	A N D	1 3 1 0	B	Dispatch: 26.4.12	Journal: AND	CE: Roopa Vardhini G.
	Journal Name	Manuscript No.		Author Received:	No. of pages: 6	PE: Suganya

SHORT COMMUNICATION

Catalase improves motility, vitality and DNA integrity of cryopreserved human spermatozoa

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Keywords
Antioxidant—DNA damage—swim-up

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Accepted: March 20, 2012

doi: 10.1111/j.1439-0272.2012.01310.x

Summary

Cryopreservation of human spermatozoa offers a pre-therapeutic possibility of preserving progenity in patients with testicular tumours. We aimed to investigate effects of cryopreservation and addition of catalase on sperm motility, vitality and DNA integrity in fresh and swim-up spermatozoa. Semen samples were collected from 50 fertile men. Each sample was divided into two parts. First part was subdivided into two equal aliquots: both cryopreserved with and without catalase. The second part was subdivided into two equal aliquots: both processed by swim up and then cryopreserved with or without catalase. Semen analyses, sperm vitality and sperm DNA integrity were performed. Sperm concentration showed significant decrease while percentage of progressive motility, sperm vitality and % of DNA damage showed significant increase in processed and cryopreserved processed samples when compared with fresh and cryopreserved fresh samples. There was no significant difference in sperm concentration while there was significant increase in % of progressive motility and sperm vitality and % of DNA damage showed significant decrease in samples with catalase when compared with samples without catalase (either fresh or processed). Catalase supplementation (fresh and processed) during cryopreservation results in better post-thawing percentage of progressive motility and percentage of sperm vitality and improved DNA integrity.

Introduction

Cryopreservation of spermatozoa is proven to be the only effective method to circumvent the sterilising effect of cytotoxic therapy in patients with malignant diseases (Neal *et al.*, 2007). Nevertheless, the cryopreservation process can lead to structural and functional alterations in spermatozoa, impairing fertilisation potential. And there is still no effective method available for preventing this cryodamage (Medeiros *et al.*, 2002).

Reactive oxygen species has been suggested as a major contributing factor for cryodamage to spermatozoa (Anger *et al.*, 2003). Accordingly, a variety of cryoprotective media, mostly supplemented with antioxidants, have been employed in an attempt to overcome cryodamage (Yoshimoto *et al.*, 2008). Antioxidant supplementation has been shown to yield significantly improved quality of cryopreserved spermatozoa (Grossfeld *et al.*, 2008). Sperm DNA is being recognised as an independent measure of sperm quality that may have better diagnostic and prognostic capabilities than standard sperm parameters (Erenpreiss *et al.*, 2006).

To date, there are some contrasting results about the value of antioxidants in sperm protection against oxidative stress. Some studies revealed that *in vitro* antioxidant supplementation can protect against oxidative damage of the sperm DNA. In support, sperm processing with removal of seminal plasma (rich in antioxidants) results in injury to the sperm DNA (Twigg *et al.*, 1998a). Ben Abdallah *et al.* (2011) reported that dimethoate (pesticides) caused a significant induction of oxidative damage in spermatozoa and a significant decrease in sperm mobility, viability and activities of catalase and addition of vitamins reduced the damage probably due to its strong antioxidant property. In contrast, other studies reported that antioxidants (e.g. vitamin C and, catalase) have limited value in protecting sperm DNA against ROS production (Taylor *et al.*, 2009). Therefore, this study was conducted to evaluate the effects of cryopreservation, processing (swim-up technique) and catalase supplementation (antioxidant) on the semen

parameters (motility, vitality and DNA integrity, that is, percentage of DNA damage).

Subjects and methods

Reagents

Swelling solution

Dissolve 0.735 g sodium citrates dehydrate ($Na_3C_6H_5O_7{\cdot}2H_2O$) and 1.351 g fructose in 100 ml distilled water. Store aliquots of this solution frozen at −20 °C and then thaw and mix well before use. Catalase was purchased from Sigma-Aldrich. SpermFreeze is a HEPES-buffered freezing medium for use with human spermatozoa; it contains 15% glycerol and o.4% human serum albumin to protect the spermatozoa from damage due to the freezing procedure manufactured by FertiPro, Belgium, Lot. FP05S). *Coulter DNA PREP* Reagents Kit, PN 6607055 (100 tests): DNA PREP LPR contains <0.1% potassium cyanide, <0.1% NaN_3, nonionic detergents, saline and stabilizers. DNA PREP Stain contains 50 μg ml^{-1} propidium iodide (PI; <0.5% PI), RNAse [Type III-A, Bovine Pancrease (4 KU ml^{-1})], <0.1% NaN_3, saline and stabilizers. Kit was supplied by Coulter (DNA Prep; Beckman Coulter Fulterton, CA, USA).

