

Protective Effects of Ascorbate and Catalase on Human Spermatozoa During Cryopreservation

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ABSTRACT: Human semen cryopreservation in the clinical management of male infertility is complicated by cryodamage to spermatozoa. We aimed to clarify the full pattern of cryodamage and evaluate the protective effects of ascorbate and catalase on cryopreserved spermatozoa. Semen samples were collected from 30 fertile males. Each sample was divided into 6 groups: fresh semen, cryopreserved semen without treatment, and samples cryopreserved with ascorbate (300 or 600 μ M) or catalase (200 or 400 IU/mL). Spermatozoa were examined for their viability, motility, reactive oxygen species (ROS) generation, mitochondrial membrane potential (MMP), apoptosis (positive for annexin V and negative for propidium iodide [ie, Ann⁺/PI⁻]), and DNA damage (Olive tail moment [OTM]) in the presence or absence of ascorbate or catalase during cryopreservation. In comparison with the fresh spermatozoa, there was a significant decrease in the viability, motility, and MMP but increase in Ann⁺/PI⁻ and OTM in the

cryopreserved spermatozoa ($P < .01$ and $P < .05$, respectively). Concurrently, ROS levels in the postthaw spermatozoa also increased significantly, and this elevation was well correlated with the quality variations of postthaw spermatozoa ($P < .01$ for all). Ascorbate (300 μ M) and catalase (200 and 400 IU/mL) reduced the ROS levels in postthaw spermatozoa significantly, compared with those in the control ($P < .05$). Furthermore, these antioxidants also prevented those characteristics from being adversely affected ($P < .05$). This study demonstrated that cryopreservation results in cryodamage to human spermatozoa, possibly through the mechanism of ROS. Appropriate ascorbate or catalase supplementation of cryoprotective medium restrains ROS levels and the resultant cryodamage.

Key words: Reactive oxygen species, mitochondrial membrane potential (MMP), DNA damage.

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Cryopreservation of human semen has been widely used as a vital method for the fertility preservation of male patients before they undergo chemotherapy, radiotherapy, and/or surgery that may lead to testicular failure or ejaculatory dysfunction (Anger et al, 2003; Tournaye et al, 2004). Freezing of sperm before initiation of treatment provides patients with “fertility insurance” and may allow them to father their own children through the use of intrauterine insemination, especially by cryopreserved donor semen, conventional in vitro fertilization or intracytoplasmic sperm injection. The use of frozen semen allows for the storage of semen during the quarantine period until donors can be rescreened for infectious diseases such as human immunodeficiency virus and hepatitis B before release for insemination (British Andrology, 1999; Payne and Lamb, 2004). Cryopreservation is also employed in the

storage of spermatozoa retrieved from azoospermic patients after testicular sperm extraction or percutaneous epididymal sperm aspiration, thereby avoiding repeat biopsies or aspiration (Anger et al, 2003). Nevertheless, the cryopreservation process can lead to structural and functional alterations in spermatozoa, impairing fertilization potential. And there is still no effective method available for preventing this cryodamage (Medeiros et al, 2002).

To date, many studies have assessed the structural and functional integrity of the sperm plasma membrane and DNA, as well as the viability and motility characteristics of frozen-thawed spermatozoa. The most common types of cryodamage reported are membrane disruption, diminished motility, and deteriorated viability (O’Connell et al, 2002); DNA damage (Donnelly et al, 2001a; Chohan et al, 2004), and apoptosis in frozen-thawed spermatozoa (Paasch et al, 2004). Most previous studies employed only a single or a few of the relevant parameters in identifying cryodamage and thus possibly have limited value. Concurrent examination of all relevant parameters for each sample may be required to better understand cryodamage in spermatozoa.

Although mechanisms behind the cryodamage to spermatozoa are thought to be multifactorial, the excessive generation of reactive oxygen species (ROS)

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has been suggested as a major contributing factor (Anger et al, 2003; Gadea et al, 2004). Accordingly, a variety of cryoprotective media, most 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 in some animal experiments (Grossfeld et al, 2008). However, limited information exists about the effects of cryoprotection on human spermatozoa.

