RESEARCH

Is pre-freeze sperm preparation more advantageous than post-freeze?

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Abstract

Human sperm cryopreservation is characterised to this day by sub-optimal success rates. Interestingly, a traditional approach to improving post-thaw outcome has been to integrate standard sperm preparation techniques into freezing protocols as a means of selecting sperm with the highest fertilisation potential prior to insemination. However, no consensus has been reached yet regarding the optimal timing (before or after freezing) of this selection step. Following analysis of a total of 20 human semen samples, which were divided into two aliquots prepared by density gradient centrifugation either before or after freezing, this study demonstrated higher post-thaw total ($P < 0.0001$), progressively motile ($P = 0.005$) and vital ($P < 0.0001$) sperm counts for frozen-prepared semen samples. The present study suggests that direct insemination with frozen-prepared sperm with minimal intervening post-thaw processing might be a more advantageous approach to current clinical practices, particularly for donor and patient intrauterine insemination programmes. Further research into cryopreservation-induced coiled sperm tail morphology is also warranted.

Lay summary

Freezing and storing of sperm in liquid nitrogen ('sperm cryopreservation') is the current method of choice for preserving the fertility of a wide scope of men. Nevertheless, sub-optimal sperm survival is still associated with traditional cryopreservation methods, namely 'slow freezing', and may affect fertility treatment success rates. Interestingly, a widely applied approach for selecting high-quality sperm before treatment has been to incorporate 'sperm preparation' techniques, such as density gradient centrifugation, in slow freezing protocols. There is, however, an ongoing debate regarding which is the optimal timing of this selection step: before or after freezing. In this study, we collected 20 human semen samples which were divided into two portions and subjected to density gradient centrifugation either before or after freezing. Post-thaw semen analyses demonstrated significantly improved sperm counts ($P < 0.05$) when density gradient centrifugation was performed before freezing, thus suggesting this approach to be more advantageous for current clinical practices.

Key Words: ▶ sperm ▶ fertility preservation ▶ andrology ▶ assisted reproduction

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Introduction

Sperm cryopreservation has achieved widespread recognition as an invaluable medical intervention for preserving male fertility, particularly before sterilising cancer therapy, owing to the simplicity and low costs associated with its application. To this day, the clinical indications for sperm cryopreservation have widened and include donor semen storage as well as 'back-up' sperm storage for oligozoospermic patients or men who cannot ensure the timely provision of an ejaculate for treatment, thus enabling for a patient-tailored approach in the use of frozen sperm.

Despite its clinical advantages and ease of access, human sperm cryopreservation remains an under-represented technique in assisted reproduction technology, with sub-optimal success rates of marked variability being commonly reported in the literature. These have been largely attributed to the extensive cryopreservation-induced sperm damage which is multifactorial and as such minimisation strategies are difficult to determine. Even more so, the advent of ICSI and robust nature of human sperm have persistently stood as favourable arguments to the theory that most frozen semen samples can now support assisted conception, thus delaying further advancements in this field.

A traditional method for improving post-freeze outcome has been to integrate standard sperm preparation techniques into cryopreservation protocols as a final step after freezing–thawing in order to select sperm with the highest fertilisation potential as well as discard cryoprotectant (CPA) media prior to insemination. Even more so, this has been postulated to allow the inherent protective action of seminal plasma against oxidative stress to take its place during cryopreservation (Grizard *et al.* 1999, Oehninger *et al.* 2000). Seminal plasma is known to be enriched in several antioxidant enzymatic and non-enzymatic compounds whose sum constitute the total antioxidant capacity of a semen sample which can, in turn, be particularly important under the increasing reactive oxygen species (ROS)-conditions associated with freezing–thawing cycles (Adeel *et al.* 2012, Martinez-Soto *et al.* 2013).

