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Research Article

Density Gradient Centrifugation Alone or the Combination of DGC with Annexin V Magnetic-Activated Cell Sorting Prior to Cryopreservation Enhances the Postthaw Quality of Sperm from Infertile Male Patients with Poor Sperm Quality

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Objective. To examine whether density gradient centrifugation (DGC) alone or its combination with annexin V magnetic-activated cell sorting (DGC-MACS) can be used to process semen samples from infertile male patients with poor sperm quality prior to subjecting these to freeze/thaw process in order to optimize the outcomes of sperm freezing. *Methods*. This study enrolled sixteen patients with sperm concentration $\geq 20 \times 10^6$/mL, sperm motility < 30%, and/or <4% normal sperm morphology. Sperms were processed by DGC or DGC-MACS prior to the freeze/thaw process. Sperm motility, hyperosmotic swelling test (HOS), TUNEL test, and morphological analysis were performed before and after the freeze/thaw process. *Results*. The freeze/thaw process had a detrimental effect on sperm motility, viability, morphology, and DNA integrity in all three groups (RAW, DGC, and DGC + MACS groups). The DGC and DGC + MACS groups showed increased sperm motility, viability, and normal morphology following freeze/thaw than untreated frozen controls. The motility and viability were not significantly different between DGC-MACS-CPT (cryopreservation-thawing) and DGC-CPT groups. Moreover, almost no grade A or grade B sperm was observed in the DGC-MACS-CPT groups. The sperm selected by DGC or DGC + MACS showed decreased levels of sperm DNA fragmentation than RAW samples following freeze/thaw. Moreover, the sperm DNA fragmentation following freeze/thaw in the DGC-MACS-CPT group was significantly lower than that in the DGC-CPT group. *Conclusions*. Sperm preparation by DGC before cryopreservation improved the quality of sperm postthaw in infertile males with poor sperm quality. If the sperm quality following freeze/thaw is foreseen to be insufficient for artificial insemination with husband's sperm or *in vitro* fertilization, or if there is high DNA fragmentation in RAW sperm, DGC + MACS should be used prior to cryopreservation to reduce sperm DNA fragmentation and improve the quality of sperm available for intracytoplasmic sperm injection.

1. Introduction

Semen cryopreservation is an invaluable medical procedure for preserving the fertility of males, particularly prior to cancer therapy [1, 2]. The clinical application of sperm cryopreservation has expanded over the recent years, including for storage of sperm from donors and storage of "reserve" sperm from patients with oligozoospermia. Cryopreservation is also useful to store sperm from males who are unable to provide an ejaculate for assisted reproductive treatment

(ART) in a timely manner. Previous studies have shown that patients undergoing cryopreservation usually have poor semen quality [3, 4]. In such cases, frozen-thawed sperms are employed for intrauterine insemination (IUI), *in vitro* fertilization (IVF), or intracytoplasmic sperm injection (ICSI) depending on the quality of sperm following the freeze/thaw process.

Despite the ongoing advances in this field, the biological and biochemical mechanisms underlying cryopreservation remain elusive. The unsatisfactory sperm quality postthawing is contributed by distinct elements involved in freezing, including rapid temperature changes, crystal ice formation, and osmotic stress [4]. Furthermore, freezing can induce production of reactive oxygen species (ROS), DNA fragmentation, apoptosis, and lipid peroxidation [5–7]. Previous studies have shown that human ejaculate contains sperm that exhibit typical characteristics of apoptosis such as externalization of phosphatidylserine (PS), activation of caspase, decreased mitochondrial membrane potential (MMP), and increased DNA fragmentation [4, 7]. These molecular changes are very common in sperm of infertile men and in sperm with abnormal functionality [8]. Moreover, apoptosis most likely contributes to the decrease in sperm quality following cryopreservation [7, 9]. In contrast to individuals with healthy sperm parameters [10], those diagnosed with poor sperm quality are more likely to be affected by these detrimental processes after thawing [4, 11–13].

