

Sperm Selection Using Micro Swim-Up: Improvement of Chromatin Integrity Compared to Density Gradient Centrifugation

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ABSTRACT

Background: Selecting high-quality spermatozoa with optimal nuclear integrity is a crucial step in assisted reproductive technologies (ART). While density gradient centrifugation (DGC) is widely used, the associated centrifugation steps may induce cell stress, potentially damaging sperm DNA. Conversely, micro swim-up (MSU) is an innovative, centrifugation-free procedure using autonomous gamete migration in a polyvinylpyrrolidone medium.

Objectives: This study aimed to compare the sperm selection technique MSU with the conventional method DGC in terms of sperm chromatin integrity in 90 semen samples.

Materials and Methods: Rapid progressive motility, sperm DNA fragmentation (sDF; SCD test), nuclear immaturity (aniline blue [AB] staining), and chromatin decondensation (chromomycin A3 [CMA3] staining) were evaluated before and after selection.

Results: Both procedures significantly improved motility and nuclear parameters compared to unselected samples. However, MSU outperformed DGC across all nuclear integrity markers. Specifically, sDF decreased from 18.50% (14.63–26.25) to 9.25% (6.00–16.00) with DGC and 1.00% (0.00–2.00) with MSU ($p < 0.0001$ MSU vs. DGC). Regarding nuclear immaturity, values improved from 21.00% (15.00–27.50) to 15.00% (8.00–18.00) with DGC and 5.00% (4.00–10.00) with MSU ($p = 0.012$ MSU vs. DGC). Similarly, chromatin decondensation decreased from 18.25% (10.88–23.63) to 13.50% (7.00–15.63) with DGC and 5.50% (2.75–10.00) with MSU ($p = 0.0001$ MSU vs. DGC).

Discussion and Conclusion: MSU selects spermatozoa with significantly superior nuclear integrity compared to DGC, markedly reducing DNA fragmentation and improving chromatin maturation. Given the direct impact of these parameters on ART outcomes, MSU might represent a valuable alternative to traditional centrifugation-based methods in clinical settings.

1 | Introduction

In assisted reproductive technologies (ART), the preparation of semen is a fundamental step to enhance the chances of successful

fertilization and pregnancy. The main objective of sperm processing is to isolate motile, morphologically normal sperm with intact DNA, while removing debris, dead cells, and spermatozoa with compromised genomic integrity. Several techniques have been

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developed to achieve optimal sperm selection, and their choice depends on the semen quality, the ART procedure used, and the clinical context.

Traditional sperm processing methods, including swim-up and density gradient centrifugation (DGC), have long been the cornerstone of semen preparation [1]. The swim-up technique is a simple and effective method that relies on the migration of motile spermatozoa into a fresh culture medium. This technique is commonly used in IVF and intracytoplasmic sperm injection (ICSI) protocols. Similarly, DGC separates sperm by their density, enriching the sample with morphologically normal sperm, but it requires centrifugation, which may damage spermatozoa, particularly in poor-quality semen samples [2]. Despite their widespread use, both methods show some limitations, particularly when sperm DNA integrity is compromised, which is a frequent issue in subfertile men [3, 4].

In response to these limitations, more advanced sperm selection techniques have been developed. A particularly promising development in sperm selection has been the use of microfluidic systems [5, 6]. These systems allow spermatozoa to be selected based on their motility and functionality by mimicking the natural conditions of the female reproductive tract, without the need for centrifugation. Microfluidic sperm sorting has been shown to reduce sperm DNA fragmentation (sDF) and improve sperm motility and morphology, which are crucial factors for successful fertilization [7]. However, the technique has some limitations, including lower sperm recovery rates and the need for specialized equipment, which may limit its widespread application in clinical settings [8].

Another emerging method, the micro swim-up (MSU) technique, is a variation of the traditional swim-up, offering a more controlled approach to sperm selection [9]. MSU is easier to implement than microfluidic systems, making it a viable option for a broad range of clinical scenarios [10]. In addition, a previous study reported that MSU showed better reproductive outcomes than DGC in couples treated by ICSI, significantly increasing the rates of blastulation per fertilized oocyte (58.5% vs. 41.7%), Day-3 embryo development (63.7% vs. 46.1%), and clinical pregnancy (41.9% vs. 25.8%) [10]. However, so far, it is not known whether MSU selects sperm with optimal chromatin integrity, a parameter known to impact reproductive outcomes in ART [11–13], similar to what has been shown with microfluidics [14].

Given these considerations, this study aimed to compare the sperm selection technique MSU with the conventional method DGC, in terms of sperm chromatin integrity. Specifically, sDF, nuclear maturity, and chromatin condensation were evaluated before and after selection using both procedures.

2 | Materials and Methods

2.1 | Study Population and Sample Collection

This study was conducted with semen samples obtained from 90 patients (mean age 37.8 ± 5.8 years) attending the Reproductive

Physiopathology Unit at Cervesi Hospital in Cattolica to undergo routine semen analysis. Semen collection was carried out after obtaining informed consent and approval by the Local Ethical Committee (Comitato Etico della Romagna, CEROM), granted on December 12, 2024, authorization number 3830.

