Total motile sperm count is improved in prepare-first samples compared to freeze-first samples

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Abstract
Introduction: Sperm cryopreservation is used for a range of clinical applications. While there are numerous protocols available, there is no gold standard for sperm cryopreservation. The goal of sperm cryopreservation is to recover as many motile sperm as possible, as high sperm motility and concentration are linked with successful pregnancies in assisted reproductive technology. This study investigated which order of protocol (freezing before or after preparation using density gradient centrifugation) and which density gradient (double-layered 95%/50% or single-layered 60%) resulted in higher post-thaw sperm motility and concentration.
Methods: Semen samples (n=42) were divided into four equal aliquots. Two aliquots were frozen first, then prepared with either a double-layered 95%/50% or single-layered 60% Puresperm® density gradient. The final two aliquots were separately prepared with the two density gradients, followed by freezing. All samples were frozen by liquid nitrogen vapor and thawed in an incubator at 37°C for 10 minutes. Post-thaw motility and concentration were assessed.
Results: Total motile sperm count was higher in samples that were prepared first (49% increase; p=0.009). Although progressive motility was higher in samples that were frozen first (30% increase; p<0.001), the order of protocol had no effect on total motility (p=0.158). Preparing samples before cryopreservation elevated sperm concentration (44% increase; p=0.044). Use of the 95%/50% gradient improved total motility (53% increase; p<0.001), while the 60% gradient increased concentration (4-fold increase; p<0.001).
Conclusions: Samples prepared before freezing yielded more motile sperm than samples frozen before preparation; and the 60% gradient increased sperm concentration, compared to the 95%/50% gradient. This study contributes to the limited literature on whether sperm should be cryopreserved before or after the density gradient centrifugation method of sperm preparation, though further studies are warranted to investigate clinical pregnancy outcomes.

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Introduction

Sperm cryopreservation is routinely utilized in assisted reproductive technology (ART) and to preserve male fertility. Sperm is cryopreserved for many reasons, including for donation and fertility preservation prior to cancer treatment. Despite this, the optimal procedure for sperm cryopreservation is yet to be elucidated. The goal of sperm cryopreservation is to recover as many motile sperm as possible. The use of samples with high sperm motility and concentration is linked with successful pregnancies in ART procedures (Chapuis et al., 2017). However, cryopreservation has many negative effects on the sperm cell, including

intracellular ice formation (Bagchi et al., 2008), increased oxidative stress (Thomson et al., 2009) and chromatin instability (Hammadeh et al., 1999), all of which can have adverse implications for successful fertilization and ongoing embryo development (Royere et al., 1991).

Compared to other cells, the human sperm cell is able to withstand a range of cooling and warming rates due to its low water content (WHO, 2010) and high membrane fluidity from unsaturated fatty acids in the lipid bilayer (Darin-Bennett & White, 1977). Nonetheless, the process of sperm cryopreservation causes major reductions in sperm quality; about 50% of live sperm are damaged during cryopreservation (Holt, 2000; Oehninger et al., 2000; Nangia et al., 2013) and this damage is especially apparent in individuals with poor initial semen quality (Hammadeh et al., 1999). Given the increasing need for ART, the optimization of sperm freezing protocols is warranted.

In ART, semen samples must undergo sperm preparation before it is utilized for procedures such as intrauterine insemination (IUI), in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI); the motile sperm population with the greatest fertilization potential is separated from the constituents of the seminal plasma.

Density gradient centrifugation (DGC) is a method of sperm preparation routinely used in fertility clinics. The gradient layers act as a filter to separate motile sperm from the seminal plasma. Semen samples are layered on top of density gradients, composed of colloidal silica with saline (WHO, 2010), and centrifuged. During centrifugation, sperm are separated according to their buoyant density (Lessley & Garner, 1983). Mature and motile sperm are highly dense (1.10 g/ml), while suboptimal and immature sperm are less dense (1.06-1.09 g/ml) (Keskin & Karabulut, 2017). A double-layered discontinuous gradient is often utilized in DGC; varying gradient concentrations have been reported with similar efficacy such as 90%/35% (Nanassy and Carrell, 2011), 95%/45% (Chen and Bongso, 1999; Ali et al., 2022) or 80%/40% (WHO, 2010). Additionally, single-layered discontinuous gradients (of around 60% silica) can also be used. These percentages can often vary between clinics, and clinics typically use density percentages that they previously deemed suitable.

