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The effect of cysteine and glutathione on sperm and oxidative stress parameters of post-thawed bull semen ☆

Pürhan Barbaros Tuncer [a], Mustafa Numan Bucak [a,*], Serhat Büyükleblebici [a], Serpil Sarıözkan [b], Deniz Yeni [c], Ayşe Eken [d], Pınar Peker Akalın [e], Hüseyin Kinet [a], Fatih Avdatek [c], A. Fatih Fidan [c], Mustafa Gündoğan [c]

[a] Tarım ve Köyişleri Bakanlığı, Lalahan Hayvancılık Merkez Araştırma Enstitüsü, Lalahan, Ankara, Turkey
[b] Erciyes Üniversitesi, Safiye Çıkrıkçıoğlu Meslek Yüksekokulu, Kayseri, Turkey
[c] Afyon Kocatepe Üniversitesi, Veteriner Fakültesi, Afyonkarahisar, Turkey
[d] Gülhane Askeri Tıp Akademisi, Etlik, Ankara, Turkey
[e] Tarım ve Köyişleri Bakanlığı, Merkez Veteriner Kontrol ve Araştırma Enstitüsü, Etlik, Ankara, Turkey

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ABSTRACT

The aim of this study was to determine the effects of antioxidants such as reduced glutathione (GSH) and cysteine in Laiciphose® extender on semen parameters, fertilizing ability, lipid peroxidation (LPO) level and glutathione peroxidise (GPx) activity of post-thawed bull semen. Totally 54 ejaculates of three bulls were used in the study. Five groups, namely; GSH (0.5 and 2 mM), cysteine (5 and 10 mM) and control group, were conducted to test the antioxidants in Laiciphose®. Insemination doses were processed that each 0.25-mL straw contained 15×10^6 sperm. The addition of antioxidants did not present any significant effect on the percentages of post-thaw sperm morphology (acrosome and total abnormalities), subjective, CASA and progressive motilities, as well as sperm motility characteristics (VAP, VSL, VCL, LIN and ALH), compared to the control groups ($P > 0.05$). GSH 0.5 mM (55.5 ± 7.38%) and cysteine 10 mM (48 ± 5.65%) led to lower rates of DNA damage, compared to control ($P < 0.05$). As regards to MDA level, cysteine at 10 mM dose gave the highest level (4.99 ± 0.44 nmol/L) ($P < 0.001$). GPx activity was demonstrated to be higher level upon the addition of 5 mM cysteine when compared to the other groups ($P < 0.05$). With respect to fertility results based on 60-day non-returns, the supplementation of antioxidants did not present significant differences ($P > 0.05$). The results of this study may provide an useful information for the future studies in this area. So, further studies could be suggested to achieve better information in terms of the DNA damage and fertilizing capacity of bull sperm frozen with effective antioxidants.

Introduction

Oxidative stress significantly damages sperm functions due to lipid peroxidation (LPO) induced by reactive oxygen species (ROS). Lipid peroxidation occurs readily in the tissues rich in highly oxidizable polyunsaturated fatty acids (PUFA) [17,10]. Sperm cells contain high concentrations of PUFA, and therefore are highly susceptible to LPO. In addition, every stage of cryopreservation (cooling, freezing and thawing) causes physical and biochemical stress on sperm plasma membrane and structure, which results in a subsequent loss of motility, membrane integrity, fertilizing capability and metabolic changes of sperm [32]. Besides, due to high PUFA concentration of sperm membrane, mammalian sperm cells may be insufficient in preventing LPO, because of having limited antioxidant system [3,27].