Nevertheless, the protective action of seminal plasma is thought to vary between individuals as well as positively correlate with semen quality, possibly explaining why subfertile patients are most vulnerable to the sperm damaging effects of cryopreservation (Hammadeh *et al.* 2001, Kalthur *et al.* 2008, Martinez-Solo *et al.* 2013). Even more so, its contradictory potential of containing cellular sources of ROS, such as leukocytes and abnormal spermatozoa, has added to the controversy around the extent of its cryoprotective role. Coupled to the wide variation in viscosity/rheological properties between samples and the obvious effect that this may have on the uniform dilution and penetration of CPA, sperm cryopreservation in the presence of seminal plasma may be more complex than previously thought. In view of this, different authors have investigated the effect of sperm preparation before freezing as a potentially superior sperm selection approach. However, studies are conflicting and lack consistency especially in relation to:I. The number of pre-freeze and post-thaw processing steps subjected to sperm of each test group;II. The concept that the entire sperm cryopreservation process remains incomplete until the CPA is removed by final sperm washing;III. The cooling rates offered by manual freezing methods;IV. Variation in methods used for the measurement of pre-freeze and post-thaw sperm parameters;V. Lack of consideration and assessment of sperm morphological defects possibly induced by sperm freezing.

In view of the above, the aim of this study was to test the hypothesis that sperm selection before freezing leads to improved quality of the cryopreserved sperm treatment unit immediately prior to insemination.

Materials and methods

Semen samples

A total of 20 semen samples were donated for the purposes of this study by men enrolled in the Fertility Unit, University Hospital, Nottingham, UK, who had previously consented to sperm donation using standard HFEA forms. Ethics approval was additionally achieved through a system of risk assessment and long-standing agreement between the hospital and University of Nottingham for running student projects, training and method development. The age of participants was between 25 and 50 years old at the time of study, and their ejaculates satisfied the minimum seminal requirements of 15×10^6 sperm/mL concentration and 1 mL volume (the median total count at fresh collection was 70.3×10^6 sperm/mL). The experiment followed a paired, repeated measures design by treating each sample as its own control. As such, after liquefaction at 37°C, each specimen was individually treated by being subdivided into two aliquots that were subjected to almost identical processing except for the timing of sperm preparation, that is, prepared before freezing (PBF) or prepared after freezing (PAF).

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More specifically, either protocol consisted of identical freezing and thawing methods with an equal number of sperm wash/centrifugation steps as well as re-suspension volumes, and was coupled to consecutive semen analyses allowing for data collection at three distinct time points of the cryopreservation process: (1) fresh collection and after pre-freeze sperm preparation (DC1); (2) post-thaw (DC2); (3) post-thaw sperm preparation and/or CPA removal (DC3) (Fig. 1).

Sperm quality assessment

Each semen analysis consisted of a sperm concentration ($\times 10^6$ sperm/mL), progressive motility and average motile speed (AMS) (μm/s) assessment using CASA (computer-assisted semen analysis) and the Sperminator™ system (Procreative Diagnostics, Staffordshire UK), previously validated for clinical use against the WHO 2010 recommended manual methods (Tomlinson *et al.* 2010). Vitality measurements were also included and collected manually by performing the Eosin-Nigrosin test (Sperm VitalStain™ stain, Nicadon Internation AB, Gothenburg, Sweden). Additionally, a preliminary sperm tail morphology assessment was carried out by counting a total of 200–300 motile and immotile sperm upon close examination of CASA-recorded 1 second video loops collected throughout this study. Sperm tail assessment was included in order to particularly investigate the degree of coiling or damage induced by freeze–thaw. Finally, changes in sperm parameter values during the first half of the experiment (DC1–DC2) were quantified as: (Median Value at DC2) – (Median Value at DC1), and the second half of the experiment (DC2–DC3) as: (Median Value at DC3) – (Median Value at DC2). A positive or negative value of change corresponds to an increase or decrease in the sperm parameter, respectively.

