

ASSESSING SPERM SELECTION TECHNIQUES: THE IMPACT ON CELL VIABILITY USING FLOW CYTOMETRY

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ABSTRACT

Introduction: This study aimed to evaluate the effects of different sperm selection techniques on seminal parameters and structural characteristics using flow cytometry, with a focus on cell viability, reactive oxygen species (ROS) production, and acrosome integrity. **Methods:** Semen samples from eight normospermic volunteers were subjected to two sperm selection techniques: simple washing and density gradient centrifugation. Following processing, the samples were analyzed using flow cytometry to assess motility, viability, ROS production, lipid peroxidation, the acrosome reaction, and plasma membrane stability. **Results:** The density gradient method significantly increased total motility ($70.75\% \pm 12.87\%$) compared with both raw semen ($55.63\% \pm 8.55\%$) and the washing technique ($53.13\% \pm 4.61\%$). Additionally, the percentage of live spermatozoa was higher in the density gradient group ($71.63\% \pm 13.72\%$) than in the washed group ($54.50\% \pm 9.53\%$). There was no significant difference in ROS production between the techniques; however, the density gradient technique reduced the number of ROS-producing dead cells ($0.04\% \pm 0.06\%$) compared with the washing technique ($0.13\% \pm 0.23\%$). There was an increase in the number of cells with a reacted acrosome after density gradient centrifugation ($25.79\% \pm 15.07\%$) compared with raw semen ($8.80\% \pm 4.28\%$). **Conclusion:** Density gradient centrifugation is superior to the washing technique in selecting viable and motile sperm: It significantly improved total motility and viability. Although the density gradient technique did not increase oxidative stress, it was associated with a higher incidence of the acrosome reaction, which may have implications for fertility outcomes.

Keywords: *Sperm selection; Flow cytometry; Density gradient; Oxidative stress; Acrosome reaction.*

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INTRODUCTION

Millions of couples around the world are unable to conceive a child naturally, with male factor infertility alone accounting for up to 50% of all cases of infertility¹. In such cases, many couples turn to assisted reproduction techniques. Over the years, with the advancement of assisted reproduction, several protocols have been developed to separate motile sperm with an apparently normal morphology from seminal plasma². Currently, three main methods are used for this separation: the *swim-up* technique, which selects spermatozoa based on their motility and ability to swim; the *density gradient centrifugation* technique in which the semen is centrifuged and separated from the plasma by media of different densities; and the *simple wash* technique, where the semen is diluted in cell medium and centrifuged, the supernatant is withdrawn, and the pellet, which contains the cells of interest, is resuspended^{3,4}.

Despite the wide clinical use of these procedures, many published studies on the effectiveness have been unable to determine which technique is superior, considering the commonly used parameters such as morphology, motility, and vitality^{2,5}. Conversely, the results from other studies have demonstrated that the parameters of concentration, motility, and morphology are positively correlated with successful conception⁶.

The microscopic analysis of semen can be used to determine which technique is most appropriate for sperm selection, as the chosen processing method directly influences the quality of the sample to be used. The ideal

sample should contain many moving spermatozoa that have a normal morphology, intact membranes, no dead cells, and minimal or no production of reactive oxygen species (ROS)⁷. Despite the goal of selecting the best cells, sperm capacitation can also interfere with the quality of the remaining spermatozoa. The currently used methods involve a series of steps, such as pipetting between tubes and multiple centrifugation cycles, which expose all cells, including healthy ones, to elevated levels of ROS, thereby inducing oxidative stress that may cause DNA damage and negatively affect fertility^{8,9}. Therefore, selection based solely on motility and morphology overlooks other critical factors, such as membrane alterations, DNA fragmentation, and ROS production¹⁰.

