

Original

# Supplementation of Cryomedium with Catalase and N-acetylcysteine Improves Human Sperm Post-thaw Motility and DNA Integrity

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## SUMMARY

**Purpose** : Reactive oxygen species have been suggested as a major contributing factor to cryodamage of the spermatozoa. Accordingly, antioxidant supplementation has been used to yield significantly improved quality of frozen sperm post-thaw. We sought to investigate outcomes with a combination of antioxidants catalase and N-acetylcysteine respectively. **Methods** : The cryomedium supplemented with 200U/ml catalase, 5 mM N-acetylcysteine and their combination was used. Semen samples were collected from normozoospermic men (n=20) and aliquots frozen in each dose of antioxidant tested. Post-thaw semen analysis by computer aided sperm analysis was performed. In addition, sperm viability (Eosin-Y staining) and sperm DNA fragmentation (TUNEL assay) were quantitated. **Results** : Combined catalase and N-acetylcysteine supplementation during cryopreservation resulted in significantly improved post-thaw recovery of total motility (from  $39.4 \pm 15.8\%$  to  $49.2 \pm 16.1\%$ ) and percentage of sperm vitality (from  $46.2 \pm 13.3\%$  to  $53.2 \pm 11.8\%$ ) (mean  $\pm$  SD,  $p < 0.05$ ). Catalase improved post-thaw DNA integrity analysis for double-stranded breaks using TUNEL assay. **Conclusions** : These results indicated that the combination of intracellular and extracellular antioxidants resulted in the pronounced effect in improving post-thaw quality of human spermatozoa.

**Key Words** : Antioxidant ; DNA damage ; Sperm cryopreservation

## INTRODUCTION

Cryopreservation of human spermatozoa has now become a routine procedure in assisted reproductive technology. It has been widely used as an effective

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<sup>1</sup>. In the case of azoospermia, cryopreservation of testicular spermatozoa can avoid repeated testicular biopsy and it is useful for effective treatment and management<sup>2</sup>. However, traditional freezing techniques are not suitable for spermatozoa from the testis because of their low number and poor in-situ motility. In addition, the cryopreservation process can lead to structural and functional alterations in spermatozoa, impairing fertilization potential<sup>3</sup>.

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Many studies have evaluated 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, deteriorated viability<sup>4</sup>, DNA damage<sup>5</sup>, and apoptosis in frozen-thawed spermatozoa<sup>6</sup>. Although mechanisms behind the cryodamage to spermatozoa are thought to be multifactorial, the excessive generation of reactive oxygen species (ROS) has been suggested as a major contributing factor to cryodamage to spermatozoa<sup>7</sup>. ROS are fundamental for intracellular signaling. In spermatozoa, they are involved both to apoptosis and to capacitation, and changes in ROS levels can alter the balance between these two processes<sup>8</sup>. The excessive ROS production has been associated with reduction of sperm motility, decreased capacity for sperm-oocyte fusion and infertility. Accordingly, antioxidant supplementation has been used to yield significantly improved quality of frozen sperm post-thaw<sup>9</sup>.

Several antioxidant agents, such as vitamins C and E, dimethylsulphoxide, taurine, hypotaurine, melatonin and catalase have already been tested in vitro or in vivo studies concerning human<sup>10</sup>, bovine<sup>11</sup>, boar<sup>12</sup>, canine<sup>13</sup>, goat<sup>14</sup>, rabbit<sup>15</sup>, and stallion<sup>16</sup> semen with controversial efficacy and usefulness. N-acetylcysteine (NAC) is a thiol antioxidant, which is regarded as excellent radical scavengers and blockers of lipid peroxidation<sup>17</sup>. However, there are no available references relative to the protection of NAC in human frozen semen. We sought to investigate outcomes with a combination of antioxidants catalase and NAC in the present study.