It is currently unclear whether sperm preparation prior to, or after freezing will yield higher sperm motility and concentration. The aim of the present study was to determine which order of protocol (freezing before or after preparation) and which density gradient (double-layered 95%/50% or single-layered 60%) would yield higher post-thaw sperm motility and concentration.

Materials and Methods

Ethics Approval

Ethics approval was granted by the Joondalup Health Campus Research Ethics Committee (Perth, Western Australia) on 5 April 2018.

Study Population

Samples were sourced from men undergoing semen analysis at the clinic, following provision of an information sheet and written consent. Men with a record of sexually transmitted disease (human immunodeficiency virus, hepatitis B, hepatitis C or other sexually transmissible infections) and sperm collected by surgical retrieval were excluded from this study. Samples were collected by masturbation after an abstinence period of two to five days. After immediate incubation in a warming incubator for semen liquefaction (37°C, 30 minutes), a pre-preparation routine semen analysis was performed by clinic staff. The remaining unused semen was utilized in this study.

Each sample was required to be above the World Health Organization 5th edition lower reference limits for progressive motility (32%) and concentration (15×10^6/ml) (WHO, 2010). A total of forty-two samples were analyzed. In the case of insufficient sample parameters, semen from different participants were pooled. To ensure equal distribution of each sample across the four study arms, 4 ml of semen was required per sample, which was then split into 4×1 ml aliquots.

Experimental Study Design

Four sample preparation protocols are defined: (i) sperm freezing before preparation with a 95%/50% gradient (freeze-first double gradient); (ii) sperm freezing before preparation

with a 60% gradient (freeze-first single gradient); (iii) sperm preparation with a 95%/50% gradient before freezing (prepare-first double gradient); and (iv) sperm preparation with a 60% gradient before freezing (prepare-first single gradient). A volume of 1 ml of neat semen was allocated for each group. Figure 1 illustrates the study's four experimental arms.

Sperm Cryopreservation Before Density Gradient Centrifugation

Semen allocated for the two freeze-first groups were frozen simultaneously. For each group, 1 ml of cryoprotective media (Quinn's Advantage™ Sperm Freeze Media, Cooper Surgical, USA) containing 5% glycerol was added dropwise to 1 ml of neat semen, for a final volume of 4 ml to be frozen (2 ml per group). The total volume was drawn by syringe into eight 500 µl labelled straws (CBS™ High Security Sperm Straws, Cryo Bio Systems, France). Four straws were used for each group.

Samples were frozen in liquid nitrogen (LN2) vapor by rapid freezing (Palomar Rios and Molina Botella, 2019). Straws were placed 12 cm above LN2 and positioned parallel to the LN2 surface (15 minutes), then 2 cm above LN2 (30 minutes). Samples were then immediately plunged into LN2 in the liquid phase and stored in tanks until analysis. Straws were thawed in a 37°C incubator for 10 minutes as per clinic protocol.

Density gradient centrifugation was performed as described previously (Peirce et al., 2015). To pre-prepare the density gradients, the 100% Puresperm® stock solution (Nidacon, Gothenburg, Sweden) was diluted with a HEPES buffered medium supplemented with 5% human serum albumin, to form 95%, 50% and 60% Puresperm® concentrations. Gradients were prepared while straws were thawing. To prepare the 95%/50% Puresperm® gradient for FP-95/50, 0.5 ml of the 50% Puresperm® upper layer was transferred into a conical-bottomed centrifuge tube (Thermo Fisher Scientific, Australia). Subsequently, 0.5 ml of the 95% Puresperm® lower layer was laid under the 50% Puresperm® layer, to produce a final volume of 1 ml. For FP-60, 1 ml of 60% Puresperm® gradient was transferred into a separate centrifuge tube.

The thawed semen was evenly distributed on top of each Puresperm® gradient. Tubes were immediately centrifuged at 350×g for 15 minutes (Mortimer 2000), and the sperm pellet was extracted into a new conical centrifuge tube. The sperm pellet was washed by addition of 0.5 ml of G-MOPS™ (Vitrolife, Sweden) and centrifuged at 500×g for 5 minutes (Mortimer 2000). Supernatants were removed, and pellets were washed by addition of 0.5 ml of G-MOPS™ and re-centrifuged (500×g, 5 minutes). Supernatants were removed down to a final volume of 0.5 ml for both tubes. Pellets were resuspended in preparation for motility and concentration assessment.