Researchers have shown that the manipulation of seminal samples, such as pipetting, centrifugation, and the use of syringes and needles, can cause structural and functional damage to the spermatozoa^{8,9,11}. Some studies have demonstrated that the centrifugations performed during the sperm selection process increase the level of ROS in the sample because the cells are separated from antioxidant-rich seminal plasma^{12,13}. The density gradient technique appears to be more strongly associated with cellular alterations¹⁴. Although this technique can select spermatozoa with greater viability and separate them from leukocytes and other agents that induce oxidative stress, centrifugation alone is considered a source of cellular stress. Consequently, these cells may produce ROS, leading to self-inflicted oxidative damage^{15,16}.

The aim of this study was to evaluate the effects of different sperm selection protocols on seminal parameters and structural characteristics through the use of flow cytometry.

MATERIALS AND METHODS

An experimental study was conducted using samples from volunteers and patients undergoing infertility investigation at Generar Human Reproduction Clinic in Porto Alegre, Brazil, between March and May 2018. The inclusion criteria were based on participant age and semen quality. For the initial analysis, a semen sample was collected from men aged 18–40 years who agreed to participate by signing a written informed consent form. Only those whose samples presented normal volume, pH, motility, vitality, and morphology, as defined by the World Health Organization (WHO)¹⁷ were included in the study, totaling eight volunteers. This research was approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre and followed the Guidelines and Regulatory Standards for Research Involving Human Subjects of the National Health Council.

Seminal analysis

Samples were collected by masturbation after 3–5 days of abstinence, and were left at room temperature for 30 minutes to 1 hour for sample liquefaction. For each aliquot, volume, pH, concentration, motility, vitality, and morphology of the spermatozoa were analyzed according to the standard procedures described in the WHO manual¹⁷. Then, the sample was divided into three parts: one portion was used for seminal plasma analysis, while the other two were subjected to seminal processing using either the simple washing technique or density gradient centrifugation. After processing, both processed aliquots were re-evaluated using the same WHO criteria. Finally, all three aliquots were assessed for acrosome reaction, reactive oxygen species (ROS) production, plasma membrane integrity, and lipid peroxidation (LPO) by flow cytometry.

Sperm preparation

Washed semen

The raw semen sample was mixed at a 1:1 ratio with Modified HTF Medium – HEPES (Irvine Scientific, Santa Clara, CA, USA) supplemented with 10% Synthetic Serum Substitute (Irvine Scientific) and centrifuged at 300 × g for 10 minutes. Subsequently, the supernatant was carefully removed, and the resulting pellet was resuspended in 0.75 mL the same medium.

Density gradient

The density gradient was prepared in a conical tube by sequentially layering 1 mL of the 90% gradient solution, followed by 1 mL of the 45% gradient solution (Irvine Scientific). The semen sample was carefully placed on top, and the gradient was centrifuged at 300 × g for 20 minutes. After centrifugation, the supernatant was discarded, and the spermatozoa recovered from the 90% layer were washed HEPES supplemented with 10% Synthetic Serum Substitute by centrifugation for 10 minutes at 300 × g. Then, the supernatant was removed, and the pellet was resuspended in 0.75 mL of the same medium.

