in the post-wash groups (DGC, DGC+CCECM, SU, and SU+CCECM). Spontaneous and calcium ionophore–induced acrosome reaction rates were determined among viable spermatozoa, and ARIC scores were subsequently calculated for each group (Figure 4).

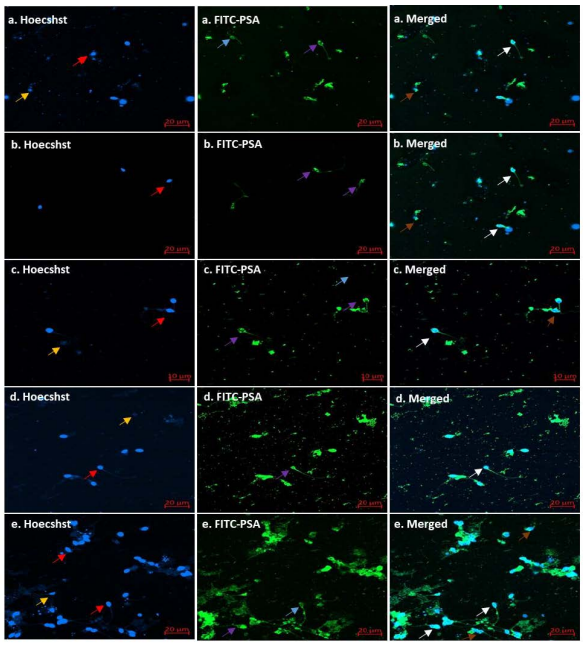

Figure 4.

Sperm viability (Hoechst) and acrosome reaction (FITC-PSA) analysis across study groups. (a–e) In Hoechst-stained micrographs, the red arrows indicate dead spermatozoa (intense blue staining), and the orange arrows indicate viable spermatozoa (pale blue staining). (a–e) In FITC-PSA–stained micrographs, purple arrows indicate viable acrosome-intact spermatozoa (acrosomal cap with intense staining), whereas blue arrows indicate viable acrosome-reacted spermatozoa (equatorial band-like staining). (a–e) In merged images, brown arrows indicate viable spermatozoa that have undergone the acrosome reaction, and white arrows indicate non-viable spermatozoa without acrosome reaction. Panels represent (a) Control, (b) DGC, (c) DGC+CCECM, (d) SU, and (e) SU+CCECM groups.

ARIC scores differed across groups. The median ARIC score in the control group was 2.5 (1.6–4.7), compared with 2.8 (1.7–6.9) in DGC, 5.7 (2.7–14.0) in DGC+CCECM, 3.7 (2.5–6.9) in SU, and 5.7 (3.2–18.0) in SU+CCECM (Table I). In Bonferroni-corrected pairwise comparisons, ARIC was significantly higher in DGC+CCECM and SU+CCECM than in the control group, whereas no differences were observed between the control group and DGC or SU. ARIC also differed between DGC and DGC+CCECM, while no significant differences were found between SU and SU+CCECM, DGC and SU, or DGC+CCECM and SU+CCECM. Overall, these results suggest that CCECM supplementation, particularly within the density-gradient protocol, may be associated with higher acrosome reaction–inducing capacity.

TUNEL assay

Sperm DNA damage was evaluated on smears prepared before and after sperm processing using the TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) method. Following HRP–DAB visualization, spermatozoa showing dark brown nuclear staining were classified as TUNEL-positive (T^+), indicating DNA fragmentation. In contrast, those with light brown/absent nuclear staining were classified as TUNEL-negative (T^-), indicating preserved DNA integrity (Figure 5).

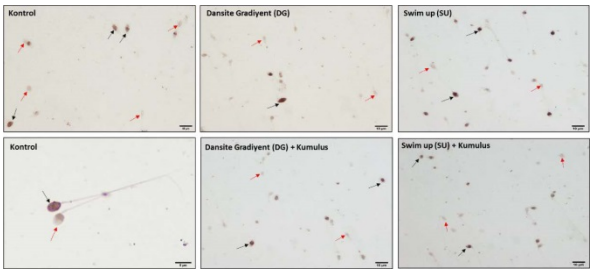

Figure 5.

Representative TUNEL staining results across sperm preparation protocols. Micrographs show TUNEL staining in the Control, DGC, DGC+CCECM, SU, and SU+CCECM groups. Black arrows indicate TUNEL-positive (T^+) spermatozoa with DNA damage, whereas red arrows indicate TUNEL-negative (T^-) spermatozoa with preserved DNA integrity.

DFI (%) differed across groups. The median DFI in the control group was 35 (18–43), compared with 17 (12–32) in DGC, 9 (3–15) in DGC+CCECM, 16 (9–20) in SU, and 2 (1–4) in SU+CCECM (Table I). In Bonferroni-corrected pairwise comparisons, DFI was significantly lower in DGC+CCECM and SU+CCECM than in the control group. In contrast, no significant differences were observed between the control group and DGC or SU. Within-protocol

comparisons showed a borderline difference between DGC and DGC+CCECM, while SU+CCECM had lower DFI than SU. No differences were observed between DGC and SU or between DGC+CCECM and SU+CCECM. Overall, these findings suggest that CCECM supplementation may be associated with lower DFI, particularly within the swim-up protocol.