## SUBJECTS and METHODS

### *Semen preparation and analysis*

The semen samples were obtained by masturbation and collected into sterile containers from 20 normozoospermic men, who were clinically free and within normal parameters and were recruited from the Andrology lab in University of Illinois at Chicago from September 2014 to April 2015. This study was approved by the Institutional Review Boards of the University of Illinois at Chicago (Chicago, USA). Patient's age ranged between 24 and 52 year with

average of  $34.5 \pm 8.8$ . Samples were left to liquefy for 30 min at 37 °C and were analyzed within an hour by computer aided sperm analysis (CASA) of Sperm Class Analyzer from Microptic (Barcelona, Spain). Semen analysis was carried out according to the criteria mentioned in the World Health Organization guideline (2010), with volumes  $\geq 1.5$  ml, concentration  $\geq 15 \times 10^6$  sperm/ml, normal viscosity, normal sperm count, viability  $\geq 40\%$  and leucocytes  $< 1 \times 10^6$ /ml.

### *Study design*

Each semen sample was divided into four parts. Each sample was cryopreserved without antioxidants, with catalase (200 U/ml), with NAC (5 mM) and using combination of catalase (200 U/ml) and NAC (5 mM). Catalase and NAC were purchased from Sigma-Aldrich (St. Louis, USA). The concentration of catalase and NAC in this study was chosen on the basis of published literature<sup>18</sup> and our preliminary study. Cryopreservation in Liquid nitrogen for at least 24 h for all samples was performed.

### *Sperm cryopreservation*

According to the manufacture's instructions

**Freezing :** Liquefied semen samples were diluted with equal volume of SpermFreeze Solution. SpermFreeze Solution is a bicarbonate and MOPS buffered freezing medium for use with human spermatozoa ; it contain 15% glycerol as cryoprotective agent, human serum albumin and cholesterol to protect the spermatozoa from damage due to the freezing processes from Vitrolife (Englewood, USA). The mixture was left for 10 min at room temperature for equilibration. The sample/medium mixture sucked into the freezing tubes. The tubes were frozen vertically for 30 min, 3 cm above the level of liquid nitrogen. Then, the tubes were immersed and stored in liquid nitrogen.

**Thawing :** The tubes were removed as required from the liquid nitrogen. The tubes were kept in the water bath at 37°C for 30 min, and evaluation of the different items was carried out after complete thawing of the samples.

### *Evaluation of sperm concentration, motility and viability*

When the frozen-thawed spermatozoa were

**Table 1** The recovered (post-thaw/pre-freeze  $\times 100\%$ ) semen parameters of cryopreservation with catalase and N-acetylcysteine

	without antioxidant	with catalase	with NAC	with catalase + NAC
Concentration ( $\times 10^6/\text{ml}$ )	42.5 $\pm$ 15.4	44.1 $\pm$ 16.3	42.5 $\pm$ 11.2	42.9 $\pm$ 10.8
Total motility (%)	39.4 $\pm$ 15.8	47.0 $\pm$ 16.4	48.3 $\pm$ 15.1	49.2 $\pm$ 16.1 *
Progressive motility (%)	22.8 $\pm$ 15.2	30.9 $\pm$ 14.6 *	30.7 $\pm$ 14.3	30.7 $\pm$ 15.9 *
Viability (%)	46.2 $\pm$ 13.3	49.2 $\pm$ 12.6	51.6 $\pm$ 12.3	53.2 $\pm$ 11.8 *

Note : Data presented as mean  $\pm$  SD

\* : significant difference vs. without antioxidant  $p < 0.05$

n = 20

warmed, sperm concentration, total motility and progressive motility were assessed using CASA. Progressive sperm motility was extensively known to be related to pregnancy rates. Progressive motility was categorized as sperm that are moving and actually getting somewhere. Total motility covered progressive and non-progressive motile sperm. Sperm viability was determined according to the World Health Organization guideline (2010). The semen samples recovered from the tubes were transferred on the microscope slide, then 0.5% (w/v) eosin Y staining (Sigma-Aldrich, St. Louis, USA) was added to the samples and mixed well. The samples were covered with a coverslip and left for 30s at room temperature. The slides were examined for the viability of spermatozoa using microscope at  $\times 400$  magnification. The spermatozoa with red or dark pink head were regarded as dead and damaged, while the spermatozoa with white or light pink head were alive and intact.

