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Impact of Sperm Cryopreservation on Semen Parameters in Asthenozoospermic Men

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Abstract

Background: *Human sperm cryopreservation has proven to be very valuable. Cryopreservation has effects on function, morphology and percentage of fertilization capacity of human sperm. Also, it has been revealed that cryopreservation has a role in sperm DNA fragmentation and infertility. In this study, viability, motility, DNA fragmentation and for the first time, intracellular nitric oxide assessed before and after cryopreservation process of human semen samples in asthenozoospermic men.*

Methods: *Semen samples were collected from 50 asthenozoosprmic men and divided into2 groups: 25 fresh semen samples as a control group, 25 frozen–thawed semen samples. Viability was assessed by eosin-negrosin staining. Motility was evaluated with a phase contrast microscope and intracellular nitric oxide was measured by flowcytometry. For evaluation of DNA fragmentation in sperm, apoptosis was assessed by flowcytometry.*

Results: *Cryopreservation of asthenozoospermic semen samples decreased sperm viability and motility and increased the intracellular nitric oxide concentration and DNA damage significantly (P<0.001).*

Conclusions: *Cryopreservation process has detrimental effects on viability and motility, intracellular nitric oxide concentration and DNA integrity in human semen samples.*

Keywords: Cryopreservation, Asthenozoospermic, Viability, Motility, Nitric oxide, DNA fragmentation, Flowcytometry.
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Introduction

The cryopreservation of human sperm is an important routine process used for various aims such as donor insemination and protection of sperm from chemotherapy patients. However, the adverse effects of cryopreservation on viability, motility, morphology, and DNA integrity of spermatozoa and fertilization capacity have been demonstrated.[1-4]

Cryopreservation leads to the elevation of lipid peroxidation rate in the sperm plasma membrane by an excessive increase in the reactive oxygen species (ROS) concentration in the semen sample.[5-11] The over production of ROS, by the suppression of oxidative phosphorylation or glycolysis, declines ATP concentration and leads to decrease in the sperm motility.[12,13] Cryopreservation may change the sperm glycocalyx by physical damage and may modify the carbohydrate component of the sperm membrane, which is necessary for physiological functions such as capacitation,[14] sperm membrane permeabilization, acrosomal reaction,[5] and fertilization.[7-9]

The mammalian chromatin integrity is one of the vital parameters for the paternal genetic contribution to the normal fetus development and offspring. Cryopreservation and oxidative stress (OS) induce DNA damage, associated with DNA fragmentation, base oxidation, chromatin cross-linking, and other modifications. Male infertility and sperm parameter defects are related to DNA fragmentation; therefore, it is crucial to assess the sperm chromatin after the freezing-thawing process to assure the success of assisted reproductive technologies (ARTs).[2] Intracellular nitric oxide (NO) plays a role in the sperm function; it is a free radical generated from the oxidation of L-arginine to L-citrulline by nicotinamide adenine dinucleotide phosphate (NADPH)-dependent NO synthase (NOS).[15]

In vitro studies have shown that low concentrations of NO enhance the motility of mouse, hamster, and human spermatozoa. On the other hand, higher NO concentrations seem to exert opposite effects on the motility, viability, and metabolism of human spermatozoa.[16]

The aim of this study was to perform an investigation of the adverse effects of the cryopreservation process on sperm viability, motility, intracellular nitric oxide, and DNA integrity in asthenozoospermic men.

Materials and Methods

Collection and preparation of samples: Semen samples were obtained by masturbation, after 3-5 days of abstinence from sexual activity, and collected in sterile containers in the infertility center of Sharhati Hospital. After liquefaction at 37°C and 5% CO2, the 50 asthenozoospermic semen samples were collected and examined for sperm concentration and motility according to the World Health Organization guidelines (Asthenozoospermic=Sperm motility <25% or <50% progression in a semen sample and fresh sperm concentration>20×106 sperm/ml). The samples were transferred to the embryology center of anatomy department of Tehran University of Medical Science.[17] In this applied basic research, semen samples of asthenozoospermic (N=50) men were randomly divided into two groups: fresh semen sample (N=25), as a control group, and frozen-thawed semen samples (N=25). For the assessment of sperm viability and motility, intracellular nitric oxide concentration, and DNA

fragmentation, each semen sample was aliquoted in four separate cryotubes according to the total sperm concentration, then an equal volume of sperm freezing solution (Vitrolife, Sweden) was added to each tube.

Freezing and thawing process: Samples were inserted into the liquid nitrogen vapor at −180°C (15-30 cm above the liquid nitrogen) for 20-30 min and then transferred to liquid nitrogen and stored for two weeks (all parameters in the control group were freshly assessed). After this time, cryotubes containing semen samples were thawed at room temperature for 5 min, and incubated at 37°C for 20 min. The freezing medium was removed by centrifugation at 1000 rpm for 5 min and appropriate medium was added according to protocol, which will be mentioned for each assessment.