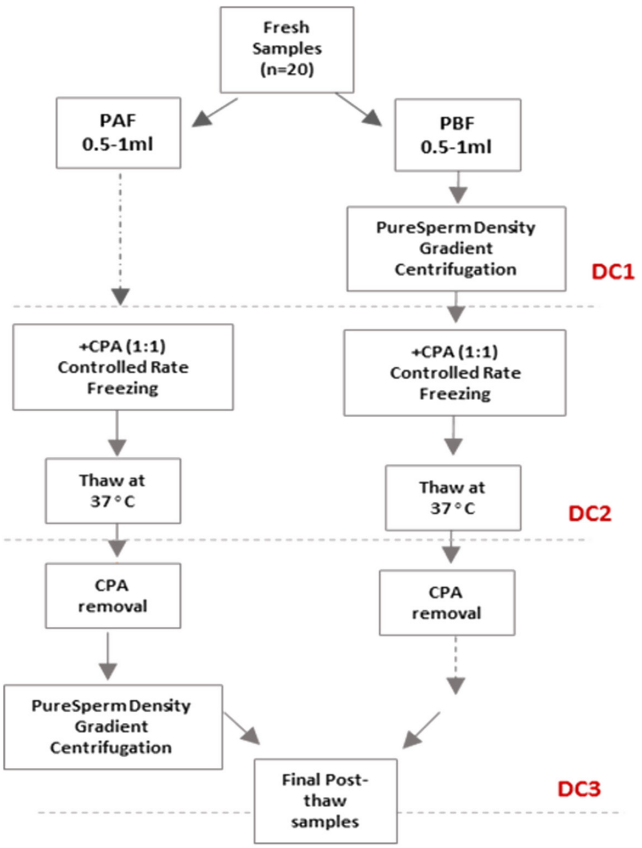

Figure 1 Flow chart of experimental design illustrating the processing of the two sample groups – prepared before freezing (PBF) and prepared after freezing (PAF) – and the timing of data collection (DC1–DC2–DC3) (median total sperm count of fresh samples = 70.3 × 106/mL).

Sperm preparation

Fresh and thawed semen samples were prepared by a two-step PureSperm density gradient centrifugation (DGC) procedure (80–40%; Nicadon International AB, Gothenburg, Sweden). This involved carefully placing 0.5–1 mL of sample on top of the two-layered gradient, centrifuging at 300 ***g*** for 20 min at room temperature (RT) and re-suspending the resulting pellet in 1 mL PureSperm wash media before centrifuging at 300 ***g*** for an additional 5 min at RT. The final sperm preparation was suspended in 0.5–1 mL wash media, depending on pellet size.

Sperm cryoprotection, slow-freezing and thawing

Fresh semen samples and prepared sperm suspensions were thoroughly mixed at a 1:1 ratio with HEPES-buffered cryopreservation medium, containing 15% glycerol and 0.4% human serum albumin (SpermFreezeTM, FertiPro, Belgium). The mixtures were then allowed to equilibrate for 5 min at RT prior to being loaded by aspiration into 0.5 mL cryostraws (CBS high security straws, IVM, France). These were then press-sealed (SYMS thermal sealer, Cryo Bio Systems) and finally frozen by slow controlled rate freezing (CRF) (PLANER Kryo 550-16 programmable controlled rate freezer, Planer Products Ltd, UK) using a pre-specified cryoprofile: Freezing commences at 20°C with a cooling rate of −2°C/min, and when −5.7°C is reached, the cooling rate is decreased to a −10°C/min until a holding temperature of −160°C. Upon completion of this freezing programme, the straws were retrieved and stored in a LN_2 dry shipper tank for a maximum of 7 days.

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All straws were thawed by rapidly rubbing between the hands followed by incubation at 37°C for 5 min. CPA removal was achieved by a two-step sequential 1:1 dilution of the retrieved CPA-sperm suspension with PureSperm wash media and incubation at RT for 5–10 min. Finally, centrifugation at 300 ***g*** for 5 min was performed and the resulting sperm pellet was re-suspended in 0.5–1 mL wash media.