Therefore, in the present study, we examined the pattern of structural and functional damage in human spermatozoa consequential to cryopreservation. In addition to evaluation of viability, motility, mitochondrial function as expressed by its membrane potential (MMP), DNA damage as determined by Olive tail moment (OTM), and phosphatidylserine (PS) externalization of membrane (apoptosis, annexin V⁺/propidium iodide⁻ [Ann⁺/PI⁻]), we evaluated these changes in relation to ROS level. We also evaluated the protective effects of antioxidants (ascorbate and catalase) on human spermatozoa against ROS during cryopreservation.

Materials and Methods

Reagents

All chemicals used were of analytical grade. Ascorbate, catalase, rhodamine 123 (Rh123), PI, 1% Triton X-100, and ethidium bromide were purchased from Sigma-Aldrich (St Louis, Missouri). 2'-7'-dihydro-dichlorofluorescein diacetate (H₂DCF-DA) was obtained from Invitrogen (Carlsbad, California). Human tubal fluid (HTF)-HEPES and serum substitute supplement were from Irvine Scientific (Santa Ana, California), and the annexin V-enhanced green fluorescent protein apoptosis detection kit was from Kaiji Co Ltd (Nanjing, China).

Semen Preparation

Semen samples were collected by masturbation after 3 to 7 days of sexual abstinence from fertile men, namely healthy male volunteers with no history of fertility problems with partners who were pregnant or had delivered a child in the past year. The average age of the couples attending the Reproductive Medicine Center at the First Affiliated Hospital of Shantou University Medicine College for infertility treatment owing to female factors was 29.9 ± 2.8 years (range, 25–35 years). After the semen was allowed to liquefy at room temperature, routine semen analysis was performed to rule out sperm abnormalities, and the remaining semen was used for further analysis. Thirty semen samples were selected for cryopreservation according to World Health Organization criteria (World Health Organization, 1999), with volumes ≥ 2.0 mL, normal viscosity, sperm count $\geq 100 \times 10^6$ spermatozoa/mL, viability $\geq 60\%$, grade a

motility $\geq 25\%$ or grade a and b motility $\geq 50\%$, and leukocytes $\leq 1 \times 10^6$ /mL. This study was approved by the Institutional Review Board of the First Affiliated Hospital of Shantou University Medical College. All participants gave informed written consent.

Study Design

Each semen sample was equally divided into 6 portions: 2 control groups (fresh semen and untreated frozen-thawed samples) and 4 experimental groups, which received the antioxidants ascorbate (300 or 600 μ M) or catalase (200 or 400 IU/mL). The antioxidant concentrations in the present study were chosen on the basis of published literature (Lewis et al, 1997; Roca et al, 2005) and our preliminary study (data not shown). Each semen sample was first cryopreserved and then thawed. Before and after freeze-thaw treatment, spermatozoa were examined for their general quality, ROS generation, DNA damage, variances of MMP, and membrane redistribution of PS (early marker of apoptosis).

Sperm Cryopreservation

As previously described (Donnelly et al, 2001b), raw (unprocessed) semen samples were mixed slowly and gently with an equal volume of modified cryoprotective medium (10% serum substitute supplement, 14% glycerol, HTF-HEPES buffer), and then antioxidant was added to the semen-freezing medium mixture. Freezing medium was equilibrated to room temperature before use. The mixtures (1.5 mL) were pipetted into cryovials (Nalge Co, Rochester, New York) and then immersed in a container filled with approximately 600 mL of water at room temperature. After that, the container holding the samples was kept in a refrigerator at 4°C for 30 minutes. The samples were then frozen as follows: aliquots were suspended in liquid nitrogen vapor (10 cm above the level of liquid nitrogen at -80°C) for 10 minutes, plunged into liquid nitrogen (-196°C) completely, and stored in liquid nitrogen until used.

