Human semen cryopreservation reduces the seminal antioxidant reservoir

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ABSTRACT

Background The effect of cryopreservation on particular antioxidants in human semen has been examined in a number of published studies. However, the effect of cryopreservation on total antioxidant capacity of human semen has not yet been investigated.

Aim: To study how total antioxidant capacity of human semen samples, from males of couples who failed to achieve a clinical pregnancy after at least one year, is affected by cryopreservation.

Methods: Semen samples (n = 77), with and without the addition of cryoprotectant medium, were cryopreserved for at least 72 hr and tested for total antioxidant capacity before and after cryopreservation using the cupric ion reducing antioxidant capacity method.

Results: Cryopreservation of human semen samples significantly decreased (P < 0.05) their total antioxidant capacity levels. In addition, cryopreservation of the cryoprotectant medium decreased its total antioxidant capacity. No significant correlation was found between seminal total antioxidant capacity before cryopreservation and the decrease in seminal cryopreservation after cryopreservation, and male age.

Conclusions: Cryopreservation of human semen reduces the seminal total antioxidant reservoir, and this reduction was found not to correlate with age. Further studies are required to standardise the level of total antioxidant capacity for uppermost sperm quality after cryopreservation.

Key words: Cryopreservation; human semen; sperm; total antioxidant capacity.

INTRODUCTION

Human sperm cryopreservation, cooling of sperm to sub-zero temperatures (~196 °C), is a viable option for fertility preservation of patients diagnosed with cancer or requiring orchidectomy prior to undergoing medical intervention (1,2). Moreover, sperm cryopreservation is crucial in certain cases with poor semen quality (i.e., testicular failure) where assisted reproductive technologies, such as in vitro fertilization, are desired (3).

In men, the ejaculated semen is well endowed with various antioxidants in order to resist possible oxidative injury to sperm. This oxidative damage is found to be handled primarily by reactive oxygen species such as hydroxyl radical, superoxide ion, and hydrogen peroxide (4). The imbalance between reactive oxygen species and antioxidants to the favor of reactive oxygen species leads to oxidative stress (5), which in turn causes sperm injury or death (6). Therefore, an effective antioxidant defense mechanism surrounding the sperm appears indispensable for adequate sperm function.

The antioxidant mechanism surrounding human sperm encompasses enzymatic and non-enzymatic antioxidants (7,8). The enzymatic mechanism includes antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and catalase (8,9). While the non-enzymatic mechanism typically includes antioxidant molecules such as ascorbic acid, glutathione, α-tocopherol, L-carnitine, urate, ubiquinol, pyruvate, taurine, and carotenes (7,8,10,11).

The effect of cryopreservation on some antioxidants in human semen has been documented in a number of published studies. For example, cryopreservation of human semen was found to reduce the antioxidant activity of L-carnitine (12,13). Another study by Gadea et al. demonstrated a significant reduction in glutathione content after human semen cryopreservation (14).

Moreover, a significant reduction in the activity of superoxide dismutase was noted after cryopreservation of human semen (15). Although the evidence above shows the effect of cryopreservation on certain antioxidants in human semen, the study that shows the effect of cryopreservation on seminal total antioxidant capacity has not yet been done to our knowledge. We hypothesized that cryopreservation decreases the level of total antioxidant capacity of human semen, and this decrease is correlated with age. In this study we investigated the level of reduction in seminal total antioxidant capacity upon semen cryopreservation -thawing, compared this reduction with the one by the cryoprotectant medium alone, and correlated this reduction with age. We performed this study on patients who attended the andrology laboratory as their samples are the target of the cryopreservation.

METHODS

Subjects and sample collection

Semen samples were randomly collected over a 9-month period in 2014 from 75 males who attended the andrology laboratory at the King Abdullah University Hospital in the north of Jordan. All enrolled males failed with their spouses to achieve a clinical pregnancy after one year or more of regular unprotected intercourse. Semen samples were collected by masturbation after 72 hours of sexual abstinence and analysed according to the guidelines of the World Health Organization (2010). The study was explained to all of the enrolled subjects by the researchers, and written informed consent was obtained prior to enrollment. Approval for the study was provided by the IRB Committee at Jordan University of Science and Technology, Irbid, Jordan.