Density Gradient Centrifugation Before Sperm Cryopreservation

Neat semen samples were first prepared, as above, followed by cryopreservation. For the two prepare-first groups, 1 ml of neat semen was layered above the respective Puresperm® gradients, centrifuged and the pellet removed to a new tube as described above. A single wash followed, in which each pellet was resuspended in 0.5 ml of G-MOPS™ wash and centrifuged, as described.

Following the single wash, the supernatant was removed, and 1 ml of G-MOPS™ was added to the pellet, followed by drop-wise addition of 1 ml of cryoprotective media to each prepared sample. Four 0.5 ml straws were frozen for each group, as described above.

Cryopreservation and thawing of both prepared samples were undertaken following the protocols described above. Once thawed, samples were washed with 0.5 ml of G-MOPS™ and centrifuged, as described. Finally, the supernatant was aspirated down to 0.5 ml, and the pellet resuspended for motility and concentration assessment for each group.

Sperm Motility and Concentration Assessment

After the protocols described, sperm motility was immediately evaluated on the Makler counting chamber (Sefi Medical Instruments Ltd, Israel). A 10 µl drop of the final sperm suspension was dispensed on the Makler and placed on a heated stage (37°C). At least 200 sperm were counted at 200× magnification under a phase-contrast microscope (Olympus,

Model: BX50F). Motility was scored manually as percentages of progressive motility, non-progressive motility and immotility in at least 200 sperm. Progressive motility and total motility (progressive + non-progressive sperm) were noted. Total motile sperm count was calculated as the product of concentration and total motility.

Sperm concentration was assessed on the Improved Neubauer Hemocytometer – the gold standard for sperm concentration evaluation (WHO, 2010). For each group, 50 µl of the final solution was mixed in 950 µl of distilled water for a 1/20 dilution. Following this, 10 µl of the diluted volume was placed under each chamber of the hemocytometer. The loaded hemocytometer was placed in a humidified chamber for 5 minutes then viewed under phase-contrast microscope at 200× magnification.

Statistical Analysis

Three-way analysis of variance (ANOVA) between groups was performed using order of protocol (freeze or prepare first), density gradient preparation (95%/50% or 60%) and categories of motility (progressive and total motility) as sources of variation. If interactions were significant, further two-way and one-way ANOVAs were performed.

Other sperm outcome variables (concentration and total motile sperm count) were performed by two-way ANOVA, with order of protocol and density gradient preparation as sources of variation. Additional one-way ANOVA was performed whenever significant interactions were indicated. If no significant interaction was identified, Fisher's least significant difference (LSD) test was used to identify significant differences between groups at a significance level of 0.05. All data was analyzed using GenStat Version 18 (VSN International, Hemel Hempstead, UK) with statistical significance considered at $p<0.05$.

Results

Study Population

Semen samples, either pooled or from a single participant, were used in this study (n=42). Informed consent was obtained from 77 participants; though not all samples were utilized due to poor quality, low volume, or unavailability of other samples for pooling to reach the required 4 ml volume. The mean age of donor males was 36.8±0.7 years. Baseline characteristics before samples were split into the four experimental groups are given in Table 1.

Progressive and Total Motility

Samples in the freeze-first 95%/50% gradient group yielded the highest percentage of progressively motile sperm (Figure 2). Overall, there was a 30% increase in progressive motility when samples were frozen first, compared to samples that were prepared first ($p<0.001$), and a 91% increase when using a 95%/50% gradient, in comparison to a 60% gradient ($p<0.001$). There was also an interaction between order and gradient ($p=0.026$). Specifically, when using the 95%/50% gradient, freeze-first samples yielded a 39% increase in progressive motility compared to the prepare-first samples ($p<0.001$); an effect not seen with the 60% gradient preparation.

The use of the 95%/50% gradient yielded higher total motility by 53% (Figure 3) compared to the 60% gradient ($p<0.001$). The order of protocol had no effect on total motility ($p=0.158$).

Concentration

Prepare-first 60% gradient samples resulted in the highest sperm concentration (Figure 4). The concentration of samples that were prepared first were higher than those that were frozen first (44% increase; $p=0.044$). Furthermore, use of the 60% gradient significantly increased sperm concentration by 4-fold in comparison to the 95%/50% gradient, irrespective of protocol order ($p<0.001$).