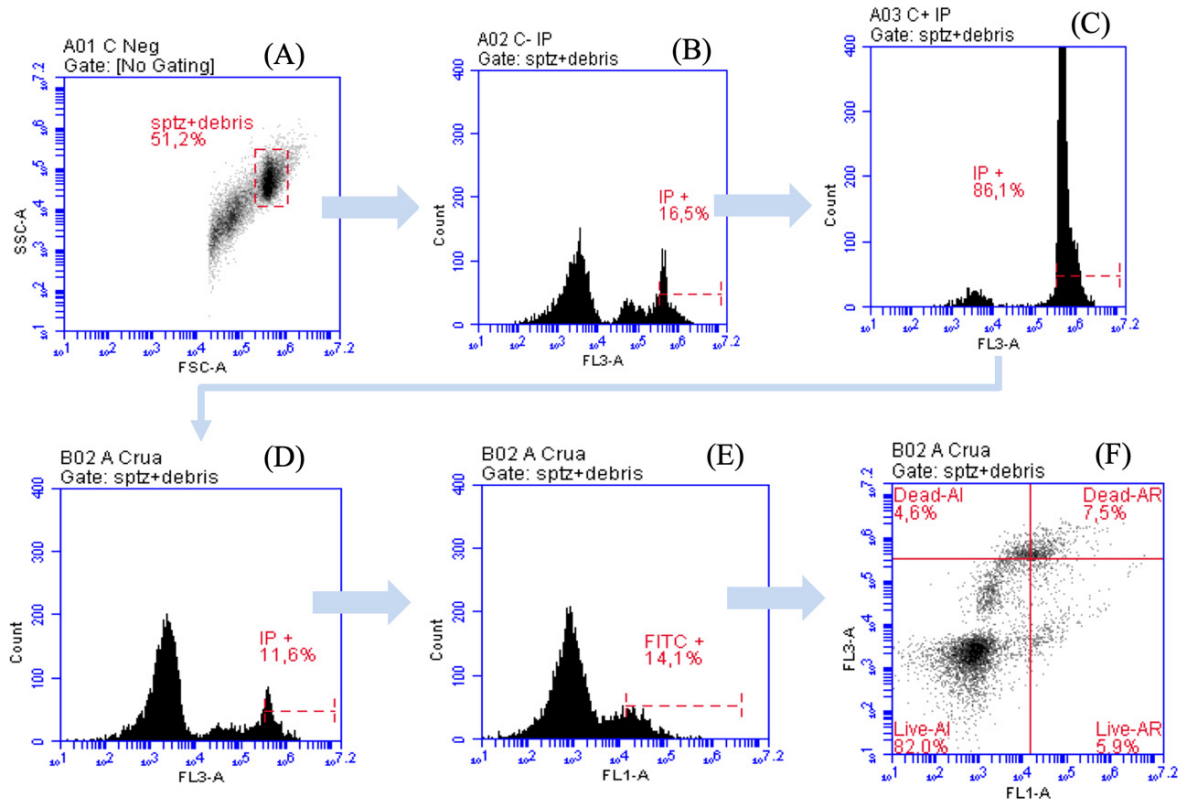
Flow cytometry analysis

Flow cytometry was performed using a BD Accuri C6 flow cytometer (Becton Dickinson, San Jose, CA, USA) operated with BD Accuri C6 Software v1.0. Fluorescently labeled spermatozoa were simultaneously excited by an argon laser (488 nm) and a red laser (670 nm). Fluorescence was detected through FL1 (533/30 nm band-pass), FL2 (585/40 nm band-pass), and FL3 (>670 nm long-pass) channels. Debris was excluded by gating on forward versus side scatter plots (Figure 1). Single-stained controls

were used for manual fluorescence compensation, and untreated samples served as negative controls for

autofluorescence. Data were acquired at 600–1000 events/s, with 10,000 events recorded per analysis.

Figure 1: Schematic representation of the flow cytometry gating strategy. A: Selection of the sperm population; B: Negative control with propidium iodide (sample + probe); C: Positive control with propidium iodide (sample subjected to repeated freeze-thaw cycles in liquid nitrogen + probe); D: Analysis of the sample with propidium iodide (PI+); E: Analysis of the sample with FITC+; F: Overlay of FL1 (FITC) and FL3 (PI) filters, allowing the separation of four populations: dead spermatozoa with intact acrosome; dead spermatozoa with reacted acrosome; live spermatozoa with intact acrosome; and live spermatozoa with reacted acrosome.