Samples were collected consecutively, including subjects with severe oligozoospermia (< 5 million/mL). Conversely, azoospermic subjects—confirmed after sample centrifugation at 249 g for 10 min—were excluded. Subjects with an insufficient ejaculate volume (less than 1.2 mL) to execute both routine semen analysis and selection by density gradient centrifugation (DGC, requiring 1 mL) were also excluded.

All patients observed a sexual abstinence period of 2–3 days. Samples were collected by masturbation into sterile containers and processed for routine semen analysis within 1 h of collection. Each sample underwent evaluation of sperm concentration, total motility, rapid progressive motility, slow progressive motility, non-progressive motility, and morphology [15].

2.2 | Experimental Design

The raw semen sample was processed using two selection procedures: DGC and MSU. Consequently, three aliquots were prepared for each sample: one for pre-selection assessments and two for selection via DGC and MSU. During the selection process, the first aliquot was stored in an incubator at 37°C until analysis (about 1 h from starting routine semen analysis).

The following parameters were evaluated in the raw sample and in both selected sperm populations: sDF via the SCD test ($n = 36$); nuclear immaturity via aniline blue (AB) staining ($n = 24$); and chromatin decondensation via chromomycin A3 (CMA3) staining ($n = 30$). All the analyses were conducted in a blinded manner. Due to the limited sperm yield obtained via MSU, it was not possible to perform SCD, AB, and CMA3 tests on the same semen samples; therefore, these parameters were evaluated across different sample sets.

In the selected populations, sperm number and rapid progressive motility were also determined. All sperm selection procedures were performed under a sterile laminar flow hood.

2.3 | DGC Selection

The gradient layers (95%, 70%, and 50%) were prepared by diluting SpermGrad solution (Vitrolife, Gothenburg, Sweden)—containing silane-coated colloidal silica particles—with G-MOPS PLUS medium (Vitrolife, Gothenburg, Sweden). For each sample, two gradients were prepared. After labeling the tubes to ensure traceability, the gradient phases (0.5 mL each) were layered in the following order: 95%, 70%, and 50%. The volume of the 50% phase was increased to 0.7 mL for samples containing high levels of non-sperm elements.

The semen sample was then gently layered dropwise on top of the gradient, with 0.5 mL added to each of the two tubes, for a total

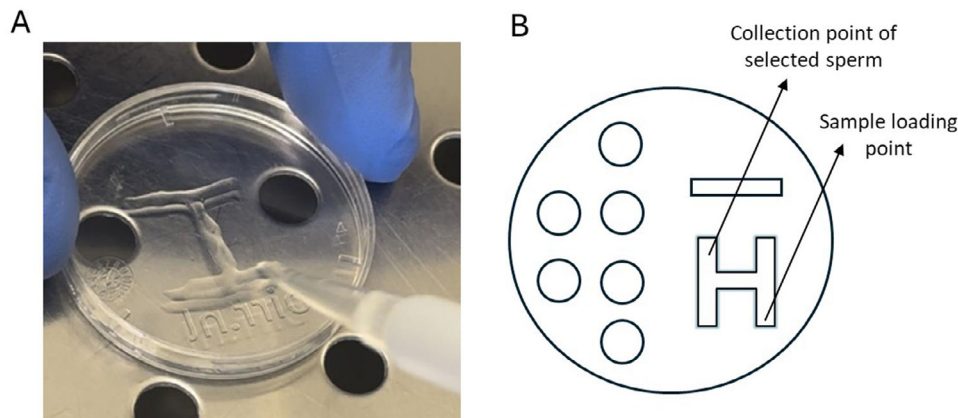


FIGURE 1 | MSU selection. (A) A volume of 10 μ L of ICSI-PVP was used to create an “H-pattern” in a 3.5 cm Petri dish. (B) A 5- μ L aliquot of the raw semen sample was deposited at one end of the H-path. Spermatozoa were allowed to migrate from the loading site to the diagonally opposite collection site.

volume of 1 mL of sample per patient. The tubes were sealed and centrifuged at 249 g for 10 min.

Following centrifugation, up to half of the 95% phase was aspirated and discarded from both tubes. The remaining portions of the 95% layers from both tubes were pooled into a single tube, supplemented with 5 mL of G-MOPS buffer, and gently resuspended. After a second centrifugation at 249 g for 5 min, the supernatant was removed, and the pellet was resuspended in 1 mL of G-MOPS. The entire procedure took 20 min.

A 10- μ L aliquot of the selected sample was deposited onto a slide or a Neubauer chamber and examined under a 40X objective to assess sperm motility and number, respectively. Once processed, the DGC-selected samples were stored in an incubator at 37°C until further use.

2.4 | MSU Selection

A volume of 10 μ L of ICSI-PVP (Vitrolife, Gothenburg, Sweden) was used to create an “H-pattern” in a 3.5 cm Petri dish (Figure 1A). ICSI-PVP is a viscous solution used for sperm handling that contains polyvinylpyrrolidone and recombinant human albumin. When constructing the “H”, careful attention was paid to the dimensions, using the diameter of the Petri dish as a reference.