Assessment of viability: For the assessment of sperm viability, the eosin-nigrosin dye exclusion staining was used. Briefly, two drops of 1% eosin stain (BDH laboratory supplies, UK) were mixed with one drop of the thawed spermatozoa. After 30 s, three drops of 10% nigrosin (BDH) were added to each solution. A drop of each fraction was smeared onto glass microscope slides and allowed to air dry. The smears were assessed by oil immersion light microscopy at × 1000 magnification. Live spermatozoa appear white, whereas dead spermatozoa with disrupted membranes appear red. Vitality was quantified by counting a minimum of 200 spermatozoa on each slide and the proportion of live spermatozoa was expressed as a percentage of total cell number.

Motility assessment: Assessment of motility was done according to the fifth edition of the World Health Organization guidelines (2010).[17] In brief, 10 µl of each semen sample was put on a microscope slide and covered with coverslip and then assessed with a phase contrast microscope at 40× magnification in multiple views.

Measurement of intracellular nitric oxide in spermatozoa: For NO measurements, DAF-2/DA (4, 5-diaminofluorescein-2/diacetate, Sigma, D225, USA) was used. In brief, each sample was mixed with DAF-2/DA and incubated in the dark for 120 min at 37°C, then, it was analyzed by fluorescence-activated cell sorter.[18] Excitation wavelength (488 nm) and emission wavelength (530 nm) were used at the single-cell level and data were analyzed using CellquestTM version 3.3 software (Becton dickinson, San Jose, CA, USA). After gating the cell population by forward and side scatter light signals, the mean fluorescence intensity of the analyzed sperm cells was detected. The final gated populations usually consisted of 8000-12000 sperm cells. Fluorescence in these cells was recorded on a frequency histogram by logarithmic amplifiers.

Measurement of DNA fragmentation in spermatozoa: In situ cell death detection kit, fluorescein (Roche, 11684795910, Germany) was used for detection and quantification of apoptosis (Programmed cell death) at the single cell level, per manufacturer's instruction. Briefly, for apoptosis measurement, after 3 times washing of test sample in PBS, adjusted to 2 × 107 cells/ml and transferred 100 µl/well cell suspensions into a V-bottomed 96-well microplate. Freshly prepared fixation solution was added to the cell suspension. Then incubated 60 min at 15–25°C. After that, centrifuged microplate and removed fixative by flicking off or suction. Then cells were washed with PBS and were centrifuged and PBS was removed by flicking off or suction. Cells were resuspended in permeabilisation solution on ice (+2 to +8°C) for 2 min and were washed with PBS. Then cells were resuspended in TUNEL reaction mixture. Added lid and incubated for 60 min at 37°C in a humidified atmosphere in the dark. Samples were washed twice in PBS and transferred cells in a tube to a final volume of 250–500 µl in PBS. Samples directly analyzed by flowcytometry.

Statistical analysis: In the present study, SPSS 16.0 software was used and data were expressed as the mean±SEM. For detection of viability and motility percentage, intracellular nitric oxide, and DNA fragmentation concentration in asthenozoospermic men, data were analyzed by t-test. Differences were regarded as statistically significant at $P<0.05$.

Results

Effect of cryopreservation process on the percentage of human sperm viability: In asthenozoospermic men, the viability of frozen-thawed group (11±1.19) decreased significantly compared with fresh semen samples (34±1.82) ($P<0.001$) (Table 1).

Effect of cryopreservation process on the percentage of human sperm motility: In asthenozoospermic men, the percentage of total motility of sperm in the frozen-thawed group decreased significantly compared with the fresh group ($P<0.001$) (Table 1).

Effect of cryopreservation process on intracellular nitric oxide concentration in spermatozoa: In asthenozoospermic men, intracellular nitric oxide concentration of frozen-thawed group (13.48±0.50) showed significant elevation compared with the fresh group (2.74±0.23) ($P<0.001$) (Figure 2a).

Effect of cryopreservation process on DNA fragmentation in spermatozoa: After cryopreservation of asthenozoospermic semen samples, the percentage of DNA damage of frozen-thawed group (24.85±0.46) significantly elevated compared with the fresh group (5.21±0.41) ($P<0.001$) (Figure 3a).