Acrosome reaction

Acrosome status was evaluated according to the method of D'Cruz and Haas with minor modifications¹⁸. Semen (5×10^6 spermatozoa/mL) was incubated with fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA, 10 μ g/mL; L0770, Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes at room temperature. The suspension was washed ($600 \times g$, 5 minutes) and resuspended in PBS. Propidium iodide (PI, 10 μ g/mL; P4170, Sigma-Aldrich) was added 5 minutes prior to analysis. Flow cytometry was performed using a 488 nm laser, with FITC fluorescence detected in FL1 and PI in FL3. Data were expressed as median fluorescence intensity for both the total sperm population and viable cells (PI-negative). Positive controls were obtained by subjecting samples to successive freeze-thaw cycles in liquid nitrogen.

ROS production

Reactive oxygen species (ROS) levels in semen after different processing methods were

evaluated according to Martinez-Soto et al.¹⁹. Spermatozoa (5×10^6 /mL) were incubated with 25 μ M dichlorodihydrofluorescein diacetate (H₂DCFDA; DCF, D6883, Sigma-Aldrich, St. Louis, MO, USA) at 25 °C for 40 minutes. Propidium iodide (PI, 10 μ g/mL; P4170, Sigma-Aldrich) was used as a counterstain to exclude non-viable spermatozoa. Green fluorescence from DCF was detected in FL1, while red fluorescence from PI-stained cells was detected in FL3. Data were expressed as median fluorescence intensity, considering both the total sperm population and only the viable (PI-negative) fraction. As a positive control, spermatozoa were incubated with 500 μ M hydrogen peroxide (H₂O₂).

Stability of the plasma membrane

To evaluate plasma membrane lipid packing disorder, spermatozoa were stained with YO-PRO-1 and merocyanine 540 (M540) following Gadea and Martinez-Soto²⁰. Samples (5×10^6 /mL) were incubated with YO-PRO-1 (25 nM; Y3603, Molecular Probes

Inc., Eugene, OR, USA) for 15 minutes at 37 °C. During the last 5 minutes, M540 (2.7 µM; 323756, Sigma-Aldrich, St. Louis, MO, USA) was added. After incubation, fluorescence was analyzed by flow cytometry, detecting YO-PRO-1 in FL1 and M540 in FL2. Data were expressed as mean fluorescence intensity for both the total sperm population and the viable (YO-PRO-1-negative) fraction. Positive controls were prepared by repeated freeze–thaw cycles in liquid nitrogen (for YO-PRO-1) and incubation with 500 µM H₂O₂ (for M540).

Plasma membrane LPO

Lipid peroxidation (LPO) was assessed using the fluorescent probe BODIPY 581/591 C11 (D3861, Molecular Probes Inc.), following Aitken and Koppers^{21,22} with minor modifications. This probe incorporates into sperm membranes and shifts its emission spectrum from red to green upon oxidation²³. Spermatozoa (5 × 10⁶/mL) were incubated with 5 µM BODIPY C11 for 30 minutes at 37 °C, washed (600 × g, 5 minutes), and resuspended in PBS. Viability was determined using propidium iodide (PI, 10 µg/mL) added 5 minutes before flow cytometric analysis. Green fluorescence (oxidized BODIPY) was detected in FL1, and red fluorescence (non-oxidized BODIPY) in FL3. Data were expressed as mean fluorescence intensity (MFI), considering both the total sperm population and viable (PI-negative) cells. Positive controls were prepared by incubating spermatozoa

with ferrous sulfate (640 µM) and hydrogen peroxide (500 mM).

Statistical analysis

Each ejaculate was treated as an individual case (n = 8). Data analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). The Shapiro–Wilk test was initially applied to assess the normality of the data distribution. Subsequently, comparisons were made using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, with statistical significance set at $p \leq 0.05$.

RESULTS

The effect of sperm selection protocols on seminal characteristics

Both sperm selection protocols evaluated in this study significantly influenced seminal parameters (Table 1). The total sperm count decreased approximately 5.6-fold following either simple washing or density gradient selection ($p < 0.0001$). An increase in total and progressive (a+b) motility was observed only in the samples processed with the density gradient technique ($p < 0.05$), whereas no improvement was seen in the washed samples. Total motile sperm count (TMSC) and progressive motile sperm count (PMSC) were significantly reduced after both sperm selection protocols ($p < 0.0001$).