After forming the H-pattern, the dish was overlaid with OVOIL paraffin oil (Vitrolife, Gothenburg, Sweden) and pre-warmed to 37°C. A 5- μ L aliquot of the raw semen sample was deposited at one end of the H-path. Spermatozoa were allowed to migrate from the loading site to the diagonally opposite collection site (Figure 1B). The migration time was set at 5 min. Following migration, the H-pattern dish was placed on the stage of an inverted microscope to roughly count the number of retrieved spermatozoa and assess rapid progressive motility via a connected monitor. The entire procedure took 10 min. In samples where the number of recovered spermatozoa was lower than approximately 200 (the minimum number required to score sDF, chromatin immaturity, and decondensation), a second MSU selection

was performed. Hence, the recovery fractions were pooled onto the same slide (see below for the description of sample deposition).

2.5 | DNA Fragmentation Evaluation via SCD Test

SCD test was performed with Halosperm kit (Halotech DNA, Madrid, Spain), according to the manufacturer’s instructions with some modifications as described here. A volume containing 12,000 spermatozoa (for raw and DGC-selected samples) was adjusted to a final volume of 2.3 μ L using G-MOPS Plus (Vitrolife, Gothenburg, Sweden), pre-heated to 37°C, followed by the addition of 4.9 μ L of low-melting-point (LMP) agarose. The total volume (7.2 μ L) was deposited onto a slide pre-treated with normal melting point (NMP) agarose and pre-heated to 37°C. The sample was then covered with a 13-mm diameter round coverslip and allowed to solidify at 4°C for 5 min. Subsequently, the coverslip was removed by gently sliding it outwards.

For MSU-selected samples, due to the significantly lower sperm count compared to raw and post-DGC samples, the procedure was performed directly on the slide. For each sample, a droplet formed by 2.3 μ L of pre-heated HTF and 4.9 μ L of LMP agarose was deposited on a slide. Spermatozoa were then transferred into the droplet using a micromanipulator and covered with a 13-mm coverslip. Hence, samples were allowed to solidify, and the coverslip was removed, as described above.

Slides were then treated with a denaturing solution for 7 min and then with a lysis solution for 25 min. After washing with double-distilled water for 5 min, the slides were dried and dehydrated in a graded ethanol series (70% and 100%) for 2 min each. After air-drying under a hood, slides were stained by sequential immersion in the two staining solutions provided by the kit (for 15 min each). Excess stain was removed, and the samples were dried. Microscopic observation was performed at 40 $\times$ magnification, analyzing 200 spermatozoa. Spermatozoa with small or no halos were classified as fragmented, while those with large or medium halos were considered non-fragmented.

2.6 | Nuclear Immaturity Assessment via Aniline Blue Staining

For raw and post-DGC samples, 100,000 spermatozoa were smeared onto a slide and air-dried. The slides were then completely covered with 5% aqueous AB (Sigma-Aldrich, Milan, Italy) mixed with 4% acetic acid (pH 3.5) for 5 min in a staining jar. Excess stain was removed, and slides were kept in a vertical position until completely dry.

For MSU-selected samples, spermatozoa were retrieved from the H-pattern and deposited directly onto the slide using a micromanipulator needle, ensuring distribution within the droplet area. The droplet was air-dried and stained with the AB solution.

Scoring was performed under a 100× oil-immersion objective: spermatozoa with unstained (white) heads were classified as mature, whereas those with blue-stained heads were considered immature. A total of 200 spermatozoa were counted across multiple fields.

2.7 | Chromatin Decondensation Assessment via CMA3 Staining

Samples were mixed with LMP agarose and deposited onto NMP agarose-pretreated slides following the protocols and sperm counts described for the SCD test.

After coverslip removal, slides were washed with PBS for 5 min, dehydrated using a graded ethanol series (70% and 100%) for 2 min each and stored until staining. For CMA3 staining, slides were placed in a humidified chamber and covered with 40 µL of CMA3 solution (250 µg/mL) (DBA Italia, Milan, Italy) in McIlvaine buffer (0.06 M CH₃COOH, 0.28 M Na₂HPO₄, and 0.02 M MgCl₂). Incubation was performed for 20 min in the dark. Subsequently, slides were washed with PBS for 5 min and twice with distilled water (ddH₂O) for 5 min, followed by dehydration in 70% and 100% ethanol for 2 min each.

Fluorescence microscopy was performed using a FITC filter and a 100× oil-immersion objective to analyze 200 spermatozoa across multiple fields. Spermatozoa exhibiting bright, fluorescent staining were considered to have reduced protamination, whereas non-fluorescent spermatozoa were associated with highly condensed chromatin.

2.8 | Statistical Analysis

The study was originally dimensioned using the difference in sDF between DGC and MSU as a primary endpoint. The sample size was calculated with G*Power software (version 3.1.9.7) and applying the following standard statistical parameters for a paired Student *t*-test: effect size (Cohen's *d*) of 0.50 (representing a medium expected difference between DGC and MSU); type I error probability of 0.05 and statistical power (1 – beta) of 0.80. Based on these criteria, a minimum sample size of 34 patients was required. This sample size resulted adequate to identify statistically significant differences when applying repeated measures tests, such as the Friedman test. Fewer patients than originally planned for

the primary endpoint were enrolled for the secondary exploratory endpoints (difference in AB and CMA3 staining between DGC and MSU).