Cryopreservation of sperm induces structural changes in phospholipids in the plasma membrane leading to externalization of PS, which is a well-studied marker of early phase apoptosis. This change occurs prior to the changes in subsequent apoptotic phases such as DNA fragmentation. PS is a phospholipid with negative charge and exhibits strong affinity for annexin V. Externalization of the PS enables its specific interaction with annexin V, which is confirmed by immunofluorescence (the annexin V binding assay). Magnetic-activated cell sorting (MACS) is a widely used tool to select nonapoptotic sperm from a heterogeneous cell population by identifying the specific marker of PS in the membranes of apoptotic sperm [14]. MACS has been used to separate apoptotic sperm from nonapoptotic sperm and can therefore enable selection of populations of sperm with greater motility and reduced DNA fragmentation [14–16]. Previous studies suggest that isolation of a pure population of nonapoptotic sperm with intact membranes from healthy donor samples prior to subjecting it to the freeze/thaw process may improve the clinical results [17, 18]. This process appears to enhance sperm motility, cryosurvival rate (CSR), and the proportion of sperm with intact transmembrane mitochondria following cryopreservation. However, some parameters of semen quality before freezing, such as sperm motility, can also influence the survival rate of thawed sperm [12, 19]. Sperm with limited motility is known to be particularly susceptible to freeze/thaw damage, leading to reduced fertilization capability. In a previous study, sperm with abnormal morphology showed high rate of DNA damage during cryopreservation when compared to the normal sperm [20]. Therefore, we hypothesized that density gradient centrifugation (DGC), a method that can select good quality sperm from a sample of raw semen, alone or the combination of DGC with MACS (DGC-MACS), can be used to process samples from infertile male patients with poor sperm quality prior to the freeze/thaw process and therefore optimize sperm quality after thawing.

2. Materials and Methods

The research was approved by the ethics committees of the Reproductive Hospital of Shandong University (IRB #2021-37). Written informed consent was obtained from all subjects. Sixteen patients (age range, 21–39 years) provided semen samples by masturbation after 3–4 days of abstinence. The samples were collected in sterile containers, and sperm concentration, motility, and morphology were evaluated in the original raw sample in accordance with the WHO protocols [21]. After liquefaction, 7 μL of each sample was placed into a disposable analysis slide (Goldcyto, Spain) and examined with CASA (SCA, Spain). Sperm morphology was assessed according to strict criteria at 1000 × magnification using light microscope (OLYMPUS BX43, Japan). Each sample (5 μL) was spread along the slide and allowed to dry for 20 minutes before staining with modified Papanicolaou method. At least 200 spermatozoa per slide were assessed under microscope with a 100 × oil immersion objective lens. Inclusion criteria were as follows: duration of infertility, 2 years; sperm concentration $\geq 20 \times 10^6$/mL; sperm motility < 30%, and/or normal morphology < 4%; no history of medical or surgical treatment in the immediately preceding 3 months prior to enrolment; normal physical examinations and endocrine hormonal profiles. However, if we cannot obtain enough sperm to accomplish the multiple experiments after the DGC-MACS and the freeze/thaw process, the semen samples should be excluded in our study.

2.1. Density Gradient Centrifugation. We used DGC to prepare the semen samples after being liquefied (PureSperm: Nidacon, International AB, Gothenburg, Sweden). Generally, semen plasma was loaded onto a 40% and 80% discontinuous gradient and then centrifuged at 300 g for 20 min. The left 80% pellet that indicates mature fraction was then centrifuged for another additional 7 min and then resuspended in human tubal fluid media (HTF: Irvine Scientific, Santa Ana, CA, USA). The isolated sperm was then separated into two parts, one subjected to MACS before subsequent freeze/thaw, and the other directly subjected to the freeze/thaw procedure.

2.2. Isolation of Sperm with Membrane Deterioration by MACS. The resuspended sperm samples after DGC flowed through a magnetic field (MiniMACS: Miltenyi Biotec, Bergisch Gladbach, Germany). Sperm were divided into two distinct subpopulation with or without annexin V binding. Briefly, the suspensions after DGC were incubated with annexin V microbeads (Miltenyi Biotec) (100 μL, 15 min, room temperature) and then placed in a separation column with iron beads that is fixed in a magnet. Apoptotic cells with impaired membrane integrity that remained in column were annexin-positive, while the passed cells with normal

membranes were annexin-negative. The power of the magnetic field was measured as 0.5 tesla between the poles of the magnet and up to 1.5 tesla within the iron globes of the column. Lastly, the column was separated from magnet, and the retained sperm was washed by annexin-binding buffer.

2.3. Cryopreservation/Thawing Protocol. All semen samples were cryopreserved using Sperm Freezing Medium (Origio, Denmark). An aliquot of the freezing medium equal to 25% of sperm sample volume was added to the specimen and gently mixed for 5 min using a mixer. This was repeated to obtain a final 1 : 1 (v/v) ratio of freezing medium and sperm samples. Cryovials with samples were frozen at -20°C for 8 minutes in the freezer, -80°C for 2 hours in liquid nitrogen vapor, and finally stored in liquid nitrogen tank at -196°C. Twenty-four hours after the samples were frozen, the vials were retrieved and thawed at 37°C for 20 minutes. The sperm was suspended in HTF medium after mild washing.

2.4. Hyperosmotic Swelling (HOS) Test. The HOS test was carried out in accordance with an established protocol [22]. Briefly, samples were first allowed to liquefy at room temperature before mixing 100 μL of semen with 1000 μL of hypoosmotic solution (equal volumes of sodium citrate (150 mosmol) and fructose (150 mosmol)). Then, the mixture was incubated at 37°C for 30 minutes and placed onto a glass slide and examined using phase-contrast microscopy (×400) (Olympus BX43F, Tokyo, Japan). For each patient, two hundred sperms were counted, and the percentage of sperm with swollen and tortuous tails was estimated. Sperm samples containing ≥ 58% of sperm with swollen and tortuous tails were considered to be normal.

