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Efficacy of Ultra-Rapid Freezing by Direct Immersion into Liquid Nitrogen for Sperm Cryopreservation and Its Potential for Male Fertility Preservation

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Abstract:

Sperm cryopreservation is a valuable technology to preserve male's fertility in many species. The success rates of current cryopreservation techniques remain mediocre and are beset by unexplained inter-individual variation. The study aims to devise and evaluate novel ultra-rapid freezing (URF) protocol to improve sperm cryopreservation utilizing approaches suggested to prevent intra- and extra-cellular ice formation relative to conventional slow controlled rate freezing (CRF) and vapour freezing. Experiments initially focused on optimising the entire protocol by reappraising each constituent part of the process in addition to initial evaluation of sperm URF comparing dry ice to liquid nitrogen (LN_2) direct plunging. Results showed that URF is feasible and can provide enhanced post-thaw survival over the CRF ($P<0.01$ sperm progression; $P<0.05$ motile sperm yield) using no more than 7.5% glycerol added at ambient conditions, particularly if sperm are first gradient prepared. Although findings indicated no difference in sperm motility and morphology between URF and conventional slow vapour freezing, URF using LN_2 plunging resulted in increased sperm DNA integrity preservation ($P<0.05$).

It was concluded that the URF protocol showed promising results and minimises the CPA and osmotic stress exposure period and other associated risks and suggestions to overcome the issue of sterility in LN_2 were justified. Thus, sperm URF/vitrification is worthy of further development and optimization of the technique.

Keywords: ***cancer, human, spermatozoa, cryopreservation, slow freezing, Ultra-rapid freezing, vitrification.***

Introduction

Although the field of medicine has embraced sperm freezing with relative success achieved using the current slow rate freezing methods, the post-thaw survival rate remains mediocre reproductive regardless of the technique used or indeed initial sperm quality (Watson 2000, Balaban et al. 2008, Brugnon et al. 2013, Kuznyetsov et al. 2015). Thus, many patients require an ICSI procedure with their frozen-thawed sperm (Crha et al. 2009). In addition, slow rate cooling has been reported inappropriate for freezing sperm samples of small numbers and poor quality such as testicular biopsied spermatozoa, which are minimum and often associated with decreased in-situ motility (AbdelHafez et al. 2009, Endo et al. 2012). The rapid freezing observed during vitrification is now considered one of the most accepted and applied strategies in the field of cryopreservation that can avoid ice crystallisation (AbdelHafez et al. 2010). Although successful pregnancies and live births are now a routine expectation after treatment using rapidly frozen "vitrified" embryos and oocytes, rapid sperm freezing has only recently been reported as successful (Isachenko et al. 2003, Isachenko et al. 2004a). However, the reported toxic and osmotic effects of high cryoprotectant (CPA) concentrations (Gilmore et al. 1997) used in vitrification has contributed in limiting the development of sperm rapid cooling.

Few recent reports have suggested that rapid sperm freezing may be as effective, yet less time consuming than conventional slow freezing (Isachenko et al. 2004b, Isachenko et al. 2008, Isachenko et al. 2012). Isachenko and his group reported the birth of two healthy babies in 2011 after treatment using sperm frozen by direct immersion into liquid nitrogen (LN_2) without the use of permeable CPA claiming they achieved sperm vitrification (Isachenko et al. 2012). Moreover, ultra-rapid freezing (URF) has been reported to sufficiently preserve the sperm motility and deoxyribonucleic acid (DNA) integrity over the conventional slow rate freezing (Isachenko et al. 2004a). However, there is little evidence to suggest that these methods have been verified by others or indeed that the technique has been adopted elsewhere.

One of the major difficulties in developing sperm freezing is the reliability of the end-point measurement, usually the post-thaw recovery of progressively motile sperm. Traditional semen analysis is poorly reproducible and associated with a high level of uncertainty (Tomlinson et al. 2010a). As such, attempts at measuring the influence of cryopreservation variables including; manual versus automated freezing, cooling rate, cryoprotectant (type, exposure and concentration) and thaw rate on post-thaw sperm quality are extremely difficult.

The objective of this study is to develop and investigate the efficiency of a robust method for sperm rapid cooling attempting to corroborate the findings of previous publications (Isachenko et al. 2004a, Isachenko et al. 2012), which reported successful sperm vitrification without the use of permeable CPA. This will be through evaluation of various forms of ultra-rapid freezing (URF) by direct plunging into liquid nitrogen and dry ice and compare it to current available SF methods. This will be achieved after determination of the robust CPA in addition to testing the effect of sperm preparation prior to freezing.

In order to avoid the inconsistency of the post-thaw sperm quality assessment, the endpoint quality analysis will ultimately be sperm motility as it is the first parameter affected by cryopreservation. To achieve this approach, the post-thaw measures will be assessed using a validated computer assisted semen analysis (CASA) system (Sperminator™), developed in the same laboratory (Tomlinson et al. 2010b).

Materials and Methods

Experimental design

Using the CASA system as a platform for initial and post-thaw semen analysis, series of experiments were conducted to make preliminary evaluation of an URF procedure. The first experiment examined the cryoprotection using either conventional 15% (v/v) glycerol 0.05M sucrose mixture or 0.5M sucrose only prior to liquid nitrogen (LN_2) rapid cooling on the same set of samples (n=23) aliquoted into equal amounts and assigned randomly for treatment. The samples were first divided evenly prior to CPA addition to either undergo sperm washing to remove seminal plasma or cryoprotected as neat semen sample. The second trial assessed the effect of sperm preparation prior to cryoprotection by 15% (v/v) glycerol and cryopreservation using the LN_2 freezing method on the post-thaw survival. Each sample (n=20) was evenly split into 2 parts whereby one of them was cryopreserved after washing to remove the seminal plasma while the other underwent density gradient (DG) preparation. The third experiment compared the efficiency of two rapid cooling methods; LN_2 plunging versus dry ice in parallel on aliquots from same set of samples (n=20) that were DG prepared and mixed with 15% (v/v) glycerol as a CPA prior to freezing. The LN_2 rapid freezing method was then examined in relation to the conventional slow controlled rate freezing (CRF) on a set of samples (n=22) frozen as neat sperm or washed from seminal plasma in parallel. All samples were mixed with 15% (v/v) glycerol prior to freezing. The final experiment compared the efficiency of rapid freezing using liquid nitrogen to the classical vapour SF simultaneously on DG prepared samples (n=16) cryoprotected with 15% (v/v) glycerol. This experiment has also examined the efficiency of cryostraws as a potential closed system for the LN_2 rapid freezing method. In addition to assessing the standard post-thaw sperm quality and morphology, the effect of each freezing protocol on the post-thaw DNA integrity was also evaluated in the last experiment.

