Comparisons between Two Antioxidant Butylated Hydroxytoluene and Glutathione Supplemented Cryopreservation Medium on Human Sperm DNA Integrity

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ABSTRACT

Introduction: Cryopreservation of semen is routinely used in a variety of circumstances including before assisted reproduction treatments, pre- radiation or chemotherapy treatment and etc. The aim of this study was to compare the effect of Butylated hydroxytoluene (BHT) and Glutathione supplemented cryopreservation medium on sperm parameters and amount of DNA fragmentation during the freeze-thaw process.

Methods: Semen samples were obtained from 60 donors. After the determination of basic parameters, groups of three sample with similar parameters were pooled and processed by Pure Sperm gradient centrifugation. The semen samples were then diluted with normal freezing medium (control) or a medium containing 5mM glutathione (test) and 0.5 mM BHT (test) stored in liquid nitrogen. Frozen cryovials were thawed individually for 20 seconds in a water bath (37 ˚C) for evaluation.

Results: Significant differences were observed in motility, viability and DNA fragmentation. Motility and viability were significantly higher in treated groups with 0.5 Mm in 5 min BHT than the control group and Glutathione 5mM ($P<0.001$).

Conclusion: Significant differences were observed in motility, viability and DNA fragmentation. Motility and viability were significantly higher in treated groups with 0.5 Mm in 5 min BHT than the control group and Glutathione 5mM ($P<0.001$).

Key Words:
Butylated hydroxytoluene, Glutathione, Cryopreservation, DNA fragmentation, Spermatozoa

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1. Introduction

Cryopreservation is the procedure that stabilizes and preserve the cells at low temperatures and it is commonly used in infertility clinics and sperm banks. Cryopreservation was discovered in 1948 by polg-smith-parks to demonstrate that sperm of poultry and cattle can survive at 70 °C in the presence of glycerol, which was in fact the theory of freezing sex cells and tissues. Sperm freezing is routinely used for pre- radiation, chemotherapy treatment, for men undergoing vasectomy, cancer, storage of donor semen for HIV and Hepatitis patients. It is also used for Azoospermic patients who have done the practice testicular sperm extraction (TESE) or percutaneous epididimal sperm aspiration (PESA) [1]. Despite tremendous efforts, Cryopreservation leads to incomplete morphological, biochemical, chromosomal changes (formation of polyploidy), ionic disturbances and damages the membrane [1]. It is proved that the freezing process leads to reduced motility and viability and increased sperm DNA damage [2, 3].

Damage in sperm cell and membrane injury may be related to cold shock, intracellular ice crystal formation and osmotic stress during cryopreservation [4]. It seems that freezing and thawing gamete results in an increased amount of reactive oxygen species (ROS) which could damage cellular compounds including DNA, lipids, and proteins [2, 3].

Recently scientists have tried to reduce the effects of ROS and improve quality of thawed sperms with the addition of antioxidant supplements to the freezing medium. For this purpose, antioxidants such as ascorbate [5], vitamin E and reduced glutathione [6] have been used. Butylated hydroxytoluene (BHT), synthetic phenolic antioxidant with Lipophilic properties, could control peroxy radicals to hydro peroxides and reduce auto-oxidation. BHT has been tested in animal studies [7-9].

Aitken and Clarkson [1988] showed that the addition of BHT into the sperm freezing media decreases ROS production, thereby reducing damage to DNA. Another antioxidant which used is glutathione non-protein thiol with low molecular weight in plant and animal cells and its intracellular concentration 5-10 mmol while the extracellular one is third of this amount. Glutathione is a tripeptide which has gamma peptide linkage and therefore it is almost stable in the intracellular since the intracellular peptidases can cleave peptide bonds formed by the alpha-carboxyl groups of amino acids [6, 10].

The main objective of this study was to evaluate DNA fragmentation in sperm after thawing in the presence of BHT antioxidant and glutathione.

2. Materials and Methods

Semen collection and processing

The study was approved by the Ethical Committee of Hamadan University of Medical Sciences and written consent was obtained from all participants. Semen samples were obtained from 60 donors with normal semen analysis by masturbation according to WHO [11] guidelines and 60 semen samples with similar characteristics were randomly divided into 20 sets of three semen samples.

Aliquots of 1ml of the liquefied pooled semen samples were layered on top of the upper layer of 40% and 80% Pure Sperm gradient (Nidacon International, Sweden) and centrifuged at 300 g for 20 minutes. After removal of the supernatant, the pellets were washed with 5ml of cook sperm medium (COOK IVF, Australia) and centrifuged at 500 g for 10 minutes [12].

Then, the pellets were resuspended in 0.5 ml of cook sperm medium. Finally, sperm samples were transferred into a series of cryotubes (5 tubes) containing 0.5 mM BHT concentrations and tubes were incubated for 5 minutes at 37 ºC to allow uptake of BHT by spermatozoa. Then, semen samples were slowly frozen in liquid nitrogen vapors [7] and stored at -196 ºC.

In the another group, 5 mM Glutathione was added to freezing medium. After 10 min equilibration of sperms at room temperature in this solution, the mixture are slowly frozen in liquid nitrogen vapors for 15 min and placed in special containers and they are stored in the (−196 °C liquid N_2). In the last group (control group), sperms were freezed in freezing medium without addition of any antioxidant.