2.5. Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labelling (TUNEL) Assay. The level of DNA fragmentation in sperm was examined by TUNEL assay using Fluorescein FragEL™ DNA Fragmentation Detection kit. Sperm were resuspended in PBS and fixed in 4% paraformaldehyde for 10 minutes at room temperature. Samples were then transferred onto glass slides coated with 0.01% poly-L-lysine. Sperm membrane was penetrated in 20 μg/mL proteinase K for 10 minutes at room temperature. After washing with TdT equilibration buffer, sperm were gently resuspended in TdT reaction solution containing TdT enzyme and FITC-labelled nucleotides. A negative control was used to eliminate the inconsistency through excluding TdT enzyme. The DNase I enzyme was used to establish a positive control. The samples were placed in a humidified incubator for 60 minutes at 37°C away from light. More than 500 sperms were examined in each slide and examined under a fluorescent microscope for the presence of green fluorescence indicating DNA fragmentation.

2.6. Statistical Analysis. Data were analyzed using SPSS 13.0. Normally, distributed variables were presented as mean ± standard deviation. Univariate analysis was performed with paired t test between samples. Parameters were compared between groups using one-way analysis (ANOVA). P values < 0.05 were considered indicative of statistical significance.

3. Results

Semen analysis was performed on samples provided by 16 infertile males. Sperm concentrations were all within the normal range and sperm motility ranged from 14.4% to 26.4% (mean 20.47 ± 3.47%). The motility of grade A + B ranged from 6.9% to 19.5% (mean 14.75 ± 3.77%). The mean proportion of sperm with normal morphology ranged from 0.6% to 7.3% (mean: 2.4 ± 1.6%).

Sperm motility in the RAW, DGC, and DGC-MACS groups was 20.47 ± 3.47%, 49.15 ± 10.47%, and 53.63 ± 10.35%, respectively (Table 1). The motility of Grade A + B in the RAW, DGC, and DGC-MACS groups was 14.75 ± 3.77%, 45.98 ± 10.99%, and 0.19 ± 0.40%. The sperm motility was significantly increased following DGC and DGC-MACS compared to the RAW control group ($P < 0.05$). Moreover, there was a significant increment in sperm motility following the combined DGC-MACS procedure in comparison to the DGC procedure alone ($P < 0.05$). However, although sperm motility was improved, almost no grade A or grade B sperm was observed in the sample after the DGC-MACS procedure.

The freeze/thaw process resulted in a significant reduction in sperm motility in all three groups. The sperm motility in the DGC-CPT and DGC-MACS-CPT groups was significantly increased compared to the RAW-CPT group (34.14 ± 9.64% and 31.50 ± 9.40% versus 15.54 ± 3.17%) ($P < 0.05$). However, there was no significant difference in sperm motility between the DGC-MACS-CPT and DGC-CPT groups. The motility of grade A + B in the RAW-CPT, DGC-CPT, and DGC-MACS-CPT groups was 12.04 ± 2.64%, 29.61 ± 10.33%, and 0%. Moreover, no grade A or grade B sperms were observed in the DGC-MACS-CPT groups. The proportion of freeze/thaw-induced damage to sperm motility was identical in RAW and DGC groups. However, the proportion of freeze/thaw-induced damage to sperm motility was higher in the DGC-MACS group compared to that in the RAW and DGC groups(Table 2).

HOS test results in the RAW, DGC, and DGC-MACS groups were 47.56 ± 18.61%, 60.31 ± 15.30%, and 58.94 ± 12.04%, respectively (Table 1). There was a significant increment in the HOS test results following DGC and DGC-MACS compared to the RAW control ($P < 0.05$). However, there was no significant difference in this respect between the DGC-MACS and DGC groups ($P < 0.05$).

The freeze/thaw process led to a significant reduction in HOS test results for sperm in the RAW, DGC, and DGC-MACS groups. There was a significant increase in HOS test results for sperm in the DGC-CPT and DGC-MACS-CPT groups when compared to the RAW-CPT group ($P < 0.05$). However, there was no significant difference in this respect between the DGC-MACS-CPT and DGC-CPT groups. The proportions of sperm with freeze/thaw-induced damage in the HOS test results were identical across all three groups (RAW, DGC, and DGC + MACS) (Table 2).

Table 1: Comparison of sperm motility, HOS test, TUNEL staining, and morphological analysis in RAW, DGC, and DGC + MACS along with RAW + CPT, DGC + CPT, and DGC + MACS + CPT groups.