Sample collection and sperm analysis

All semen samples used in this research were obtained from consenting patients and donors attending the Fertility Unit, University Hospital, Nottingham, UK, and signed consent forms were provided. Ethical approval was obtained for all validation work associated with the CASA development. Ejaculates were collected according to the World Health Organisation (WHO) guidelines (WHO 2010) by masturbation into a sterile non-toxic polypropylene container (Alpha Laboratories, Eastleigh, UK) after at least 48 hours of sexual abstinence. Included samples had a minimum sperm count of $10x10^6$ sperm ml^{-1}, sperm motility of at least 40%, and minimum semen volume of 1ml. Semen samples were received and assessed according to the WHO grading system (WHO 2010) using the Sperminator™ (Procreative Diagnostics, Staffordshire UK), a prototype computer assisted semen analysis (CASA) system developed in Nottingham and validated against current recommended manual methods (Tomlinson et al. 2010b). The Sperminator was fitted with a heated stage set to 37^0C (Linkham Scientific, Guildford UK).

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Sperm processing and cryoprotection

For samples that underwent washing for seminal plasma removal, the sample was suspended in 2ml (22-25°C) PureSperm Wash® (PSW; Nidacon, Mölndal, Sweden) and centrifuged at 500xg for 10 minutes. The pellet was re-suspended in 0.5-1ml PSW depending on the pellet size. For density gradient preparation, 0.5-2ml of the sample was layered on the top of the gradient layers using a two-step Isolate gradient (80%-40%; Irvine Scientific, Santa Ana, CA, USA), centrifuged (300xg, 20 minutes) before transferring and washing of the pellet in PSW for a further 5 minutes.

Sperm cryoprotection was achieved by adding drops of increasing volumes (14, 19, 27, and 40µl) of the CPA (22-25□C), sequentially at 30-seconds intervals to give a 1:1 ratio and final glycerol concentration of 7.5% of glycerol or 0.25M of sucrose in line with previous protocols (Donnelly et al. 2001, Widiasih et al. 2009). Apart from the experiment using sucrose as sole CPA, all samples were frozen using 15% glycerol (7.5% v/v final concentration) in HEPES buffered with 0.4% human serum albumin and supplemented with 0.05M sucrose and 1% glycine (SpermFreeze™; FertiPro; Beernem, Belgium). Sucrose was prepared in PureSperm® Wash (Nidacon, Mölndal, Sweden) to obtain 0.5M solution.

Sperm slow freezing

The sample-CPA mixture was packaged and sealed in cryostraws (CBS high security straws, 0.5ml, IMV, Paris, France). For the static vapour SF, straws were placed 25cm above the bottom of a full charged dry shipper and suspended in nitrogen vapour (~90^{O}C) for 15 minutes to achieve a 10^{O}C per minute cooling rate (previously validated with a thermocouple). Straws were then lowered in the container while ensuring their remaining in the upper space for 10 minutes to be then stored in liquid nitrogen.

Slow controlled rate freezing (CRF) was performed using a PLANER programmable controlled rate freezer (Planer, Kryo 360M-1.7, Planer Products Ltd, Sunbury-on-Thames, Middlesex, UK). The programme was adapted from previous publication (Morris et al. 1999), in which it initiated at 22°C with a cooling rate of -2°C/minute until it reaches -10°C. After that, the cooling rate increased to -10°C/minute until 100°C. Samples were then retrieved form the freezing chamber and stored in LN^{2}.

Rapid sperm freezing

For dry ice freezing protocol, uniform 3cm deep wells were drilled in a block of crushed dry ice to hold the samples (Berndtson & Foote 1969). Drops (50µl each) of the cryoprotected sample were placed into each well, rapidly forming a solid sphere, and left in dry-ice at least for 30 minutes prior to thawing.

The LN_2 direct plunging protocol was performed as described by (Isachenko et al. 2008). Briefly, aliquots of 50µl of the cryoprotected sperm suspension were plunged directly into liquid nitrogen contained in a foam box and samples were preserved by a metal strainer to facilitate sample collection afterwards. The sample forms a sphere upon contact with liquid nitrogen and remains floating on its surface for short period (4-9 seconds). The sphere then solidifies and sinks to bottom of the strainer and kept there for at least 30 minutes prior to thawing. Frozen spheres were collected using forceps. The same protocol was exactly applied for examining the cryostraws (CBS) as a potential closed system in which cryostraws packaged samples were plunged directly into LN_2 and collected after at least 30 minutes.

Sperm thawing and post-thaw processing

All samples were thawed rapidly after holding for 30 seconds in air to initiate the thawing process. For all rapidly frozen sperm samples; frozen sperm spheres were transferred into 1.7ml Eppendorf tube. For CBS straws sealed samples; straws were placed into falcon tubes filled with warm media (37^{O}C) still sealed within their straws. Both containers were submerged into a 40^{O}C water bath in order to achieve 37^{O}C inside the tube (according to initial temperature testing performed in the lab) and kept for 1 minute for complete rapid thawing. Samples were then decanted from container into a tube and once are completely liquefied they were assessed using the CASA.

Assessment of sperm morphology

This was achieved using pre-stained morphology slides (CELL•VU DRM900; Millennium Sciences, Inc; NY; USA), which are conventional microscope slides containing a dried layer of methylene blue and cresyl violet stains combined. A total of 200 sperm were classified and % of normal or abnormal was reported.