Sperm thawing

Frozen sperm vials were placed in water bath (37 °C) for 4-5 minutes. Once the specimen were completely thawed, then gently were mixed and washed with sperm medium to eliminate cryosperm [7]. After thawing, sperm motility, viability (detected by staining with eosin 0.5%), and DNA fragmentation were evaluated.

Viability test

The viability of the sperm in the sample was assessed by means of eosin 0.5% stain. The sperm smears were

Table 1. Level of sperm motility, viability and DNA fragmentation in three groups. Sample (n=20)

Group	Motility (%)	Viability (%)	Damaged DNA(%)
Control	34.7±5.7	46.56±5.9	49±7.6
BHT 0.5 mM/5 min	44.4±5.9	56.04±5.7	25.3±7.6
Glutathione 5 mM	35.39±5.24	49.56±8.51	36.13±8.21

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prepared by mixing a drop of semen with one drop of the stain on a warm slide. The viability was assessed by counting 200 cells under 400*magnification. Sperms with partial or complete red colorization were assigned non-viable or dead whereas colorless sperms were designated as alive [11].

Evaluation of sperm DNA fragmentation

Determination of sperm DNA fragmentation was carried out using Cell Death Detection Kit (Roche Diagnostics, Deutschland GmbH, Germany). Thawed sperms were smeared on microscope slides, air-dried, fixed with 4% paraformaldehyde in phosphate-buffered saline for 60 min and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Then, smears of sperm were further processed for TUNEL assay. Spermatozoa with fragmented DNA were visualized using fluorescence microscope in 488 nm (Figure A,B). A negative control (sperm without the addition of the Tdt enzyme) and a positive control (sperm treated with a 141 mM solution of H_2O_2) were also assessed by TUNEL assay [10].

Determination of DNA fragmentation in the thawed sperm of control group (A), no addition of Antioxidant) and in the thawed sperm with Antioxidants (B) using the TUNEL test. Spermatozoa with fragmented DNA were visualized using fluorescence microscope in excitation wavelength 488 nm and detection in the 540 nm. The spermatozoa with DNA fragmentation are shown in green and spermatozoa having intact DNA are shown in red.

Statistical analysis

Results were expressed as the mean±SD. Sperm motility, motion characteristics motility, viability and DNA fragmentation were analyzed by analysis of variance, followed by Tukey's post hoc test to determine significant differences between groups. Differences with values of $P<0.05$ were considered to be statistically significant. Statistical analyses were performed by using SPSS 13 package program.

3. Results

Semen parameters of all participants are illustrated in Table 1. As shown in the table, significant differences were observed in motility, viability and DNA fragmentation between the groups. Motility were significantly higher in treated group with 0.5 Mm in 5 min BHT (44.4±5.9) than the control group (34.7±5.7) and Glutathione 0.5mM (35.39±5.24) ($P<0.001$). Viability were significantly higher in treated group with 0.5 Mm in 5 min BHT (56.04±5.7) than the control group (46.56 ±5.9) and Glutathione 0.5mM (49.56±8.51) ($P<0.001$). DNA fragmentation were significantly lower in treated groups (25.3±7.6) than the control (49±7.6) and Glutathione 0.5mM (36.13±8.21).

4. Discussion

The results of this study demonstrate that the addition of 0.5 mM BHT to the freezing medium decreased DNA fragmentation compared to the controls and Glutathione 0.5mM ($P<0.001$). Furthermore, freezing medium supplemented with 0.5 mM BHT led to higher sperm motility and viability compared with control Glutathione 0.5 mM ($P<0.001$).

Freezing and thawing process has deleterious effects on sperm DNA integrity by inducing DNA fragmentation [3]. DNA integrity of sperm is necessary for the correct transmission of genetic information to the next offspring and therefore, DNA damage may result in male infertility [13]. Studies have proved that high levels of ROS that are created during freezing and thawing process modify the occurrence of single- and double-strand DNA breakage in the sperm nucleus. Here, we showed that the addition of BHT into the freezing media reduces the percent of sperms with DNA damage from 49% in non-treated cells to 25% in BHT-received cells. Therefore, it can be concluded that the addition of BHT to the freezing media reduces spermatozoa ROS formation and DNA fragmentation. Tuncer et al., 2010 reported that adding Glutathione to the freezing medium have an impact on the DNA integrity and reduction of DNA damage [14-16]. Freezing and thawing process, leading to loss of DNA integrity and induces single- and double-strand DNA break of sperm cells. Our

result indicated that the addition of BHT(0.5 mM) to the sperm freezing media, significantly reduced sperm DNA damage compare to control and Glutathione groups.

We conclude that these antioxidants can be good scavenger of free radicals in the spermatozoa freezing medium and can compensate reduced activity of superoxide dismutase and glutathione peroxidase after a cycle of freezing and thawing. It seems that BHT could be an important scavenger of free radicals in the spermatozoa freezing medium and can compensate reduced activity of superoxide dismutase and glutathione peroxidase after a cycle of freezing and thawing. In conclusion, the addition of BHT to the freezing medium could be of advantage in reduction of ROS formation and prevents detrimental effect of ROS to human sperm function.

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