Table 1: Seminal parameters according to the World Health Organization guidelines. Data shown as mean ± standard deviation. Values followed by different lowercase letters within the same row are significantly different (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$). Identical letters indicate no statistically significant difference.

Seminal characteristics	Raw	Washed	Density gradient
Sperm concentration (× 10 ⁶)	46.31 ± 13.16	49.56 ± 11.88	42.73 ± 12.44
Total sperm number (10 ⁶)	198.37 ± 76.64 ^a	37.17 ± 8.91 ^b	32.04 ± 9.34 ^b
Sperm motility (a+b) (%)	43.88 ± 8.63 ^b	44.13 ± 7.47 ^b	59.50 ± 11.90 ^a
Total sperm motility (%)	55.63 ± 8.55 ^b	53.13 ± 4.61 ^b	70.75 ± 512.87 ^a
Progressive motile spermatozoa (× 10 ⁶)	20.62 ± 7.27	21.17 ± 5.06	25.43 ± 8.80
Total motile spermatozoa (10 ⁶)	26.69 ± 9.05	25.36 ± 5.66	29.85 ± 11.30
Progressive motile sperm count (× 10 ⁶)	90.06 ± 41.88 ^a	16.61 ± 5.31 ^b	19.85 ± 6.99 ^b
Total motile sperm count (× 10 ⁶)	115.72 ± 53.94 ^a	19.02 ± 4.24 ^b	21.95 ± 7.85 ^b
Live sperm (%)	63.63 ± 7.61 ^{ab}	54.50 ± 9.53 ^b	71.63 ± 13.72 ^a
Normal sperm morphology (%)	12.88 ± 4.19	13.25 ± 3.01	17.88 ± 4.82

Although the percentage of viable spermatozoa differed between treatments with statistical significance ($p < 0.05$), the absolute magnitude of these differences was modest and do not reflect a clinically or biologically

relevant improvement compared with raw semen. Regarding morphology, neither of the sperm selection protocols affected the total number of morphologically normal spermatozoa.

Effect of sperm selection protocols on oxidative stress parameters

The combination of DCF and PI probes demonstrated that neither washed nor density-selected spermatozoa induced increased ROS production. However, samples processed with

the density gradient exhibited a significantly lower proportion of dead spermatozoa producing ROS ($p < 0.05$) (Table 2). Regarding sperm membrane lipid peroxidation, as assessed using the BODIPY probe, no significant differences were observed between the two processing protocols (Table 3).

Table 2: Sperm viability and ROS (H₂DCFDA/PI) subpopulations in raw, washed, and density-gradient processed samples from normospermic men (n = 8). Data shown as mean $\pm$ standard deviation.

Sperm subpopulations*	Raw	Washed	Density gradient
Live-NoROS	67.42% $\pm$ 10.53%	65.06% $\pm$ 12.55%	75.57% $\pm$ 13.25%
Live-ROS	1.92% $\pm$ 1.64%	3.05% $\pm$ 3.52%	0.75% $\pm$ 1.45%
Dead-NoROS	30.05% $\pm$ 9.46%	31.77% $\pm$ 12.24%	23.65% $\pm$ 13.03%
Dead-ROS	0.61% $\pm$ 0.69% ^a	0.13% $\pm$ 0.23% ^{ab}	0.04% $\pm$ 0.06% ^b

*Live-NoROS - live sperm with no reactive oxygen species (ROS) production; Live-ROS - live sperm with ROS production; Dead-NoROS - dead sperm with no ROS production; Dead-ROS - dead sperm with ROS production. Lowercase letters indicate a significant difference (one-way analysis of variance followed by the Tukey test, $p < 0.05$).