Discussion and Conclusion

This study evaluated whether supplementing routine sperm preparation media with cumulus cell extracellular matrix (CCECM) could improve conventional semen parameters and functional and genomic sperm quality in an ART-relevant setting. Using a within-sample design, each normozoospermic ejaculate was split into five aliquots (control, DGC, DGC+CCECM, swim-up, swim-up+CCECM), thereby minimizing inter-individual variability and enabling direct protocol-to-protocol comparisons.

Semen parameter changes after processing

As expected, sperm processing reduced sperm concentration compared with raw semen, reflecting the removal of debris, immotile sperm, and other non-target cells during selection[15]. Consistent with this, concentration was significantly lower than control in all processed groups. Importantly, CCECM supplementation did not change concentration in either technique (DGC vs DGC+CCECM; swim-up vs swim-up+CCECM), indicating that the observed concentration loss is primarily attributable to the method's selection principle rather than CCECM addition.

In contrast, sperm processing enriched for higher-quality fractions with improved motility and viability. Swim-up–based selection typically yields a highly motile upper-layer fraction, whereas density-gradient centrifugation (DGC) provides broader recovery while effectively removing debris[16,17]. In our dataset, motility increased significantly compared with control in DGC+CCECM, swim-up, and swim-up+CCECM, whereas DGC alone showed a non-significant trend. Notably, CCECM supplementation significantly increased motility within swim-up (swim-up vs swim-up+CCECM), supporting a protocol-dependent benefit of CCECM on sperm movement.

Morphology did not differ significantly across groups, suggesting that—within normozoospermic samples—neither the preparation method nor CCECM addition produced a detectable shift in strict morphology distributions, which is consistent with the limited sensitivity of morphology for capturing functional competence in otherwise normal ejaculates[2,11].

Functional competence: acrosome reaction capacity (ARIC)

Because fertilization requires timely capacitation and acrosome reaction, we assessed induced acrosome responsiveness using the ARIC score (ionophore-induced AR minus spontaneous AR). The acrosome reaction is a key functional endpoint and has been linked to fertilization outcomes[18,19]. In our study, ARIC was significantly higher in the CCECM-supplemented groups compared with control (control vs DGC+CCECM; control vs swim-up+CCECM), whereas control did not differ from DGC or swim-up without CCECM. These findings suggest that CCECM may enhance acrosome responsiveness beyond that achieved with standard selection alone, potentially by providing a more physiologic microenvironment that resembles cumulus-associated cues involved in sperm activation during fertilization[13,18,19].

Genomic quality: sperm DNA fragmentation (DFI)

Sperm DNA integrity is increasingly recognized as a clinically relevant dimension of male fertility and ART outcomes[8]. Sperm preparation can reduce DNA-damaged sperm by selecting more competent subpopulations; however, centrifugation-associated oxidative stress and iatrogenic handling may also influence DNA integrity[20,21].

In our results, DFI decreased markedly in the CCECM-supplemented groups (control vs DGC+CCECM and control vs swim-up+CCECM). By contrast, control did not differ from DGC and showed only a borderline difference versus swim-up. This pattern indicates that CCECM addition, rather than processing alone, was the main driver of the observed improvement in DFI in this cohort. The significant reduction in DFI within swim-up when CCECM was added (swim-up vs swim-up+CCECM) supports a genuine adjunct effect. Similar to prior split-sample studies, swim-up and/or DGC can reduce DNA-damaged sperm, but the magnitude and direction may vary by patient population, endpoints, and laboratory conditions[22-25,26].

Mechanistically, cumulus-associated extracellular matrix is rich in hyaluronan and related components that interact with mature sperm and may preferentially support functionally competent, genomically intact subpopulations[5-7]. In ART, hyaluronan-based approaches have been associated with selecting spermatozoa with lower DNA damage and improved nuclear quality[7,4,27]. Our findings extend this concept by suggesting that incorporating CCECM into routine media may confer additional protection/selection advantages, particularly in swim-up.

Strengths and limitations

A key strength is the within-ejaculate, five-arm design, which reduces confounding from inter-individual semen variability. In addition, we assessed conventional parameters together with functional (ARIC) and genomic (TUNEL-based DFI) outcomes, enabling a more comprehensive evaluation. This study has several limitations, including the modest sample size (n=30), the inclusion of only normozoospermic cases, and the use of a single assay (TUNEL) to assess DNA fragmentation without parallel validation by other methods, such as SCD or Comet. In addition, the labor-intensive nature of CCECM isolation may limit its immediate clinical applicability unless simplified and standardized protocols are established.

In normozoospermic ejaculates processed for ART, adding cumulus cell extracellular matrix to standard sperm preparation media did not alter the expected reduction in sperm concentration after processing. Still, it was associated with higher motility and viability (protocol-dependent) and, significantly, with improved functional and genomic quality. Specifically, CCECM supplementation increased ARIC scores compared with raw semen and produced substantial reductions in DFI, with the most significant benefit observed with the swim-up protocol. These findings support CCECM as a promising, more physiologic additive to routine sperm preparation; however, validation in larger cohorts—including oligo/teratozoospermic populations—and with additional DNA integrity assays and clinical endpoints (fertilization, embryo development, pregnancy outcomes) is warranted.

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