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Comparison between Sperm Preparation before Cryopreservation and Post-Thawing Sperm Preparation using Density Gradient Centrifugation in Normozoospermic Semen Samples

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Abstract

Sperm cryopreservation has been considered as an efficient procedure for management of male fertility. Different freezing protocols have been developed to maximize the post freeze quality. The current study investigates the differences between sperm preparation before and after freeze. 120 Semen analysis reports from 18 sperm donors were analysed. 60 semen analysis reports were from samples that were frozen then washed (Group 1: Post-wash) while the other 60 semen analysis reports were obtained from samples that were washed then frozen (Group 2: Pre-wash). Sperm concentration and progressive motility were evaluated for each group and compared. Sperm concentration was higher in the pre-wash group compared to post-wash (33.05 ±12.9 vs 26.13 ±13.36, respectively). Progressive motility was higher in the post-wash group (82.3 ±14.9 vs 51.6 ±8.2). There was a significant decrease in sperm concentration between fresh samples and both (post- and pre-wash) results. Significant decreases in progressive motility were found in both methods. Sperm freeze using fresh semen samples and washed samples resulted in significant decrease in post freeze progressive motility. However, fresh samples had a higher post freeze recovery rate compared to washed samples (72.15% vs 56.32%). Semen preparation by density gradient centrifugation before freezing resulted in better sperm concentration, while the preparation of spermatozoa after freezing had a higher progressive motility. Therefore, sperm freezing methods should be adapted according to semen samples quality.

Keywords: Concentration, Cryopreservation, Freezing, Progressive motility, Sperm

Introduction

Cryopreservation permits long-term storage of semen and, when cryopreserved correctly, the sperm maintains a state of metabolic arrest that prevents cellular ageing and retains the viability and fertilising ability of the sperm for an essentially unlimited period (1). Cryopreservation of human spermatozoa is a highly efficient procedure for managing male fertility, and much of its successful application seems to have a crucial impact on the reproductive outcome of assisted reproduction technologies (2). Despite the success of human sperm cryopreservation, this procedure still induces sperm cryoinjuries (3, 4). During cryopreservation, the dramatic physical and environmental changes of sperm detrimentally affect the sperm quality. This may result in alterations in sperm membrane integrity and fluidity (5, 6, 7), decrease in metabolic activity of sperm mitochondria (8), consequently leading to a decrease sperm motility (9, 10), normal morphology (11, 12, 8). Many different standardized commercially available cryopreservation media have been developed to optimize the outcome of sperm freezing (13, 14, 15, 8). Furthermore, different freezing protocols have been developed to provide a high sperm recovery rate (16, 17, 18, 19). Another approach to optimize sperm quality consists of preparing semen samples before freezing. For sperm cryopreservation, pre-frozen sperm quality baseline, has been previously demonstrated to be the crucial factors relating to post-thawing sperm outcome (20, 21, 22). Sperm preparation using the swim-up method before freezing improves sperm motility and reduces apoptosis in post-freezing-thawing sperm compared with post-thawing sperm preparation using the swim-up (22). However, a more recent study has found that sperm preparation using the swim-up method after freezing improves sperm count, motility and viability in frozen-thawed sperm compared with sperm preparation before freezing (23). Data on sperm preparation before or after freezing using density gradient centrifugation is limited. Density gradient centrifugation before cryopreservation and hypotaurine supplementation were found to improve post-thaw quality of sperm from infertile men with oligoasthenoteratozoospermia (21). It was previously found that semen quality is better preserved in fresh and cryopreserved semen prepared with density gradient compared to swim-up (24).

The aim of this study is to compare sperm preparation before cryopreservation and post-thawing sperm preparation using density gradient centrifugation in normozoospermic semen samples.

Materials and methodologies

Study population

This was a retrospective cohort study evaluating semen analysis reports (n=120) of qualified sperm bank donors recorded at Androcryos Andrology Laboratory & Sperm Bank, Parktown, from January 2022 to February 2022. The current study was approved by the general manager of Androcryos Andrology Laboratory & Sperm Bank. All the participants were normozoospermic healthy sperm bank donors, living in Johannesburg. Semen samples were processed following the requirements stipulated by the World Health Organization

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guidelines (WHO, 2010). Sperm morphology was in accordance with the Tygerberg Strict criteria for sperm morphology (WHO, 2010; Menkveld, 2013).