Group	n	Motility (%)	HOS (%)	TUNEL (%)	Morphology (%)
RAW	16	20.47 ± 3.47	47.56 ± 18.61	35.56 ± 12.36	2.42 ± 1.63
DGC	16	49.15 ± 10.47^{a}	60.31 ± 15.30^{a}	16.44 ± 7.05^{a}	4.94 ± 2.98^{a}
DGC + MACS	16	53.63 ± 10.35^{ab}	58.94 ± 12.04^{a}	12.44 ± 5.70^{ab}	5.55 ± 3.49^{ab}
RAW + CPT	16	15.54 ± 3.17^{abc}	33.88 ± 14.94^{abc}	39.25 ± 13.99^{abc}	1.72 ± 1.07^{abc}
DGC + CPT	16	34.14 ± 9.64^{abcd}	44.19 ± 16.91^{bcd}	20.00 ± 7.41^{abcd}	3.55 ± 2.29^{abcd}
DGC + MACS + CPT	16	31.50 ± 9.40^{abcd}	43.63 ± 11.87^{bcd}	14.94 ± 6.23^{abcde}	4.39 ± 3.05^{abcde}

Data presented as mean ± standard deviation ($n = 16$). [a]$P < 0.05$ versus RAW group. [b]$P < 0.05$ versus DGC group. [c]$P < 0.05$ versus DGC + MACS group. [d]$P < 0.05$ versus RAW + CPT group. [e]$P < 0.05$ versus DGC + CPT group.

Table 2: Effect of prefreeze sperm processing by DGC and DGC + MACS on percentage motility, HOS, morphology, and TUNEL staining in 16 infertile males before and after cryopreservation.

Sperm parameter	RAW	DGC	DGC + MACS	F	P
Motility (%)					
Prefreeze	20.47 ± 3.47	49.15 ± 10.47	53.63 ± 10.35		
Postthaw	15.54 ± 3.17	34.14 ± 9.64	31.50 ± 9.40		
Percentage change (%)	-23.71 ± 10.54	-30.81 ± 11.91	$-41.77 \pm 11.74^{*}\#$	10.167	<0.01
HOS (%)					
Prefreeze	47.56 ± 18.6	60.31 ± 15.30	58.94 ± 12.04		
Postthaw	33.88 ± 14.94	44.19 ± 16.91	43.63 ± 11.87	0.128	0.880
Percentage change (%)	-27.08 ± 16.25	-28.68 ± 12.32	-26.38 ± 10.20		
Morphology (%)					
Prefreeze	2.42 ± 1.63	4.94 ± 2.98	5.55 ± 3.49		
Postthaw	1.72 ± 1.07	3.55 ± 2.29	4.39 ± 3.05		
Percentage change (%)	-27.97 ± 14.41	27.30 ± 11.86	-20.66 ± 10.86	1.682	0.198
TUNEL (%)					
Prefreeze	35.56 ± 12.36	16.44 ± 7.05	12.44 ± 5.70		
Postthaw	39.25 ± 13.99	20.00 ± 7.41	14.94 ± 6.23		
Percentage change (%)	10.36 ± 7.88	$26.77 \pm 22.02^{*}$	$24.23 \pm 17.99^{*}$	4.301	0.020

Data presented as mean ± standard deviation ($n = 16$ patients). $^{*}P < 0.05$ versus RAW group. $^{\#}P < 0.05$ versus DGC group.

The mean proportions of sperm with normal morphology (as determined by Papanicolaou staining) in the RAW, DGC, and DGC-MACS groups were $2.42 \pm 1.63\%$, $4.94 \pm 2.98\%$, and $5.55 \pm 3.49\%$, respectively (Table 1). The percentage of normal sperms (morphological) was significantly increased after DGC and DGC-MACS when compared to the RAW group ($P < 0.05$). In addition, the proportion of morphologically normal sperm was significantly improved after combined DGC-MACS procedure compared to the DGC procedure alone ($P < 0.05$).

The freeze/thaw process significantly reduced the proportion of normal (morphology) sperm in the RAW, DGC, and DGC-MACS groups. The morphology of sperm was significantly ameliorated in the DGC-CPT and DGC-MACS-CPT groups compared to the RAW-CPT group ($P < 0.05$). Moreover, the morphology of sperm in the DGC-MACS-CPT group was also significantly improved compared to the DGC-CPT group. The proportion of damage to sperm morphology following freeze/thaw was identical across all three groups (RAW, DGC, and DGC + MACS) (Table 2).

Finally, TUNEL results showed that the mean proportions of sperm with DNA fragmentation in the RAW, DGC, and DGC-MACS groups were $35.56 \pm 12.36\%$, $16.44 \pm 7.05\%$, and $12.44 \pm 5.70\%$, respectively (Table 1). The percentage of sperm with DNA damage was significantly reduced after the DGC and DGC-MACS treatment compared to the RAW group ($P < 0.05$). In addition, the proportion of sperm with DNA damage after combination of DGC-MACS was significantly lower than that after the DGC procedure.