Total Motile Sperm Count

Overall, prepare-first 60% gradient samples yielded the highest total motile sperm count (Figure 5). There were overall effects of order (prepare > freeze; 49% increase; $p=0.009$) and gradient (60% > 95%/50%; 2.5-fold increase; $p<0.001$).

Discussion

This study has shown that freezing semen samples first increased progressive motility whilst preparing first increased concentration. However, total motility did not significantly differ with each respective order of protocol. From the higher concentration in prepare-first groups, total motile sperm counts were accordingly greater in

Table 1. Baseline semen characteristics of initial pooled samples (*n*=42) before samples were split into four experimental groups.

Parameter	Mean	SEM	Range
Progressive motility (%)	66.0	2.3	33 – 92
Non-progressive motility (%)	1.7	0.3	0 – 8
Total motility (%)	67.7	2.3	35 – 90
Concentration ($\times$ 10^6/ml)	72.8	8.8	23.5 – 299
Total motile sperm count ($\times$ 10^6/ml)	49.1	5.8	11.5 – 134.9

Figure 1. Representation of protocol steps in four experimental groups. Semen samples were split into four groups of 1 ml each and were frozen or prepared first with a double 95%/50% or single 60% density gradient.

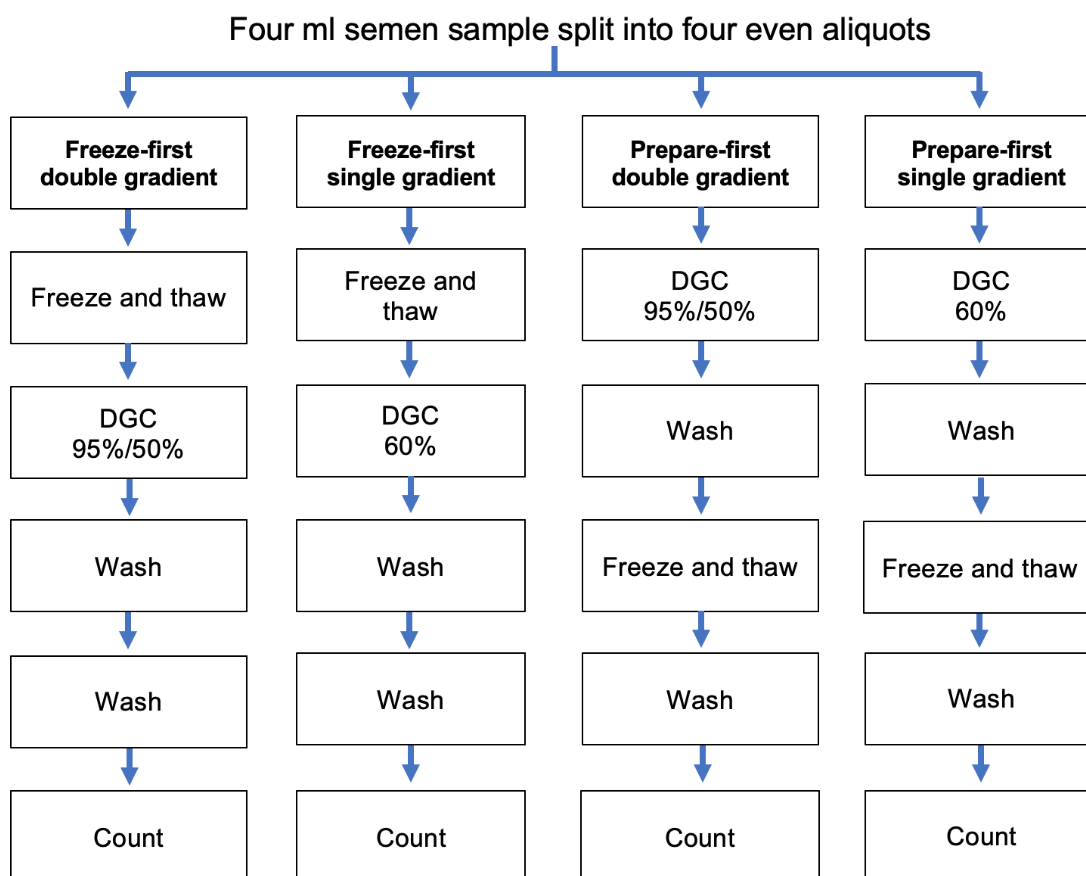


Figure 2. Progressive motility (%) of sperm in semen samples that were frozen first (white bars) or prepared first (grey bars) with either a 95%/50% or 60% density gradient. Values represent mean ± SEM (*n*=42/group). ***Overall effect of gradient (95%/50% > 60%); $p<0.001$ (two-way ANOVA). Overall effect of order (freeze > prepare); $p<0.001$ (two-way ANOVA). Interaction between gradient and order; $p=0.026$ (two-way ANOVA). §§§$p<0.001$ compared to corresponding freeze first group (one-way ANOVA).