Assessment of sperm DNA fragmentation (Halosperm)

The DNA fragmentation of sperm was assessed using the Halosperm® test (Halotech DNA, S.L.; Madrid, Spain), an in vitro detection kit based on the sperm chromatin dispersion (SCD) test (Fernández et al. 2005, Muriel et al. 2006). The test was performed immediately after thawing to avoid any delay that can interfere with the analysis outcome. Briefly, each sample was diluted (10million/ml) and 50µl of the diluted sample were mixed with equilibrated agarose gel (37^{O}C). Samples were then placed within a slide (8µl) on a metal surface (5 minutes at 4^{O}C) to allow solidification. Slides were then washed by horizontal immersion in acid solution for 7 minutes at room temperature (20-25^{O}C)

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followed by immersion in lysis buffer for 20 minutes at room temperature and then washed in distilled water for 5 minutes. The washing steps aimed to remove the nuclear proteins to allow the appearance of nucleoids with a central core surrounded by a halo of DNA loops (Fernández et al. 2005).

Sperm were dehydrated in increasing ethanol concentrations (70%, 90%, 100%) and air-dried for 2 minutes. Positive control sperm (DNA fragmented sperm, without a halo) was processed in parallel with each sample. Slides were then immersed in Wright's staining solution for 7 minutes (Solution 3; Halotech DNA, S.L.; Madrid, Spain) followed by phosphate buffer saline (PBS) staining solution for 7 minutes (Solution 4; Halotech DNA, S.L.; Madrid, Spain). After drying, visualisation was performed using a bright field microscopy. A minimum of 200 sperm were scored for each sample under x100 objective. The percentage of sperm with fragmented DNA (following the criteria explained in Table 1) was calculated from the total scored spermatozoa.

Table 1: Criteria of sperm DNA fragmentation according to the halo presented. These criteria were adopted from the official Halotech DNA instruction manual.

Sperm without DNA fragmentation		**Sperm with DNA fragmentation**	
Big halo	The halo width is ≥ the minor diameter of the core	Small halo	The halo is ≤ 1/3 of the minor diameter of the core
Medium halo	The halo zise falls between those with big halo and with very small halo	Without halo	No halo is observed
		Degraded	No halo and irregular stained core

Statistical analysis

For the statistical analysis, the Kolmogorov-Smirnov test and normality histograms were used to explore data normality. As most of the motile sperm count (million/ml), progressive motility (%), and normal morphology (%) data were not normally distributed and transformation was not applicable, the non-parametric Kruskal-Wallis or Mann-Whitney U test was used as appropriate to determine the statistical significance between treatment comparisons. Data presented corresponds to the median and first and third quartiles (Q1-Q3). The DNA fragmentation results were subjected to the One-way ANOVA test after being log-transformed and were expressed as mean values and the standard error of the mean (mean±SEM). Tukey's and Dunnett's tests were used for post-hoc analysis as appropriate following ANOVA. A P-value of <0.05 was considered statistically significant. Computer statistical software was used to perform the analysis (IBM SPSS version 19.0, Chicago, IL, USA).

Cryoprotection of neat and washed sperm using 0.5M sucrose versus conventional 15% (v/v) glycerol and 0.05M sucrose prior to rapid cooling in liquid nitrogen

In an attempt to reduce cytotoxicity suggested to be imposed by penetrative CPA, sucrose, which is a non-penetrating CPA, was evaluated for its cryoprotection efficiency in relation to the conventional glycerol-sucrose combination (n=23). Both cryoprotectants were evaluated for providing better post-thaw sperm survival either when added to neat sperm or after seminal plasma removal. Cryopreservation with CPA containing glycerol proved significantly superior to sucrose (P<0.001; Kruskal-Wallis test) with higher numbers of motile sperm in both neat and washed samples (**Error! Reference source not found.**). Furthermore, post-thaw progressive motility was also higher (**Error! Reference source not found;** P<0.001) when cryoprotected by glycerol, in neat (7.2, 4.6-13%; median, Q_1-Q_3) and washed (10.7, 7.7-15.1%) sperm compared to sucrose (3.4, 1.9-4.2% and 3.5, 1.6-5.6% for neat sperm and washed sperm respectively).

Results

Table 2: Initial and post-thaw motile sperm concentration (millions/ml), cryoprotected with either glycerol or sucrose only with and without seminal plasma using LN_2 as the coolant (n=23). Different letters indicate significant differences at P<0.001.

	Median (Q_1-Q_3)	
Initial	11.38 (5.2-35.6)	a
Near Sperm +	2.13 (1.7-4.13)	b
Neat Sperm + Sucrose	1.25 (0.64-2.1)	c
Washed Sperm + Glycerol	3.07 (1.83-4.9)	b
Washed Sperm + Sucrose	0.76 (0.36-1.9)	c

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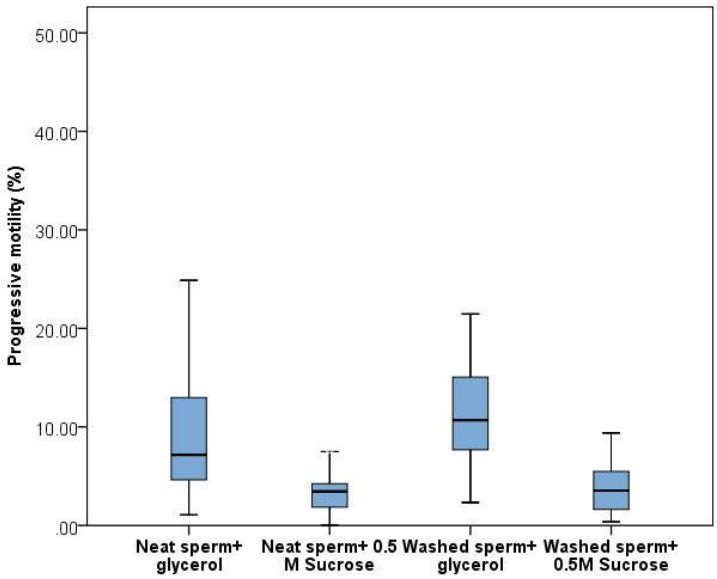

Figure 1: Comparison of progressive sperm motility (%) cryoprotected with either glycerol (15% v/v) or sucrose alone (0.5M) frozen with and without seminal plasma using liquid nitrogen ultra-rapid freezing protocol (n=23). Box and whisker plots are representative of medians and quartiles.