#### Detection of sperm DNA fragmentation

Sperm DNA fragmentation was examined by TUNEL assay using In Situ Cell Death Detection Kit according to the manufacture's instructions (Roche, Mannheim, Germany). The sperm DNA integrity was assessed when thawed spermatozoa were cultured in 37°C for 30 min. The recovered spermatozoa were transferred to microscope slides and air-dried. The slides were fixed 4% paraformaldehyde for 1h at room temperature. The samples were then permeabilised by 0.5% Triton X-10 solution for 2 min on ice. After that, the permeabilised spermatozoa were incubated with the TUNEL reaction mixture in the dark at 37°C for 1h. The slides were rinsed with PBS three

times to remove excess stain and counterstained with 4,6-diamidino-2-phenylindole (DAPI, Roche). The DNA fragmentation of spermatozoa was examined with a fluorescence microscope. Spermatozoa with or without DNA fragmentation present green or blue fluorescence respectively.

#### Statistical analysis

For comparing seminal parameters and DNA fragmentation Wilcoxon signed rank test was used for intra-group comparisons, and differences in mean values were assayed. In each case, the level of statistical significance was set at  $p < 0.05$  and each domain scores using the Statcel 3 program (OMS Publishing, 2011, Japan).

## RESULTS

#### Recovered total motility, progressive motility and viability of frozen-thawed spermatozoa

As shown in Table 1, in the presence of catalase and NAC, there was no significant difference in sperm concentration when compared with the each group. However, the recovered (post-thaw/pre-freeze  $\times 100\%$ ) progressive motility of spermatozoa cryopreserved with catalase alone and the combination of catalase and NAC was significantly higher than that of spermatozoa cryopreserved without antioxidants. The recovered total motility and sperm viability of spermatozoa cryopreserved with the combination of catalase and NAC was significantly higher than that of spermatozoa cryopreserved without antioxidants.

#### DNA fragmentation of spermatozoa

As shown in Table 2, compared with the pre-freeze

**Table 2** Effects of cryopreservation, catalase and N-Acetylcysteine supplementation on the DNA integrity

	pre-freeze	post-thaw			
		without antioxidant	with catalase	with NAC	with catalase + NAC
TUNEL (%)	18.4 ± 4.9	58.3 ± 12.9 <sup>a</sup>	43.2 ± 12.7 <sup>b</sup>	55.5 ± 14.2	45.8 ± 17.3 <sup>c</sup>

Note : Data presented as mean ± SD

a : significant difference vs. pre-freeze  $p < 0.05$

b : significant difference vs. without antioxidant  $p < 0.01$

c : significant difference vs. without antioxidant  $p < 0.05$

n = 10

group, the process of freezing and thawing caused an increased spermatozoa DNA fragmentation. The spermatozoa DNA fragmentation in the catalase alone group and the combination of catalase and NAC group were less than that without antioxidants group.

## DISCUSSION

This study represents the first attempt to supplement NAC with catalase to aid in the cryopreservation of human sperm. Our data shows that sperm concentration, percentage of motility and sperm viability decreased and DNA damage in cryopreserved samples increased significantly when compared with pre-freezing samples. In addition, catalase and NAC supplementation improves sperm motility, viability and DNA integrity preservation through the frozen-thawed process when compared with the group not using antioxidant.

These results 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<sup>19)</sup>.