Sperm concentration and motility evaluation

The evaluation of sperm concentration was performed by transferring a drop of the immobilized well-mixed specimen in the centre of the Makler counting chamber and covered with the special cover glass. Then, the cover glass was grasped with a finger opposite the black dots allowing the drop to spread on the entire area of the disc into a thickness of 10 µm. The chamber was then lifted by its handles and placed on the stage of the microscope. The sperm count was evaluated using Primo Star microscope (Zeiss) at X20 objective and X10 eyepiece. Sperm motility parameter was assessed by dropping approximately 10 µl of semen sample on a warm, unfrosted slide (Labstar Plain, Lot no.: 09152018, Lasec, Johannesburg, South Africa) using the non-pyrogenic serological pipette. Then, the slide was covered with a cover slip and allowed to stand for a few seconds before being evaluated using the Primo Star, phase contrast microscope (Zeiss, Carl Zeiss, Göttingen, Germany, 195-42826) at 20X objective, phase 2, according to the WHO 2010 Manual criteria. An estimation of the total percentage of motile spermatozoa per field was determined.

Sperm cryopreservation

Cryopreservation was performed using the single step freezing medium SpermFreeze™ (FertiPro, Beemem, Belgium) and following the manufacturer guidelines. SpermFreeze™ requires 0.7mL of medium for each millilitre of semen. Semen samples were frozen in vaporous nitrogen for 15 minutes then in liquid nitrogen (-196 degrees C) for 24 hours.

Sperm washing

The density gradient centrifugation (DGC) sperm wash was performed. The DGC technique by means of 80% PureSperm® (Nidacon, cat no: PS80-100), 40%PureSperm® (Nidacon, cat no: PS40-100) and PureSperm Wash (PSW, cat no: PSW-100), was used according to the manufacturer's guidelines. Semen samples were divided into two groups: Group 1, consisted of post-washed spermatozoa (washed after freeze) while Group 2 (pre-washed) were spermatozoa treated before freezing (See Figure 1). Sperm concentration and progressive motility were determined.

Statistical analyses

Statistical analysis was performed using the MedCalc® statistical software version 19.5 (MedCalc Software Ltd, Ostend, Belgium; https:www.medcalc.org; 2020). Descriptive statistics for variables were presented as mean ± SD. The normality of data was evaluated using the Chi-squared test. The t-test was used to evaluate the statistical differences between groups. For all statistical tests, a P-value of < 0.05 was considered statistically significant.

Results

One hundred and twenty semen samples were included in the study. Sixty semen samples for Group 1 and sixty semen samples for Group 2. The average age for Group 1 and Group 2 were 31 ±5.03 and 33 ±2.3, respectively.

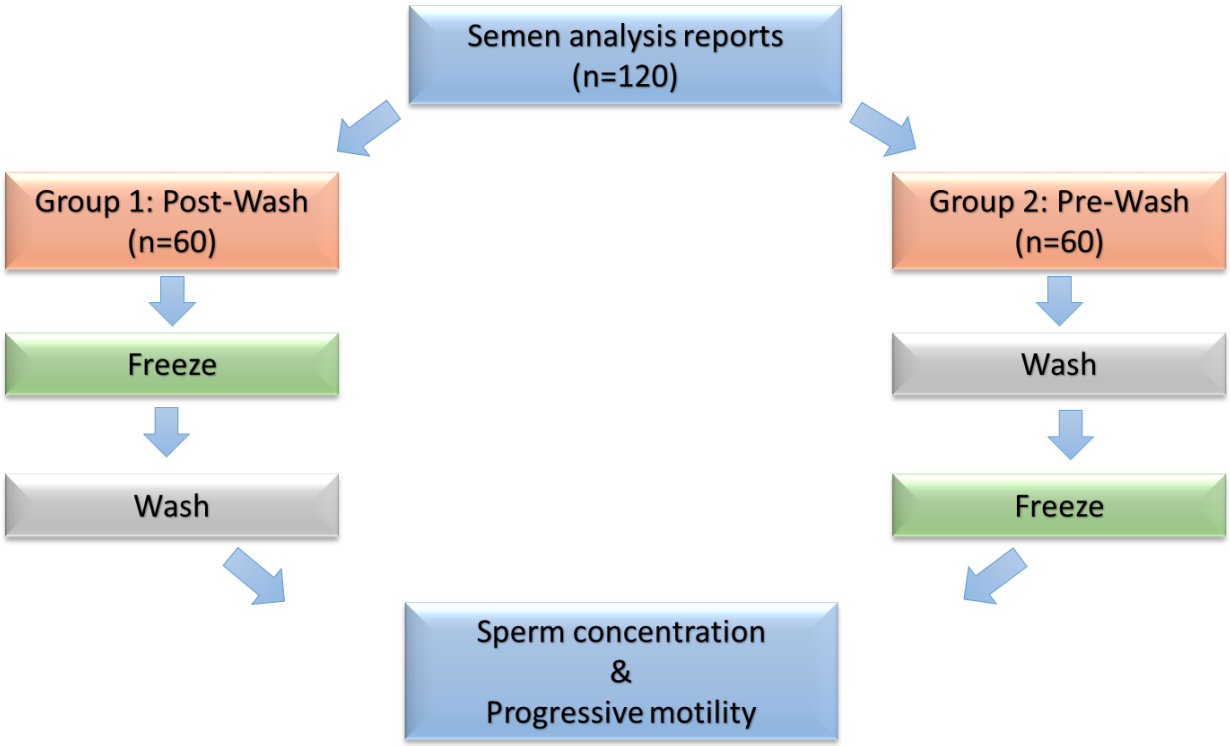

Figure 1: Diagrammatic illustration of the comparison between washed and unwashed pre and post freeze semen samples