Sperm cells, when freezed and thawed for artificial insemination (AI), have been exposed to oxygen and light radiation which could irreversibly affect sperm functions. Under these conditions, it was stated that supplementation of antioxidants improved the post-thaw motility, viability, membrane integrity and fertility of boar [14], bull [24], ram [30] and goat [2] sperm cells. Cysteine, which has antioxidant capacity, stimulates glutathione synthesis, thus enhancing the maintenance of intracellular glutathione levels and scavenging ROS. Additionally, cysteine has been shown to prevent loss of sperm functions during sperm liquid storage or in the frozen state [22,19]. Glutathione is a large class of antioxidants and has –SH (thio) groups and antioxidant features. Therefore, it is able to react with many ROS directly and is also a co-factor for glutathione peroxidase (GPx) that catalyses the reduction of toxic H_2O_2 and hydroperoxides [7].

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* Corresponding author. Address: Ministry of Agriculture and Rural Affairs, Lalahan Livestock Central Research Institute, Lalahan, Ankara, Turkey.
E-mail address: mustafanbucak@yahoo.com (M.N. Bucak).

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In a previous study of bull sperm freezed with Laiciphose extender, the non-return (60–90 days) obtained from field inseminations and in vitro fertilization rates resulted in 60.4% and 83.4–87.1%, respectively [8]. There is a lack of information regarding the effectiveness of commercial Laiciphose extender containing salt buffer and milk powder on sperm functions, LPO level and GPx activity. According to our knowledge, the evaluation of the influence of antioxidants, such as cysteine and GSH, in this extender was not reported. The novelty of this study was to determine the effect of cysteine and GSH, prior to cryopreservation, on semen parameters, fertilizing ability, oxidative stress parameters (MDA and GPx) for frozen-thawed bull semen.

Materials and methods

Animals and semen collection

In this study, three Holstein bulls (3 and 4 years of age) were housed at the Lalahan Livestock Central Research Institute (Ankara, Turkey), and maintained under uniform feeding and housing conditions. A total number of 60 (20 ejaculates for each bull) ejaculates were collected from these bulls with the aid of an artificial vagina twice a week, according to artificial insemination standard procedures. Ejaculates containing spermatozoa with >80% forward progressive motility and concentrations higher than 1.0×10^9 spermatozoa/mL were used in the study. Nineteen ejaculates for each bull met these criteria. The first and second ejaculates from the same bull were combined. The study included nine independent experiments for each bull.

Semen processing

The volume of ejaculates was measured in a conical tube graduated at 0.1 mL intervals and sperm concentration was determined by means of an Accucell photometer (IMV, L'Aigle, France). The sperm motility was estimated by using a phase-contrast microscopy (200×). A extender (Laiciphose: IMV, L'Aigle, France) was used as the base freezing extender. Each ejaculate was split into five equal experimental groups and diluted to a final concentration of 60×10^6/mL spermatozoa with the extender containing cysteine (5 and 10 mM), GSH (0.5 and 2 mM) and no additive (control). Diluted semen samples were loaded into 0.25-mL French straws and cooled down to 4 °C in 2 h, frozen at a programmed rate of −3 °C/min from +4 °C to −10 °C; −40 °C/min from −10 to −100 °C; −20 °C/min from −100 to −140 °C in a digital freezing machine (Digitcool 5300 ZB 250, IMV, France). Thereafter, the straws were plunged into liquid nitrogen. Sperm samples from three bulls were frozen for nine replications. After 24 h, frozen straws were thawed in a 37 °C water bath for 20 s immediately before use.