Many studies have tested the effects of antioxidants on spermatozoa as a scavenger of ROS, with variable results. While there is a general agreement that spermatozoa are highly vulnerable to the oxidative stress<sup>20)</sup>, and that the ROS level in the sperm suspension was significantly reduced by the addition of antioxidants to the sperm preparation medium<sup>21)</sup>. So the use of antioxidants could improve the results of arti-

cial reproductive techniques<sup>22)</sup>.

It is well known that the integrity of sperm DNA is also affected by ROS<sup>23)</sup>, which has been associated with poor semen quality, low fertilization rate, impaired implantation, increased abortion rate and even an elevated incidence of disease in the offspring<sup>24)</sup>. In this study, we found that adding catalase and NAC as antioxidants reduced the fragmentation of sperm DNA.

Catalase is a common antioxidant, it directly catalyzes the dismutation of extracellular hydrogen peroxide into water and molecular oxygen. Hydrogen peroxide is considered to be the most toxic element because of its ability to cross membranes freely and to inhibit enzyme activities and cellular functions, thus decreasing the antioxidant defenses of the spermatozoa<sup>25)</sup>. Therefore, using catalase appears to protect DNA integrity from the powerful attack by hydrogen peroxide. In the past report, it is reported that catalase alone supplementation during cryopreservation resulted in better post-thawing percentage of progressive motility and percentage of sperm vitality and improved DNA integrity<sup>26)</sup>.

NAC is a derivative of the naturally occurring amino acid L-cysteine. NAC is a precursor of cysteine/glutathione and as a stimulator of cytosolic enzymes participating to glutathione metabolism. NAC increases the concentration of the endogenous reducing agent intracellular glutathione and/or acts directly as a free radical scavenger<sup>27)</sup>. These antioxidant properties are explained by NAC's structure and chemical reaction, which is similar to glutathione's. Also NAC may act through direct chemical interaction with radical element and/or ROS-dependent byproducts. In

human semen when tested in vitro it was proven that NAC had a distinct scavenging effect against ROS<sup>28</sup>.

The addition of catalase and NAC during cryopreservation results in improvement of post-thaw total and progressive motility, sperm viability, and protects the DNA from damage. Therefore, the synergistic effects of the combined treatment of catalase and NAC that were shown only upon simultaneous treatment with the two compounds might be a result of reduction in both intracellular and extracellular ROS in protecting semen sample against cryodamage.