Table 3: Sperm subpopulations by viability and lipid peroxidation (LPO, BODIPY C11/PI) in raw, washed, and density-gradient processed samples from normospermic men (n = 8). Data shown as mean $\pm$ standard deviation.

Sperm subpopulations*	Raw	Washed	Density gradient
Live-NoOx	44.63% $\pm$ 23.88%	47.77% $\pm$ 24.03%	64.01% $\pm$ 17.38%
Live-Ox	30.48% $\pm$ 20.09%	28.59% $\pm$ 19.32%	16.63% $\pm$ 11.42%
Dead-NoOx	14.48% $\pm$ 8.02%	15.19% $\pm$ 8.48%	13.22% $\pm$ 9.41%
Dead-Ox	10.42% $\pm$ 6.70%	8.46% $\pm$ 5.35%	6.14% $\pm$ 3.66%

*Live-NoOx - Live sperm with no LPO; Live-Ox - Live sperm with LPO; Dead-NoOx - Dead sperm with no LPO; Dead-Ox - Dead sperm with LPO.

Effect of sperm selection protocols on cell structural integrity

Incubation of semen samples with FITC-PSA and PI probes enabled the categorization of sperm subpopulations based on plasma membrane integrity and acrosome status (Table 4). Semen processing did not affect the number of cells exhibiting both intact plasma membranes and acrosomes. However, a significant increase in the number of spermatozoa

with reacted acrosomes was observed following density gradient treatment ($p < 0.05$), regardless of membrane integrity. Using M540 and Yo-Pro1 probes, it was found that although density gradient treatment reduced the population of live spermatozoa with destabilized plasma membranes ($p < 0.05$), the average percentage of live cells with stabilized membranes remained similar between experimental groups (Table 5).

Table 4: Sperm subpopulations by viability and acrosome reaction (FITC-PSA/PI) in raw, washed, and density-gradient processed samples from normospermic men (n = 8). Data shown as mean $\pm$ standard deviation.

Sperm subpopulations*	Raw	Washed	Density gradient
Live-IA	72.28% $\pm$ 12.44%	56.99% $\pm$ 19.98%	52.97% $\pm$ 23.11%
Live-RA	8.80% $\pm$ 4.28% ^b	20.50% $\pm$ 9.13% ^{ab}	25.79% $\pm$ 15.07% ^a
Dead-IA	12.45% $\pm$ 6.38% ^a	1.43% $\pm$ 0.64% ^b	1.22% $\pm$ 0.76% ^b
Dead-RA	6.48% $\pm$ 2.96% ^b	21.07% $\pm$ 11.31% ^a	20.03% $\pm$ 11.62% ^a

* Live-IA - live sperm with an intact acrosome; Live-RA - live sperm with a reacted acrosome; Dead-IA - dead sperm with an intact acrosome; Dead-RA - Dead sperm with a reacted acrosome. Lowercase letters indicate a significant difference (one-way analysis of variance followed by the Tukey test, $p < 0.05$).

Table 5: Sperm subpopulations by viability and membrane integrity (Merocyanine 540/Yo-Pro-1) in raw, washed, and density-gradient processed samples from normospermic men (n = 8). Data shown as mean $\pm$ standard deviation.

Sperm subpopulations*	Raw	Washed	Density gradient
Live-Ord	54.43% $\pm$ 10.58%	50.60% $\pm$ 15.50%	65.15% $\pm$ 16.43%
Live-Disord	21.03% $\pm$ 7.87% ^a	19.17% $\pm$ 7.90% ^a	10.01% $\pm$ 5.31% ^b
Dead-Ord	11.45% $\pm$ 9.57%	14.88% $\pm$ 11.98%	11.73% $\pm$ 9.09%
Dead-Disord	13.09% $\pm$ 15.27%	15.35% $\pm$ 17.71%	13.11% $\pm$ 16.86%

* Live-Ord - live sperm with an ordered membrane; Live-Disord - Live sperm with a disordered membrane; Dead-Ord - dead sperm with an ordered membrane; Dead-Disord - dead sperm with a disordered membrane. Lowercase letters indicate a significant difference (one-way analysis of variance followed by the Tukey test, $p < 0.05$).