Evaluation of in vitro semen parameters

Subjective motility was assessed using a phasecontrast microscope (100×), with a warm stage maintained at 37 °C. A wet mount was made using a 5-μL drop of semen placed directly on a microscope slide and covered by a coverslip. Sperm motility estimations were performed in three different microscopic fields for each semen sample by the same researcher. The mean of the three successive estimations was recorded as the final motility score. Besides estimating subjective sperm motility, a computer-assisted sperm motility analysis (CASA; IVOS version 12; Hamilton-Thorne Biosciences, MA, USA) was also used to analyze sperm motion characteristics. CASA was set up as follows: phase contrast; frame rate 60 Hz; minimum contrast 80; low and high static size gates 0.10–3.40; low and high intensity gates 0.30–1.70; low and high elongation gates 8–97; default cell size 5 pixels; default cell intensity 70. Thawed semen was diluted (5 mL semen + 95 mL extender) in a Tris-based extender (without egg yolk and glycerol) and evaluated immediately after dilution. A 4-mL sample of diluted semen was put onto a prewarmed chamber slide (20 mm; Leja 4; Leja Products BV, Holland), and sperm motility characteristics were determined with a 10× objective at 37 °C. The following motility values were recorded: motility (%), progressive motility (%), VAP (average path velocity, μm/s), VSL (straight linear velocity, μm/s), VCL (curvilinear velocity, μm/s), ALH (amplitude of lateral head displacement, μm), LIN (linearity index; LIN = [VSL/VCL] × 100). For each evaluation, 10 microscopic fields were analyzed which include at least 300 cells.

For the evaluation of sperm abnormalities, at least three drops of each sample were added to Eppendorf tubes containing 1 mL of Hancock solution [25]. One drop of this mixture was put on a slide and covered with a cover slip. The percentages of acrosome and total abnormalities were determined by counting a total of 400 spermatozoa under phase-contrast microscopy (1000× magnification, oil immersion).

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane. This was performed by incubating 30 μL of semen with 300 μL of a 100 mOsm hypo-osmotic solution at 37 °C for 60 min. After incubation, 0.2 mL of the mixture was spread with a cover slip on a warm slide. Two hundred sperms with swollen or coiled tails were recorded under phase-contrast microscopy (400× magnification) [21].

For assessment of sperm DNA damage, diluted semen samples were centrifuged at 300*g* for 10 min at 4 °C. Seminal plasma was removed and remaining sperm cells were washed with (Ca^{2+} and Mg^{2+} free) PBS to yield a concentration of 1×10^5 spermatozoa/cm^3 [1]. Sperm DNA damage was investigated using the single cell gel electrophoresis (comet) assay that was generally performed at high alkaline conditions.

Embedding of sperm in agarose gel

Each microscope slide was pre-coated with a layer of 1% normal melting point agarose in PBS and thoroughly dried at room temperature. Next, 100 μL of 0.7% low melting point agarose at 37 °C was mixed with 10 μL of the cell suspension and dropped on top of the first layer. Slides were allowed to solidify for 5 min at 4 °C in a moist box. The coverslips were removed and the slides were immersed in freshly prepared cold lysis buffer containing 2.5 M NaCl, 100 mM Na_2–EDTA, 10 mM Tris, 1% Triton X-100 and 40 mM dithiothreitol (pH 10) for 1 h at 4 °C. Then the slides were incubated overnight at 37 °C in 100 μg/mL proteinase K and added to the lysis buffer. The slides were removed from the lysis buffer, drained and placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution, containing 300 mM NaOH and 1 mM EDTA, (pH 13), for 20 min to allow the DNA to unwind. Electrophoresis was performed for 20 min at room temperature at 25 V and was adjusted to 300 mA. Subsequently, the slides were washed with a neutralizing solution of 0.4 M Tris, pH (7.5), in order to remove alkali and detergents. After neutralization the slides were stained with 50 μL of 2 μL/mL ethidium bromide and covered with a coverslip. All steps were performed under dim light to prevent further DNA damage [16,26]. The images of 100 randomly chosen nuclei were analyzed visually. Observations were made at a magnification of 400× using a fluorescent microscope (Olympus, Japan). Each image was classified according to the intensity of the fluorescence in the comet tail, and given a value of 0, 1, 2, 3 or 4 (from undamaged class 0 to maximally damaged class 4), so that the total score of the slide would range from 0 to 400 arbitrary units (AU) [31]. Damage was detected by a tail of fragmented DNA that migrated from the sperm head, causing a 'comet' pattern, whereas whole sperm heads, without a comet, were not considered damaged.