Semen preparation and analysis

The semen samples were obtained by masturbation after at least 3 days of sexual abstinence and collected into sterile containers from 50 fertile men (within the last year), who are clinically free and with normal parameters and were recruited from the outpatient clinic of the Department of Dermatology and Andrology, Assiut university hospital from January 2009 to January 2010. All the participants were nonsmokers, and their age ranged between 18 and 54 year with average of 37.94 ± 10.35. Twenty-six participants were farmers while the remaining had administrative work. Samples were left to liquefy for 30 min at 37 °C and were analysed within an hour. Semen analysis was carried out according to the criteria mentioned in the World Health Organization manual (1999), with volumes $\geq$2.0 ml, normal viscosity, normal sperm count, viability $\geq$60%, grade a motility $\geq$25% or grade a and b motility $\geq$50%, and leucocytes $\leq 1 \times 10^6$ ml^{-1}. The experimental design was approved by the Ethics Committee at the Faculty of Medicine, Assuit University. Informed consents were obtained from all the participants.

Study design

Each semen sample was equally divided into two parts. The first part of the sample was further divided into two equal aliquots. Catalase (200 u ml^{-1}) was added to one aliquot during cryopreservation. The second aliquot was cryopreserved without catalase treatment. The second part of the sample was processed by swim-up technique. The processed sample was divided into two equal aliquots. The first aliquot was cryopreserved with addition of catalase (200 u ml^{-1}). The second aliquot was cryopreserved without addition of catalase. The antioxidant concentrations in this study were chosen on the basis of published literature (Roca *et al.*, 2005). Cryopreservation in liquid nitrogen for at least 24 h for all samples (fresh and processed) was performed.

Hypo-osmotic swelling (HOS) test

Warm 1 ml swelling solution in a closed Eppendorf tube at 37 °C for about 5 min. Add 0.1 ml liquefied semen and mix gently with the pipette. Keep at 37 °C for at least 30 min (but not longer than 120 min) and examine the sperm cells with a phase-contrast microscope. Swelling of spermatozoa is identified as changes in the shape of the tail. Count in duplicate the number of swollen cells in a total of 200 spermatozoa counted and calculate the mean percentage (WHO, 1999).

Steps of swim-up technique

Ham's F10 (1.5 ml) is gently layered over semen (1 ml) in a sterile 15-ml conical based centrifuge tube. The tube is inclined at angle of 45 and incubated for 1 h at 37 °C. The tube is then gently returned to the upright position and uppermost 1 ml removed, and 0.5 ml of Ham's F10 medium was added for assessment of sperm quality before using it.

Sperm cryopreservation

According to the manufacturer's instructions

Freezing: One millilitre of liquefied semen mixed with 0.7 ml of SpermFreeze. The mixture was left for 10 min at room temperature for equilibration. The sample/medium mixture sucked into the freezing straws. Shaking was carried out to remove the air bubble to the centre of the straw. The straws were frozen vertically for 15 min, just above the level of the liquid nitrogen. Then, the straws were immersed and stored in liquid nitrogen.

Thawing: The straws were removed as required from the liquid nitrogen. The straws were placed in tap water for 5 min. Then, the straws were kept in the incubator (at 37 °C for 5–20 min), and evaluation of the different items was carried out after complete thawing of the samples.

DNA damage analysis

The sperm DNA damage was performed on flow cytometry model PAS DAKO-Cytomation. Determination of DNA

damage was based on the fluorescence emission from individual sperm cells after staining with PI and excitation with a 488-nm argon laser. The measurement is based upon the ability PI to bind histochemically to DNA under appropriate staining conditions. The liquefied fresh and processed semen samples were diluted with phosphate buffered saline (PBS) (pH 7.4) to 2×10^6 sperm ml^{-1}. Fifty microlitres (50 µl) of semen samples was incubated with 100 µl of lysing reagent for 15 s. Two millilitres of DNA Prep stain was added and mixed with tube. Immediately after staining, tube acquisition was performed by flow cytometry. The intensity of the fluorescence emission corresponds to the DNA content. Sperm DNA damage was calculated after acquisition of 5000 cells (spermatozoa). Analysis of human spermatozoa displays a constant and characteristic bimodal nonartifactual DNA pattern confirming the existence of two distinct populations. The main population is represented by a peak followed by a shoulder that is the marginal population. The marginal population represents a sperm group altered in the nuclear condensation (DNA damage), yielding unstable chromatin that appears more stainable (Shapiro, 1995).