Sperm Thawing

After storage for approximately 7 days, sperm thawing was performed according to the protocol described by Donnelly et al (2001a) with slight modification. The cryovials were removed from liquid nitrogen and immediately immersed in a 37°C circulating water bath. Once totally thawed (1–2 minutes), the vials of thawed semen were inverted to thoroughly mix the contents prior to sampling, and an aliquot from each cryovial was removed for semen analysis. The rest was diluted with an equal volume of HTF-HEPES buffer and then centrifuged at $300 \times g$ for 7 minutes. After supernatant removal, the pellet was resuspended in HTF-HEPES buffer and adjusted to 5×10^6 spermatozoa/mL for subsequent analysis.

Semen Analysis

Semen analysis was performed on all samples by the same technician to minimize variations. Semen analyses were performed using light microscopy ($\times 400$ magnification),

combined with a computer-assisted semen analysis system, and a Makler chamber was used for motility scoring (CASA A-QH-3; Qinghua Tongfang, Beijing, China). The settings were as follows: spermatozoa with an average path velocity (VAP [average velocity of sperm movement]) $>10 \mu\text{m}/\text{ms}$ were considered rapid with middle progressive motility. A minimum of 5 fields per sample were evaluated, with a minimum of 200 spermatozoa counted per sample. Only spermatozoa with rapid and middle progressive motility (World Health Organization categories a and b) were assessed. Sperm viability was assessed using eosin Y stain. A total of $20 \mu\text{L}$ of each aliquot was mixed with $20 \mu\text{L}$ of 0.5% eosin Y stain on a glass microscope slide and viewed using light microscopy to determine the percentage of viable sperm. Live sperm remained white, and dead sperm stained red because the integrity of their plasma membranes had been compromised, causing an increase in membrane permeability that led to uptake of the dye. The percentage of live spermatozoa was expressed as viability (%).

Flow Cytometric Analysis of ROS Generation

Flow cytometry (Beckman Coulter, Brea, California) has been proven to be a specific, accurate, and reproducible method for evaluating sperm ROS. $\text{H}_2\text{DCF-DA}$ was used to measure the amount of ROS in thawed specimens. This dye is a stable cell-permeable nonfluorescent probe that intracellularly turns to highly fluorescent 2',7'-dichlorofluorescein upon oxidation (Gadea et al, 2005). The fluorescent signal detected at 488 nm excitation and 525 nm emission is expressed as mean green intensity fluorescence units (mean channel in the FL1) and was used as an index of ROS level. Twenty microliters of $\text{H}_2\text{DCF-DA}$ was added to $200 \mu\text{L}$ of the sperm suspension to get a final concentration of $10 \mu\text{g}/\text{mL}$ and incubated for 15 minutes at room temperature in the dark. Then the sample was centrifuged at $300 \times g$ for 7 minutes in HTF-HEPES buffer to wash away $\text{H}_2\text{DCF-DA}$. The supernatant was removed, and the remaining sperm suspension (approximately $200 \mu\text{L}$) was analyzed for mean fluorescence. A minimum of 10 000 spermatozoa was examined for each assay. The sample without $\text{H}_2\text{DCF-DA}$ staining and centrifugation served as a negative control.

DNA Damage Analysis

For the detection of sperm DNA damage, a modified alkaline single-cell gel electrophoresis (comet) assay was performed as previously described (Anderson and Plewa, 1998; Singh et al, 2003). The following procedure was carried out under yellow light to prevent further induced damage to DNA. Fully frosted microscope slides were covered with $100 \mu\text{L}$ of 1% normal melting point agarose and then dried at 4°C for 15 minutes. Approximately $10 \mu\text{L}$ of sperm suspension (approximately 10^5 sperm cells) was mixed with 0.7% low melting point agarose ($90 \mu\text{L}$) and then pipetted onto the first agarose layer, spread, and solidified on ice. The slides were immersed in the lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH adjusted to 10 before adding 1% Triton X-100 and 10 mM dithiothreitol) at 4°C for 1 hour and then incubated in alkaline solution at room temperature for 20 minutes to unwind the

DNA. Electrophoresis was conducted at 1 V/cm, 100 mA at 4°C for 30 minutes in freshly prepared electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH >13). After electrophoresis, slides were immersed in Tris (0.4 M, pH 7.5) for 15 minutes of neutralization and then in ethidium bromide solution ($5 \mu\text{g}/\text{mL}$) for 15 minutes and mounted with coverslips.