Descriptive statistics have been reported as median and interquartile range (IQR). The normality of data distribution was assessed using the Shapiro–Wilk test. Statistical differences among the three groups (SCD, AB, and CMA3) were assessed using the Kruskal–Wallis test, while comparisons within each group (raw semen, DGC, and MSU selected spermatozoa) were performed using the Friedman test. In both cases, Dunn's multiple comparisons test was used to adjust for multiple testing. When we compared the fold increase or fold decrease obtained by DGC and MSU, a paired-samples *t*-test was used since data after standardization show an approximately normal distribution. The precision of the estimates was reported using 95% confidence intervals (95% CI) of the median, calculated using a non-parametric approach based on the binomial method in GraphPad, accounting for the slight and expected mathematical approximations inherent to this method. The magnitude of the experimental effect was quantified by calculating the effect size using Kendall's *W* coefficient. A *p*-value of less than 0.05 was considered statistically significant for all tests. Statistics were performed using GraphPad Prism 8.4.2 (GraphPad Software, Inc., San Diego, USA).

3 | Results

A total of 90 patients were recruited for this study. Across the three sample sets, we could compare only the baseline semen characteristics (sperm concentration, morphology, progressive and total motility) and the rapid progressive motility after selection (Table 1).

No significant differences among the three groups were evidenced for any of the parameters evaluated (*p* > 0.05, Kruskal–Wallis test). These results statistically suggest that the three subgroups can be considered comparable and free from selection bias regarding semen quality.

Each semen sample was simultaneously processed using both DGC and MSU. Since the volume loaded in DGC (1 mL) is higher than that used in MSU (5 µL), the total number of retrieved spermatozoa differed significantly, ranging in the millions for DGC (median (IQR): 8 million [3.35–16.00]) (recovery rate of 22%) and in the hundreds for MSU (median (IQR): 200 units [140.00–300.75]), where the recovery rate could not be quantified reliably.

The two procedures were first compared in terms of sperm rapid progressive motility. The Friedman test revealed a significant difference among the unselected, DGC, and MSU groups [$\chi^2(2) = 170.0$, *p* < 0.0001]. The effect size (Kendall's *W*) was 1.00, indicating a perfect consistency in the processing effect across all 90 subjects. Figure 2A shows that both selection methods—DGC and MSU—yielded a marked and significant improvement in sperm rapid progressive motility as compared to the unselected sample (unselected: median 20.00% [95% CI: 15.00–20.00], DGC: median 87.00% [95% CI: 85.00–90.00], MSU: median 100.0% [95% CI: 100.0–100.0]). Notably, the improvement of motility by MSU

TABLE 1 | Baseline semen characteristics and rapid progressive motility after selection across the different sample sets used to perform the SCD test, AB, and CMA3 staining.

Parameter	Total (<i>n</i> = 90)	SCD (<i>n</i> = 36)	AB (<i>n</i> = 24)	CMA3 (<i>n</i> = 30)	Reference values [15]
Concentration (10 ⁶ /mL)	43 [30–66]	43 [25–74]	42.5 [23–59]	47 [33–70]	≥ 16
Morphology (% normal forms)	4 [3–6]	5 [4–6]	4 [3–4]	4 [2–5]	≥ 4
Progressive Motility (%)	35 [30–48]	35 [25–50]	40 [35–48]	35 [27–45]	≥ 30
Total Motility (%)	50 [40–60]	55 [40–67]	50 [45–60]	50 [40–56]	≥ 42
Rapid progressive motility (%)					
Unselected	20 [10–25]	20 [8–25]	19 [15–25]	15 [8.75–20]	—
DGC	87 [80–90]	90 [80–90]	82.5 [70–90]	88.5 [80–90]	—
MSU	100 [100–100]	100 [100–100]	100 [100–100]	100 [100–100]	—

Note: Data are presented as median [IQR].

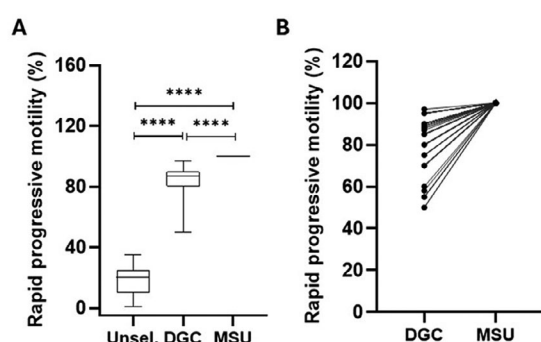


FIGURE 2 | Comparison of sperm rapid progressive motility among selection methods. (A) Box-and-whisker plots representing rapid progressive motility (%) before (unselected) and after selection with DGC and MSU (*n* = 90). *****p* < 0.0001 (Friedman test followed by Dunn's multiple comparisons test). (B) Individual data points illustrating rapid progressive motility (%) using DGC and MSU. Each line represents a paired sample from the same individual processed using both methods.

was greater than that by DGC (*p* < 0.0001). Indeed, in all sperm populations recovered from MSU, rapid progressive motility was 100% (Figure 2B).

To verify whether similar results would be obtained starting from different patients, subjects were stratified into two subgroups based on their median initial rapid progressive motility (20.0%) (Table 2). In the lower-quality subgroup (<20.0%, median value 10%, *n* = 45), MSU significantly outperformed DGC, achieving a median motility of 100% compared to 80% for DGC (*p* < 0.0001). Similarly, in the higher-quality subgroup (≥20.0%, median value 25%, *n* = 45), MSU reached the maximum motility threshold in all samples (median 100%), maintaining a statistically significant superiority over the DGC group (median 90%, *p* < 0.0001). We calculated the improvement of rapid progressive motility also as a fold increase of baseline value (Table 2). Both selection procedures improved the parameter much more in the lower- than in the higher-quality subgroup (DGC: 12.53 vs. 2.72, respectively, *p* < 0.0001; MSU: 16.70 vs. 3.27, respectively, *p* < 0.0001).