Assessment of seminal plasma removal compared to density gradient preparation prior to freezing applying LN_2 rapid-cooling

This experiment (n=20) examined whether the seminal plasma imposes any inhibitory effect on CPA penetration and if it alone impairs the post-thaw survival indicated by sperm progressive motility (%) and motile count (million/ml). Moreover, the density gradient (DG) prepared samples prior to freezing were also included in the comparison showing merit over both freezing neat sperm or washed sperm in post-thaw motile sperm concentration (11.6, 0.7-34.0; median, Q1-Q3; P<0.01) and progressive motility (23.6, 4-33.2; median, Q1-Q3; P<0.001). The results (**Error! Reference source not found.**) also revealed that washing samples prior to freezing to remove seminal plasma had no significant effect on (P>0.05) post-thaw motile sperm concentration (3.0, 0.97-6.3) and progressive motility (8.5, 4.8-13.9) over freezing neat sperm contained within the semen (2.6, 0.9-5.2 and 10.5, 4.5-15.4 respectively).

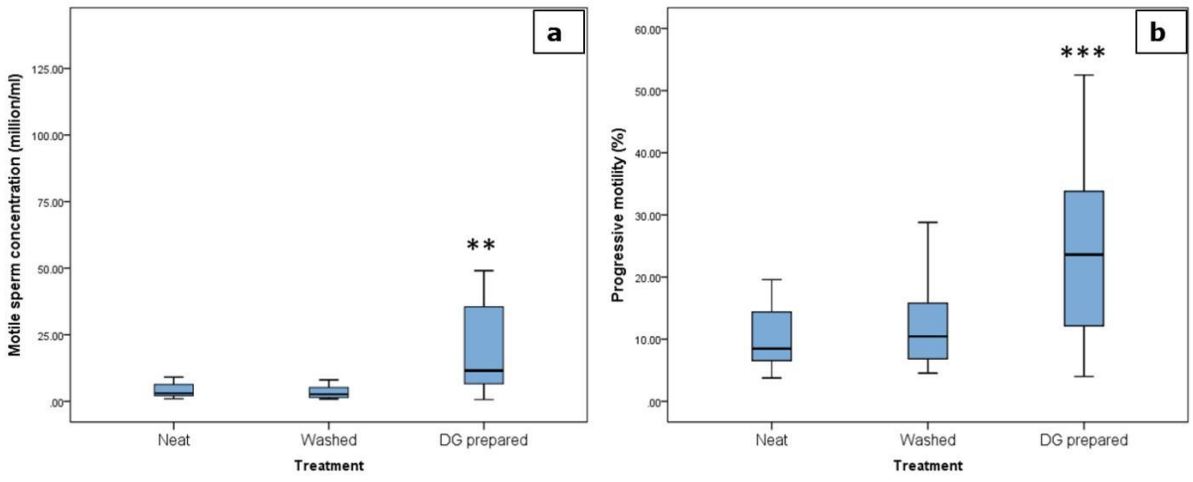

Figure 2: Post-thaw motile sperm count (a) and progressive motility (b) comparing freezing with and without seminal plasma and after DG preparation using liquid nitrogen ultra-rapid freezing protocol (n=20). **P<0.01, ***P<0.001 (Kruskal-Wallis test).

Liquid nitrogen rapid freezing against dry ice rapid cooling protocol

The post-thaw analysis (n=20) showed no effect (P>0.05) on the initial total sperm count (76.3, 51-98.8; median, Q_1-Q_3) after both LN_2 (63.13, 42.2-10.1) and dry ice (63.11, 38.2-94.9) rapid freezing (**Error! Reference source not found.**). However, the motile sperm concentration (million/ml) was significantly reduced (P<0.05; Kruskal-Wallis test) from 35.6 (17.18-54.23) to 11.6 (7.04-34.01) after LN_2 and 14.9

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(5.1-20.7) after dry ice, with no difference between the two methods in the post-thaw assessment (**Error! Reference source not found. a;** P>0.05). This was also reflected on the post-thaw sperm progressive motility (**Error! Reference source not found. b**), which as expected showed a decrease (P<0.05) from the pre-freeze progression (47.5, 39.5-57) after both LN_2 (23.6, 12.6-33.2) and dry ice (17.7, 12.0-30.5) freezing. However, post-thaw sperm of liquid nitrogen and dry ice frozen samples showed that motile sperm count and progressive motility were equally preserved by both freezing methods (P>0.05; Mann-Whitney test).

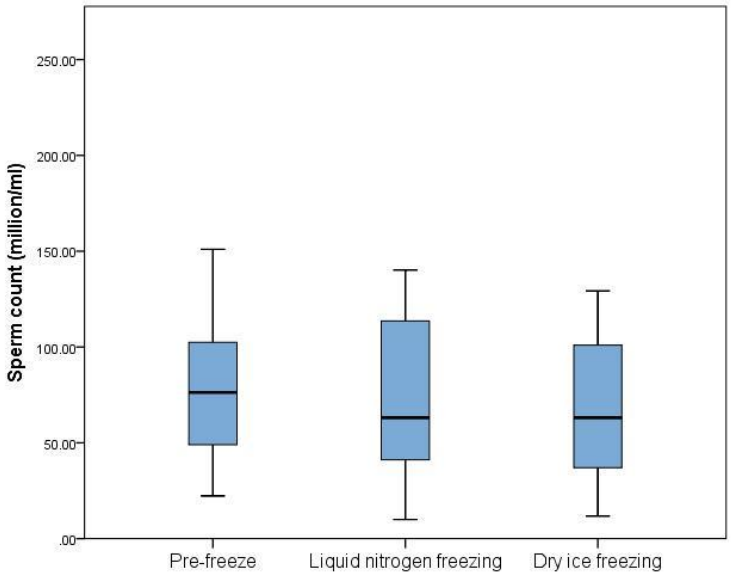

Figure 3: Boxplots of total sperm count (million/ml) before and after random cryopreservation by two different ultra-rapid freezing protocols; liquid nitrogen method (LN_2) and dry ice method. P>0.05 (Krusla-Wallis test).