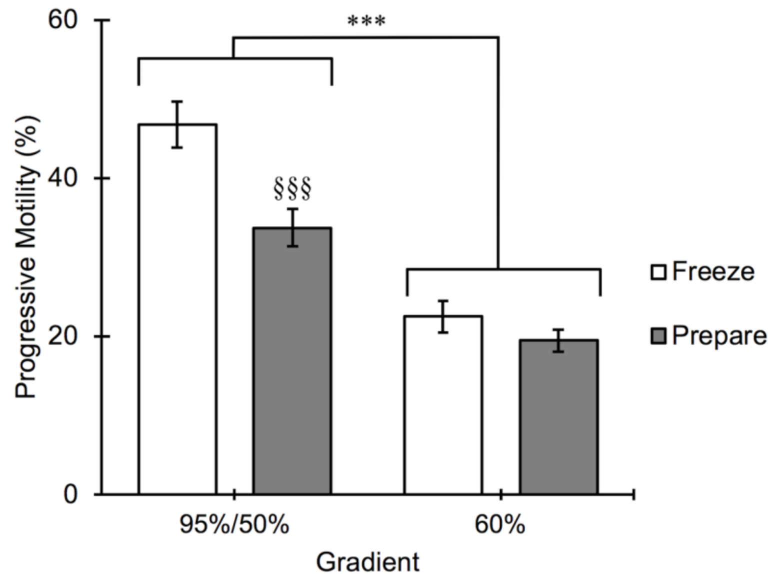


Figure 3. Total motility (%) of sperm in semen samples that were frozen first (white bars) or prepared first (grey bars) with either a 95%/50% or 60% density gradient. Values represent mean ± SEM (*n*=42/group). ***Overall effect of gradient (95%/50% > 60%); $p<0.001$ (two-way ANOVA).

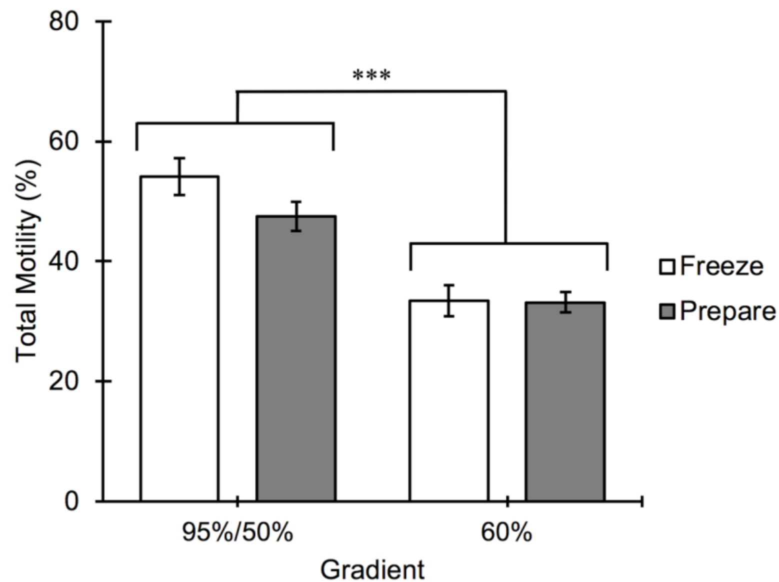


Figure 4. Concentration (million/ml) in semen samples that were frozen first (white bars) or prepared first (grey bars) with either a 95%/50% or 60% density gradient. Values represent mean ± SEM (n=42/group). ***Overall effect of gradient (95%/50% > 60%); $p<0.001$ (two-way ANOVA). Overall effect of order (prepare > freeze); $p=0.044$ (two-way ANOVA).

