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Magnetic-activated cell sorting before density gradient centrifugation improves recovery of high-quality spermatozoa

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SUMMARY

Recent studies have evaluated the use of magnetic-activated cell sorting (MACS) to reduce apoptotic spermatozoa and improve sperm quality. However, the efficiency of using MACS alone, before or after sperm processing by density gradient centrifugation (DGC) has not yet been established. The purpose of this study is to determine the optimal protocol of MACS in assisted reproduction techniques (ART). Thus, we compared sperm quality obtained by DGC alone (DGC), DGC followed by MACS (DGC-MACS), MACS followed by DGC (MACS-DGC), and MACS alone (MACS), and found that the combined methods (MACS-DGC and DGC-MACS) led to retrieval of less spermatozoa with fragmented DNA compared to the single protocols. However, MACS-DGC protocol led to a significantly higher percentage of spermatozoa with progressive motility and normal morphology than DGC-MACS protocol. These findings suggest the potential clinical value of using MACS-DGC to improve sperm quality in seminal preparation for ART.

INTRODUCTION

Assisted reproduction techniques (ARTs) are widely used for treatment of human infertility. However, the success rates of these procedures are less than desired (Ishihara *et al.*, 2015). Studies of infertile couples indicated that male factors are a major cause of infertility (Nadalini *et al.*, 2014). Male subfertility is commonly associated with high rates of DNA damage in the spermatozoa, and such damage has, in turn, been correlated with wide range of adverse clinical outcomes including impaired fertility, disordered embryonic development, high rates of miscarriage, and an increased congenital malformation and childhood diseases (Aitken & Koppers, 2011; Shamsi *et al.*, 2011; Beshay & Bukulmez, 2012). When ART is used to address defective sperm function, fertilization is being achieved in vitro with spermatozoa that would have been excluded from this process in vivo (Beshay & Bukulmez, 2012).

DNA damage in spermatozoa can affect both mitochondrial and nuclear DNA (Sakkas & Alvarez, 2010). Alterations in chromatin remodeling during the process of spermiogenesis could result in DNA fragmentation and the presence of DNA nicks in ejaculated spermatozoa may be indicative of incomplete maturation during spermiogenesis (Sakkas & Alvarez, 2010). Activation of caspases, disruption of mitochondrial membrane potential, and increased DNA fragmentation are some of the apoptotic features that have been identified in ejaculated spermatozoa (Gorczyca *et al.*, 1993; Said *et al.*, 2008). In the presence of efficient apoptosis, abnormal germ cells are eliminated so that only normal sperm cells are present in the ejaculate (Sakkas *et al.*, 1999). For some reason, certain germ cells marked for apoptosis 'escape' the elimination mechanism, and complete the remodeling process during spermatogenesis. Therefore, production of ejaculated spermatozoa that possess apoptotic markers indicate that, in some men with abnormal sperm parameters, an 'abortive apoptosis' has taken place (Sakkas *et al.*, 1999).

Basic techniques of seminal processing are used to select sperm cells that have normal morphology and motility. Sperm preparation techniques are based on sperm sedimentation or migration, density gradient centrifugation (DGC), and 'swim up' (SU) behavior (Makker *et al.*, 2008). However, these procedures have limitations, because they do not use molecular criteria to assess reproductive function, so the selected cells do not necessarily have the greatest functional competence. Although basic seminal processing methods, such as DGC, show great potential

for ART (Makker *et al.*, 2008), additional processing methods may complement these methods and provide selection of functionally superior spermatozoa. Recent data indicate that techniques that allow selection of non-apoptotic sperm cells may overcome some of the limitations of these other methods (Said *et al.*, 2006; Nasr-Esfahani *et al.*, 2012; Sakkas, 2013; Mcdowell *et al.*, 2014; Rappa *et al.*, 2016).