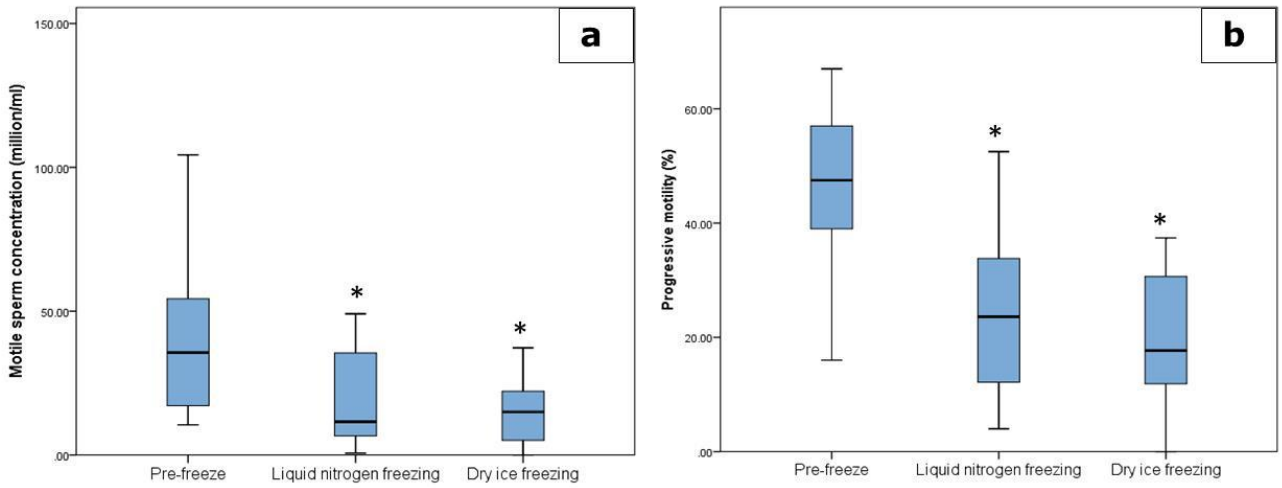

Figure 4: Boxplots showing the motile sperm count (a) and progressive motility (b) before and after random cryopreservation by two different ultra-rapid freezing protocols; liquid nitrogen method and dry ice method. *P<0.05 (Kruskal-Wallis test).

Liquid nitrogen rapid freezing compared to the slow controlled rate freezing (CRF) protocol using both neat and washed resuspended sperm

For further evaluation of the liquid nitrogen rapid freezing protocol, it was compared to the conventional slow CRF method using the standard glycerol-based CPA. Samples (n=22) were frozen by both methods in parallel as neat and washed (seminal plasma free) sperm. Both freezing protocols showed similar highly significant (P<0.001) reduction of the initial progressive motility (27.3; 19.8-39.1%) in both neat semen and washed sperm samples post-thaw. The post-thaw sperm count and motile sperm count (**Error! Reference source not found.** and **Error! Reference source not found. b**) were similarly preserved by both freezing methods (P>0.05). Moreover, the percentage of recovery (yield) of the motile sperm count post-thaw as a proportion of survival from the initial sample was significantly higher (P<0.05) for the rapid freezing method (**Error! Reference source not found.**) when compared to the slow freezing protocol. However, the progression of those motile sperm showed that LN_2 protocol

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has provided significantly more (P<0.01) progressive spermatozoa either frozen with or without seminal plasma (Figure 5 a). Within each freezing method, applying the washing step prior to freezing to remove seminal plasma showed no significant benefit (P>0.05; Mann-Whitney test between neat and washed sperm) on all post-thaw parameters, which coincides with our previous findings.

Table 3: Initial and post-thaw sperm count and motile sperm concentration (millions/ml) frozen either using liquid nitrogen ultra-rapid freezing (LN_2) or conventional controlled slow rate freezing protocol (CRF) as neat sperm or washed from seminal plasma (n=22). P>0.05 (Kruskal-Wallis test).

	Sperm Count (Million/ml)	Motile Sperm count (Million/ml)
	Median (25^{th}-75^{th} Quartile)	Median (25^{th}-75^{th} Quartile)
LN (near sperm)	37.21 (25.7 50.01)	3 (2.07-6.3)
CRF (near sperm)	33.07 (27.8-47.5)	1.9 (1.34-2.73)
LN (washed sperm)	29.3 (22.1-49.03)	2.94 (1.6-5.23)
CRF (washed sperm)	39.75 (25.3-47.9)	2.04 (1.34-3.5)