Statistical analysis

Statistical analyses of data were carried out using IBM SPSS 26.0 software. Prior to selecting the appropriate statistical test, normality of all collected data for each sperm parameter was assessed using the Shapiro–Wilk normality test. Among the non-normally distributed measurements, the Friedman Test was employed, as a non-parametric equivalent to a repeated measures (RM) ANOVA analysis, for the investigation of within-group statistical differences in sperm parameters analysed at the three different time points (DC1 vs DC2 vs DC3) of the cryopreservation protocol. Comparisons between PBF and PAF groups were performed using a two-tailed Wilcoxon matched-pairs signed-rank test for non-normally distributed data, and a two-tailed Student's paired *t*-test for normal data. Finally, Pearson's correlation coefficient was calculated to assess relationships between selected sperm parameters. Values are reported as Median (25%, 75%) for non-normally distributed data, and as mean ± S.D. for normal data. In all cases, a value of $P < 0.05$ was considered statistically significant.

Results

Overall cryopreservation outcome following post-thaw DGC and/or CPA removal (DC3)

As expected, within both prepared after freezing (PAF) and prepared before freezing (PBF) groups, cryopreservation resulted in an overall significant decrease in the median motility index (MI = Average Motile Speed (AMS) x Progressive Motility (PM)/100) (PAF: $P < 0.0001$; PBF: $P < 0.0001$), vitality (%) (PAF: $P < 0.0001$; PBF: $P < 0.0001$) and, most notably in a significant increase in coiled tail defects (%) (PAF: $P < 0.0001$; PBF: $P < 0.0001$). Interestingly, however, comparative analyses between the PAF and PBF group showed no significant differences in their median DC3 vitality (%), coiled tail forms (%) and MI scores (Table 1). Likewise, the differentially timed DGC but equal number of sperm washing steps, coupled to identical freezing–thawing protocols, resulted in an expected overall significant decrease in the total sperm count of both groups (PAF: $P < 0.0001$; PBF: $P = 0.001$). However, 16 out of the 20 samples analysed in the present

Table 1 Summary list of seminal parameters obtained from samples prepared after freezing (PAF) or before freezing (PBF) and their comparative evaluation across different time-points of the cryopreservation process (DC1–DC2–DC3). Data are presented as median (25–75%).

Sperm parameters	Prepared after freezing	Prepared before freezing	*P*-value*
Total sperm count (×10⁶/mL) (*n* = 20)			
DC1	70.3 (56.3–102.4)	32.9 (16.5–55.7)	<0.0001
DC2	65.8 (48.3–106.5)	29.4 (26.1–48.5)	<0.0001
DC3	8.3 (6.9–14.5)	19.1 (15.0–40.0)	<0.0001
Progressive Motility (%) (*n* = 20)			
DC1	39.4 (27.5–59.2)	67.9 (30.5–71.6)	0.0094
DC2	12.1 (7.6–18.5)	18.7 (8.7–24.9)	0.0136
DC3	11.0 (6.2–16.7)	10.5 (4.3–17.7)	NS
Vitality (%)			
DC1	79.7 (72.8–83.9)	89.5 (77.6–91.8)	NS
DC2	37.7 (33.9–47.7)	36.9 (23.3–42.7)	NS
DC3	34 (27.5–39.5)	34 (27.5–46.0)	NS
Motile Index (*n* = 20)			
DC1	12.0 (8.6–26.3)	32.7 (13.9–37.1)	<0.0001
DC2	3.8 (1.5–5.9)	6.7 (3.2–10.7)	<0.0001
DC3	3.3 (1.7–7.1)	3.3 (1.6–6.1)	NS
Coiled Tail Morphology (*n* =15) (% abnormal forms)			
DC1	5 (4–7)	6 (4–8)	NS
DC2	10 (13–16)	21 (19–30)	0.0001
DC3	38 (35–50)	39 (34–45)	NS

*Statistical Significance at $P < 0.05$.