The translocation of phosphatidylserine from the inner leaflet to the outer leaflet of the spermatozoa plasma membrane is an early event of sperm apoptosis (Martin *et al.*, 1995), and is considered one of the signs for recognition and removal of apoptotic cells by phagocytosis (Makker *et al.*, 2008). Annexin V (a marker of apoptosis) has a high affinity for phosphatidylserine, and when combined with magnetic microspheres and subjected to a magnetic field in an iron matrix, can separate apoptotic and non-apoptotic spermatozoa. This procedure is called magnetic-activated cell sorting (MACS) (Ainsworth *et al.*, 2005), and is based on electrophoretic separation of apoptotic (Annexin-positive fraction) and non-apoptotic (Annexin-negative fraction) spermatozoa, without compromising viability so they can be used in ART (Ainsworth *et al.*, 2007). Recent studies have evaluated the use of MACS to reduce apoptotic spermatozoa and improve sperm quality, but the efficacy of MACS in achieving pregnancy after ART is controversial (Dirican *et al.*, 2008; Romany *et al.*, 2010, 2012, 2014; Khalid & Qureshi, 2011a,b; San Celestino *et al.*, 2011; Gil *et al.*, 2013; Troya & Zorrilla, 2015).

Although the externalization of phosphatidylserine is considered an early sign of apoptosis in spermatozoa (Asrm, 2013), some studies have shown that externalization of phosphatidylserine may be part of the physiological capacitation process and an acrosome reaction that occurs in spermatozoa subjected to DGC with albumin (Tavalaee *et al.*, 2012). One study with small sample size (Tavalaee *et al.*, 2012) showed that the use of MACS before DGC was more efficient than MACS after DGC for the recovery of non-apoptotic spermatozoa (Tavalaee *et al.*, 2012). However, the efficiency of using MACS alone or before or after sperm processing by classical methods to improve sperm quality in terms of sperm motility and/or concentration has not yet been established (Lee *et al.*, 2010; Khalid & Qureshi, 2011b; San Celestino *et al.*, 2011; Romany *et al.*, 2012; Tavalaee *et al.*, 2012; Bucar *et al.*, 2015; Cakar *et al.*, 2016).

The purpose of this study is to determine the optimal use of MACS in an ART protocol. Thus, we compared sperm quality obtained by DGC alone (DGC), DGC followed by MACS (DGC-MACS), MACS followed by DGC (MACS-DGC), and MACS alone (MACS). We used the TUNEL assay to assess sperm chromatin integrity and also measured the cell concentration, motility, and morphology of spermatozoa selected by these four procedures.

MATERIALS AND METHODS

We performed a prospective experimental study at the Sector of Human Reproduction, Department of Obstetrics and Gynecology, Ribeirao Preto School of Medicine, University of São Paulo (FMRP-USP). From May 2014 to March 2015, all men who went to the Human Reproduction service of the University Hospital (HCFMRP) for semen analysis were assessed for eligibility. We included men aged between 18 and 50 years old (Brahem *et al.*, 2011) with 3–5 days of sexual abstinence, without history of cancer or use of chemotherapy or other medications likely to affect spermatogenesis. As we proposed to evaluate four different methods of semen processing at the same sample, samples with seminal volume lower than 2.5 mL and semen concentration lower than 15.0 million/mL were excluded from this study. Thus, based on these eligibility criteria, samples from 15 men were considered eligible. Initially, seminal fluid was removed (Tavalaee *et al.*, 2012; Bucar *et al.*, 2015) by washing with 2 mL of HTF-modified HEPES buffer (Irvine Scientific, Santa Ana, CA, USA), followed by centrifugation at 300 ***g*** for 10 min. Spermatozoa were than resuspended in 2.0 mL of HTF-modified HEPES buffer and divided into four separate fractions and processed by DGC alone, DGC followed by MACS, MACS followed by DGC, and MACS alone. Cell concentration, cell motility, and cell morphology were evaluated according to the 5th edition of the World Health Organization Manual guideline (WHO, 2010). The percentage of apoptotic cells was evaluated based on the TUNEL method. Each of the 15 samples was processed by the same four methods.