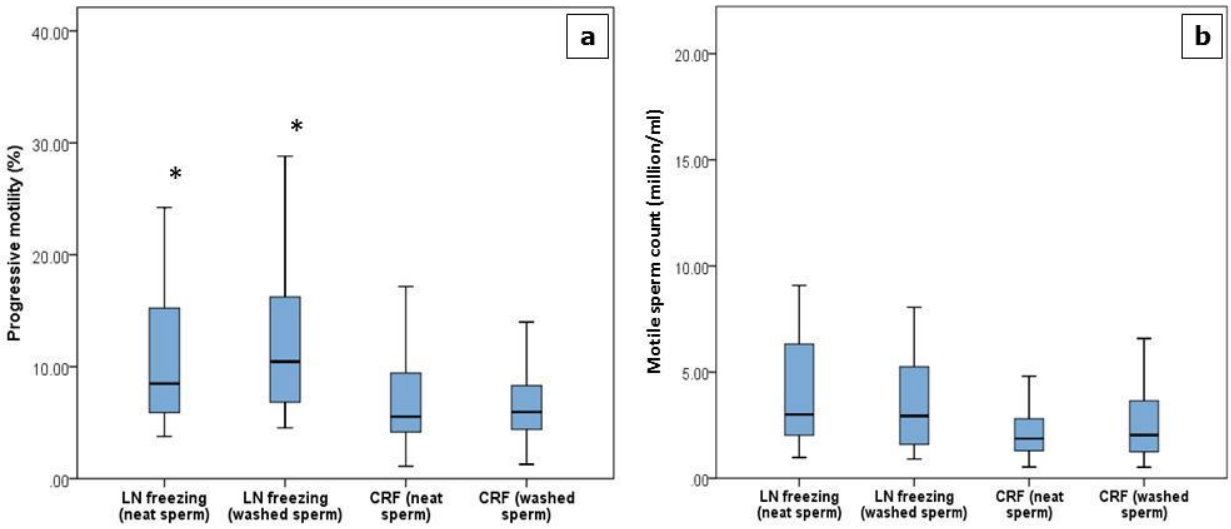

Figure 5: Box plot showing post-thaw progressive motility (%) and motile sperm count (millions/ml) frozen with and without seminal plasma comparing liquid nitrogen (LN_2) ultra-rapid freezing protocol with the conventional controlled slow rate freezing (CRF) method (n=22). *P<0.01 (Kruskal-Wallis test; Mann-Whitney test between each two groups).

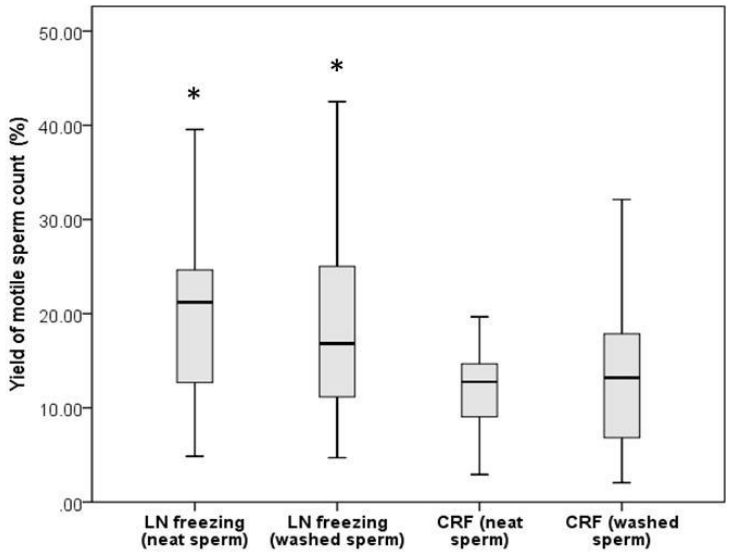

Figure 6: Box plot showing the proportional recovery (yield%) of motile sperm from the pre-freeze sample frozen with (neat) and without seminal plasma (washed) comparing liquid nitrogen (LN_2) ultra-rapid freezing protocol with the conventional controlled slow rate freezing (CRF) method (n=22). *P<0.05 (Kruskal-Wallis test; Mann-Whitney test between each two groups).

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Liquid nitrogen rapid freezing against the conventional vapour static slow freezing

This experiment compared the recently developed LN_2 rapid freezing protocol to the conventional vapour slow freezing (n=16), which is often used in clinics. In terms of sperm motility, although using conventional straws in URF resulted in the lowest motile sperm count (1.34, 0.5-2.9) and progression (5.3, 4-6.63; P<0.001), applying the initially proposed approach of dropping sperm suspension directly into LN_2 provided similar (P>0.05) motile count (17.84, 4.7-46) and progression (42.2, 31.38-53.24) to the slow vapour freezing protocol (14.42, 2.7-46.1; 32.81, 22.21-50.3, respectively; Figure 7 a and b). However, there was no effect of the freezing method on morphology (**Error! Reference source not found. c**).

The degree of fragmentation in the sperm deoxyribonucleic acid (DNA) post-thawing was examined after all freezing methods and they all showed <30% fragmentation (**Error! Reference source not found.**), which is often found in sperm from normal ejaculates (Fernández et al. 2003). However, applying the drops direct plunging URF was observed to result in significantly reduced DNA fragmentation (6.14±1.6; mean±SEM) compared to both straws URF (15.6±2.3; P<0.05) and slow vapour freezing (22.3±3.23; P<0.01; One-way ANOVA).