To evaluate sperm chromatin integrity, the two procedures were first compared in terms of sDF, measured using the SCD test.

The Friedman test revealed a significant difference between the three groups [$\chi^2(2) = 62.70$, *p* < 0.0001]. The effect size (Kendall's *W*) was 0.87, indicating a strong effect. As reported in Figure 3A, the unselected samples exhibited a median sDF of 18.50% (IQR: 14.63–26.25, 95% CI: 15.50–25.00), which was significantly reduced to 9.25% (IQR: 6.00–16.00, 95% CI: 6.00–15.00) and 1.00% (IQR: 0.00–2.00, 95% CI: 0.00–2.00) following DGC and MSU, respectively. Notably, the MSU procedure shows a sharp decrease in fragmented cells, representing a dramatic improvement not only over the unselected ejaculate but also over the standard DGC method (*p* < 0.0001). As shown in Figure 3B, all values found in the MSU-selected samples were lower than their corresponding DGC-selected values. Using the fifth percentile value observed in the unselected samples as a threshold (9.25%), no MSU-selected samples had higher values (0%), while the percentage rose to 50% among DGC-selected samples.

We also performed a subgroup analysis based on the median baseline sDF level (18.5%) (Table 3). In the higher-sDF subgroup (≥18.5%, *n* = 18), where the baseline genomic damage was more severe (median value 26.00%), MSU drastically reduced sDF to a median of 1.00%. This reduction was significantly greater than that achieved by DGC (median 10.25%, *p* = 0.0026). In the lower-sDF subgroup (<18.5%, median value 14.75%, *n* = 18), MSU similarly achieved a sharp decrease of damaged cells (median 1.00%), again significantly outperforming DGC (median 6.00%, *p* = 0.0026). We also compared the mean fold decrease in sDF between the higher- and lower-sDF groups (Table 3), finding no significant difference for either DGC (*p* = 0.322) or MSU (*p* = 0.559).

We then evaluated the effect of selection with both procedures on chromatin immaturity detected as AB staining. The Friedman test revealed a significant difference between the three groups [$\chi^2(2) = 44.09$, *p* < 0.0001]. The effect size (Kendall's *W*) was 0.96, indicating a very strong effect. Figure 3C shows that the MSU sperm selection procedure significantly reduced the percentage of immature spermatozoa (median 5.00% [IQR: 4.00–10.00, 95% CI: 4.00–9.00]) compared to both unselected samples (median 21.00% [IQR: 15.00–27.50, 95% CI: 15.00–27.00]) and the conventional DGC method (median 15.00% [IQR: 8.00–18.00, 95% CI: 8.00–18.00]). As shown in Figure 3D, all values found in

TABLE 2 | Subgroup analysis of rapid progressive motility (%) following DGC and MSU selection, stratified by baseline levels.

Cut-off	Groups	Median	IQR	95% CI	<i>p</i> -value*	Mean fold increase	95% CI	<i>p</i> -value**
<20.00%	Unselected	10.00	5.00–15.00	8.00–15.00	<i>p</i> < 0.0001	—	—	—
	DGC	80.00	70.00–89.00	75.00–85.00		12.53	8.24–16.82	<i>p</i> < 0.0001
	MSU	100.00	100.00–100.00	100.00–100.00		16.70	10.85–22.54	
≥20.00%	Unselected	25.00	20.00–25.00	20.00–25.00	<i>p</i> < 0.0001	—	—	—
	DGC	90.00	85.00–90.00	89.00–90.00		2.72	2.52–2.92	<i>p</i> < 0.0001
	MSU	100.00	100.00–100.00	100.00–100.00		3.27	3.05–3.50	

*Friedman test followed by Dunn's multiple comparisons test.
 **MSU vs. DGC, paired-sample *t*-test.