The slides were viewed using a BX 51 fluorescent microscope (Olympus, Tokyo, Japan) that was equipped with an excitation filter of 515 to 560 nm from a 100-W mercury lamp and a barrier filter of 590 nm. Fifty cells were captured randomly and analyzed by an image analysis system using the program Komet 5.5 (Kinetic Imaging Ltd, Liverpool, United Kingdom). Each cell has the appearance of a "comet," with a brightly fluorescent head and a "tail" to one side, formed by the DNA that contains strand breaks being drawn away from the comet head into a tail during the electrophoresis. OTM was recorded, which represents the integrated value of percentage tail DNA multiplied by migration distance between the positions of the head and tail mean; OTM is proportional to the levels of damaged DNA and is considered a sensitive parameter of the comet assay for DNA damage (Olive et al, 1990).

Analysis of MMP

Rh123, a widely used mitochondria-specific probe, accumulates in mitochondria depending on MMP, and the fluorescence of the accumulated fluorochromes corresponds to the transmembrane potential. MMP was assessed using flow cytometry as described previously (Troiano et al, 1998). The mitochondrial activity and viability of living, highly motile spermatozoa were assessed after staining with Rh123 and PI, respectively. Human spermatozoa, suspended in $200 \mu\text{L}$ of HTF-HEPES buffer at a final cell concentration of $5 \times 10^6/\text{mL}$, were incubated in the presence of Rh123 (at a final concentration of $10 \mu\text{g}/\text{mL}$) for 10 minutes at room temperature in the dark. After washing with HTF-HEPES buffer, the supernatant was removed. The sperm suspension ($200 \mu\text{L}$) was incubated with $10 \mu\text{L}$ of PI for another 10 minutes in the dark. The proportion of sperm that acquired Rh123 staining was examined using flow cytometry with 488-nm excitation and 525-nm emission settings, and MMP was expressed as the percentage of $\text{Rh123}^+/\text{PI}^-$ cells. A minimum of 10 000 spermatozoa were analyzed for each sample. Semen samples fixed with 75% ethanol were included as a negative control.

Analysis of Apoptosis by Annexin V/PI Staining Assay

Externalization of PS to the outer side of the plasma membrane is one of the earliest features of cells undergoing apoptosis, which can be directed by annexin V, a calcium-dependent phospholipid-binding protein with a very high affinity for PS (Glander and Schaller, 1999; Paasch et al, 2004). In combination with annexin V, PI staining helps to differentiate apoptotic spermatozoa from necrotic ones.

Early apoptosis in spermatozoa, as evaluated by membrane redistribution of PS, was examined using an annexin V-enhanced green fluorescent protein apoptosis detection kit (Kaiji Co Ltd). Intact cells (Ann^-/PI^-), apoptotic cells

Table 1. Adverse effect of freezing/thawing on spermatozoal functions

Sperm	Viability %	Motility %	MMP %	Ann ⁺ /PI ⁻ %	OTM %
Fresh	78.0 ± 4.9	64.7 ± 4.7	40.7 ± 5.8	10.8 ± 6.4	7.4 ± 2.3
Postthaw	58.8 ± 10.1 ^a	38.1 ± 7.9 ^a	27.5 ± 10.8 ^a	18.1 ± 3.9 ^b	10.5 ± 3.1 ^b

Abbreviations: MMP, mitochondrial membrane potential; OMT; Olive tail movement.

^a $P < .01$, ^b $P < .05$ (significance of difference from the fresh control group).

(Ann⁺/PI⁻), and necrotic cells (PI⁺) were observed among a minimum of 10 000 spermatozoa from each sample. Spermatozoa only treated with buffer without annexin V and PI served as the negative control. The excitation wavelength was 488 nm supplied by an argon laser at 15 mW. Green fluorescence (480–530 nm) was measured in the FHL-1 channel and red fluorescence (580–630 nm) in the FHL-2 channel. The percentage of positive cells and the mean fluorescence were calculated on a 1023-channel scale by Epics software (Beckman Coulter).