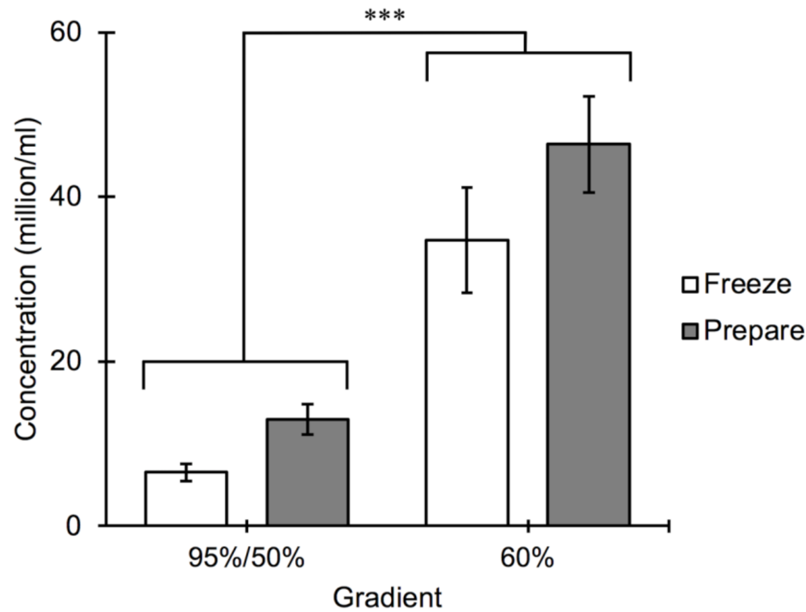
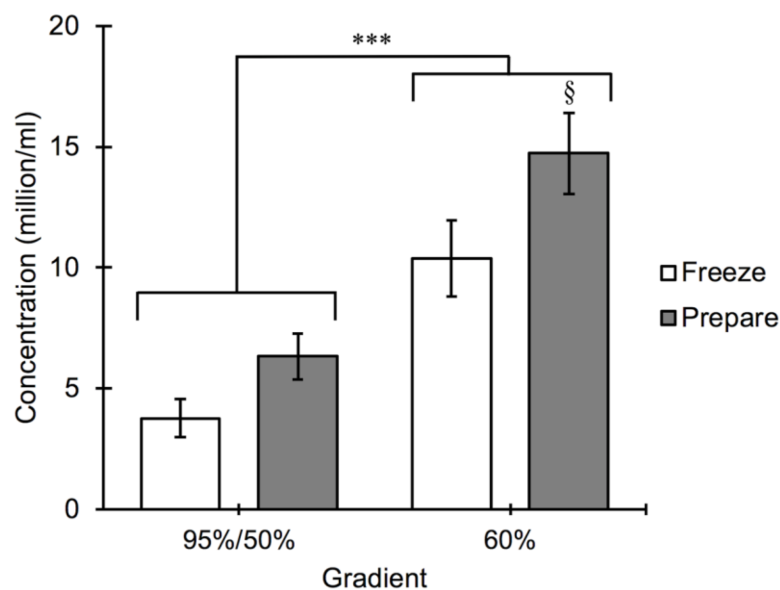


Figure 5. Total motile sperm count (million/ml) in semen samples that were frozen first (white bars) or prepared first (grey bars) with either a 95%/50% or 60% density gradient. Values represent mean ± SEM (n=42/group). ***Overall effect of gradient (60% > 95%/50%); $p<0.001$ (two-way ANOVA). Overall effect of order (prepare > freeze); $p=0.009$ (two-way ANOVA). §$p\leq0.05$ compared to corresponding freeze first group (LSD).



the prepare-first cohort. Secondly, the use of the 95%/50% density gradient lead to increased progressive motility but decreased concentration, compared to the 60% density gradient. There is conflicting literature on whether semen samples should be prepared before or after freezing. Previous studies have reported semen should be prepared before freezing (Brugnon et al., 2013; Petyim et al., 2014; Androni et al., 2021), while another has found the opposite (Palomar Rios et al., 2018). However, it is difficult to directly compare our data with these studies due to numerous disparities in methodology, study population and final parameters considered. To our knowledge, this study is the first to investigate different density gradient percentages and the order of protocol for freezing using DGC.