Density gradient centrifugation

Samples were loaded into a discontinuous gradient (Isolate-Sperm Separation Medium, Irvine Scientific) on 45 and 90% columns and centrifuged at 300 ***g*** for 30 min (WHO, 2010) at room temperature (25 °C). The resulting pellet was centrifuged for additional 10 min and resuspended in 0.5 mL of HTF-modified HEPES buffer (Irvine Scientific). All processing was performed in the absence of serum.

Magnetic-activated cell sorting

A 0.5 mL aliquot of spermatozoa suspended in HTF-modified HEPES buffer, obtained either after sperm wash to remove the seminal plasma or after DGC, was centrifuged and the pellet (maximum of 10^7 cells) was resuspended in 80 μL of binding buffer with 20 μL of Annexin V-conjugated microspheres, both from the Annexin V microbead kit (Miltenyi Biotec, Huburn, CA, USA), for 15 min at room temperature. After addition of 400 μL of binding solution, the suspension was placed in the separation column (MiniMACS, Miltenyi Biotec). Labeled (apoptotic) cells were retained on the column and non-labeled (viable) cells passed through the column. This non-labeled fraction was recovered and processed as described previously (Bucar *et al.*, 2015).

Sperm DNA fragmentation detection (TUNEL method)

DNA fragmentation was assessed by the TUNEL assay using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics GmbH, IN, USA), as described previously (Tavalaee *et al.*, 2012), and modified by the authors. The sperm suspension was first centrifuged for 5 min at 300 ***g***. The supernatant was discarded and the pellet was washed in phosphate-buffered saline (1× PBS), Sigma-Aldrich, SP, Brazil. A drop of the suspension was spread on slides, air-dried, fixed by immersion in 80% methanol (LS Chemicals, São Paulo, SP, Brazil), and then placed in a freezer for 20 min. The slides were permeabilized with 0.01% Triton X-100, Sigma-Aldrich, SP, Brazil for 2 min, washed in 1× PBS solution to adjust the pH to 7.4, and then dried at room temperature. A mix of rTdT nucleotides was prepared for the test and control slides. The DNA strands breaks were labeled with fluorescein-12-dUTP and maintained for 60 min at 37 °C in a humidified chamber protected from light. The reactions were stopped by immersing the slides in 1× PBS, dried at room

temperature in the dark, stained with a Vectashield solution containing DAPI (H 1200 Vector Laboratories, Inc., Burlingame, CA, USA), and covered with a cover slip. Samples were analyzed using a fluorescence inverted microscope Nikon Eclipse E200 (Nikon Corp., Tokyo, Japan). A total of 200 cells were evaluated on each slide (Tavalaee *et al.*, 2012; Delbes *et al.*, 2013; Fortunato *et al.*, 2013). Cells with DNA damage were considered TUNEL positive (apoptotic) (Fig. 1).

Statistical analysis

Continuous variables without normal distributions, determined by graphical analysis and the Kolmogorov–Smirnov test, are summarized as medians and interquartile ranges (IQRs) and compared by the Friedman test. Pairwise comparisons employed Dunn's multiple comparisons test. A $p < 0.05$ was considered significant. All statistical analyses were performed using GRAPHPAD PRISM (version 6.0, GraphPad Software, La Jolla, CA, USA).