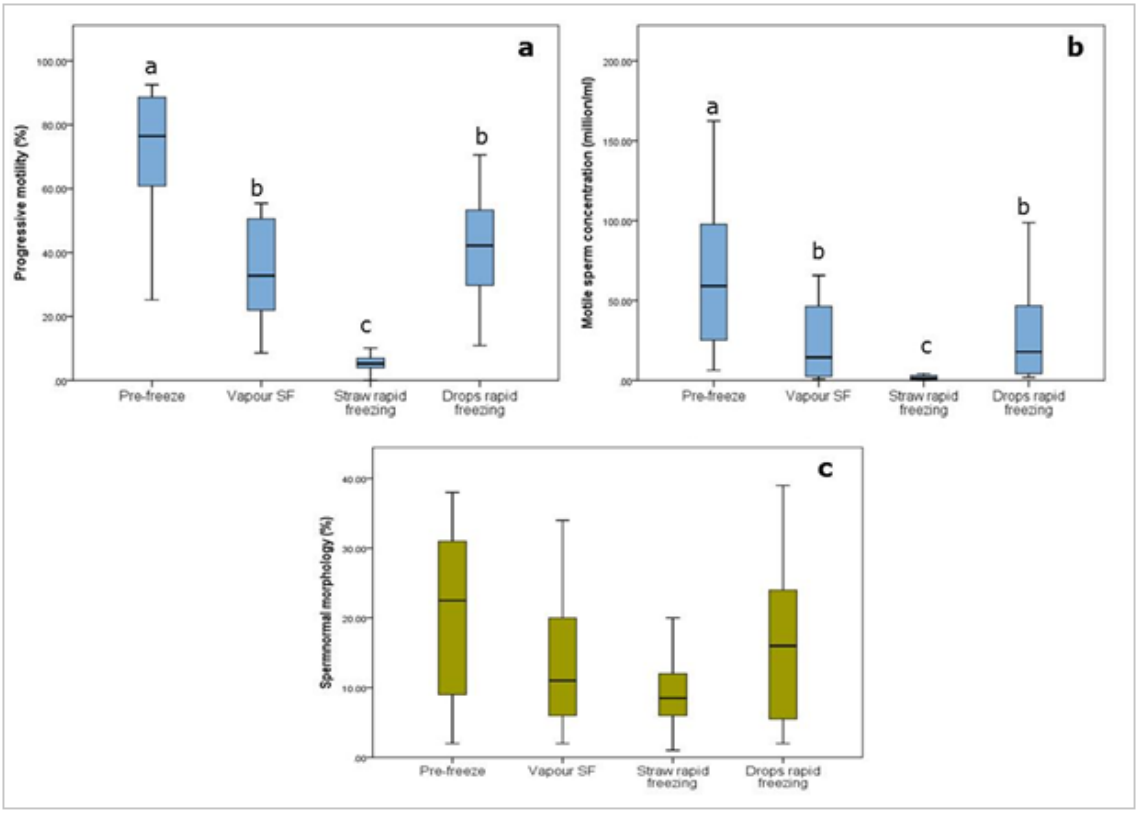

Figure 7: Boxplots showing the progressive motility (a) and the motile sperm count (b) before and after slow and rapid freezing. Different letters represent statistical significance at P<0.001 (Kruskal-Wallis test). The bottom graph (c) illustrates the effect of both slow and rapid freezing on the sperm morphology (P>0.05; Kruskal-Wallis test).

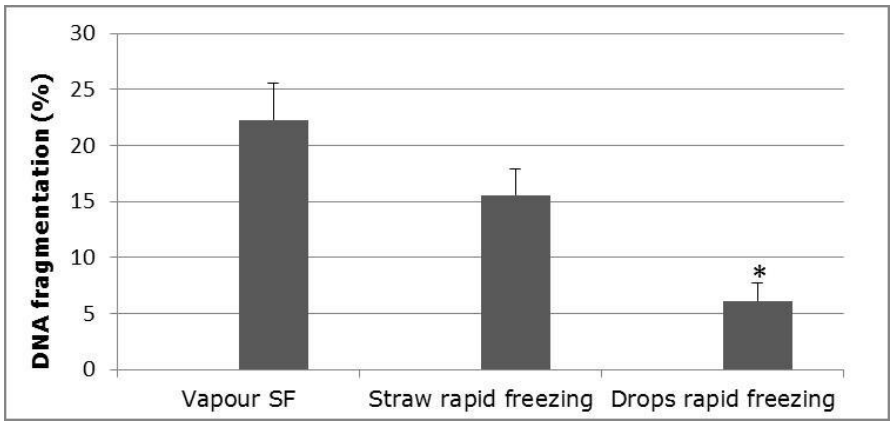

Figure 8: The effect of both slow and rapid freezing on the DNA integrity, expressed as mean±SEM, demonstrating the significant reduction in DNA damage using the drops LN_2 freezing. * P<0.05 (One-way ANOVA).

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Discussion

This study was designed to examine several aspects of sperm cryopreservation protocols in series of comparative experiments and determine whether a robust sperm cryopreservation method could be developed. The clear limitations are associated with the available of samples and size of human ejaculates which restricts the number of parallel comparisons which can be made and as such required a number of smaller discrete experiments.

In general the findings showed that non-penetrative CPA has no merit when used alone and thus glycerol (15%) was adopted as the CPA of choice for the study. Density gradient (DG) separation provided significantly higher post-thaw motile sperm concentration and progression which helped further study and was therefore applied to following experiments. Applying the robust sperm preparation and cryoprotection protocol, ultra-rapid freezing (URF) was attempted using small volumes of sperm suspension-CPA mixture placed directly into either dry ice wells (-75°C) or liquid nitrogen (-196°C). As the experiment showed similar results using either coolant, for reasons of simplicity and availability, the latter was adopted as the method of choice. The hypothesis that URF is effective because it avoids ice formation whilst minimising extended exposure to the damaging effects of osmotic stress and toxicity of CPA would appear to have merit over the controlled slow rate freezing. Despite the commonly observed inter-individual variation, seminal plasma-free sperm and those frozen in seminal fluid recovered similarly. The optimised LN_2 URF protocol for human sperm has resulted in sufficient sperm motility, normal morphology, and improved sperm DNA integrity over the conventional vapour freezing protocol. The use of the validated CASA system gives us confidence that all pre- and post-freezing sperm quality data was measured with a high degree of consistency and reliability.

The current experiments could not agree with recent findings using only sucrose and human serum albumin (HSA) as CPA (Isachenko et al. 2008, Isachenko et al. 2012). This earlier work appeared to provide sufficient surviving sperm for use only with ICSI thus perhaps the study aims were entirely different (Isachenko et al. 2012).