### REFERENCES

- 1) Anger JT, Gilbert BR, Goldstein M : Cryopreservation of sperm : indications, methods and results. *J Urol* **170** : 1079–1084, 2003.
- 2) Okada H, Dobashi M, Yamazaki T, et al : Conventional versus microdissection testicular sperm extraction for nonobstructive azoospermia. *J Urol* **168** : 1063–1067, 2002.
- 3) Medeiros CM, Forell F, Oliveira AT, et al : Current status of sperm cryopreservation : why isn't it better? *Theriogenology* **57** : 327–344, 2002.
- 4) O'Connell M, McClure N, Lewis SE : The effects of cryopreservation on sperm morphology, motility and mitochondrial function. *Hum Reprod* **17** : 704–709, 2002.
- 5) Chohan KR, Griffin JT, Carrell DT : Evaluation of chromatin integrity in human sperm using acridine orange staining with different fixatives and after cryopreservation. *Andrologia* **36** : 321–326, 2004.
- 6) Paasch U, Sharma RK, Gupta AK, et al : Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. *Biol Reprod* **71** : 1828–1837, 2004.
- 7) Gadea J, Selles E, Marco MA, et al : Decrease in glutathione content in boar sperm after cryopreservation. Effect of the addition of reduced glutathione to the freezing and thawing extenders. *Theriogenology* **62** : 690–701, 2004.
- 8) Del Olmo E, García-Álvarez O, Maroto-Morales A, et al : Oestrous sheep serum balances ROS levels to supply in vitro capacitation of ram spermatozoa. *Reprod Domest Anim* **51** : 743–750, 2016.
- 9) Grossfeld R, Sieg B, Struckmann C, et al : New aspects of boar semen freezing strategies. *Theriogenology* **70** : 1225–1233, 2008.
- 10) Kessopoulou E, Powers HJ, Sharma KK, et al : A double-blind randomized placebo cross-over controlled trial using the antioxidant vitamin E to treat reactive oxygen species associated male infertility. *Fertil Steril* **64** : 825–831, 1995.
- 11) Pang YW, Sun YQ, Jiang XL, et al : Protective effects of melatonin on bovine sperm characteristics and subsequent in vitro embryo development. *Mol Reprod Dev* **83** : 993–1002, 2016.
- 12) Pena FJ, Johannisson A, Wallgren M, et al : Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. *Anim Reprod Sci* **78** : 85–98, 2003.
- 13) Michael A, Alexopoulos C, Pontiki E, et al : Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozen-thawed canine spermatozoa. *Theriogenology* **68** : 204–212, 2007.
- 14) Shafiei M, Forouzanfar M, Hosseini SM, et al : The effect of superoxide dismutase mimetic and catalase on the quality of postthawed goat semen. *Theriogenology* **83** : 1321–1327, 2015.
- 15) Alvarez JG, Storey BT : Taurine, Hypotaurine, Epinephrine and Albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility. *Biol Reprod* **29** : 548–555, 1983.
- 16) Aurich JE, Schonherr U, Hoppe H, et al : Effects of anti-oxidants on motility and membrane integrity of chilled stored stallion semen. *Theriogenology* **48** : 185–192, 1997.
- 17) Deneke SM : Thiol-based antioxidants. *Curr Top Cell Regul* **36** : 151–180, 2000.
- 18) Roca J, Rodriguez MJ, Gil MA, et al : Survival and in vitro fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. *J Androl* **26** : 15–24, 2005.
- 19) Li Z, Lin Q, Liu R, et al : Protective effects of ascorbate and catalase on human spermatozoa during cryopreservation. *J Androl* **31** : 437–444, 2010.
- 20) Aitken RJ, Sawyer D : The human spermatozoon—not waving but drowning. *Adv Exp Med Biol* **518** : 85–98, 2003.
- 21) Chi HJ, Kim JH, Ryu CS, et al : Protective effect of antioxidant supplementation in sperm-preparation

- medium against oxidative stress in human spermatozoa. *Hum Reprod* **23** : 1023-1028, 2008.
- 22) Foote RH, Brockett CC, Kaproth MT : Motility and fertility of bull sperm in whole milk extender containing antioxidants. *Anim Reprod Sci* **71** : 13-23, 2002.
- 23) Menezo Y Jr, Russo G, Tosti E, et al : Expression profile of genes coding for DNA repair in human oocytes using pangenomic microarrays, with a special focus on ROS linked decays. *J Assist Reprod Genet* **24** : 513-520, 2007.
- 24) Lewis and Aitken. DNA damage to spermatozoa has impacts on fertilization and pregnancy. *Cell Tissue Res* **322** : 33-41, 2005.
- 25) Griveau JF, Dumont E, Renard P, et al : Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. *J Reprod Fertil* **103** : 17-26, 1995.
- 26) Moubasher AE, El Din AM, Ali ME, El-sherif WT, et al : Catalase improves motility, vitality and DNA integrity of cryopreserved human spermatozoa. *Andrologia* **45** : 135-139, 2013.
- 27) Gressier B, Cabanis A, Lebeque S, et al : Decrease of hypochlorous acid and hydroxyl radical generated by stimulating human neutrophils : comparison in vitro of some thiol-containing drugs. *Methods Exp Clin Pharmacol* **16** : 9-13, 1994.
- 28) Amini MR, Kohram H, Zare-Shahaneh A, et al : The effects of different levels of catalase and superoxide dismutase in modified Beltsville extender on rooster post-thawed sperm quality. *Cryobiology* **70** : 226-232, 2015.