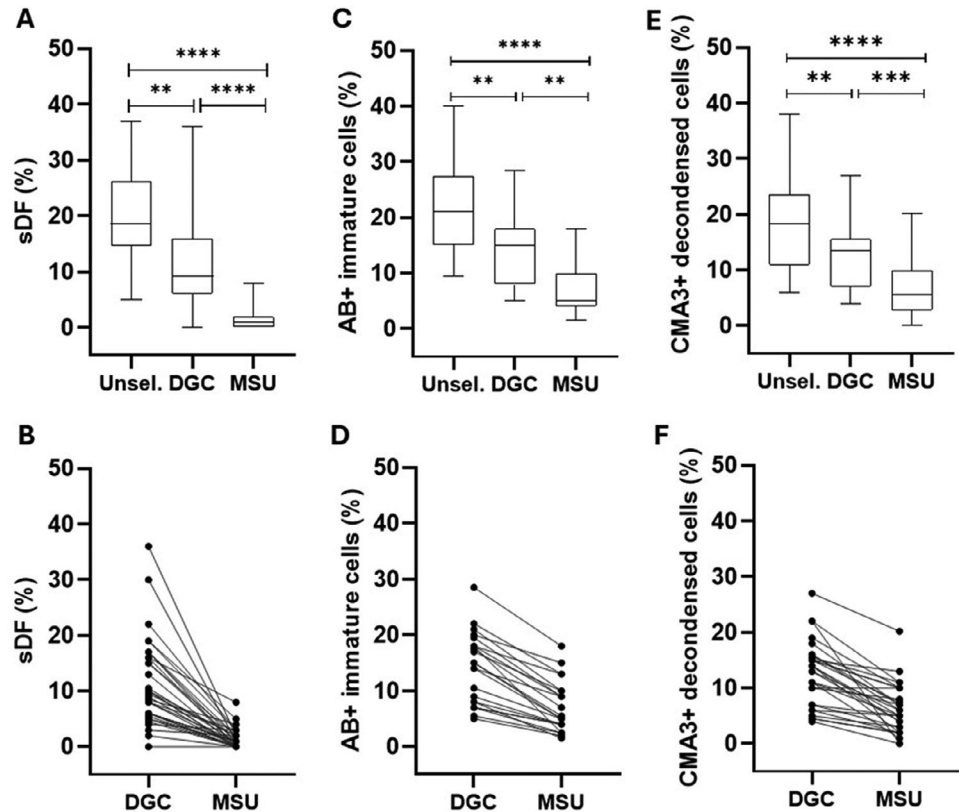


FIGURE 3 | Evaluation of sperm chromatin integrity in unselected and DGC- and MSU-selected spermatozoa. (A and B) Sperm DNA fragmentation (sDF) (*n* = 36) represented as (A) box-and-whisker plots comparing all three groups and (B) pairwise line plots showing individual sample shifts between DGC and MSU. (C and D) Aniline blue-positive (AB+), immature cells (*n* = 24), shown as (C) group comparisons and (D) pairwise line plots showing individual sample shifts between DGC and MSU. (E and F) Chromomycin A3-positive (CMA3+), decondensed cells (*n* = 30) displayed as (E) general distribution and (F) pairwise line plots showing individual sample shifts between DGC and MSU. Box plots represent the median, interquartile range (IQR), and minimum/maximum values. Asterisks indicate statistical significance: ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 (Friedman test followed by Dunn's multiple comparisons test).

the MSU-selected samples were lower than their corresponding DGC-selected values. Using the fifth percentile threshold found in unselected samples (9.90%), only 26% of MSU-selected samples had higher values, a percentage that increased to 65% among DGC-selected samples.

Then, we stratified the samples according to the median baseline percentage of chromatin immaturity (21%) (Table 4). In the

higher-immaturity subgroup (≥21%, *n* = 12), MSU reduced the median value from 27.25% to 9.50%, significantly outperforming the 18.00% achieved by DGC (*p* = 0.0429). In the lower-immaturity subgroup (<21%, *n* = 12), MSU reduced the median percentage of immature cells (15.00%) to 4.00%, showing a significant advantage over DGC, which reached a median of 8.00% (*p* = 0.0315). Also for AB staining, we calculated the mean fold decrease obtained after selection (Table 4), finding

TABLE 3 | Subgroup analysis of sDF (%) following DGC and MSU selection, stratified by baseline levels.

Cut-off	Groups	Median	IQR	95% CI	<i>p</i> -value*	Mean fold decrease	95% CI	<i>p</i> -value**
<18.50%	Unselected	14.75	10.38–17.13	10.50–17.00	<i>p</i> = 0.0026	—	—	—
	DGC	6.00	4.38–16.25	4.50–16.00		0.38	0.31–0.45	<i>p</i> < 0.0001
	MSU	1.00	0.00–2.00	0.00–2.00		0.92	0.91–0.93	
≥18.50%	Unselected	26.00	23.00–30.63	24.00–30.50	<i>p</i> = 0.0026	—	—	—
	DGC	10.25	8.00–16.25	8.00–16.00		0.49	0.45–0.54	<i>p</i> < 0.0001
	MSU	1.00	0.00–2.25	0.00–2.00		0.94	0.92–0.95	

*Friedman test followed by Dunn's multiple comparisons test.

MSU vs. DGC, paired-sample *t*-test.TABLE 4** | Subgroup analysis of sperm chromatin immaturity (AB staining, %) following DGC and MSU selection, stratified by baseline levels.

Cut-off	Groups	Median	IQR	95% CI	<i>p</i> -value*	Mean fold decrease	95% CI	<i>p</i> -value**
<21.00%	Unselected	15.00	13.00–16.50	11.50–18.00	<i>p</i> = 0.0315	—	—	—
	DGC	8.00	7.00–10.50	5.50–15.00		0.37	0.33–0.41	<i>p</i> < 0.0001
	MSU	4.00	2.50–5.00	2.00–5.50		0.72	0.70–0.75	
≥21.00%	Unselected	27.25	23.13–31.50	23.00–32.00	<i>p</i> = 0.0429	—	—	—
	DGC	18.00	15.50–20.75	15.00–21.00		0.34	0.31–0.37	<i>p</i> < 0.0001
	MSU	9.50	6.00–13.00	5.00–13.00		0.65	0.61–0.68	

*Friedman test followed by Dunn's multiple comparisons test.