DISCUSSION

Density gradient centrifugation is widely used for the preparation of spermatozoa in both *in vitro* fertilization and intrauterine insemination, primarily due to its ability to separate motile cells from other cell types, such as leukocytes, epithelial cells, dead cells, and debris present in the sample. Indeed, this protocol resulted in improved seminal parameters when compared to raw and washed semen. Semen washing by centrifugation is a simple, fast, and cost-effective preparation method that involves centrifuging the semen sample in a buffered and supplemented culture medium. Unlike other techniques, this method does not select spermatozoa of higher quality, but simply removes the seminal plasma and concentrates the sample, and is primarily employed for low-quality semen samples³. An increase in total and progressive motility, as well as in the number of live spermatozoa, was observed following density gradient treatment, consistent with previous findings²³. There was no significant change in concentration following seminal processing, as demonstrated by Fácio et al.²⁴, although the total number of cells was reduced due to the inherent effects of the protocols applied.

The use of dual-staining flow cytometry enabled the categorization of distinct sperm subpopulations, enhancing our understanding of the effects of sperm washing and density gradient centrifugation protocols. No significant differences were observed between the selection techniques regarding non-ROS-producing cells, regardless of whether the cells were alive or dead, or producing ROS. This suggests that neither washing nor density gradient centrifugation contributed to increased ROS levels in the medium, contrary to what has been reported in previous studies^{15,16,25}. However, when comparing the techniques, the density gradient was more effective in reducing the proportion of ROS-producing dead cells. As dead or dying cells are the primary

source of reactive oxygen species, and are highly detrimental due to their potential to induce damage in neighboring cells²⁶, their removal from the sample is particularly important.

ROS play an important role in several physiological processes in spermatozoa, such as capacitation, the acrosome reaction, oocyte fusion, and fertilization, when present at low levels¹³. This physiological balance is maintained by a combination of enzymes and antioxidants present in the seminal plasma. However, when ROS production exceeds antioxidant capacity, this equilibrium is disrupted, leading to oxidative stress²⁷. At elevated levels, ROS generated by spermatozoa typically exert harmful effects on the cells themselves.

One of the consequences of elevated ROS levels is lipid peroxidation (LPO)-induced damage to cell membranes²⁸. Spermatozoa are particularly vulnerable to LPO due to the high concentration of polyunsaturated fatty acids in their membranes. LPO results in membrane disruption and flagellar malformation, leading to reduced motility²⁹, which can compromise the sperm cell's ability to fertilize an oocyte^{30,31}. In this study, the absence of elevated ROS levels was associated with minimal membrane lipid peroxidation. Specifically, since no imbalance between ROS and antioxidants was detected—whether due to ROS production or the capacitation protocols employed—significant LPO was not observed. Aitken et al.²¹ demonstrated that the proportion of cells exhibiting LPO-related damage correlates with ROS generation. Furthermore, high levels of LPO have been linked to reduced sperm motility^{21,32}. In the present study, the low levels of LPO were also supported by the observation that sperm motility was not impaired; in fact, motility improved following selection by density gradient centrifugation.

Lipid peroxidation can destabilize sperm membranes, a process closely linked to the acrosome reaction, during which the acrosome fuses with the plasma membrane and releases its contents to enable

sperm–oocyte fusion^{33,34}. Elevated ROS and LPO may compromise acrosomal integrity and interfere with fertilization^{35,36}.