Glycerol was also previously reported to be robust when compared against non-penetrative sugars (Berndtson & Foote 1972) and can also provide significantly improved post-thaw sperm survival with minimum exposure (Berndtson & Foote 1969). More recent examination of glycerol against other types of CPA reported similar findings indicating its superior efficiency with highest motility using extenders containing glycerol (Malo et al. 2012) and that applying sugars as the only CPA is insufficient and results in membrane injury (Gao et al. 1993). Furthermore, using sugars only as CPA cannot be controlled given the fact that the seminal plasma already contains sugar, which differs in its concentrations among individuals and ejaculates. Whether freezing spermatozoa in raw semen or after preparation is a matter of debate (Said et al. 2010, Khalili et al. 2014). The current success of rapidly freezing density gradient (DG) prepared sperm was of considerable interest and mirrors the previous findings for slow cooling (Sharma & Agarwal 1997, Esteves et al. 2000). The reasons for the well documented inter-sample variation, even in ejaculates from the same individual, in terms of post-thaw sperm quality are largely unknown (McGonagle et al. 2002, Tomlinson et al. 2010a). The ejaculate comprises contributions from the testis and accessory glands and varies widely from patient to patient. Constituents of seminal plasma such as lipids, sugars, proteins and the viscosity of the fluid will undoubtedly lead to variation amongst men in terms of the response to CPA addition and cooling and undoubtedly contributes to the variability in sperm survival rates (Mann 1964, Chian 2010). Sperm washing or DG centrifugation potentially removes this variability and provides a more consistent sperm environment for every sample frozen. The presence of cellular particles in the seminal plasma other than spermatozoa can act as a source of ROS known to result in oxidative damage and apoptotic-like action during the cryopreservation process (Counsel et al. 2004, Said et al. 2010, Brugnon et al. 2013, Khalili et al. 2014). This may not fully explain the benefits of DG preparation which may most importantly select a sub-population of sperm that are more able to withstand the ionic and osmotic stresses associated with CPA addition, cooling and thawing.

The damaging mechanism during the freezing process may be mechanical or induced by a physiochemical factor. The physiochemical damage caused by conventional slow freezing on both sides of the sperm membrane is possibly attributable to the alterations in the lipid phase transition during the freeze-thaw process (Alvarez & Storey 1993, Mossad et al. 1994, Isachenko et al. 2004a) resulting in the subsequent reduction in sperm motility (Critser et al. 1987, Mossad et al. 1994). This indicates that the conventional slow freezing might be deleterious to the sperm (Isachenko et al. 2004a). Ultra-rapid freezing of human sperm using direct plunging into LN^2 was previously reported to reduce cryoinjury whilst maintaining important post-thaw physiological characteristics (Zhu et al. 2013). Dry ice cooling also proved as effective, indicating that 'vitrification-like' cooling rates can produce sufficient motility and that by merely reducing processing/cooling time, sperm survival can be improved. The current results are consistent with other recent studies that have also examined different rapid freezing protocols for either small or large sperm numbers and reported successful post-thaw survival of both human (Morris 2006, Jee et al. 2010, Endo

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et al. 2012, Kuznyetsov et al. 2015, Slabbert et al. 2015) and canine spermatozoa (Kim et al. 2012). Moreover, the liquid nitrogen rapid freezing method applies a very high cooling rate that was theoretically calculated to reach up to 720 000°K/minute in the initial phase, that occurs over only a few seconds (Isachenko et al. 2003). This is thought to aid in preventing the nucleation of ice crystals, which if did form is insufficiently large to harm the human spermatozoa (Isachenko et al. 2004a). Such conditions will probably also reduce the possibility of the substantial re-crystallisation that might occur during the thawing process. Therefore, the LN_2 method was adopted due to its ease of use, constant availability, rapidity, simplicity, and with no requirement for special cryobiological equipment.

The current study demonstrated that using the conventional straws as an enclosed carrier system can significantly increase DNA damage. This might be due to its effect on the cooling rate. The preference for pelleting as a method to produce improved post-thaw sample viability was also reported by Tselutin et al. (1999) when testing fowl semen. The impact of the freezing carrier has been also investigated previously and several types of sperm carrying devices used with different cooling methods have been reported including ampoules, straws, and cryovials (AbdelHafez et al. 2009) each with its own benefits and disadvantages. Other systems were aimed for the small numbers of spermatozoa (Desai et al. 1998, Gvakharia & Adamson 2001, Schuster et al. 2003, Desai et al. 2004, Isachenko et al. 2004b, Just et al. 2004, Isachenko et al. 2005, Herrler et al. 2006, Isaev et al. 2007, Sereni et al. 2008). However, developing a robust species specific freezing techniques and optimal carriers might represent a challenge due to the genetic diversity exhibited within certain species (Critser & Mobraoten 2000) and no consensus regarding a robust carrying device has been achieved yet.

Lastly it is important to state that the lack of consensus amongst those studies attempting to optimise sperm freezing quality often fail to address the uncertainty often associated with what is the most important 'end-point' measurement i.e. sperm concentration and motility. Some studies may focus on sperm ultrastructure or DNA integrity, yet importantly there is no better indicator of success than motility. Few studies will use the same well-validated methods for assessing motility or concentration nor provide quality assurance with regard to standardisation of the entire process from specimen collection to reporting of results (Tomlinson 2016). Manual motility is not only highly subjective but insufficiently sensitive to detect sperm cryo-damage which may not necessarily render them immotile but severely sap their energy potential or swimming speed. If any consensus is to be reached, in order to decide on optimum cooling rates, choice and concentration of CPA then future studies must therefore address the reliability of the measured end-point as a matter of priority.

Conclusion

Rapid freezing using either LN_2 appears effective in providing an adequate motile sperm concentration whilst preserving DNA integrity and in particular if sperm are prepared beforehand. This could not be viewed as 'vitrification' since cooling on dry ice was equally as effective. The eventual application is unclear although the rapid cryopreservation of large numbers of concentrated sperm pellets may be an advantage in patients with poor sperm quality or where freezer capacity is at a premium. However until a suitable closed-system can be developed rapid freezing could not be viewed as a replacement for current standard procedures.

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Conflict of interest

Dr. Mathew Tomlinson is a Director/Owner of Procreative diagnostics, a new company established to develop and market the Sperminator® an automated system for measuring sperm concentration and motility.

Author's contribution

All authors have contributed in this work. Aljaser FS has performed the research, analysed the data, and wrote the manuscript. Almajed FS has reviewed and directed the data analysis and figures. Tomlinson MJ has reviewed the English grammar and syntax of the manuscript. Both Campbell BK and Tomlinson MJ contributed in the scientific responsibility in addition to directing the research.

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