Glutathione adversely impacts post-thaw DNA integrity in asthenozoospermic semen samples

Muayad K. Ali[1]
Department of Applied Embryology, High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, AL-Nahrain University, Iraq.
moaed.klil@ierit.nahrainuniv.iq

Essraa M. Al-Essawe[2]
Department of Physiology, High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, AL-Nahrain University, Iraq.
essraa.m.al.essawe@ierit.nahrainuniv.iq

ABSTRACT

Sperm cryopreservation is a popular method for preserving male reproductive potential. Oxidative stress-induced cryoinjuries reduce sperm function. Glutathione has several antioxidant properties; adding glutathione after thawing could enhance the ability to maintain post-thaw sperm functionality. The aim of this study was to investigate if the addition of glutathione prevents cryodamage in asthenozoospermic spermatozoa.

Keywords: Asthenozoospermia, cryopreservation, glutathione, human, DNA spermatozoa

Introduction:

Cryopreservation of human sperm was first used in the 1960s. Since then, cryopreserved spermatozoa have been the most successful and accepted technique to retain male fertility potential on various occasions, and approaches are now a fundamental element of assisted reproduction technologies (ARTs) (1,2). This approach is advantageous for retaining male fertility prior to radiation or chemotherapy treatment, as well as for males undergoing specific surgical procedures such as vasectomy, testicular failure, or ejaculatory disorder (1,3). Furthermore, healthy men who have been subjected to ionising pollution, toxic chemicals, or organic pollutants, as well as persons who have had sexual practice reassignment operations, could be eligible for sperm banking (4).

Male infertility is frequently caused by asthenozoospermia, a condition characterised by reduced sperm motility. Previous research found that, as compared to normozoospermic men, asthenozoospermic patients have considerably greater sperm DNA fragmentation index and reactive oxygen species (ROS) levels, as well as worse mitochondrial activity and membrane integrity, making asthenozoospermic spermatozoa more vulnerable to cryopreservation-induced damage (5–7).

Despite significant effort over the last decade to avoid sperm cryodamage during the freeze thaw process, including the inclusion of various cryoprotectants, antioxidant and adjustment of cryopreservation settings, the results have never been adequate, particularly for aberrant sperm samples (8–11).

Glutathione is a thiol tripeptide (glutamyl cysteinyl glycine) present in the animal body (somatic cells and gametes) with several biological roles. This thiol is crucial in the anti-oxidation of both endogenous and exogenous components.

Glutathione is the immune system's maestro and the master detoxifier and antioxidant (12,13). The goal of this study was to evaluate the effect of a post-thaw addition of 10 mM glutathione on repairing sperm cryo-damage in asthenozoospernic semen samples.

Materials and methods:

Semen samples were collected from 57 asthenozoospermic patients with an age range of 23–46 years. The samples were collected by masturbation after 3-5 days of sexual abstinence in a clean, dry, and labelled container. After liquefaction within 30–40 minutes, semen samples were assessed macroscopically and microscopically according to the WHO 2010 guideline.

A manual, controlled freezing procedure was used to freeze sperm samples. The Sperm Freeze™ Kit (FertiPro N.V., Industrie Park Noord 32, 8730 Beernem, Belgium) was brought to room temperature for 5 minutes. After that, 1 mL of the liquefied semen sample was transferred to a cryovial, and then 0.7 mL of sperm freeze medium was applied in a dropwise manner, gently rotating within 5 minutes. After that, the cryovials were put in cryoholders and chilled (4°C) for 15 minutes. Subsequently, the samples were exposed for 10 minutes to liquid nitrogen vapour at a distance of 20 cm (−80°C) (14) before being immersed and stored in a liquid nitrogen tank. The samples were thawed a month later, the cryovials were taken from liquid nitrogen to room temperature for 10 minutes before being put in a 37°C water bath for 5–10 minutes until the ice melted (1).

After thawing, the samples were divided into two equal aliquots. The first aliquot evaluated without any addition served as a control (C group). Glutathione was added to the other aliquot, serving as the treated group (T group), at a final concentration of 10 mM. Both groups were then evaluated for motility parameters and morphology according to WHO guidelines (2010).