RESULTS

We analyzed the baseline parameters of sperm samples from the 15 enrolled men before implementation of the selection procedures with median age of 28 years old [(IIQ) 25: 26.0 e IIC 75: 34.0]. Seminal volume ranged from 2.5 to 4.2 mL [median: 3.1, (IIQ) 25: 2.8 e IIC 75: 3.5]. The total sperm count ranged from 75.0 to 458.0 $\times$ 10^6 cells [median: 172.0 $\times$ 10^6 cells (IIQ) 25: 118.0 e IIC 75: 214.0 $\times$ 10^6 cells]. The concentration of spermatozoa ranged from 25.0 to 183.0 $\times$ 10^6 cells per mL (median: 51.0 $\times$ 10^6 per mL, IQR: 39.0 to 66.0 $\times$ 10^6 per mL). The percentage of cells with normal morphology ranged from 2 to 9% (median: 5.0%, IQR: 4.0 to 7.0%). The percentage of cells with progressive motility ranged from 12 to 87% (median: 39.0%, IQR: 19.0 to 59.0%). The percentage of cells with DNA fragmentation ranged from 6.0 to 29.0% (median: 24.0%, IQR 9.0 to 26.0%). The percentage of living cells ranged from 71.0 to 95.0% (median: 85.0%, IQR: 77.0 to 88.0%) (Table 1 and Fig. 2).

Table 1 and Fig. 2 show the parameters of the same 15 samples after the different selection procedures. The percentage of cells with DNA damage was significantly lower in the MACS-DGC group than in the DGC and MACS groups, but similar to the DGC-MACS group. The sperm concentration was highest and similar in the DGC and MACS groups, and significantly lower in the MACS-DGC and DGC-MACS groups, which were similar to each other. The progressive motility of cells was highest and similar in the MACS-DGC and DGC groups, and significantly lower in the MACS and DGC-MACS groups. The percentage of morphologically normal cells was significantly higher in MACS-DGC group than the DGC-MACS and MACS groups, but similar to the DGC group.

DISCUSSION

Our comparison of four sperm processing methods (DGC, DGC-MACS, MACS-DGC, and MACS) indicated that the two combined methods (MACS-DGC and DGC-MACS) provided recovery of spermatozoa with lower percentages of fragmented DNA. MACS alone or applied after the DGC promotes a significant reduction in the progressive spermatozoa retrieved, as well

Figure 1 DNA integrity spermatozoa stained by TUNEL method (TdT-mediated dUTP nick end labeling). Blue signals indicate labeling of sperm DNA by 4′,6-diamidino-2-phenyl-indole (DAPI). Green signals indicate TUNEL-positive spermatozoa. Arrows indicate TUNEL-positive spermatozoa in overlapping images (MERGE). Samples processed by (A) DGC, (B) DGC/MACS, (C) MACS/DGC, and (D) MACS. [Colour figure can be viewed at wileyonlinelibrary.com].