The freeze/thaw process led to a significant increase in DNA damage rate in the RAW, DGC, and DGC-MACS groups. The DNA fragmentation level was significantly downregulated in sperm from the DGC-CPT and DGC-MACS-CPT groups when compared to the RAW-CPT group ($P < 0.05$). However, the DNA fragmentation in sperm from the DGC-MACS-CPT group was significantly

lower than that in the DGC-CPT group ($P < 0.05$). The proportion of damage in terms of DNA fragmentation of sperm following freeze/thaw was higher in the DGC-MACS groups and the DGC groups than the RAW groups. However, the proportions of damage in terms of DNA fragmentation of sperm following freeze/thaw were identical in the DGC groups and DGC + MACS groups (Table 2).

4. Discussion

Despite significant advances in the field of cryopreservation, current techniques remain insufficient, particularly for semen samples provided by subfertile males which exhibit greater susceptibility to cryoinjury. In addition, there is an increasing concern related to the effect of cryopreservation on a range of biological factors in sperm that could interfere with the success of downstream fertility procedures such as IVF and ICSI. Therefore, improving the sperm cryopreservation protocol to enable collection of a sufficient number of functional sperm for downstream procedures is of much clinical significance. However, the mechanisms underlying the specific effects of cryopreservation on key sperm parameters are yet to be fully elucidated. Previous research has shown that cryopreservation can induce oxidative stress and apoptosis-like phenomenon in sperm, thus causing a variety of structural alterations including disruption of the plasma membrane, externalization of PS, impairment of the MMP, and promotion of DNA fragmentation [7].

Although extensive research has been conducted to evaluate the effects of cryopreservation on sperm, there is no clear consensus on the most efficient protocol. Many different protocols have been used to cryopreserve sperm in an attempt to increase sperm survival postthawing. Most of these protocols advocate that sperm should be washed prior to freezing to select high-quality sperm and to exclude seminal plasma. Seminal plasma has been shown to contain some antioxidant enzymatic and nonenzymatic compounds; collectively, these relate to the total antioxidant capacity of the semen sample and can be particularly important under the increasing reactive oxygen species (ROS) conditions associated with freeze/thaw cycles [23, 24]. However, the protective effect of seminal plasma is believed to vary between individuals and shows a positive correlation with semen quality; this potentially explains the greater vulnerability of subfertile patients to the sperm damaging effects of cryopreservation [13, 25]. In addition, the senescent sperm and other types of cells in seminal plasma including immune cells, epithelial cells, and microbial contamination produce ROS to induce oxidative stress and apoptotic-like cell death during freeze/thaw. This phenomenon is contrary to the cryoprotective role of seminal plasma. Prefreezing strategies are aimed at optimizing sperm quality at baseline prior to cryopreservation and leading to an improved response of the sperm with regards to the osmotic and mechanical disruption associated with cryopreservation [26]. In this context, researchers have explored the preparation strategy to select normal sperm before cryopreservation. A large number of studies have demonstrated favorable postthaw cellular viability based on the prefreeze approach [26–30], whereas others have shown comparable or contradictory outcomes [31–33]. Therefore, optimization of sperm cryopreservation strategy is complex and challenging that requires collaborative exploration in future, ideally through standardized experimental studies.

In the present study, DGC was applied before the freeze/thaw process. After DGC, we observed improved sperm motility, normal morphology, more integrative membrane, and lower levels of DNA fragmentation in purified sperm from infertile males. However, we cannot conclude that all purified sperm showed highest quality. For instance, researchers have identified that the swim up (SU) and DGC methods cannot select sperm efficiently with regards to apoptosis, DNA fragments, membrane integrity, and sperm ultrastructure [34]. Previous studies and meta-analyses have demonstrated a negative correlation of sperm DNA damage with male reproduction and the probability of success following ART [35–37]. Muratori et al. first showed that DGC improved DNA damage in almost half of all adult semen samples processed with this method [38]. Furthermore, the centrifugation steps used in this technique induce ROS, which is detrimental to the activity of sperm [39, 40]. This further reinforces the notion that the currently used sperm selection methods used for ART are insufficient to select functional sperm, and the development of an alternative tool to select the best sperm for further ART procedures is a key imperative. Therefore, it may be more efficient to use MACS to prepare purified sperm from infertile males.

MACS is a tool to collect apoptotic spermatozoa via interacting PS on damaged cell membranes. By this method, the apoptotic sperms are magnetically attached to annexin V-conjugated microbeads, and normal sperms are collected in the flow through of column set in the magnetic field. The damaged sperms, characterized by PS expressed on the impaired membrane, are thus retained by magnetic beads, and the nonlabelled (nonapoptotic) cells flow into the collection column [41]. To summarize, MACS is a valuable tool for ART because of its ability to decrease the number of apoptotic cells in the heterogeneous ejaculate characterized by DNA fragmentation.