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study showed significantly higher DC3 total and vital sperm counts (Figs 2 and 3) while 14 samples showed significantly higher DC3 PM sperm counts, when DGC was performed before freezing (PBF) (Fig. 4).

The effect of freeze–thaw (DC1–DC2) and CPA removal/post-thaw DGC (DC3) on sperm survival

A statistically significant difference in the MI change was reported between the two groups during both stages of the cryopreservation protocol (DC1–DC2 and DC2–DC3), with the prepared before freezing (PBF) samples being most negatively affected (DC1–DC2: −8.4 (−13.8, −6.2) vs −21.4 (−28.5, −8.3), $P< 0.05$; DC2–DC3: 0.5 (−1.0, 3.7) vs -3.1 (−5.7, −1.0), $P< 0.05$). In addition, the PBF samples showed a more significant % vitality decrease during the first half of the experiment (DC1–DC2: −38.2 ± 12.7 vs 48.9 ± 18.2, $P< 0.05$) in comparison to the prepared after freezing (PAF) group which showed a more significant decrease during the later experimental stage (DC2–DC3: −7.0 ± 15.2 vs −1.8 ± 14.7, $P< 0.05$). Finally, a more significant % increase in coiled tails was observed in the PBF group during the DC1–DC2 stage (8.0 ± 5.2 vs 18.6 ± 7.4, $P< 0.05$) whereas in the PAF group during the DC2–DC3 stage (26.9 ± 9.7 vs 13.8 ± 10.9, $P< 0.05$).

The relationship between post-thaw progressive motility and tail coiling

Correlation analyses, using data collected from both groups, were performed to investigate the relationship between DC3 progressive motility (PM) and sperm tail coiling. Only a weak, non-significant negative correlation was observed between % PM and % total tail abnormal forms (r= −0.333, $P= 0.072$).

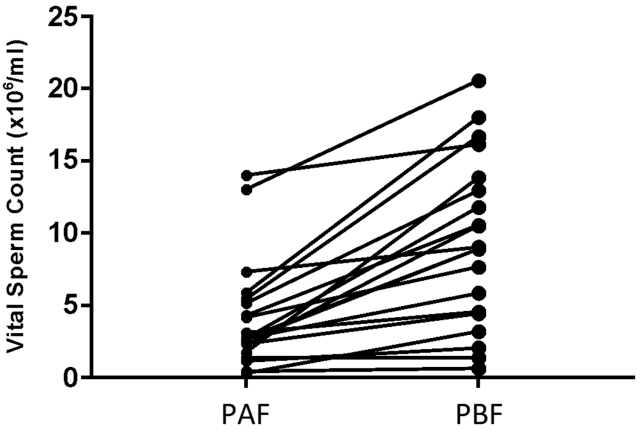

Figure 3 Line-plot comparing the DC3 median vital sperm counts between PAF and PBF samples (n =20). P = 0.015; Wilcoxon matched-pairs signed-rank test statistical significance at P <0.05.

Discussion

Different arguments have been brought forward against or in favour of the timing of sperm preparation before freezing. These have been mostly focused on the evidenced total antioxidant capacity of seminal plasma to minimise the exposure of sperm to endogenous and cryo-induced generation of free oxygen radicals as well as its paradoxical potential of containing ROS-producing non-sperm cells and therefore being cytotoxic under prolonged exposure (Alvarez & Storey 1992, Aitken *et al.* 1989, Whittington *et al.* 1999). Likewise, authors in favour of the