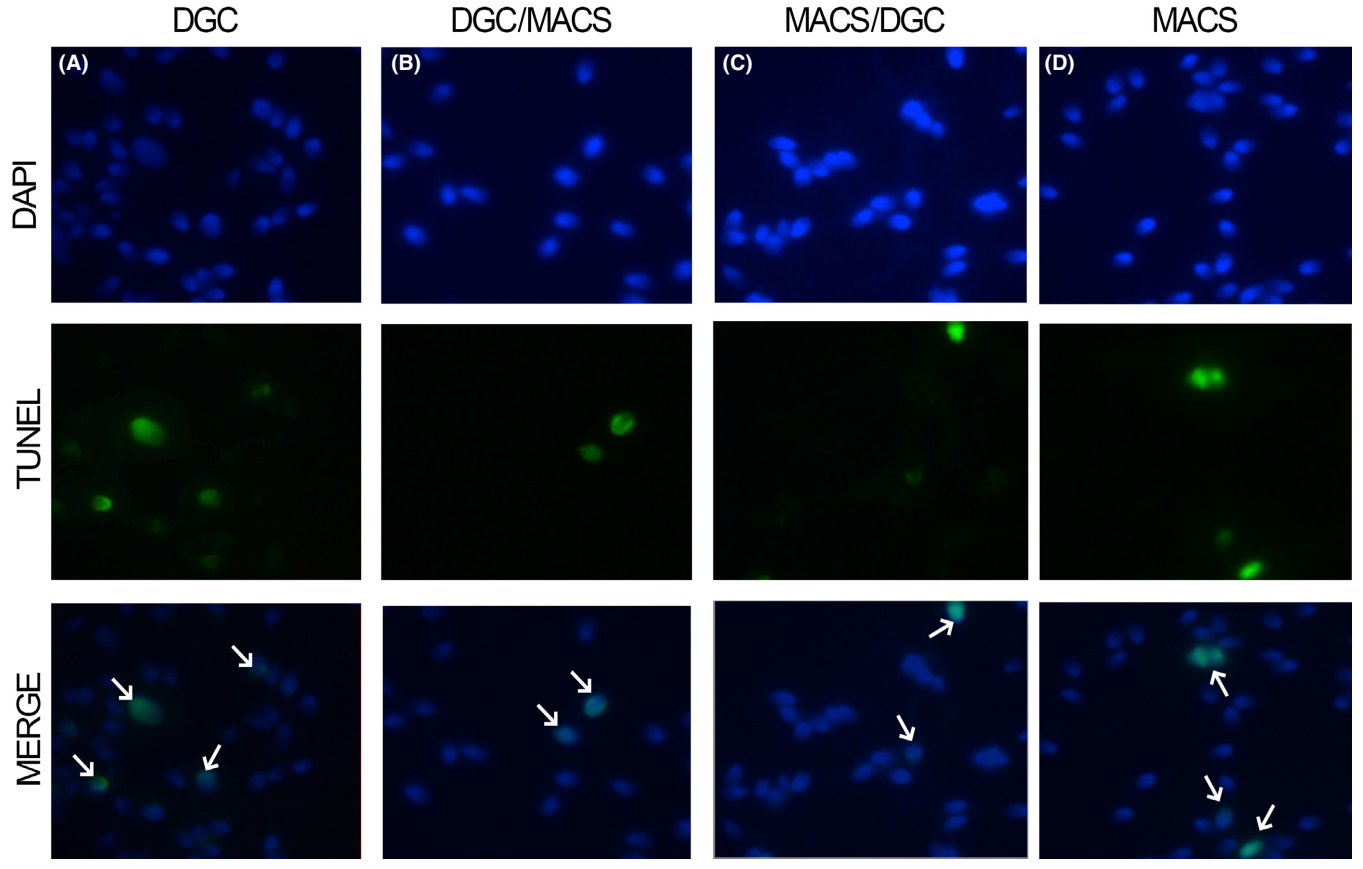


Table 1 Comparison of sperm DNA fragmentation, concentration, progressive motility, and normal morphology among samples processed by density gradient centrifugation (DGC), magnetic-activated cell sorting followed by DGC, MACS-DGC, DGC-MACS, and MACS

	Baseline parameters	DGC	DGC-MACS	MACS-DGC	MACS
DNA fragmentation, %	24 (9–26)	10 (5–16)[a]	6 (3–11)[b]	4 (2–7)[a,c]	8 (6–16)[b,c]
Concentration, %	51 (39–66)	12 (7–16)[a,b]	5 (3–9)[a,c]	4 (3–7)[b,d]	15 (13–24)[c,d]
Progressive motility, %	39 (19–59)	61 (39–76)[a,b]	10 (2–20)[a,c]	68 (44–78)[c,d]	11 (2–27)[b,d]
Normal morphology, %	5 (4–7)	6 (5–7)	5 (4–6)[a]	7 (6–9)[a,b]	5 (4–8)[b]

DNA fragmentation measured by TUNEL (TdT-mediated dUTP nick end labeling). Each entry shows the median (interquartile range). Only the four processing groups (DGC, DGC/MACS, MACS/DGC, and MACS) were compared statistically with each other by the Friedman test. Pairwise comparisons employed Dunn's multiple comparisons test. Common letters in the same row indicate a significant difference ($p < 0.05$).

as the percentage of spermatozoa with normal morphology. However, the MACS applied before the DGC promotes the recovery of samples with a higher percentage of spermatozoa with progressive motility and normal morphology, combined with low percentage of fragmented DNA.