Summary statistics for sperm concentrations (fresh, after freeze and after wash) and progressive motility (fresh, after freeze and after wash) for both evaluated groups are shown in Table 1. Fresh sperm concentration for Group 1 and Group 2 were 105.83 ±39.4 and 93.48 ±49, respectively. Table 2 summarizes the differences in progressive motility and sperm

concentration of post and pre-wash spermatozoa. The post-wash group had a higher progressive motility (82.3 ±14.9) and lower sperm concentration (26.13 ±13.36) compared to the pre-wash group (51.6 ±8.2 and 33.05 ±12.9, respectively). Although, the differences were not significant. There were no significant differences in progressive motility between fresh semen samples and post-washed spermatozoa (Group 1) and between fresh semen samples and pre-wash (Group 2) (Table 3 and Table 4). However, significant differences ($p<0.05$) in sperm concentration were observed in both post- and pre-washed methods.

Table 1: Summary statistics of the age and evaluated semen parameters

	Group 1 (post-wash)	Group 2 (pre-wash)
Age	31 ±5.03	33 ±2.3
Concentration (Fresh)	105.83 ±39.4	93.48 ±49
Concentration (After Freeze)	56.08 ±21.7	33.05 ±12.9
Concentration (After Wash)	26.13 ±13.36	59.66 ±20
Progressive motility (Fresh)	65 ±11	63.5 ±7.8
Progressive motility (After Wash)	82.3 ±14.9	91.61 ±11.48
Progressive motility (After Freeze)	46.9 ±9.08	51.6 ±8.2

Table 2: Comparison between post-wash (Group 1) and pre-wash (Group 2) progressive motility and sperm concentration

	Post-wash (Group 1)	Pre-wash (Group 2)	p-value
Progressive motility	82.3 ±14.9	51.6 ±8.2	0.075
Sperm concentration	26.13 ±13.36	33.05 ±12.9	0.084

Table 3: Comparison between fresh semen parameters and post-wash (Group 1)

	Fresh semen sample	Post-wash (Group 1)	p-value
Progressive motility	65 ±11	82.3 ±14.9	0.072
Sperm concentration	105.83 ±39.4	26.13 ±13.36	0.025

Table 4: Comparison between fresh semen parameters and post-freeze (Group 2)

	Fresh semen sample	Pre-wash (Group 2)	p-value
Progressive motility	63.5 ±7.8	51.6 ±8.2	0.072
Sperm concentration	93.48 ±49	33.05 ±12.9	0.034

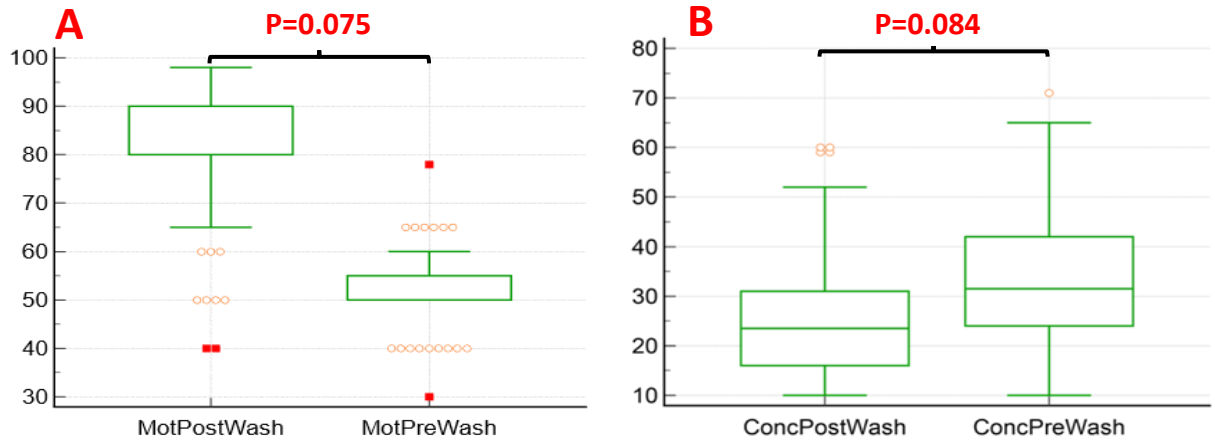

Figure 2: Graphical illustration of the differences between post- and pre-wash progressive motility (A) and post- and pre-wash concentration (B)