The cryosurvival rate of sperm motility was calculated using the formula (15):

Cryosurvival rate = post-thaw sperm motility / pre-freeze sperm motility x 100

Aniline blue staining (AB), an acidic dye with a strong affinity for non-lysine-rich histones, was used to assess sperm DNA integrity. In summary, a droplet (10 µL) of well-mixed semen was pipetted onto a labelled, pre warmed microscope slide, and then spread gently and evenly with a clean and dry round edged class slide. The smears were air dried and fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer saline for 30 minutes at room temperature, then stained with 5% aqueous AB stain in 4% acetic acid (pH = 3.5) for 5 minutes. Excessive stain was rinsed under running water and the slides were blotted on filter paper. At least 200 spermatozoa were evaluated at a magnification of (X 1000) under oil immersion using bright field optics; unstained or pale blue-stained spermatozoa were considered normal, while dark blue-stained spermatozoa were considered abnormal (16).

The study was ethically approved by the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies at Al-Nahrain University's ethical approval committee.

Data were analyzed using IBM SPSS Statistics for Windows, Version 23. The Kolmogorov Smirnov test was used to determine the normal distribution of the variables. The dependent sample t-test (paired t-test) was performed to evaluate the mean values of spermatozoal parameters before freezing and after thawing, as well as to compare the impact of 10 mM glutathione addition after thawing to the control without addition (C group vs. T group). Variables were presented as mean and standard error of the mean (Mean ± SE). The level of significance was set at $p \leq 0.05$.

Results:

All sperm motility parameters and normal sperm morphology were significantly reduced ($p \leq 0.0001$) after the thawing process compared with their values before freezing (table 1).

Table 1: The effect of cryopreservation on asthenzoospermic sperm parameters

Sperm parameters	Before freezing	After thawing	*p* -value
Total motility %	60.03 ± 1.39	40.05 ± 1.41	$p \leq 0.0001$
Progressive motility %	23.95 ± 1.35	15.64 ± 0.72	$p \leq 0.0001$
Non- progressive motility %	36.08 ± 1.00	24.42 ± 0.87	$p \leq 0.0001$
Immotile spermatozoa %	39.97 ± 1.39	59.35 ± 1.45	$p \leq 0.0001$
Morphologically normal spermatozoa %	57.06 ± 1.48	42.61 ± 1.08	$p \leq 0.0001$

Values are (mean ± SEM);
* Paired t test (dependent sample t-test; $\boldsymbol{p} \leq 0.05$).

Post-thaw sperm total motility, progressive and non-progressive, and morphologically normal spermatozoa were greater ($p \leq 0.0001$) in the treated group after adding 10 mM glutathione than in the control group without glutathione supplementation (table 2). Additionally adding 10 mM glutathione improved cryosurvival for motility rate (C, 67.10 ± 2.93 vs. T, 76.76 ± 3.28; $p \leq 0.0001$). While post-thaw addition of glutathione (10 mM) significantly impaired human sperm DNA integrity for asthenozoospermic patients (C, 19.28 ± 0.78 vs. T, 23.41 ± 0.91; $p \leq 0.0001$).

Table 2: The effect of adding 10 mM glutathione after thawing on asthenzoospermic sperm parameters

Sperm parameters	C group (0 mM without glutathione)	T group (with 10 mM glutathione)	*p* -value
Total motility %	39.30 ±1.49	44.83 ±1.62	$p \leq 0.0001$
Progressive motility %	14.99 ±0.77	17.97 ± 0.74	$p \leq 0.0001$
Non- progressive motility %	24.31 ± 0.89	26.86 ± 0.99	$p \leq 0.0001$
Immotile spermatozoa %	60.69 ± 1.49	55.17 ± 1.62	$p \leq 0.0001$
Morphologically normal spermatozoa %	42.59 ± 1.11	44.81 ± 1.11	$p \leq 0.0001$

Values shown are (Mean **±** SEM)
* Control group (C group, **0 mM** without glutathione) and treated group, when 10 mM of glutathione was added after thawing (T group, **10 mM**).
* Paired t test (dependent sample t-test; $\boldsymbol{p} \leq 0.05$).

Discussion:

The cryopreservation of spermatozoa may enable the long-term preservation and transit of viable genetic components (17). Cryo-damage to the sperm plasma membrane has been demonstrated in previous studies, which have been attributed to the formation of a large amount of ice inside the cell (18,19). Moreover, increased free radical and ROS generation diminishes membrane fluidity, increases DNA fragmentation, and impairs spermatozoa fertilization ability (20).