It is unknown whether combining MACS with DGC and/or SU separation techniques can improve sperm selection, or the optimal sequence for combining these techniques. Most studies compared the effectiveness of MACS alone with classical processing methods (DGC or SU) by measurement of DNA fragmentation, although a few studies compared the effects of combined methods on sperm quality (Tavalaee *et al.*, 2012; Nadalini *et al.*, 2014; Bucar *et al.*, 2015; Cakar *et al.*, 2016). For example, Tavalaee *et al.* (2012) collected semen samples from 15 infertile patients and processed them using the same methods used in this study. They found that DGC or MACS alone reduced DNA fragmentation by about 30%, DGC-MACS reduced DNA fragmentation by 40%, and MACS-DGC reduced DNA fragmentation by 49% (Tavalaee *et al.*, 2012). This supports our finding that the combined procedures (DGC-MACS and MACS-DGC) produced a significantly less DNA fragmentation. However, these authors did not evaluate other important parameters related to sperm quality, such as sperm concentration and motility. Our study is the first to simultaneously assess all of these variables in a protocol using MACS and DGC.

Bucar *et al.* (2015) randomly distributed semen samples from 100 men into five distinct groups, and then analyzed the effect of using combined methods of sperm processing (DGC-SU, DGC-MACS-SU, DGC-SU-MACS, MACS-DGC-SU, and MACS-SU). Relative to controls (no processing), all five groups had significantly reduced DNA fragmentation, with the greatest reduction in the MACS-DGC-SU group (83.3%). However, these authors did not evaluate seminal processing using MACS-DGC and DGC-MACS, and did not compare the effect of the five methods on cell morphology, concentration, and progressive motility. Cakar *et al.* (2016) evaluated whether combining MACS with DG or SU techniques could improve sperm selection, but they only performed MACS after DGC or SU. Compared to fresh sperm samples, sperm concentration and rapid progressive motile sperm concentration was significantly lower following both combined methods (SU/MACS and DGC/MACS). This shows that performing MACS after a classical sperm separation technique is an unsuitable protocol (Cakar *et al.*, 2016), in agreement with our findings. As progressive motility is essential feature for the success of certain ARTs, such as intrauterine insemination (IUI) and in vitro fertilization (IVF) (Van Voorhis *et al.*, 2011; Berker *et al.*, 2012; Sakkas *et al.*, 2015), it is fundamental to evaluate this parameter. Our study is the first to demonstrate that the MACS performed before DGC yields a significantly higher percentage of progressive spermatozoa than the DGC-MACS procedure (68 vs. 10%), simultaneously assessing the motility and concentration of spermatozoa in the same protocol.