Statistical analysis

Data were analysed using SPSS version 16 program. Values were expressed as mean ± standard deviations (SD) and percentages. Comparisons between two groups were analysed by Unpaired *t*-test and chi-square. Pearson correlation test was applied to analyse correlations between different quantitative variables within each group. *P* value <0.05 was considered significant.

Results

Adverse effects of cryopreservation on the semen parameters

As shown in Table 1, the sperm concentration and percentage of progressive motility, as well as sperm vitality, decreased significantly after cryopreservation compared with the fresh ejaculated spermatozoa. Also, the freezing/thawing resulted in highly significant increase in % of DNA damage in cryopreserved fresh compared with the fresh ejaculated spermatozoa. There was positive correlation between sperm vitality and progressive sperm motility ($r = 0.59$, $P = 0.001$) while there was no correlation between sperm vitality, progressive sperm motility and % of DNA damage.

Effects of swim up (processed) on semen parameters

Table 1 shows statistically significant decrease in sperm concentration and statistically significant increase in percentage of progressive motility, sperm vitality and % of DNA damage in processed (swim up) samples when compared with fresh ejaculated spermatozoa.

Protective effects of antioxidant (catalase) supplementation on the semen parameters

As shown in Table 1, in the presence of catalase, there was no significant difference in sperm concentration when compared with the samples without catalase (either fresh or swim up). In addition, there was significant increase in % of progressive motility and sperm vitality as

Table 1 Effects of cryopreservation, swim up (processed) and catalase supplementation on the semen parameters

Sperm	Concentration (mill ml^{-1})	Progressive motility (A + B) (%)	Hypo-osmotic swelling-test (%) viability	DNA damage (%)
Fresh ejaculated spermatozoa	90.0 ± 38.8	56.9 ± 7.6	74.1 ± 9.4	9.0 ± 3.3
Cryopreserved ejaculated spermatozoa without catalase	56.5 ± 24.5[a]	23.8 ± 11.7[a]	55.6 ± 11.3[a]	12.5 ± 3.4[a]
Cryopreserved ejaculated spermatozoa with catalase	55.5 ± 22.3[NS]	30.5 ± 12.0[b]	62.2 ± 11.9[c]	10.1 ± 4.4[c]
Swim-up spermatozoa	54.0 ± 23.6[d]	76.4 ± 7.2[d]	90.7 ± 7.8[d]	11.7 ± 3.2[d]
Cryopreserved swim-up spermatozoa without catalase	32.2 ± 16.7[e]	36.5 ± 15.5[e]	66.1 ± 11.7[e]	14.9 ± 4.1[e]
Cryopreserved swim-up spermatozoa with catalase	32.4 ± 17.3[NS]	46.6 ± 17.3[f]	70.0 ± 11.1[g]	12.0 ± 3.0[g]

NS, not significant.
[a]$P < 0.001$ (significance of difference from fresh ejaculated spermatozoa).
[b]$P < 0.01$, [c]$P < 0.05$, (significance of difference from Cryopreserved ejaculated spermatozoa without catalase).
[d]$P < 0.001$ (significance of difference from fresh ejaculated spermatozoa).
[e]$P < 0.001$, (significance of difference from swim-up spermatozoa).
[f]$P < 0.01$, [g]$P < 0.05$, (significance of difference from cryopreserved swim-up spermatozoa without catalase).

well as significant decrease in % of DNA damage in samples with catalase when compared with samples without catalase (either fresh or swim up).

Discussion

Although the cryopreservation of human semen is an important technique routinely employed in the clinical management of male infertility, the relevant cryodamage remains a great challenge (Medeiros *et al.*, 2002). Sperm motility and viability are the most commonly affected parameters (Donnelly *et al.*, 2001a).