Table 1. Viability and motility percentage in asthenozoospermic men

Group	viability	motility	Total motility%	immotility%	P.V
Asthenozoospermic men	Fresh	34.60±1.82	35±1.68	65±1.68	P<0.001
	Frozen – thawed	11.00±1.19*	13.2±1.28*	86.8±1.28*	P<0.001

P<0.05 were considered significant. *: Significant difference vs. fresh group (P<0.05)

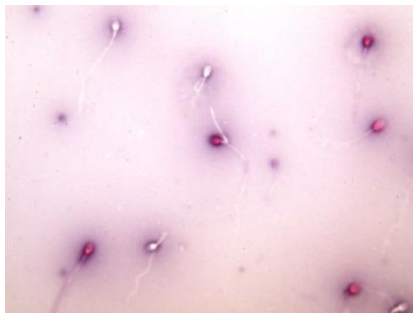

Figure 1. Spermatozoa from asthenozoospermic men staining with eosin–nigrosin dye which assessed by oil immersion light microscopy at ×1000 magnification. Live spermatozoa appear white whilst dead spermatozoa with disrupted membranes have taken up the eosin stain and appear red (a). P<0.05 were considered significant

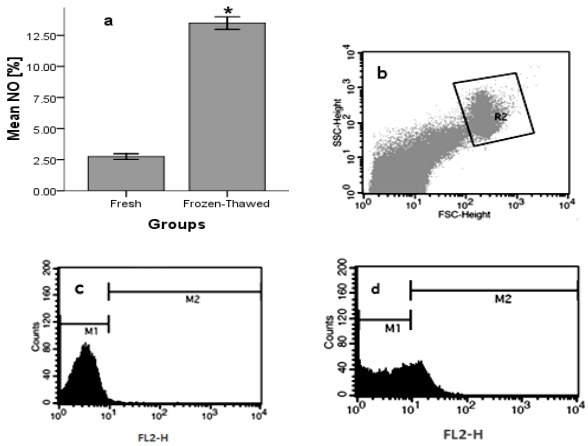

Figure 2. Effect of cryopreservation on intracellular nitric oxide content in asthenozoospermic men (a). Dot plot representing total events acquired and final gated population of spermatozoa (b). Histogram of unstaining semen sample (c). Histogram of semen samples, incubated with baseline 4,5-diaminofluorescein-2/diacetate (DAF-2/DA) fluorescence (d). P<0.05 were considered significant. *: Significant difference vs. fresh group (P<0.001). Error bars: +/- 1SE

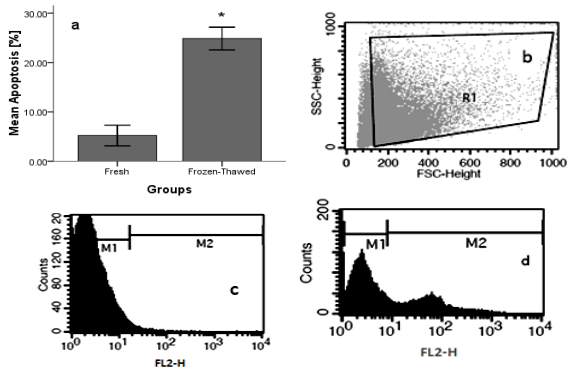

Figure 3. Effect of cryopreservation on DNA fragmentation in asthenozoospermic men (a). Dot plot representing total events acquired and final gated population of spermatozoa (b). Histogram of unstaining semen sample (c). Histogram of semen samples, incubated with a TUNEL reaction mixture (d). P<0.05 were considered significant. *: Significant difference vs. fresh group (P<0.001). Error bars: +/- 1SE

Discussion

In this study, we detected that cryopreservation process of asthenozoospermic semen samples can significantly reduce the percentage of sperm motility and viability, and elevate the intracellular nitric oxide concentration and DNA fragmentation when compared with fresh samples (P<0.001).

Cryopreservation of human sperm is used as a part of the treatment process for infertility. However, during cryopreservation, physical and chemical stresses lead to changes in the membrane lipid component, sperm viability and motility, and acrosome status. All these changes decrease the ability of human spermatozoa for fertilization after cryopreservation. Cryodamage mechanisms in human spermatozoa seem to be multifactorial. Some studies have reported direct physical damage to sperm structure or function related to ice formation and high osmotic pressure during freezing. Cold shock during sperm cryopreservation is related to oxidative stress and reactive oxygen species (ROS) production. ROS damage is mediated by oxidative attack of bis-allylic methylene groups of sperm phospholipid-bound polyunsaturated fatty acids (PUFAs) and lead to lipid peroxidation.[2,3,7,11]

Findings have showed that after cryopreservation, sperm motility and viability decrease about 25-75%.[19] Moreover, the generation of ROS, such as superoxide onion and hydrogen peroxide, led to generation of cytochrome C in mitochondria and the production of Apaf complex in cytoplasm and caspase activity. Subsequently, caspase activates endonucleases and leads to DNA fragmentation and apoptosis.[12,13]

Specific factors (Including freezing and the presence of reactive oxygen species) change sperm viability and activity of mitochondria while inducing sperm apoptosis.[20,21] Frozen spermatozoa show a shorter lifespan and a lower fertility compared with the raw fresh semen. This happens due to the great difference between fresh and frozen-thawed sperm in the generation rate of O2- and H2O2, or in the intracellular concentration of free calcium ions (Ca2+).[21,22]

Our results showed that the viability elevated significantly in fresh group compared with the frozen-thawed group (P<0.001).