In the present study, we observed a significant increase in the motility of sperm and the percentage of sperm with normal morphology after the combined DGC-MACS procedure compared to that after DGC procedure alone. However, we observed no significant difference in HOS test results between the DGC-MACS and DGC groups. Although the total motility of sperm was increased, almost no grade A or grade B sperm was observed after DGC + MACS. Our results differ, at least in part, to previous reports that claimed that DGC + MACS enhances sperm motility ([42–44] [15, 34]). Moreover, Said et al. also reported a significant reduction in sperm motility when MACS was performed on freeze/thaw samples [45]. Generally, MACS is a feasible and safe method to generate functional sperm. There is no evidence on the influence of separation columns and magnetic fields on sperm. One concern in using DGC-MACS for processing semen samples is that DGC-MACS involves repeated steps of centrifugation and resuspension, which

may have detrimental effects on the sperm. In addition to causing direct mechanical damage to the plasma membrane of sperm, centrifugation may also cause indirect adverse effects via the generation of ROS [46, 47]. Moreover, in the study by Cakar et al., MACS after DGS and SU was found to cause a significant loss in the numbers of total and rapid progressive sperm [48]. Another concern is the room temperature required for MACS, which may cause deleterious effects on sperm motility. However, increased temperature during MACS may influence the efficiency of MACS. Lastly, in our study, MACS led to a significant decrease in progressive motility; this may also be related to the initial quality of samples, as the sperm total motility in the RAW semen samples ranged from 14.4% to 26.4%.

We also observed that combined DGC-MACS procedure significantly reduced the number of sperm with DNA damage compared to DGC alone. Our results are in consistent with some previous reports. For example, in the study by Zahedi et al., MACS led to decreased DNA fragmentation in sperm from both fertile and infertile patients [49]. In addition, both Lee et al. [50] and Degheidy et al. [51] revealed that MACS led to enrichment of sperm with decreased DNA fragmentation in semen samples from individuals suffering from idiopathic infertility and varicocele [50, 51]. Collectively, the available data indicates that MACS reduces the sperm with DNA damage and can be beneficial for patients with enhanced DNA fragmentation [15].

Our study is mainly aimed at examining whether DGC alone or DGC in combination with MACS (DGC-MACS) can be used to process samples from infertile males with poor sperm quality prior to subjecting these to the freeze/thaw process, thus optimizing the outcomes. Therefore, raw semen or washed sperms (DGC and DGC-MACS) were all subjected to the freeze/thaw process. In our study, we observed some deleterious effects of the freeze/thaw process on sperm motility, viability, and sperm morphology in all three groups (RAW, DGC, and DGC + MACS groups). Our findings are consistent with those of the previous study by Raad et al. who also showed decreased sperm motility and morphology postthaw in both fertile and infertile individuals, when compared to that in fresh semen samples [52]. In another study, Kalthur et al. found a significant decline in the progressive motility in frozen-thawed samples of asthenozoospermic and normozoospermic semen compared to that in fresh samples [48]. Furthermore, in the study by Nijs et al., cryopreservation reduced the proportion of motile sperm from 50.6% to 30.3% [49]. Moreover, many studies have demonstrated disruption of sperm morphology in postfreeze/thaw samples compared to prefreeze/thaw samples [4, 55].

The DGC groups and DGC + MACS groups showed higher proportions of sperm with motility, viability, and normal morphology after the freeze/thaw process compared to that in the RAW group. Our results are consistent with previous studies in which samples processed by DGC or swim up from a washed sperm preparation prior to freezing exhibited a higher level of postthaw motility and morphology [26, 29, 56, 57]. Thus, we can speculate that removal of seminal plasma and purification of functional sperm prefreezing may limit the impairment of viability, motility, and morphology caused by cryopreservation. We found no significant difference in the motility and viability of sperm between the DGC-MACS-CPT and DGC-CPT groups. However, we observed significantly improved number of sperm with normal morphology in DGC-MACS-CPT compared to that in DGC-CPT groups. Interestingly, we observed that the proportion of damage in terms of sperm viability or morphology following the freeze/thaw process was identical across all three groups; these results are partly in accordance with a previous report [57]. Therefore, the increased sperm motility, viability, and morphology after selection by DGC or DGG + MACS procedures in our study do not seem to be a consequence of the selected sperm being more resistant to the freeze/thaw process. The selected sperm showed higher absolute changes in the sperm characteristics after cryopreservation, which can be explained by higher initial rates, but not the greater resistance to cryodamage. Moreover, we also observed that the proportion of damage in terms of sperm motility after the freeze/thaw process in the DGC-MACS groups was higher than that in the RAW or DGC groups. Our results are in contrast to those of Said et al. who reported that DGC + MACS enhanced sperm motility following cryopreservation [45]. Moreover, we also observed almost no grade A or grade B sperm following freeze/thaw process after the combined DGC-MACS procedure. These differences may be attributable to the differences in the characteristics of the study population. In our study, samples from infertile males diagnosed with poor sperm quality were subjected to DGG + MACS prior to the freeze/thaw process and not semen samples from healthy volunteer sperm donors. It is also possible that centrifugation, resuspension, and room temperature during the MACS procedure may have induced effects which have already been discussed above. Moreover, our data showed a significant difference between the DGC-CPT group and the DGC-MACS-CPT group in terms of sperm morphology. It is possible that sperm motility and sperm viability are more sensitive to the repeated steps of centrifugation and resuspension than sperm morphology and that these factors induced detrimental effects in sperm after DGC-MACS.