**MSU vs. DGC, paired-sample *t*-test.

no difference between the lower- and the higher-immaturity subgroups, either after DGC (*p* = 0.656) or MSU (*p* = 0.190).

Finally, we compared the level of protamination between the unselected and DGC- and MSU-selected sperm populations, using the CMA3 test. The Friedman test revealed a significant difference between the three groups [$\chi^2(2) = 55.27$, *p* < 0.0001]. The effect size (Kendall's *W*) was 0.92, indicating a strong experimental effect. Results confirmed that the MSU selection procedure significantly reduces the percentage of poorly condensed spermatozoa (5.50% [IQR: 2.75–10.00, 95% CI: 4.00–7.50]) compared to both unselected samples (18.25% [IQR: 10.88–23.63, 95% CI: 14.00–21.00]) and the conventional DGC method (14.00% [IQR: 7.00–15.63, 95% CI: 10.00–15.00]) (Figure 3E). All values found in the MSU-selected samples were lower than their corresponding DGC-selected values (Figure 3F). Using the fifth percentile threshold found in unselected samples (7.40%), only 29% of MSU-selected samples had higher values, compared to 71% for DGC-selected samples.

The stratification of the subjects based on median baseline percentage of decondensed cells (18.25%) confirms the superior performance of MSU in selecting cells with properly compacted chromatin, regardless of the initial values of the parameter (Table 5). In the higher-decondensation subgroup (≥18.25%, *n* = 15), MSU lowered the median level of protamine deficiency from 23.50% to 7.00%, while DGC achieved a less pronounced reduction

to 15.00% (*p* = 0.0185). In the lower-decondensation subgroup (<18.25%, *n* = 15), MSU reduced the median percentage of CMA3-positive cells from 11.00% to 5.00%, significantly outperforming DGC, which showed a median of 10.00% (*p* = 0.0078). As for the mean fold decreases obtained after selection (Table 5), no differences were found between the higher- and lower-decondensation subgroups, either for DGC (*p* = 0.385) or MSU (*p* = 0.280).

4 | Discussion

Sperm preparation is a crucial step in ART, as it directly influences the quality of spermatozoa selected for fertilization, with downstream effects on embryo development, implantation, and live birth rates. The present study compared two sperm selection procedures typically performed before oocyte insemination. DGC is one of the two standard strategies used in ART laboratories, alongside swim-up [15], yielding a relatively high number of selected spermatozoa. In contrast, MSU is a quite innovative procedure developed in recent years by Palini and collaborators [9] for application in ICSI, where a limited number of spermatozoa possessing the best possible characteristics are required. Conversely, the applicability of MSU beyond ICSI is limited by its low recovery rate. In couples undergoing ICSI, the authors have demonstrated that MSU selection improves several reproductive outcomes compared to DGC. These include the rates of blastulation per fertilized oocyte, Day-3 embryo development,

TABLE 5 | Subgroup analysis of sperm protamine deficiency (CMA3 staining, %) following DGC and MSU selection, stratified by baseline levels.

Cut-off	Groups	Median	IQR	95% CI	<i>p</i> -value*	Mean fold decrease	95% CI	<i>p</i> -value**
<18.25%	Unselected	11.00	10.00–15.50	10.00–15.50	<i>p</i> = 0.0078	—	—	—
	DGC	10.00	5.00–11.00	5.00–11.00		0.25	0.21–0.30	<i>p</i> < 0.0001
	MSU	5.00	2.00–7.00	2.00–7.00		0.60	0.55–0.64	
≥18.25%	Unselected	23.50	20.00–25.00	20.00–25.00	<i>p</i> = 0.0185	—	—	—
	DGC	15.00	14.00–18.00	14.00–18.00		0.32	0.29–0.35	<i>p</i> < 0.0001
	MSU	7.00	4.00–10.00	4.00–10.00		0.68	0.65–0.72	

*Friedman test followed by Dunn's multiple comparisons test.

**MSU vs. DGC, paired-sample *t*-test.

and clinical pregnancy, while tending to decrease the miscarriage rate [10].

Herein, we provide novel and additional insights into the MSU technique by comparing specific sperm characteristics in MSU- and DGC-selected populations to identify underlying factors possibly contributing to MSU's better clinical results previously reported by Palini and collaborators [10]. Firstly, MSU provided 100% rapid progressive motility across all samples, surpassing DGC. This finding is not unexpected, as MSU specifically selects spermatozoa capable of migrating “by swimming” from the loading site to the collection point within a set timeframe. In addition, there is no risk of contamination with less motile cells (as can happen in traditional swim-up), as MSU keeps sample manipulation to a minimum. However, motility is unlikely to be the primary clinical differentiator, as ICSI operators routinely select the most motile spermatozoa regardless of the preparation method [16].

The second key parameter assessed was sDF. Whether sDF levels negatively impact IVF outcomes remains controversial; some meta-analyses report significant correlations [17, 18], while others show negative or inconclusive results [19, 20]. Our study demonstrated that while both procedures reduce sDF compared to raw samples, MSU provides a markedly superior improvement over DGC. This enhancement was consistent across the cohort, with all subjects showing reduced DNA damage compared to DGC. In detail, the residual 9.25% sDF with DGC suggests that the mechanical stress of centrifugation may limit its ability to isolate genetically intact cells. In contrast, the MSU technique—by using a noninvasive, migration-based selection—successfully isolates a population with almost negligible DNA damage.