Statistical Analysis

Results were analyzed using SPSS version 13.0 (SPSS Inc, Chicago, Illinois), and $P < .05$ was considered significant. Adverse effects of cryopreservation on sperm function were analyzed by Student's *t* test; protective effects of ascorbate and catalase supplementation on human sperm cryopreservation were analyzed by 1-way analysis of variance (ANOVA); and the correlation between ROS and semen parameters, MMP, OTM, and Ann⁺/PI⁻ were analyzed by Pearson's correlation test. Lines in the dispersion plots were calculated by linear regression.

Results

Adverse Effects of Cryopreservation on Sperm Quality

As shown in Table 1, the percentage of viable spermatozoa and motility, as well as functional mitochondria of spermatozoa, decreased significantly after cryopreservation compared with the fresh control group. In addition, the freezing/thawing also resulted in a highly significant increase in both OTM and the percentage of Ann⁺/PI⁻ spermatozoa, indicating significant DNA damage and apoptosis, respectively.

Cryopreservation-Induced Sperm Damage Involves Excessive ROS Generation

To determine the possible mechanism underlying the quality deterioration of human spermatozoa after cryopreservation, we examined the amount of ROS production in frozen-thawed spermatozoa. The results showed that ROS levels after cryopreservation increased significantly compared with the fresh control group (36.7 ± 17.0 vs 16.8 ± 12.9 ; $P < .05$). Correlation analysis showed that the ROS levels in the postthaw

spermatozoa were negatively correlated with the viability ($r = -.577$; $P < .01$), motility ($r = -.604$; $P < .01$), and MMP ($r = -.555$; $P < .01$) but positively correlated with both Ann⁺/PI⁻ ($r = .653$; $P < .01$) and DNA damage intensity as indicated by OTM ($r = .515$; $P < .01$) (Figure 1), indicating the cryodamage of spermatozoa from excessive ROS generation.

Protective Effects of Antioxidants on Sperm Cryopreservation

Because excessive ROS was generated during the cryopreservation process as indicated in our results and other studies (Chatterjee and Gagnon, 2001; Gadea et al, 2004), we evaluated further the effects of the antioxidants ascorbate and catalase for human spermatozoa. As shown in Figure 2, in the presence of ascorbate at 300 μ M or catalase at 200 or 400 IU/mL, there was a significant decrease in the ROS levels compared with those in the control group without any antioxidant treatment. In contrast, 600 μ M ascorbate had no effect on ROS generation. We also determined and compared the sperm parameters after freezing/thawing with and without these 2 antioxidants. As shown in Table 2, ascorbate at 300 μ M and catalase at 200 or 400 IU/mL protected against cryodamage in terms of the sperm quality.

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). As previously reported, sperm motility and viability are the most commonly affected characteristics during cryopreservation and thus are the main cause for the reduced fertility after freezing/thawing (Donnelly et al, 2001a; O'Connell et al, 2002). Despite its importance, the mechanism by which motility is reduced has not been elucidated. Sperm motility is partially dependent on mitochondrial function. It has been suggested that motility, membrane integrity (viability), and mitochondrial function are similarly affected by cryopreservation (Henry et al, 1993). Our study also demonstrated that