Although an accumulated body of evidence has shown the influence of cryopreservation on the motility, morphology, and viability of sperm, there is no clear consensus on the influence of cryopreservation on DNA fragmentation [18, 53]. Some studies have indicated that cryopreservation affects sperm DNA integrity [32, 59, 60], whereas some other studies have yielded different results [58, 61, 62]. This discrepancy may be related to differences with respect to sample size, freezing procedures, study population, tests used to evaluate DNA integrity (TUNEL, sperm chromatin structure assay, sperm chromatin dispersion, and comet neutral or comet alkaline), and the sample preparation procedures precryopreservation (i.e., swim up or DGC).

For instance, Duru et al. used TUNEL assay and annexin V staining to examine the integrity of DNA and membrane after cryopreservation in 21 subjects [61]. They found that the freeze/thaw changed the symmetry of the plasma membrane and translocated the PS, but the DNA integrity was

normal. Paasch et al. also showed limited effects of freeze/thaw on sperm DNA. These studies suggested a close association between cryopreservation and impaired MMP, accompanied with upregulation of caspase 3, 8, and 9. However, the TUNEL assay showed no obvious DNA damage in 84 samples [5]. In addition, Isachenko et al. examined the influence of slow freezing and vitrification on DNA activity in sperm without cryoprotectant, and the results showed no effect of cryopreservation on DNA integrity [62]. Lusignan et al. found that cryopreservation may have a deleterious effect on the integrity of human sperm DNA and compaction. However, the sperm DFI was not affected during cryopreservation under the various methods of storage tested [58].

In the present study, we observed deleterious effects of the freeze/thaw process on the proportion of sperm with DNA fragmentation in all the three groups (RAW, DGC, and DGC + MACS groups) in infertile men. Our results are consistent with some previous reports. For example, Donnelly et al. obtained semen samples from fertile and infertile males and assessed sperm DNA integrity pre- and postcryopreservation using comet assay [32]. They found that semen from fertile men was more resistant to freezing damage than sample from infertile men. Moreover, in fertile man, there was no significant decrease in DNA integrity after cryopreservation. These findings concurred with those of Raad et al. who reported a significantly higher percentage of sperm with fragmented DNA postfreezing/thawing in all 5 cryomedia compared to fresh semen of infertile participants [52]. The high level of DNA fragmentation after cryopreservation in the infertile semen correlated with impaired sperm DNA packaging [63]. This phenomenon may be explained by the impaired sperm chromatin from the infertile ejaculated semen [64]. Moreover, some authors found that freezing/thawing procedure induces sperm DNA damage irrespective of whether the semen sample was from fertile or infertile male. Spano et al. found that cryopreservation disrupted the sperm DNA integrity, as evaluated by sperm chromatin structure assay in 19 samples [65]. These findings concurred with those of Paula et al. in a study of 77 patients. They evaluated the sperm DNA damage using TUNEL assays pre- and postcryopreservation and found a negative effect of the freeze/thaw process on the DNA integrity [60]. A recent study by Cankut et al. found increased sperm DNA fragmentation after cryopreservation, as assessed with the Halosperm technique as well as TUNEL assay [59].