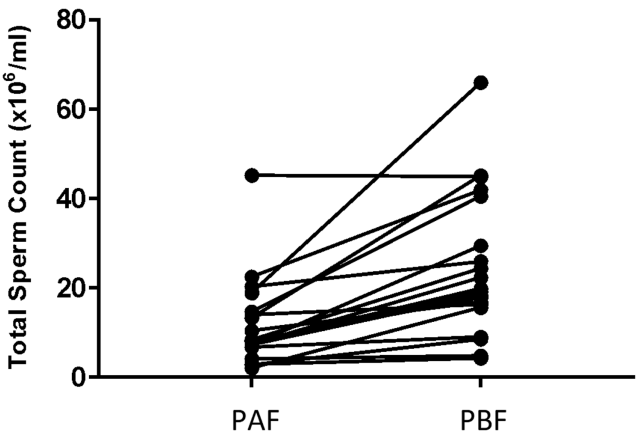

Figure 2 Line-plot comparing the DC3 median total sperm counts between PAF and PBF samples (n =20). P = 0.005; Wilcoxon matched-pairs signed-rank test statistical significance at P < 0.05.

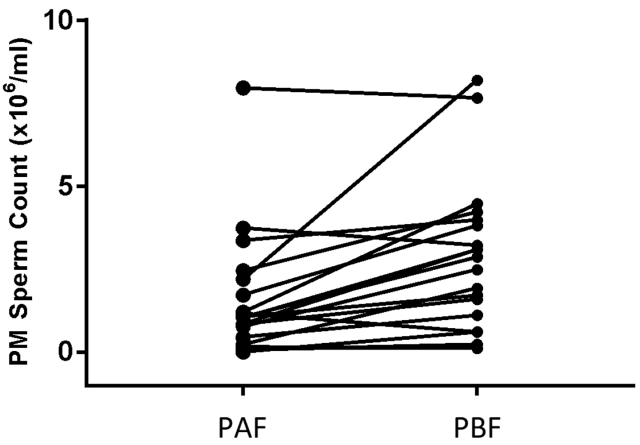

Figure 4 Line-plot comparing the median PM sperm counts between PAF and PBF samples (n = 20). P < 0.0001; Wilcoxon matched-pairs signed-rank test statistical significance at P < 0.05.

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pre-freeze approach to sperm selection have proposed that optimisation of baseline sperm quality before freezing would allow for an improved response from the prepared semen samples to the osmotic and mechanical challenges of cryopreservation. The interaction of the seminal plasma with the sperm membrane has been equally discussed, with studies suggesting that seminal plasma removal will accelerate the capacitation process, thereby decreasing sperm life-span, and aggravate membrane instability by increasing its fluidity and permeability (Medeiros *et al*. 2002). Conversely, seminal plasma removal has also been postulated to module membrane fluidity in favour of a less than- damaging increase in permeability to CPAs, thereby promoting the beneficial effects of the latter on sperm integrity during freezing–thawing cycles. Interestingly, authors have more recently argued that membrane fluidity has instead a biphasic relationship with cryogenic success and thus becomes optimal when it is balanced between preventing the cryopreservation-induced disruption of sperm membrane architecture and allowing the free entry or exit of CPAs for maximal cryoprotection (Miller *et al*. 2005, Martinez-Soto *et al*. 2013).

Interestingly, a number of studies have reported favourable post-thaw results when following the pre-freeze approach (Perez-Sanchez *et al*. 1994, Esteves *et al*., 2000, Counsel *et al*. 2004, Petyin *et al*. 2004, Brugnon *et al*. 2013), whereas additional conflicting studies have either showed comparable outcomes or the opposite to be true (Grizard *et al*. 1999, Donnely *et al*. 2001, Sharitha & Bongso 2001, Palomar Rios *et al*. 2018). Comparative evaluation of these studies remains, however, challenging owing to the methodological disparities being present between protocols used, with a number of these studies notably neglecting to complete the cryopreservation process by not including CPA removal as the final step. Optimisation of sperm cryopreservation remains therefore a complicated topic that requires further collaborative investigation ideally based on standardised experimental designs.