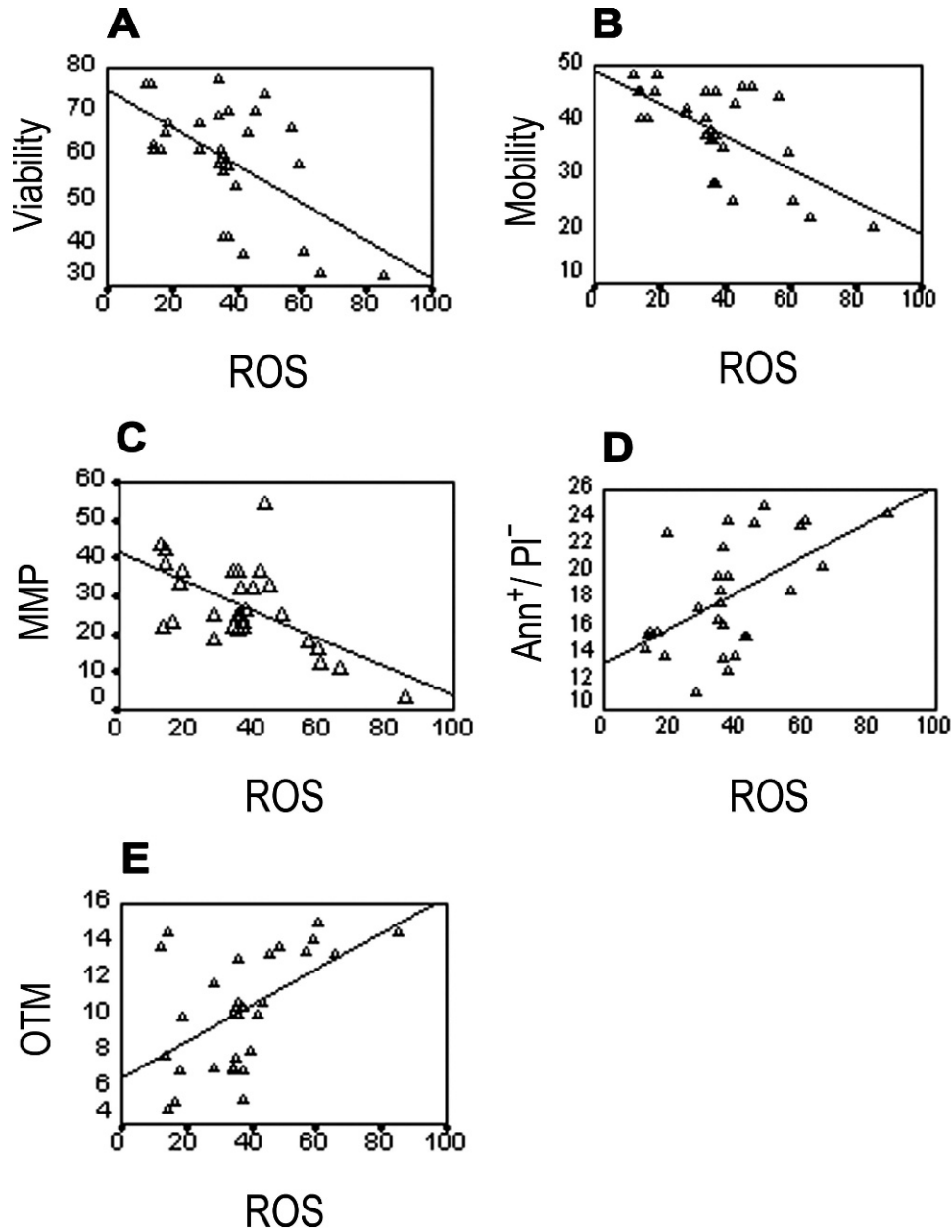


Figure 1. Correlation between reactive oxygen species levels and the (A) viability %, (B) motility %, (C) mitochondrial membrane potential %, (D) annexin V⁺/propidium iodide⁻ %, and (E) Olive tail moment % in frozen-thawed spermatozoa. Data represent means ± SEM from 30 samples.

the plasma and mitochondrial membranes were equally vulnerable. This suggests that the reduction in motility may be explained by an impairment of mitochondrial activity.

Mitochondrial function is commonly discussed in evaluating sperm cryodamage. Increasing evidence indicates that abnormal alterations of mitochondria are early indicators of apoptosis in the affected spermatozoa (O’Connell et al, 2002; Paasch et al, 2004). Because the parameter MMP measured here is

believed to reflect mitochondrial function and is a good indicator for early apoptosis, our observations imply that impaired mitochondrial function during cryopreservation may lead to pathologic variations such as apoptosis. This finding is in agreement with the observations in earlier studies of altered mitochondrial function and subsequent apoptosis (Paasch et al, 2004). Additionally, PS externalization has been generally accepted as an early event of apoptosis in human spermatozoa (Glander and Schaller, 1999). These