These observations were indeed related to the cryopreservation process, which causes changes in osmotic pressure, and changes in ion concentration, that effects mitochondrial activity, resulting in energy production failure and, finally, motility loss. The mean percent of immotile spermatozoa rose considerably after cryopreservation. These findings were in accordance those of other studies (21). Lipid peroxidation, on the other hand, reduced sperm velocity, motility, viability, and mitochondrial function (22). Cryopreservation

resulted in a considerable decrease in progressive sperm motility (23). A similar observation was reported in that sperm motility after thawing was significantly decreased when compared to the pre-cryopreservation state (24); the present study findings are consistent with those observations.

In the current investigation, we did not focus on the effect of cryopreservation on DNA stability; rather, the aim was to evaluate the effect of adding a specific concentration of glutathione as an antioxidant after thawing in an attempt to recover the DNA cryo-injury of asthenozoospermic spermatozoa. The reasoning behind adding glutathione to frozen sperm samples is based on the earlier observation that total reduced glutathione concentration is significantly reduced in frozen and thawed sperm samples (25).

Glutathione is the most abundant non-protein thiol in mammalian cells (26). Cellular glutathione is essential in biological act ivities such as protein and DNA synthesis and amino acid transport. However, its most significant function is to prevent cells from oxidation; the sulphydryl group is a powerful nucleophile that protects cells against oxidants, electrophil es, and free radicals (27).

The results of this study revealed that adding glutathione at a concentration of 10 mM after thawing enhanced motility parameters and sperm morphology when compared to a control group that did not receive glutathione. DNA fragmentation rate was dramatically raised in treated group. In an observation that is similar to our results, sperm motility was increased following the addition of glutathione to thawed semen samples. They reported that 1 to 5 mM concentrations of glutathione were associated with sperm motility improvement without affecting viability, and no deleterious effect of sperm chromatin condensation was observed (25).

Cryo-damage occurs as a result of several factors during the freezing and thawing processes. Excessive ROS generation and oxidative stress have previously been recognized as crucial contributing factors (28).

Two major mechanisms have been documented to impact cell viability under freezing the generation of ROS, which has been shown to induce alterations in membrane function and structure (29); and a shift in antioxidant defense systems (30).

ROS generation have been proposed as an independent marker of male infertility, regardless of whether the patients' sperm parameters are normal or abnormal (31), and that this concept might be extended to the spermatozoa antioxidant defense system. The levels of ROS increased during thawing, with an increase in superoxide anion generation due to a drop in superoxide dismutase (SOD) activity and a decrease in glutathione content due to a decrease in its reductase activity and oxidation by hydrogen peroxide. Glutathione doses of 5, 10, and 20 mM had no influence on sperm progressive motility or other CASA movement characteristics in both normo- and asthenozoospermic samples. While *in vitro*, glutathione supplementation of 10 mM provided protection against H_2O_2-induced sperm DNA damage in asthenozoospermic samples (32).

The results of this study indicated a significant cryosurvival improvement by adding 10 mM glutathione to thawing semen of asthenozoospermic pationts, but did not support the DNA stability. However, the disparity in outcomes among the studies might be related to the various glutathione concentration levels utilized, the cryopreserving technique used, and the type of semen samples, as well as the general health situation of the participants.

Furthermore, the harmful consequences of glycerol, which is commonly used for sperm cryopreservation, have been documented. Rapid fluctuations in osmolarity are common during freezing and thawing process, causing membranous structure deformations (23).

Asthenozoospermia, representing the majority of male infertility patients, usually requires the use of sperm cryopreservation to preserve fertility and improve motile sperm for assisted reproductive procedures (ART) (33). Asthenozoospermic spermatozoa shown to be susceptible to cryodamage, (organelle and

chromatin damage) caused by freeze-thaw cycles (33).

As a result of existing clinical practise and novel clinical needs, cryostorage of sperm is becoming increasingly important: radiotherapy or various surgical procedures; fertility preservation following chemotherapy; assisted reproduction; and confirmation of seronegativity for sexually transmitted diseases following semen banking (34–37). Reducing freezing injury of asthenozoospermic spermatozoa is still a critical issue that should be addressed. The findings of this study suggest that more research needs to be done to investigate the effects of various glutathione concentrations, either alone or in combination with other antioxidants, on sperm motility, DNA integrity, and ROS generation in order to increase DNA cryosurvaval.

Conclusions:

Post-thawing addition of 10 mM glutathione increased the cryosurvival of asthenozoospermic samples without providing protection against DNA cryoinjury.

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