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The effect of cysteine and superoxide dismutase on the quality of post-thawed chicken sperm

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ABSTRACT

The study was conducted to determine the influence of N-acetyl-L-cysteine (NAC) and superoxide dismutase (SOD) on chicken sperm motility, plasma membrane and acrosome integrity, mitochondrial activity, lipid peroxidation (LPO) and apoptotic changes after freezing–thawing process. Semen samples from fifteen Greenlegged Partridge roosters were pooled, diluted with EK extender without antioxidants (control), or supplemented with 5 mM NAC, or 200 U/mL SOD. Samples were subjected to cryopreservation. After thawing, sperm parameters evaluated by using CASA system and flow cytometry were assessed.

The extender supplemented with NAC and SOD caused the increase ($P < 0.01$) in sperm motility and provided the higher percentage of rapid sperm ($P < 0.01$) compared to control. The addition of NAC increased the progressive motility of cells ($P < 0.01$). In NAC and SOD groups post-thaw plasma membrane integrity was higher ($P < 0.05$) and less spermatozoa showed apoptotic changes ($P < 0.01$, $P < 0.05$). Post-thaw percentage of sperm with high mitochondrial activity was the greatest ($P < 0.05$) with NAC addition. The SOD supplementation only reduced ($P < 0.05$) the percentage of sperm with LPO, following the cryopreservation. These results indicate that the addition of NAC (5 mM) and SOD (200 U/mL) is beneficial for the function of frozen–thawed chicken spermatozoa. The antioxidants prevented the reduction in motility, viability and mitochondrial membrane potential, as well as protected from apoptotic changes in sperm. Lipid peroxidation in sperm plasma membrane was decreased by SOD supplementation. Therefore, these antioxidants can be recommended as an additional component of chicken freezing extender.

Introduction

Although, semen possesses an antioxidant system, which includes glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and also natural antioxidants [1,8,32,33,40], their activity is affected by cryopreservation, which increases the intensity of LPO [15,23,32]. Mechanisms of spermatozoal cryodamage affecting post-thaw sperm motility, viability, plasma and acrosome membranes integrity and subsequently fertility are multifactorial, however an oxidative stress seems to be the essential contributing factor [41].

Numerous studies have been preformed to evaluate the effect of antioxidants' addition in freezing extenders on mammalian spermatozoa. Moreover, several modifications have been introduced to diminish the occurrence of oxidative stress in sperm by changing the environmental conditions or by the use of antioxidants during freezing and thawing of semen.

The sulphur-containing amino acid cysteine scavenges free radicals through direct chemical interactions with them [6,16]. NAC is a precursor for the intracellular cysteine and glutathione biosynthesis and also participates in the glutathione metabolism, where it acts as a stimulant of cytosolic enzymes. Then, SOD, is an enzymatic biological antioxidant, which generally scavenges the reactive oxygen species (ROS), such as superoxide anion and hydroxyl radicals, and thus controls oxidative stress in the mammalian sperm [25].

In recent studies, the supplementation of freezing diluents with cysteine has been shown to improve the post-thawed sperm functions of dogs [30], cats [42], rams [9], bulls [6,38], or to provide the protective effect against the DNA bulls sperm damage [44]. Moreover, it is worth mentioning that the addition of superoxide

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dismutase to the semen extenders has had controversial effects. Some authors reported the protective effect of SOD on sperm [5,21] but the others detected the increase in sperm DNA defragmentation [4].

To our knowledge, the role of cysteine and superoxide dismutase in the cryopreservation diluent, against cryodamage of chicken sperm, has not yet been investigated. Therefore, the aim of this study was to determine the influence of the above mentioned antioxidants on chicken sperm motility, plasma membrane and acrosome integrity, mitochondrial activity, LPO and apoptotic changes.

Materials and methods

Chemicals

The fluorescent probes (Live/Dead Sperm Viability Kit: SYBR-14, propidium iodide (PI); PNA from *Arachis hypogaea* Alexa Fluor® 488 conjugate; JC-1; C_{11}-BODIPY$^{581/591}$; Annexin V) were purchased from Life Technologies Ltd, Grand Island, NY, USA. The antioxidants N-acetyl-L-cysteine (NAC, #A7250), superoxide dismutase (SOD, #S5395) and all the remaining components were purchased from Sigma–Aldrich St. Louis, MO, USA.

Extender

A EK diluent (1.4 g sodium glutamate, 0.14 g potassium citrate × H_2O, 0.7 g glucose, 0.2 g D-fructose, 0.7 g inositol, 0.1 g polivinylpyrrolidone, 0.02 g protamine sulfate, 0.98 g anhydrous sodium hydrogen phosphate, 0.21 g anhydrous sodium dihydrogen phosphate were diluted to 100 mL with distilled water; pH 7.3, osmotic pressure 390 mOsmol/kg) [34] was used as the basic extender. Such extender was used throughout the whole experiment and was supplemented with the studied concentrations of antioxidants.