In this regard, it is well-established that while DGC yields highly motile populations, it may not optimally preserve DNA quality [21]. DGC has been associated with poor sDF improvement and even the induction of de novo DNA damage [4]. The mechanisms remain unclear, though some attribute this to mechanical stress and increased oxidative stress caused by centrifugation [22]. Others suggest that heavy metal contamination in colloidal silica gradients may trigger oxidative stress and subsequent sDF [23], or that specific sperm cells are inherently vulnerable to the procedure's insults [4]. Notably, MSU lacks these centrifugation-

related stressors, which may explain its better results in terms of sperm DNA integrity.

In addition to sDF, this study evaluated nuclear maturity and chromatin condensation. Although often used synonymously, we employed two independent techniques—AB and CMA3 staining—to detect distinct aspects of chromatin immaturity: excess residual histones and protamine deficiency, respectively. The impact of chromatin maturity on ART is supported by several studies, linking high immaturity to increased miscarriage rates, reduced fertilization rates, poorer embryo quality, and lower pregnancy probabilities in ICSI cycles [24–26]. Furthermore, Ribas-Maynou et al. [13] observed a negative impact of poor protamination (CMA3) on fertilization rates in oocyte donation models, where female factors are minimized.

Our comparison of MSU and DGC regarding AB and CMA3 staining yielded consistent results. Both procedures improved these parameters compared to raw samples, yet MSU demonstrated a significantly greater improvement than DGC across nearly all patients. The effects of DGC on nuclear maturity are less well studied than those of sDF. Early research using AB showed slight improvements in DGC-selected populations [27], a finding later confirmed with more pronounced results [28]. Similar trends using CMA3 have been reported [29, 30].

In the subgroup analyses, we found that MSU performed better than DGC in terms of increasing rapid progressive motility and improving sDF levels, nuclear immaturity, and chromatin decondensation. This exploratory analysis might suggest that better MSU performance is independent of the baseline values of these parameters. Furthermore, we found that both procedures improved rapid progressive motility, especially in samples with poor baseline values. Conversely, the improvement in the other parameters (sDF, nuclear immaturity, and chromatin decondensation) was unaffected by their respective baseline values in both DGC and MSU.

While the reason why MSU's better clinical results than DGC [10] is not fully elucidated, the reported correlation between motility and nuclear maturity [31] suggests that the higher sperm motility by MSU may be inherently associated with more intact chromatin structure. In this context, microfluidic sperm sorting has also

been shown to reduce sDF and improve sperm motility [7, 14]. However, this technique has some limitations, including lower sperm recovery rates and the need for specialized equipment, which may limit its widespread application in clinical settings. Consequently, MSU may represent a better choice for routine sperm selection for ICSI in terms of simplicity, cost-effectiveness, and versatility. Indeed, MSU requires minimal technical expertise and basic equipment, making it more accessible, particularly in clinics with limited resources or where specialized microfluidic devices are not available.

A limitation of this study is that all three sperm integrity assays (SCD, AB, and CMA3) could not be performed on the same set of samples. This was due to the inherently low sperm recovery characteristic of the MSU technique, which yielded insufficient cell numbers to conduct multiple staining protocols simultaneously on a single aliquot. Such a limitation prevented us from performing a pairwise correlation between the results of the three assays, and thus verifying whether the increase in DNA integrity was accompanied by similar improvements in nuclear maturity and chromatin protamination. Nevertheless, even if the direct correlation between the three parameters is not demonstrable within this study, the results are statistically robust and provide unequivocal evidence of MSU's efficacy, as the observed trends remained consistent across all evaluated parameters. Another limitation of the study is that we did not evaluate sperm morphology after selection, a parameter used to select the spermatozoon to be injected into the oocyte in ICSI. A final limitation concerns the reason why MSU performs better than DGC in terms of the analyzed parameters. This presumably lies in a combination of factors, including the absence of centrifugation, shorter processing times, and the different principles on which the selection is based. However, this study was unable to determine the individual contribution, if any, of each factor.

5 | Conclusions

This study compared MSU and DGC in terms of rapid progressive motility, sDF, and sperm nuclear maturity in populations selected via both procedures. Although both MSU and DGC effectively improved sperm parameters compared to raw semen samples, MSU proved to be significantly superior to DGC. These enhanced results regarding sDF and nuclear maturity provide underlying mechanisms that might explain the better reproductive outcomes previously observed with MSU in couples undergoing ICSI [10]. Nevertheless, further research is required to confirm whether the improvement of DNA and chromatin quality obtained by MSU can actually explain the clinical success previously reported with this procedure [10].

Author Contributions

Simone Palini: conceptualization, methodology, formal analysis, data curation, supervision, project administration, writing – original draft, review and editing. **Serena Benedetti:** formal analysis, data curation, visualization, writing – original draft, review and editing. **Silvia De Stefani:** investigation, writing – original draft, review and editing. **Luca Galluzzi:** investigation, writing – original draft, review and editing.

Mariangela Primiterra: investigation, writing – original draft, review and editing. **Sara Rita D'Errico:** investigation, writing – original draft, review and editing. **Michele Tanturli:** formal analysis, data curation, supervision, review and editing. **Monica Muratori:** conceptualization, formal analysis, supervision, project administration, writing – original draft, review and editing.

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The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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