In our study, the DGC groups and DGC + MACS groups exhibited lower proportions of sperm with DNA fragmentation postfreeze/thaw process compared to the same ejaculates frozen in the RAW group. Our results concur with previous studies in which DGC or SU prefreezing increased the proportion of high-quality sperm after cryoinjury, as a lower proportion of TUNEL-positive or fewer apoptotic sperm was identified compared to the sperm selection procedure performed after freezing [27, 30]. These findings suggest that removal of the seminal plasma and selection of highly active sperm by DGC before freezing may decrease sperm DNA fragmentation incurred during cryopreservation. Furthermore, the DGC + MACS group exhibited lower proportions of sperm DNA fragmentation after the freeze/thaw process than the DGC group. In the current study, we assessed the integration of MACS coupled with DGC in our cryopreservation protocol. The procedure delivers two sperm fractions: annexin V-conjugated microbeads- (ANMB-) positive (labelled apoptotic spermatozoa) and ANMB-negative (unlabeled with intact membranes). Following cryopreservation and thawing, ANMB-negative sperm had the lowest levels of DNA fragmentation. This suggests that depletion of the early apoptotic sperm ameliorated the DNA damage induced by cryopreservation. However, any improvement in sperm DNA fragmentation after selection by the DGC or DGC + MACS procedures in our study does not seem to be a consequence of the selected sperm being more resistant to the freeze-thaw process. First, the sperm selected after DGC or DGC + MACS had lower absolute sperm DNA fragmentation following cryopreservation because they had higher initial values, rather than because of their greater resistance to cryoinjury. Conversely, we observed that the proportion of damage in terms of DNA fragmentation of sperm following freeze/thaw was higher in the DGC-MACS groups and DGC groups than the RAW groups. However, the proportion of damage in terms of DNA fragmentation of sperm following freeze/thaw was identical in the DGC groups and DGC + MACS groups.

There is no general agreement on the specific mechanisms that induce DNA damage. DNA integrity is challenged by freezing because cryopreservation can easily alter the function of the mitochondrial membrane and increase the generation of ROS. The peroxidative damage caused by elevated concentrations of ROS correlates with impairment of sperm plasma membrane and axoneme structure. Moreover, lack of DNA repair enzymes is another causative factor for DNA fragmentation postfreezing [66]. Additionally, freezing has been shown to abate antioxidant activity of the sperm and result in high sensitivity to ROS damage. High concentrations of ROS and reduction in the levels of antioxidant enzymes can lead to cellular apoptosis [7, 67]. The release of apoptotic inducers from the mitochondria leads to DNA damage [68]. Moreover, cryopreservation can produce an apoptosis-like event in sperm [7, 69, 70]. In our study, seminal plasma was removed after DGC, and annexin V (+) apoptotic sperm was removed after MACS. However, the proportion of damaged in the sperm DNA fragmentation following freeze/thaw was identical in the DGC and DGC + MACS groups. This suggested that DNA damage in sperm involves elevated oxidative stress during cryopreservation but not activation of caspase and apoptosis. Our findings may also be related to the protective role of seminal plasma during cryopreservation. This protective effect is still important in subfertile patients during the freeze/thaw process.

Current DGC + MACS isolation methods can enhance the number of sperm with intact DNA within the sperm population used for ICSI or isolate a single viable sperm for injection with a reduced risk of DNA fragmentation [71]. The use of sperm selected by MACS for ART has not been described or evaluated in detail. Stimpfel et al. [72]

analyzed teratozoospermic males and females aged over 30 years who were subjected to ICSI and reported that healthy blastocysts were produced when sperm was purified by MACS after DGC/SU and compared to DGC/SU alone [72]. Ziarati et al. [73] showed increased number of superior embryos and clinical pregnancies when performing DGC + MACS than DGC alone in a study of 80 infertile couples suffering from male factor [73]. In the study by Mei et al. [74], selection of nonapoptotic spermatozoa by MACS for higher sperm DFI was found to improve the live birth rate and decrease transfer cycles of IVF/ICSI [74]. Our study suggests that DGC + MACS can be performed before freezing for infertile males suffering from low-quality sperm to optimize the outcomes of ICSI. However, only 16 patients were included in our study, which, from a certain point of view, may be a quite small sample size. This is a possible limitation of our study. Further study with a larger sample size is required to obtain more robust evidence. As a consequence of outstanding success of ICSI, this method has been applied to infertile males suffering from various extents of oligoasthenoteratozoospermia. Further research is required to evaluate whether DGC + MACS should be performed before freezing in oligoasthenoteratozoospermia or to determine the degrees of oligoasthenoteratozoospermia that should be subjected to DGC + MACS before the freeze/thaw process. Moreover, we also realize that MACS-DGC or DGC-Zeta procedure has also been used to process semen samples with good results [75, 76]. Currently, for selected sperm free of seminal plasma, it is more beneficial to use sperm vitrification technique [21]. Therefore, further researches are also required to evaluate whether MACS-DGC or DGC-Zeta could be performed before freeze/thaw process and whether sperm vitrification technique could be used in related experiment to optimize the outcomes of sperm freezing.

5. Conclusions

Sperm selection by DGC before cryopreservation improved the quality of sperm postthaw in infertile males with poor sperm quality. Therefore, if the sperm quality following freeze/thaw is foreseen to be insufficient for AIH or IVF or in case of high level of sperm DNA fragmentation in the fresh untreated sample, DGC + MACS should be performed before freezing to generate functional sperm with limited DNA damage that can be used for ICSI.

Data Availability

The data are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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