In our study, freeze-induced asthenozoospermia was noticeable in both prepared after freezing (PAF) and prepared before freezing (PBF) samples. This is in line with the 25–75% motility reduction widely reported in most freeze–thaw cycles carried out to date (Keel & Black 1980, Oberoi *et al*. 2014), which is commonly pertained to a sub-lethal loss of plasma and mitochondrial sperm membrane integrity as well as to apoptotic cell death. Likewise, timing the sperm preparation step before or after freezing had no statistically significant effect on the post-thaw percentages of all sperm parameters analysed, namely vitality, AMS, MI and abnormal tail forms.

However, a highly noticeable and statistically significant increase in the total post-thaw sperm count, and hence the total count of vital and PM sperm (Figs 2, 3, 4), was reported for the PBF compared to the PAF samples, putting sperm preparation before freezing forward as a superior optimisation approach. Having made significant efforts to control for experimental sources of bias, we sought to further elucidate this finding through our stepwise comparative analyses.

Interestingly, during post-thaw processing (DC2–DC3), preparation before freezing seemed to maintain sperm vitality better as suggested by the slight but more significant decrease in sperm vitality associated with sperm preparation after freezing. Likewise, although both groups seemed to accumulate coiled tail defects during post-thaw processing, this effect was more prominent when sperm preparation was performed after freezing. It could thus be suggested that either (1) frozen-prepared samples showed an increased tolerance to the mechanical stresses and osmolarity changes associated with CPA removal, (2) the frozen fresh samples were possibly more sensitive to the above perturbations and/or (3) the post-freeze density gradient centrifugation (DGC) intervening during that time did not offer any benefit for, or even negatively affected post-thaw vitality and tail morphology outcome.

Nevertheless, when progressive speed is comparatively evaluated between the two groups, conflicting arguments may arise. Post-thaw processing (DC2–DC3) of frozen fresh samples resulted in a less significant MI reduction compared to frozen-prepared samples. As such, it seems that the intervening post-freeze DGC provides a timely benefit to the conventional cryopreservation protocol, by counteracting possible motility perturbations caused by the preceding freeze–thaw-CPA removal cycle. Likewise, the freeze–thaw process (DC1–DC2) was shown here to be more damaging to the vitality, progression and tail morphology of samples prepared prior to freezing. It could thus be suggested that seminal plasma acted as a protective barrier to free radical attack and membrane damage during the freeze–thaw process.

As such, we postulate that differences in pre-DGC baseline sperm quality and the presence or absence of seminal plasma from the freezing medium equated to disparities in sperm cryotolerance between the two groups and differentially affected the pre- and post-freeze DGC efficiency. As such, although both trial arms accumulated analogous cryo-injuries at different times during the cryopreservation process, the difference in DGC efficiency

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led to markedly different final sperm counts. Interestingly, this study is the third in our knowledge to suggest that the benefit of pre-freeze sperm selection is not the suggested improvement in sperm cryotolerance but rather an increased efficiency in recovering higher counts of functional sperm from a fresh non-stressed semen sample. Notably, a study by Graczykowski and Shiegel (1991) had brought forward the concept of sperm preparation causing a dramatic loss in motility when applied to frozen thawed rather than fresh semen samples. Further in support of this, Tomlinson *et al.* (2010), reported improved ongoing pregnancy rates of IUI and IVF donor cycles when using DGC prepared sperm.

Cryopreservation-induced sperm tail coiling

Along with the dramatic decrease in post-thaw motility, sperm tail coiling/bending has been equally documented in post-cryopreservation semen specimens (Fig. 5; Holt *et al.* 1988, O'Connell *et al.* 2002, Turan *et al.* 2017), but has lacked systematic study. A very interesting analogy is, however, made when the significant osmotic challenges, brought about by cryogenic temperatures and cryoprotection cycles, are considered in relation to tail membrane deformation due to cell-swelling, a commonly reported phenomenon following the routine hypo-osmotic swelling (HOS) test. Even more so, studies have speculated on possible sperm cryo-injuries taking place at the cytoskeletal level with likely implications on the fertilising potential of cryopreserved semen (Desrosiers *et al.* 2006), however, no clarification has been yet achieved on whether the above findings have a direct correlation with the outstanding sperm motility loss inflicted by current cryopreservation protocols.