Experimental design

Each fresh semen specimen (2-200 x 10⁶ sperm mL⁻¹) was gently homogenised using a 1 mL pipette and divided into 2
aliquots (A and B) with equal volumes. Aliquot A was further divided into 2 aliquots, the first was tested directly for total antioxidant capacity, while the second was cryopreserved in liquid nitrogen. Aliquot B was mixed with the cryoprotectant medium (Irvine Scientific, Santa Ana, CA, USA) and similarly divided into 2 aliquots; the first was tested for total antioxidant capacity while the second was cryopreserved in liquid nitrogen. After at least 72 hours, the cryopreserved aliquots were thawed at room temperature (25°C) and analysed for total antioxidant capacity. Independently, the total antioxidant capacity was measured for the cryoprotectant medium alone, before, and after cryopreservation.

Cryopreservation
An aliquot of the cryoprotectant medium was added to the specimen and gently mixed for 5 min. This step was repeated to give a final 1:1 (vol/vol) ratio of the cryoprotectant medium to the semen sample. Cryovials containing the specimen were placed in a freezer at -20 °C for 8 min and in liquid nitrogen vapor at -80 °C for 2 hours (13). The vials were transferred to the liquid nitrogen at -196 °C for at least 72 hours, and then they were removed and thawed at 25 °C for 20 min. The average sperm recovery for all tested samples was 47.8 ± 16.8% (mean ± SD).

Measurement of total antioxidant capacity
Each tested aliquot, before and after cryopreservation, was centrifuged at 300 g for 5 min and the supernatant (cell-free seminal plasma) was tested for total antioxidant capacity using the cupric ion reducing antioxidant capacity (CUPRAC) method. This method was chosen to measure the total antioxidant capacity in human semen because of its reliability, sensitivity, and suitability for biological fluids (16,17). Briefly, 1 mL of working solution (0.02 M CuCl2 solution), 0.0075 M neocuproine alcoholic solution, and 1 mL ammonium acetate buffer solution at 1:1:1 (vol/vol/vol) were mixed in a centrifugation tube with 12.5 μL of seminal plasma. All reaction tubes were centrifuged for 3 min at 750g. Carefully, the supernatant (cell-free medium) from each centrifuged sample was collected, and the absorbance of the formed colored complex was measured against the reagent blank at 450nm after 30 min incubation at room temperature.

Statistical analyses
Results are reported as the means ± SEM. Statistical analysis was performed using the Student’s paired t-test to compare the means of the 2 groups. One-way analysis of variance (ANOVA) was used when more than two groups were compared (GraphPad Prism 5.01 computer software; GraphPad Software, Inc., CA, USA). Analyses of relationships between variables were performed using Spearman’s nonparametric correlation analysis. Statistical significance was set at the p < 0.05 level.

RESULTS
Figure 1 illustrates the effect of cryopreservation on the total antioxidant capacity of human semen as evaluated by the CUPRAC method. The absorbance at 450nm is proportional to the total antioxidant capacity of semen. There was a significant decrease (P < 0.0001) in the absorbance at 450nm, and thus in total antioxidant capacity, using the CUPRAC method after semen cryopreservation with and without cryoprotectant medium supplementation (0.650 ± 0.028 vs. 0.579 ± 0.025, and 1.070 ± 0.025 vs. 0.980 ± 0.028, respectively; n = 77).

Figure 2 illustrates the correlation between the absorbance at 450nm, and between the total antioxidant capacity of human semen as evaluated by the CUPRAC method and the patient’s age. As illustrated in this figure, the total antioxidant capacity of human semen decreased slightly with age, but this was not statistically significant.

Figure 3 illustrates the correlation between the reduction in the absorbance at 450nm, and thus in the semen total antioxidant capacity, as evaluated by the CUPRAC method after cryopreservation (without cryoprotectant medium supplementation) and the patient’s age. As illustrated in this figure, the reduction in semen total antioxidant capacity after cryopreservation versus patient’s age was not statistically significant.

**Figure 1.** Effect of cryopreservation on TAC of human semen with and without cryoprotectant medium supplementation as evaluated by the CUPRAC method. The absorbance at 450nm is proportional to the total antioxidant capacity. Values are given as the means ± S.E.M (n = 77; P < 0.0001).

**Figure 2.** Correlation between age and seminal total antioxidant capacity. The absorbance at 450nm is proportional to the total antioxidant capacity (n = 77).
absorbance at 450nm after cryopreservation is proportional to cryoprotectant medium supplementation. The reduction in absorbance at 450nm after cryopreservation is proportional to the reduction in total antioxidant capacity (n = 77).