In this study, no overall increase in membrane destabilization was observed; however, specific alterations in the acrosomal region were detected. Similar to Raad et al.²⁶, we found a higher proportion of spermatozoa with reacted acrosomes after density gradient processing, even without significant changes in ROS or LPO, indicating that the alteration is not necessarily oxidative stress-related^{37,38}. Hernández-Silva and colleagues (2021) also reported increased acrosome reactions after density gradient compared to swim-up, attributing this to higher capacitation³⁹. In contrast, we observed a similar increase after simple washing/centrifugation, suggesting that mechanical stress from centrifugation and medium movement may also trigger the acrosome reaction. While density gradient centrifugation effectively selected motile and viable spermatozoa, it was also associated with an increased proportion of reacted acrosomes post-processing.

Another consequence of LPO involves the destabilization of cell membranes. Conformational changes in the membrane play a key role in the acrosome reaction, during which the acrosome fuses with the sperm plasma membrane, releasing its contents into the extracellular environment³³. Once activated, the acrosome facilitates the fusion between the sperm and oocyte membranes³⁴. Alterations in the extracellular environment - such as increased ROS levels and LPO - can compromise the acrosome, thereby interfering with the reaction and, ultimately, with fertilization^{35,36}.

Viable cells that test positive for M540 may indicate an early stage of capacitation⁴⁰, as the acrosome reaction begins with membrane destabilization. The results did not show an increase in the overall number of cells with membrane destabilization; however, a specific alteration was detected in the acrosomal region. Similarly, Raad et al.²⁶ reported a lower percentage of live spermatozoa with intact acrosomes after density gradient processing. Although there were no significant changes in ROS or LPO levels, the analysis revealed an increase in the number of cells with a reacted acrosome—even after sperm selection - indicating that this alteration is not necessarily linked to oxidative stress. These findings are consistent with previous studies^{37,38}. Hernández-Silva et al. (2021) compared the acrosome reaction after density gradient and swim-up processing, reporting an increase in reacted acrosomes with and without calcium ionophore stimulation following density gradient preparation, concluding that the increase was due to a higher capacitation level of the spermatozoa³⁹.

This suggests that although the technique enhances certain seminal parameters, it may simultaneously trigger premature acrosome exocytosis, potentially compromising the fertilization potential of spermatozoa in assisted reproduction procedures such as in vitro fertilization (IVF). This highlights the importance of evaluating not only classical seminal parameters such as motility and viability, but also the structural and functional integrity of spermatozoa when selecting processing techniques. Therefore, while density gradient centrifugation improves sample quality in terms of motility and viability, it may also introduce functional alterations that should be carefully considered when determining its application in clinical contexts.

In clinical practice, both simple washing and density gradient centrifugation are used to prepare semen samples, but their application often differs depending on the assisted reproductive technique. Simple washing is commonly employed in intrauterine insemination (IUI), where a high sperm concentration and motility are desirable, but stringent selection is less critical. In contrast, density gradient centrifugation is frequently used for in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) due to its ability to remove debris, dead cells, and leukocytes, thereby enriching the sample with highly motile and viable spermatozoa.

It is important to acknowledge that the distinction between physiological and pathological ROS levels is highly complex and dependent on the specific biological context. Moreover, the sample analyzed consisted of only eight normospermic donors, which may limit the external validity and generalizability of the results. Individual variability in seminal antioxidant capacity or baseline ROS levels could have influenced the observed outcomes. Therefore, future studies with larger cohorts and the inclusion of additional markers of oxidative stress are recommended for a more comprehensive and robust assessment.

In summary, this study demonstrated that sperm selection techniques did not exert harmful effects via oxidative stress or other molecular alterations, with the exception of an increased proportion of sperm exhibiting acrosomal alterations following density gradient centrifugation. While the density gradient method proved more effective than simple washing, resulting in a significantly greater proportion of viable spermatozoa with enhanced motility and preserved overall cellular integrity, this observation of increased acrosomal disruption warrants further investigation. A deeper understanding of the biological significance and potential reproductive implications of acrosomal changes is essential, particularly in the context of assisted reproductive technologies.

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