Freeze-first samples had higher progressive motility compared to those that were prepared first. Seminal plasma contains antioxidants, which protect against cellular damage by acting as free radical scavengers against reactive oxygen species (ROS) (Lewis et al., 1997; Garrido et al., 2004). High amounts of ROS can enter the sperm plasma membrane and cause further internal ROS production, leading to additional DNA fragmentation (Whittington & Ford, 1999; Henkel et al., 2005; Ribas-Maynou et al., 2014). Accordingly, antioxidant supplementation in cryoprotective media is documented to elevate post-thaw motility (Taylor et al., 2009; Rezaeian et al., 2016; Berkovitz et al., 2018). Brugnon et al. (2013) observed that hypotaurine supplementation during freezing increased progressively motile sperm in the post-thaw analysis, regardless of whether samples were frozen or prepared first. Thus, in the context of the current study, antioxidants present in seminal plasma may be a contributing factor to elevated post-thaw motility.

Lower progressive motility was identified in the prepare-first order of protocol. The removal of constituents of the seminal plasma, fructose and heparin binding proteins, may have attributed this finding. Fructose is a major carbohydrate source for sperm; active sperm cells utilize fructose for metabolism and motility (Biswas et al., 1978; Lewis-Jones et al., 1996; Schoenfeld et al., 1979). Moreover, heparin binding proteins may also protect sperm from lipid peroxidation during freezing (Kumar et al., 2008) and decrease generation of ROS (Patel et al., 2016). It has been reported that lipid peroxidation is enhanced in defective sperm presenting with high levels of ROS (Aitken et al., 1989). Therefore, absence of both fructose and heparin binding proteins during freezing may hinder motility in samples that were prepared first.

Although prepare-first samples had lower progressive motility, total motile sperm count was ultimately higher due to their higher concentration. Preparing before freezing may yield higher concentration of thawed sperm, as only the selected motile sperm population, which would be more resistant to cryoinjury, is frozen. While seminal plasma is recognized to protect sperm from oxidative stress, it concurrently contains sources of ROS that can cause oxidative damage and sperm apoptosis. These constituents include: leukocytes, bacteria, epithelial cells and senescent, abnormal, and immature sperm (Whittington & Ford, 1999; Allamaneni et al., 2005; Henkel et al., 2005; Ricci et al., 2009). Removing these adverse constituents may diminish sperm apoptosis, minimize cryoinjury, and ultimately increase concentration. This notion of increased apoptosis in freeze-first groups is supported by Petyim et al. (2014), though the study utilized the swim-up method. Although the authors did not find differences in concentration between samples that were frozen or prepared first, they identified reduced numbers of apoptotic sperm in the prepare-first groups after thawing (indicated by Annexin V/Propidium Iodide apoptosis binding assay) (Petyim et al. 2014). Thus, we recommend samples to be prepared before freezing as total motile sperm counts were higher in these groups.

Use of the 95%/50% density gradient lead to increased progressive motility but decreased concentration, compared to the 60% density gradient. To our knowledge, no published study has investigated post-thaw differences using different density gradients; however, our data is in agreeance with studies that utilized fresh samples. Chen & Bongso (1999) compared three-layered (90%/70%/40%) and two-layered (90%/45%) gradients. Although analysis of total motility was excluded, their progressive motility

results aligned with ours; the gradient with the greater number of layers increased percentage of progressive motility (Chen & Bongso, 1999). Similarly, another study on fresh semen reported that the single-layered (50%) gradient yielded lower total motility and greater concentration overall, compared to the double-layered gradient (90%/45%) (Zhou et al., 2010). Since there is no other published data combining use of different density gradients and investigating the optimal order of protocol, this study provides novel evidence that use of different density gradients impacts total motility, while the order of protocol does not.

While this study recommends sperm to be prepared before freezing, further validations are necessary. Other sperm parameters such as sperm morphology and DNA integrity should be considered. Comparisons on suboptimal samples are also warranted as this study only considered samples with high concentration and progressive motility. This is important as there are post-thaw differences between normozoospermic and suboptimal samples (Counsel et al., 2004; Palomar Rios et al., 2018), which also have lower levels of antioxidants within the seminal plasma (Smith et al., 1996). Additionally, we advise further longitudinal studies to investigate fertilization rate, clinical pregnancy rate and live birth outcomes.

Conclusion

This study contributes to the current limited literature and recommends that sperm should be prepared by DGC before cryopreservation. Given the results of this study, it could feasibly be recommended that for ART procedures requiring a high motile sperm population, such as IUI and IVF, a 60% gradient be used. For procedures which require a smaller cohort of motile sperm, such as ICSI, the 95%/50% gradient should be used. As the number of people utilizing ART rises, the optimization of sperm freezing protocols is essential to maximize success rates for these patients.

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