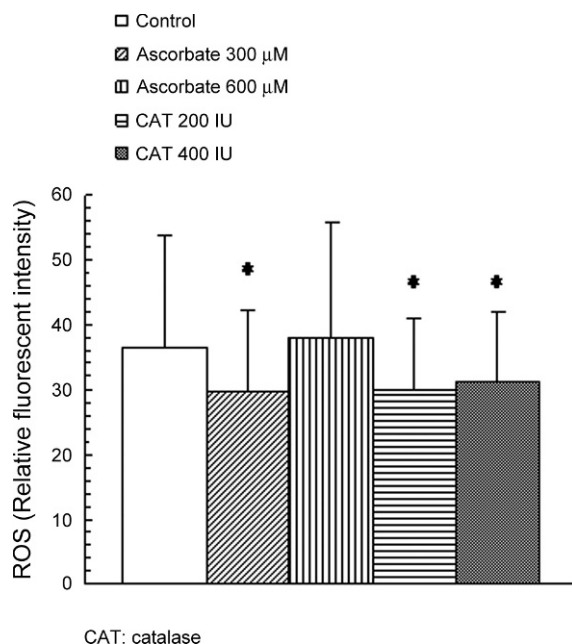


Figure 2. Effects of antioxidant supplementation on reactive oxygen species levels during cryopreservation. Data represent means \pm SEM from 30 samples. * $P < .05$, compared with the control.

alterations precede other manifestations of programmed cell death such as DNA fragmentation. Therefore, the observed alteration of MMP, together with PS externalization (another apoptotic marker), indicate early apoptosis in the frozen-thawed spermatozoa. Chromatin condensation is also vital for spermatozoa because of the fact that spermiogenesis results in the discarding of cytoplasm, causing cessation of transcription and leaving the spermatozoa incapable of undertaking DNA repair. Hence, the assessment of sperm DNA damage related to freeze/thawing is very important. The present study demonstrated that cryopreservation also leads to DNA damage.

Collectively, these results strongly indicate that the cryopreservation process indeed led to a variety of

structural and functional injury to human spermatozoa. Possible mechanisms for the cryodamage to human spermatozoa are thought to be multifactorial, but the excessive ROS production during freezing and thawing has been previously demonstrated to be a significant contributing factor (Chatterjee and Gagnon, 2001). The study showed a significant inverse correlation between MMP and ROS levels in cryothawing spermatozoa. Apart from the plasma membrane, mitochondria are a major site of intracellular ROS formation, which results in a disruption of electron transport. The coupling of electron transport with oxidative phosphorylation in sperm could be disrupted by ROS formation and result in a decrease in the number of sperm that maintain normal mitochondrial function and sperm motility (Armstrong et al, 1999). It was demonstrated previously that mitochondrial dysfunction increases production of ROS, and membrane lipid peroxidation is one outcome of ROS formation in sperm.

Oxidative stress may also lead to cell apoptosis (Wang et al, 2003). This may reflect a causal relationship in that ROS could be responsible for inducing apoptosis. Our findings demonstrated that the externalization of PS to the outer leaflet of the plasma membrane and the mitochondrial depolarization, which are 2 characteristics of apoptotic cell death, were observed in cryothawed spermatozoa. These parameters were associated with a decrease of sperm vitality. It is possible that the oxidative stress induced by cryopreservation acts as a mediator of apoptosis and that the deleterious effects observed on sperm motion are an early functional reflection of an apoptotic event.

In addition to the negative effects on motility and viability, ROS can also damage the DNA in the sperm nucleus (Baumber et al, 2003). In the present study, we reported that ROS increases DNA fragmentation in human spermatozoa. Further, a direct correlation has been observed between the percentage of DNA-damaged sperm and the quantity of ROS detected in the semen. Our results agree with those of Donnelly et al

Table 2. Protective effects of ascorbate and catalase supplementation on cryopreserved human spermatozoa