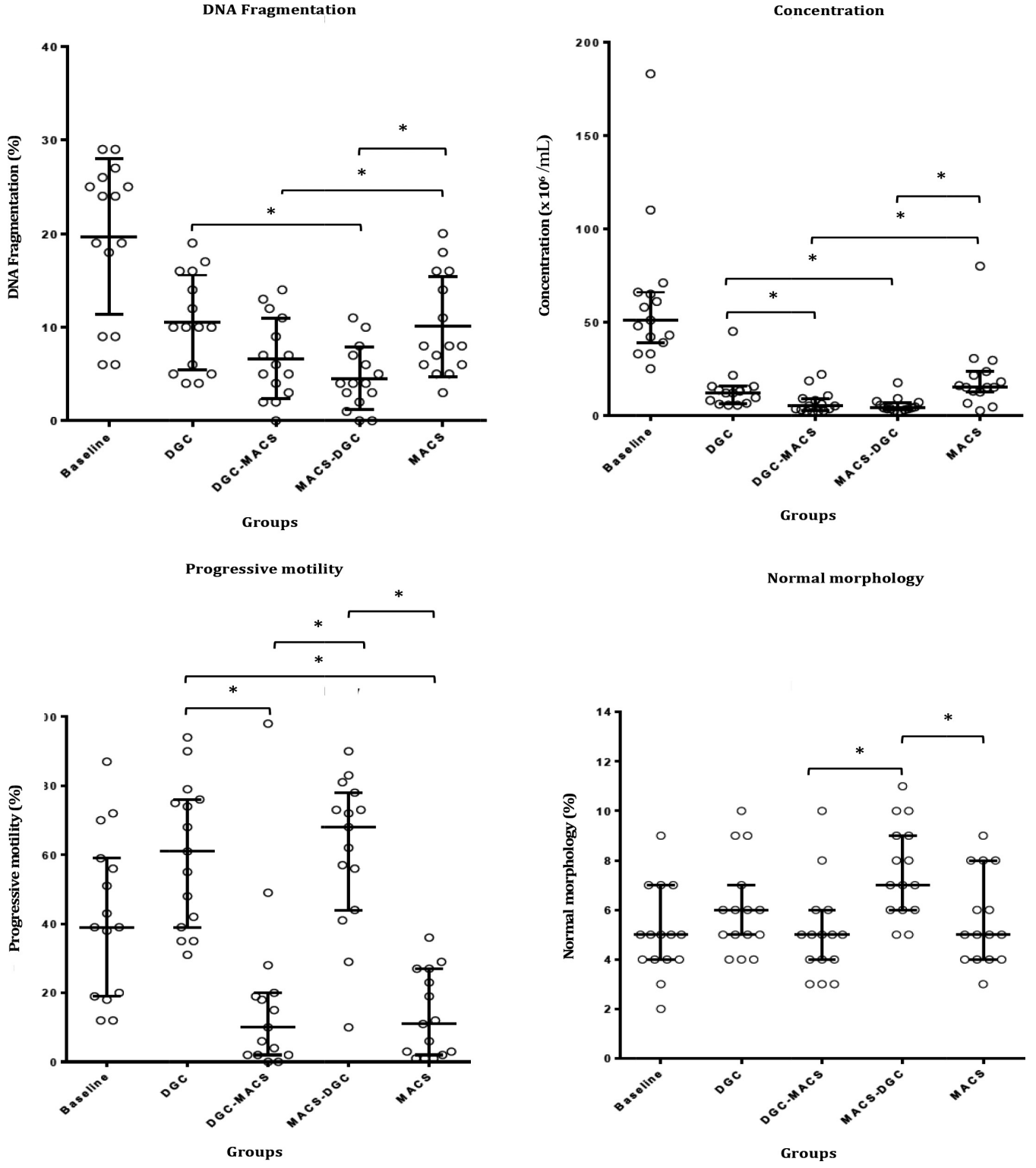
In this study, we found an increase in the percentage of cells with normal morphology only for MACS-DGC processing, unlike Tavalaee *et al.* (2012), who observed increases with the MACS-DGC and DGC-MACS procedures. Said *et al.* (2005) and Aziz *et al.* (2007) showed no improvement in the percentage of cells with normal morphology following MACS, but reported a decrease in this variable in normozoospermic patients (Delbes *et al.*, 2013). However, the impact of sperm morphology in natural fertility and the success of ART remains poorly elucidated (Nikbakht & Saharkhiz, 2011; Sakkas *et al.*, 2015).