DISCUSSION

Although many studies have examined the multifaceted effects of cryopreservation on different antioxidant parameters of semen, to the best of our knowledge, this is the first study to test directly the effect of cryopreservation on total antioxidant capacity of human semen from infertile men. We hypothesized that cryopreservation of human semen decreases the level of seminal total antioxidant capacity. The results from this study are in concordance with this assumption by demonstrating that cryopreservation of human semen samples significantly decreases the level of their total antioxidant capacity.

Cryopreservation has been found to induce the formation of reactive oxygen species in human semen samples (18). Studies show that sperm cryopreservation-thawing leads to alterations in NADPH oxidase in the plasma membrane and to alterations in the electron transport chain of the mitochondria, which enhance the generation of reactive oxygen species (19,20). The generated reactive oxygen species in the cryopreserved semen could be behind the decrease in seminal total antioxidant capacity. Besides, the accumulation of reactive oxygen species leads to oxidative stress, an imbalance between oxidants and antioxidants, and lipid peroxidation, and thus to a reduction in the post-thaw recovery of sperm (1,2).

Alternatively, in cryopreservation, due to removal and dilution of seminal plasma, sperm experience a substantial reduction in the antioxidant reservoir (i.e., ascorbate, urate, alpha tocopherol, hypothaurin, taurin, carotenoids, and glutathione). Such weakening in the antioxidant defense mechanism surrounding the sperm increases the chance of exposing sperm membranes to oxidative injury. In fact, the plasma membrane of human sperm contains considerable amounts of polyunsaturated fatty acids that can easily be oxidised by reactive oxygen species (21).

The evidence above explains why manufactured cryoprotectant media are supplemented with antioxidants. However, the amount of these antioxidants, and thus the total antioxidant capacity in the cryoprotectant medium is not properly standardized for premium sperm recovery after cryopreservation. For example, in this study, the absorbance at 450nm utilising seminal plasma after cryopreservation was 0.65, and the absorbance at 450nm for seminal plasma supplemented with cryoprotectant medium after cryopreservation was 0.98; indicating a 69% increase in the absorbance, and thus in the total antioxidant capacity reservoir surrounding the sperm. Such increases should be standardised to obtain the uppermost sperm recovery.

In fact, standardising the increase in total antioxidant capacity reservoir surrounding the human sperm after cryopreservation is very important because antioxidants, in general, are a double-edged sword with opposing effects if the safety threshold dose is exceeded. For example, an in-vitro study on human sperm by Donnelly et al. demonstrated that higher concentrations (> 20 μM) of vitamin C, a key antioxidant in human semen, has a negative effects on sperm quality, especially sperm motility (22). Another in-vitro study by Banhani et al. showed that higher doses (50 mM) of L-carnitine, a potent antioxidant present in human semen, significantly decreased human sperm motility and viability (11). Therefore, a random (i.e., unstandardised) increase in the antioxidant activity surrounding the sperm after cryopreservation may not provide the desired sperm recovery.

Independently, in this study, we investigated the correlation between age and the level of seminal total antioxidant capacity (before and after cryopreservation). A previous study conducted on infertile men (n = 52) aged between 21-52 found a negative correlation between seminal total antioxidants and age (23). The results from this study did not show a significant decrease in total antioxidant capacity versus age. The contradiction between both studies may due to the method of assaying the seminal total antioxidants and the size of the population involved (18-70 yr vs. 21-52 yr). Furthermore, our study for the first time investigated the correlation between the decrease in total antioxidants by the effect of cryopreservation and age. The results here did not show a significant correlation between these two variables.

CONCLUSIONS

In summary, the results from this study suggests that cryopreservation of human semen reduces the effectiveness of the antioxidant defense mechanism surrounding the sperm, and this reduction is not associated with age. Further studies are required to optimise the seminal total antioxidant capacity after cryopreservation to reach the uppermost sperm quality. This optimisation should be primarily achieved by standardising the amount of antioxidants that are added to the cryoprotectant media before cryopreservation.

ACKNOWLEDGMENTS

This study was supported by the Deanship of Research at Jordan University of Science and Technology; grant number 20130097. The authors declare no conflicts of interest.

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