	Viability %	Motility %	MMP %	Ann ⁺ /PI ⁻ %	OTM %
Control ^a	58.8 \pm 10.1	38.1 \pm 7.9	27.5 \pm 10.8	18.1 \pm 3.9	10.1 \pm 3.1
Ascorbate, μ M					
300	67.2 \pm 14.1 ^b	43.5 \pm 10.0 ^b	30.1 \pm 10.3 ^b	15.3 \pm 3.0 ^c	7.7 \pm 1.2 ^c
600	55.9 \pm 16.4	36.2 \pm 11.1	29.5 \pm 9.2	17.8 \pm 5.9	9.9 \pm 2.8
Catalase, IU/mL					
200	68.4 \pm 13.8 ^c	43.9 \pm 8.2 ^c	34.6 \pm 12.9 ^c	15.6 \pm 2.2 ^c	7.5 \pm 1.6 ^c
400	68.8 \pm 14.8 ^c	44.3 \pm 9.4 ^c	32.9 \pm 11.2 ^b	17.2 \pm 3.2	7.8 \pm 1.9 ^c

Abbreviations: MMP, mitochondrial membrane potential; OMT; Olive tail movement.

^a Sample without antioxidant supplementation.

^b $P < .05$, ^c $P < .01$ (significance of difference from the fresh control group).

(2001a). In contrast, 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 males 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.

Therefore, ROS has been suggested as a cryopathogenic factor, especially after evidence of a correlation between abnormal ROS levels and defective cryothawed sperm function. Our study also supports this viewpoint. 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; Thuwanut et al, 2008). The newer cryoprotective media have been shown to be useful for inhibition of ROS generation in some experiments, but to date, limited information exists for the protective effects of ascorbate or catalase on the quality of human spermatozoa (Rossi et al, 2001). In the present study, we evaluated the potential benefits of ascorbate or catalase for human sperm cryopreservation. The results showed that ascorbate (300 μM) or catalase (200 or 400 IU/ml) supplementation greatly improved the motility and viability, inhibited DNA damage and early apoptotic events, and protected the mitochondrial function, while concurrently reducing the amount of ROS in frozen-thawed spermatozoa. It is therefore evident that ascorbate or catalase supplementation in cryoprotective medium reduces the production of ROS during cryopreservation and thus protects human spermatozoa from cryodamage. This finding is in agreement with the findings in other species (Roca et al, 2005; Bucak et al, 2007; Michael et al, 2007).

Although the present experiment was not designed to provide evidence of whether either ROS generation or lipid peroxidation develops during cryopreservation, the levels of ROS detected by flow cytometry in our experimental samples of thawed spermatozoa increased significantly compared with the fresh control group. This suggestion is in agreement with that of Maldjian et al (2005), who evaluated the changes in lipid composition undergone by boar spermatozoa during cryopreservation, suggested that cryopreservation enhances lipid peroxidation, and concluded that there is a need for ROS scavengers. Moreover, the fact that the levels of ROS were significantly reduced by the addition of ascorbate or catalase to the sperm freezing extender reflects that this enzyme and nonenzyme 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.

It should be noted however that 600 μM ascorbate did not show any effect on ROS generation. In fact, ascorbate acts as a scavenger of ROS mainly through counteracting the adverse effects of H_2O_2 and O^- and also as the major antioxidant in seminal plasma of fertile men (Lewis et al, 1997). However, ascorbate presents in liquid as both ascorbate and ascorbyl radical A, and the latter, if accumulated excessively, will induce a series of free radical reactions that finally lead to a higher level of ROS. Therefore, although ascorbate at suitable doses is completely oxidized to dehydroascorbic acid, this agent at excessive amounts will cause intermediate product A accumulation and induce oxidative damage. This may explain why 600 μM ascorbate, presumably at an inadequate concentration, might counteract its antioxidative effect and even worsen produce sperm cryodamage.

In conclusion, our study indicated that cryopreservation produces a variety of injuries to human spermatozoa and that this cryodamage is due to, at least in part, ROS. Moreover, both ascorbate and catalase supplementation in cryoprotective media reduces ROS levels, thereby protecting against cryodamage to human spermatozoa. Our work is ongoing to determine further whether these antioxidants can improve the fertilization capacity of frozen-thawed human spermatozoa in clinical settings.

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