Studies comparing pregnancy rates following different seminal processing methods (MACS with different additional protocols) obtained variable results (Dirican *et al.*, 2008; Romany *et al.*, 2010, 2012, 2014; Khalid & Qureshi, 2011a,b; San Celestino *et al.*, 2011; Troya & Zorrilla, 2015). Among other things, this could be because of the lack of protocol standardization. It should be noted that previous studies using MACS for sperm processing utilized this methodology alone (Dirican *et al.*, 2008; Alvarez Sedó *et al.*, 2010; Nikbakht & Saharkhiz, 2011; Van Thillo *et al.*, 2011), MACS after SU (Romany *et al.*, 2010, 2014), or MACS after DGC (Rawe *et al.*, 2010; Khalid & Qureshi, 2011b; Nadalini *et al.*, 2014). Only one study tested the effect of MACS before DGC (Tavalaee *et al.*, 2012), but they did not evaluate the efficiency of this combined processing method in terms of sperm motility and concentration.

Our findings indicate that the MACS-DGC protocol was the most effective in that it produced cells with the lowest percentage DNA fragmentation, the highest percentage of progressive motility, and the highest percentage of normal morphology.

As previously pointed out, it has been shown that externalization of phosphatidylserine may be part of the physiological capacitation process and an acrosome reaction that occurs in sperm subjected to DGC (Tavalaee *et al.*, 2012), which physiologically justify the use of MACS before DGC. Some studies have shown that MACS column seems cause a slight decrease in motility (Paasch *et al.*, 2003; Grunewald *et al.*, 2009; Lee *et al.*, 2010). Moreover, sperm population exposed to the MACS column seems to be more susceptible to the mechanical and magnetic forces within the column, which may result in tail defects (Paasch *et al.*, 2003). These defects can be removed and slight decrease in motility can be improved by applying DGC after MACS. In addition, microbeads remaining after MACS can be separated by using DGC after MACS, which may make the procedure safer for clinical purpose (Tavalaee *et al.*, 2012).

Figure 2 Comparison of sperm DNA fragmentation, concentration, progressive motility, and normal morphology among samples processed by density gradient centrifugation (DGC), magnetic-activated cell sorting followed by DGC (MACS-DGC), DGC-MACS, and MACS. *Note*: Only the four processing groups (DGC, DGC/MACS, MACS/DGC, and MACS) were compared statistically with each other. *$p < 0.05$



This study has no direct clinical implications, but it raises the possibility that use of the MACS-DGC protocol for seminal processing may improve live birth rates following IUI or IVF. This hypothesis requires investigation in future clinical trials.

A limitation of our study is the small sample size. This was because we considered eligible for our study only semen samples with at least 2.5 mL of volume and concentration of at least 15.0 million/mL in order to evaluate the four processing

methods in the same samples. This procedure reduced biases related to the potential impact of different samples on assessed outcomes. However, as we recruited men among those investigating infertility in our IVF center, most of them had abnormal semen parameters limiting our sample size. Thus, studies evaluating larger series are needed to confirm our results. Another limitation in the present findings can only be extrapolated to men with the adopted eligibility criteria and new studies are needed to validate our findings in men with abnormal semen parameters.

In conclusion, we compared four sperm processing methods (DGC, DGC-MACS, MACS-DGC, and MACS) and found that the combined methods (MACS-DGC and DGC-MACS) led to retrieval of fewer spermatozoa with fragmented DNA. However, MACS-DGC protocol led to a significantly higher percentage of spermatozoa with progressive motility and normal morphology than the DGC-MACS protocol. These findings suggest that for assisted reproduction procedures in which progressive motility is related to reproductive success, such as IUI and IVF, use of the MACS-DGC protocol may provide better outcomes.

AUTHORS' CONTRIBUTIONS

Paula A. Navarro was the principal investigator. She participated in conception and design of the study, interpretation of data, and revision of the article. Thalita S. Berteli was responsible for data collection, interpretation of data, and drafted the manuscript. Wellington P. Martins contributed to the data analysis and interpretation of data. Michele G. Da Broi and Rui A. Ferriani contributed to interpretation of data and revision of the article. All authors participated in the manuscript revision.

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