Table 5: Comparison between fresh semen samples and post-freeze motility (Group 1) and between washed samples and post freeze motility (Group 2)

	Fresh samples	Post-freeze(Group 1)	Recovery rate	p-value
Prog. motility	65 ±11	46.9 ±9.08	72.15%	P =0.036
	Washed samples	**Post-freeze(Group 2)**	**Recovery rate**	**p-value**
Prog. motility	91.61 ±11.48	51.6 ±8.2	56.32%	<0.005

Table 5 highlights the difference in progressive motility in both fresh and washed semen samples. Significant ($p<0.05$) decreases in progressive motility were found in both methods. Sperm freeze using fresh semen samples had a higher recovery rate compared to using washed samples (72.15% vs 56.32%).

Discussion

The current study evaluated the differences between preparing (washing) spermatozoa after freezing and washing spermatozoa before freezing. Several studies have investigated the effect of freezing on semen quality by comparing pre- and post-freeze sperm parameters (8, 20, 21, 25, 26). Furthermore, following the increasing need to optimize sperm recovery after cryopreservation, previous studies have compared sperm selection or washing before and after freezing (20, 22, 23, 27). However, most of the studies focus on the swim-up method as a sperm selection method (20, 22, 23). The current study compared sperm concentration and progressive motility evaluated from pre- and post-washed cryopreserved semen samples, using density gradient centrifugation method for sperm preparation.

The results demonstrate that sperm preparation after cryopreservation improves progressive compared to sperm preparation before freezing. Similar results were reported previously by Donnelly et al. (2000), Esteves et al. (2000) and Palomar et al. (2018) (20, 23, 28), who found that sperm preparation method after freezing resulted in higher sperm motility. However, the results observed in the current study differ from previous findings by Petyim et al. (2014) who reported higher progressive motility percentages when cryopreservation was performed after sperm preparation.

The increase motility observed can be due to the protective action of seminal plasma during cryopreservation. Cryopreservation can induce sperm DNA damage (11) mediated by oxidative stress (4). A negative correlation between sperm DNA Fragmentation and sperm motility was previously reported (29, 30). The seminal plasma biochemical composition consists of different antioxidant systems which protect sperm from reactive oxygen species and DNA damages produced by cryopreservation (31, 32).

It was previously demonstrated that sperm preparation before freezing resulted in higher sperm concentration compared to sperm preparation after freezing (22). Similar results were obtained in the current study. Cryopreservation can adversely affect apoptotic markers such as DNA fragmentation and caspase-3 activation (33). Sperm selection procedures can eliminate sperm with fragmentated DNA (34, 35). Consequently, preparing spermatozoa before freezing, increase the number of sperm that will survive the apoptotic mechanism which occurs during cryopreservation.

Several sperm freezing protocols have been developed to prevent damage to the sperm cells and to maximize sperm recovery rate (2, 36-41). Some studies compared vapor freezing and freezing in liquid nitrogen (38-42) while other investigated the effects of cryoprotectants on sperm recovery (37, 43, 44) or the use of vitrification as a possible advanced cost-effective method for the storage of human spermatozoa (45-49). The current study suggested that short freezing of spermatozoa in vapor nitrogen followed by a 24h freezing in liquid nitrogen does not significantly decrease post-freeze sperm progressive motility and concentration.

The comparison between freezing of washed vs unwashed semen samples is discussed in the literature (50-52). Washed semen samples using density gradient centrifugation were found to have better sperm parameters than unwashed groups (52). However, older studies found that the recovery rates for sperm motility were higher for unwashed samples compared with washed semen samples (50). This is consistent with the results obtained in the current study. Although, freezing of both unwashed and washed semen samples resulted in significant decreases in sperm motility, freezing of unwashed samples has a higher recovery rate.

Conclusion

Sperm wash by density gradient centrifugation after freeze results in higher progressive motility while semen samples prepared by density gradient centrifugation before freeze had a higher sperm concentration after freeze. Although the differences between the two methods were not significant. Furthermore, cryopreservation of unwashed semen had a higher recovery rate in term of progressive motility. This study reinforces the protective role of seminal plasma during cryopreservation. The current study suggests that sperm freezing methods should be adapted according to semen samples quality. For instance, for asthenozoospermic samples, washing after freeze might yield higher number of motile spermatozoa. The current study only focuses on normozoospermic samples, consequently, could not evaluate the differences between other semen parameters categories. Additionally, the current study is limited to sperm concentration and progressive motility. This represents limitations of this study. Further investigations are required to support the current findings.

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Conflicts of interest

The author declare that they have no conflict of interest.

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