Sperm motility is one of the most important features that play role in the fertilization ability of spermatozoa.[8] Baumber J and colleagues showed that exposure to high concentration of ROS can lead to disruption of mitochondria and plasma membranes, resulting in chromosomal and DNA fragmentation by suppression of glycolysis pathway and ATP production. These events subsequently cause a reduction in sperm motility.[22,23] Results showed that infertile men have abnormal sperm morphology, low scores of motility and sperm concentration.[8] In bovine sperm cell PI3K/NOS, pathway enhanced motility range and administration of PI3K inhibitor led to the decrease of sperm motility.[24,25] In our study, the total sperm motility percentage decreased significantly after cryopreservation compare with fresh samples (P<0.001).

Another factor that has a role in the sperm function is the intracellular nitric oxide (NO). NO, a highly reactive gas with a short half-life, is synthesized by NADPH-dependent NO syntheses (NOSs) from the enzymatic conversion of L-Arginine to L-Citrulline.[15] The NO-generating system is been demonstrated in the human reproductive tract, where NO plays a role in a variety of reproductive functions, such as capacitation and acrosomal reaction.[26,27] In vitro studies showed that low concentrations of NO enhance the motility of mouse,[28] hamster,[29] and human spermatozoa.[16] On the other hand, higher NO concentrations seem to have opposite effects on the motility, viability, and metabolism of human spermatozoa.[16,18] Miraglia et al. reported that NO stimulates human sperm motility via activation of soluble guanylate cyclase, cGMP synthesis, and activation of cGMP-dependent protein kinases.[30] In stallion spermatozoa, NO was detected both in fresh and freeze-thaw sperm suspensions, but its production increased after cryopreservation process.[31]

In the present study, results showed that the level of intracellular nitric oxide was significantly elevated in the frozen-thawed group compared with the fresh group (P<0.001).

DNA fragmentation in spermatozoa is another factor that was assessed in this study.

Various biological positions in human spermatozoa that result in DNA damage could be caused by the induction of an apoptotic cascade.[1] Also, changes in the permeability, integrity, and symmetry of the plasma membrane are related to apoptosis. Sperm apoptosis occurs after cryopreservation due to oxidative stress and creation of reactive oxygen species (ROS), and may lead to the decreased life-span of the surviving population.[1,11]

To decrease the impact of ROS, prosurvival factors, such as antioxidants, have an important role, and the withdrawal of prosurvival factors could induce apoptosis in human spermatozoa. Survival factors through activation of Pl3-kinase-Akt phosphorylation prevent the spermatozoa from apoptosis that leads to the cell death. In vitro incubation of spermatozoa in simple culture medium without any prosurvival factors leads to apoptosis characterized by mitochondrial ROS generation. On the other hand, most ARTs (Cryopreservation process) culture media only have balanced salt solutions supplemented with energy substrates and, possibly, serum albumin. There are no preparations to support the spermatozoa from apoptosis. In contrast, in vivo long life-span of human spermatozoa may be related to the presence of prosurvival factors in the epididymal plasma and uterotubal fluids that reduce apoptosis.[1] During the freezing and thawing of sperm in buffalo, human, bull, and stallion, DNA integrity was unchanged.[11]

Despite many other researches' findings, or results indicated that the generation of excessive oxidative stress (OS) during the cryopreservation process leads to the DNA fragmentation.[2] As a result, because of the detrimental effects of cryopreservation on sperm chromatin, attention to the assessment of the DNA integrity of frozen-thawed spermatozoa is important.[11]

In the present research, DNA fragmentation was determined in asthenozoospermic men. The results showed that DNA damage significantly increased in frozen-thawed semen samples compared with the fresh group (P<0.001).

Generally, our results clearly demonstrated that the cryopreservation process reduces the sperm viability and motility and elevates the intracellular NO concentration and DNA damage in asthenozoospermic human spermatozoa. Further examination may elucidate detailed mechanisms of cryopreservation process and its effect on human spermatozoa functions.

To the best of our knowledge, this is the first evidence that demonstrates the effect of the cryopreservation process on motility, viability, intracellular nitric oxide, and DNA integrity in asthenozoospermic men.

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Conflict of Interest

The authors declared that they have no conflict of interest.

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