Animals and semen collection

The experiment was conducted on 15 mature males of Green-legged Partridge breed kept individually in cages (60 × 50 × 75 cm) at 18–20 °C, under 14L:10D photoperiod. Birds were fed with commercial feed for breeding flocks, without additional antioxidant supplementation. Water was provided *ad libitum*.

The pooled semen was collected twice a week, by the dorso-abdominal massage method [12]. The collection was always performed by the same people and under the same conditions. Only clean ejaculates were used for further analysis.

Dilution and cryopreservation procedure

Each pooled semen sample was divided into three aliquots and subjected to cryopreservation. Before freezing the three aliquots were diluted with the basic extender containing 5 mM of NAC, 200 U/mL of SOD and no antioxidant (control) at the ratio of 1:2.

Semen samples were frozen in accordance with the procedure adapted from Tselutin et al. [43]. Diluted samples were stored for 15 min at −8 °C, then dimethylacetamide (DMA) was added to a final concentration of 6% and after 3 min of equilibration the solution was pipetted and plunged drop-by-drop directly into the liquid nitrogen. After 3 months of storage frozen semen was thawed in water bath at 60 °C for 6 s.

Fifteen semen collections and freezing procedures were performed

Assessment of sperm quality

Sperm motility parameters

Sperm motility was evaluated in semen diluted 1:100 in DMEM (Dulbecco modified medium low glucose) [7] using computer assisted semen analyzer (CASA) Hamilton-Thorne Sperm Analyser IVOS version 12.2 l (Hamilton Thorne Biosciences, MA, USA) under 1.89 × 10 magnification. 3 μl aliquot of semen was placed in Leja4 analysis chamber (Leja, Nieuw–Vannep, Netherlands) at 35 °C and evaluated. Five field randomly selected by the computer were analyzed for each semen sample. The parameters measured were: the percentage of motile sperm (MOT), the percentage of progressively motile spermatozoa (PROG), path velocity (VAP, average velocity/smoothed average position of the spermatozoa), progressive velocity (VSL, straight line distance between the beginning and the end of the track), curvilinear line velocity (VCL, average velocity measured over the actual point-to-point track followed by the cell), straightness (STR, a measure of VCL side to side movement determined by the ratio VSL/VAP × 100), linearity (LIN, a measure of the departure of the cell track from a straight line; the ratio VSL/VCL × 100), percentage of rapid spermatozoa (RAPID).

Plasma membrane integrity

Sperm membrane integrity was determined by double-fluorescent labeling technique, according to the protocol described by Partyka et al. [34]. Briefly, 300 μL of the diluted samples were stained with 5 μL of SYBR-14 (commercial solution diluted 50-fold) and 5 μL of PI. The PI negative and SYBR-14 positive population showing green fluorescence was considered live, with sperm plasma membrane intact (PMI).

Acrosome integrity

Sperm acrosome status was assessed by lectin PNA from *Arachis hypogaea* Alexa Fluor® 488 conjugate. Diluted semen samples were mixed with 10 μL of PNA working solution (1 μg/mL) and incubated for 5 min in room temperature in dark. After incubation, the samples were washed and 5 μL of PI were added before cytometric analysis [34].

Mitochondrial activity

Sperm mitochondrial activity was determined using staining with the JC-1 and PI. The 3 mM stock solution of JC-1 in DMSO was prepared. From each sample, 500 μL of a sperm solution containing 50 × 10^6 cell/mL was stained with 0.67 μL JC-1 stock solution. The samples were incubated at 37 °C in the dark for 20 min before flow cytometric analysis. Sperm emitting orange fluorescence were classified as high mitochondrial activity, emitting both green and orange fluorescence as medium, and emitting only green fluorescence as low mitochondrial activity.

Lipid peroxidation

Lipid peroxidation was evaluated using a fluorescent lipid probe C_{11}-BODIPY$^{581/591}$ as we described before [31]. This probe emits orange fluorescence in its non-oxidized state, shifting to green fluorescence when peroxidized. Diluted samples were incubated with 1 μL of 2 mM C_{11}-BODIPY$^{581/591}$ in ethanol and incubated for 30 min at 37 °C in the dark. Samples were then centrifuged at 500*g* for 3 min and the sperm pellets were resuspended in 500 μL of EK. To determine viability sperm was loaded with PI and incubated further for 5 min at room temperature before cytometric analysis.

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