In this study, there was a statistically significant decrease in sperm concentration, percentage of progressive motility and sperm vitality in cryopreserved fresh samples when compared with fresh samples. These findings are in accordance with previous reports (Li *et al.*, 2010; Zribi *et al.*, 2010). The excessive ROS production during freezing and thawing has been demonstrated to be a significant contributing factor. Generation of ROS induces peroxidation of the plasma lipid membrane of the spermatozoa (Ozkavukcu *et al.*, 2008; Zribi *et al.*, 2010). The freezing/thawing process exposes the spermatozoa to damaging physical and chemical environments including crystal ice formation, which physically affects the cell. Ice formation around the cells concentrates the surrounding matrix rapidly, leaving the cells in fluids containing high solute content (Ozkavukcu *et al.*, 2008). Furthermore, the glycerol, which is used during cryopreservation, had toxic effect on spermatozoa. The rapid changes in osmolarity, which occur during freezing–thawing, cause deformations of the membranous structures (Buhr *et al.*, 2001).

The assessment of sperm DNA damage related to freeze/thawing is very important. This study demonstrated significant increase in percentage of DNA damage in cryopreserved fresh samples when compared with fresh samples. This is in the agreement with Li *et al.* (2010) who reported that ROS increases DNA fragmentation in human spermatozoa. On the contrary, Jiang *et al.* (2005) did not report any adverse effect of cryopreservation on sperm DNA, and Donnelly *et al.* (2001b) reported that only spermatozoa from infertile men demonstrated a significant increase in DNA fragmentation following cryopreservation. Cryopreservation protocols and extender formulations vary among laboratories and among species and may account for the differences observed.

The most commonly used sperm preparation techniques for ARTs are the swim up and density gradient centrifugation. Our results demonstrate significantly lower sperm concentration but significantly higher percentage of progressive motility and sperm vitality in processed (swim up) samples when compared with fresh samples. This finding is in agreement with Younglai *et al.* (2001) who reported, significant decrease in sperm concentration and significant increase in motility and morphology of spermatozoa after swim-up method. Adiga & Kumar (2001) reported that enhancement of sperm motility after swim up was seen among normal sperm count. A higher recovery rate of total motile spermatozoa was observed in the semen specimens processed by swim-up method. The swim-up method selects the highly motile spermatozoa depending only on its active upward migration through the interface between semen and culture medium (Donnelly *et al.*, 2001a; Jameel, 2008). The recovery of spermatozoa after swim up varies according to the various modifications of the techniques.

We found a significantly higher percentage of DNA damage in the processed (swim up) samples when compared with the fresh samples. These findings are in agreement with Donnelly *et al.* (2001a) and Marchesi *et al.* (2010) and they suggest the use of gentle methods of sperm preparation and centrifugation at 300 ***g*** to minimise the production of ROS that damage DNA. The washing step and the separation of spermatozoa from seminal plasma (source of antioxidants) are responsible for harmful ROS production and further DNA damage (Twigg *et al.*, 1998b). On the other hand, Younglai *et al.* (2001) reported that swim-up separation does not increase the level of DNA damage. Techniques revealing sperm DNA damage are numerous and often not equivalent (i.e. not revealing the same kind of DNA damage). Even when the same technique is used, an important pitfall is the lack of standardised protocols to which all users can adhere to minimise inter-laboratory variations.

In recent years, a variety of cryoprotective media, mostly supplemented with antioxidants, have been designed in an attempt to overcome the cellular damage caused by cryopreservation (Chi *et al.*, 2008). In this study, we evaluated the potential benefits of catalase for human sperm cryopreservation. Our results clearly show in agreement with others (Chi *et al.*, 2008; Li *et al.*, 2010) that catalase supplementation is associated with a higher percentage of progressive motility, improved sperm vitality and DNA integrity (i.e. decrease in DNA damage) in fresh and processed semen. Catalase supplementation can inhibit apoptotic DNA damage by protecting the mitochondria. The levels of ROS were significantly reduced by the addition of catalase to the sperm freezing extender reflects that this enzyme may be capable of scavenging the ROS generated during the cryopreservation process, which reduces the damaging effects of oxidative stress and subsequently improves the quality of cryothawed human spermatozoa (Li *et al.*, 2010).

In conclusion, addition of antioxidant (catalase) during cryopreservation (fresh and swim-up semen) results in

better post-thawing percentage of progressive motility and sperm vitality and decreases the percentage of DNA damage.

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