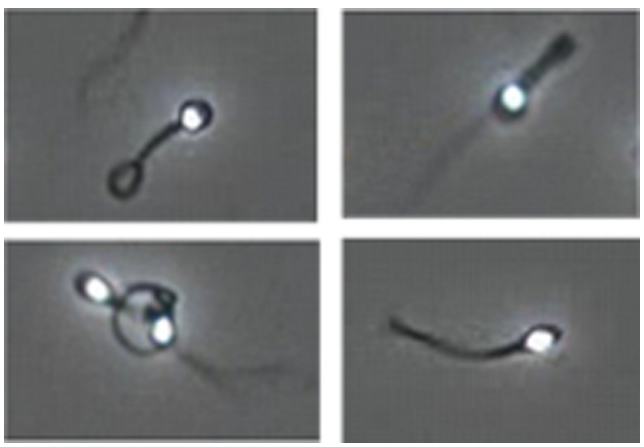

Figure 5 CASA output screen showing representative examples of documented tail coiling in thawed semen.

Interestingly, sperm flagella defects have been considered primary culprits behind idiopathic or genetic motility disorders, with an architectural disorganisation of the tail cytoskeleton being a proposed causative factor (Chemes & Alvarez Sedo 2012). Nevertheless, our current understanding of the mechanisms causing this tail deformation has been largely limited to transgenic mouse models and bull studies, pointing either to an inherent genetic fragility characterising sperm with a coiled or bent flagellum (Suzuki-Toyota *et al.* 2007, Zheng *et al.* 2007) or to an osmotically dysregulated epididymal environment.

With the earlier described in mind, we hypothesised that our samples, irrespective of sperm selection approach, would show an increased frequency of coiled tails, which would, in turn, be characterised by a negative relationship with progressive motility. As such, although the expected statistically significant increase in % Tail Coiling was reported in both groups, a negative but only weak and not statistically significant correlation was observed between the percentage of total tail coiling and sperm progression. Nevertheless, it is noteworthy that in both groups, the non-progressive/static spermatozoa accounted for the highest frequency of tail abnormalities (DA Androni, S Dodds, M Tomlinson & W Maalouf, unpublished observations).

Conclusion

The present study showed that sperm preparation before freezing confers an advantage over the more traditional post-freeze approach by yielding higher counts of vital and progressively motile sperm. Detailed comparative analyses showed the present outcome to be explained by differences in the DGC efficiency being present between frozen fresh and frozen-prepared sperm. In other words, we demonstrated that timing most of semen processing prior to the stress-inducing freeze–thaw cycle likely ensures a more efficient sperm selection to take place and an avoidance of over-handling cryo-damaged semen samples. A preliminary analysis of the overlooked cryopreservation-induced sperm tail coiling was also highlighted and only a weak relationship with sperm motility was reported. Nevertheless, its further study is warranted as a potential novel candidate for future research avenues in this field. Finally, it should be noted that the accuracy of our data analyses was strongly limited by the significantly reduced post-thaw motile sperm counts observed in both groups. This should, however, act as a strict reminder that despite optimisation efforts, sperm cryopreservation can be to this

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day a limiting factor during treatment decision making for fertility preservation patients.

Declaration of interest
Dr Tomlinson is a co-director of a company which is involved in the research, development and commercialisation of the CASA system, Sperminator™.

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Author contribution statement
D A A and S D performed experiments and statistical analyses of data. D A A wrote the manuscript with support from M T. M T conceived and supervised the study. W M coordinated and helped supervise the study. W M and M T contributed to the review and editing of final manuscript.

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