EFFECT OF GLUTATHIONE SUPPLEMENTATION TO SEMEN EXTENDER ON POST-THAWED ROOSTER SPERM QUALITY INDICES FROZEN AFTER DIFFERENT EQUILIBRATION TIMES

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Abstract

BACKGROUND: Avian sperm is susceptible to lipid peroxidation, compromising their fertility. The semen antioxidant system protects sperm plasma membrane against reactive oxygen species. OBJECTIVE: The study evaluates the effect of glutathione (GSH) addition to semen extender during different equilibration times (ET) on rooster sperm cryopreservation. MATERIALS AND METHODS: Semen samples were weekly collected from 60-week-old broiler breeder roosters. Collected samples were pooled and divided to six equal parts and frozen according to a randomized design (2 × 3 factorial arrangement). Treatments included adding two levels of GSH [0 (GSH-0) or 1 (GSH-1) mM] to semen extender during three ET: 0 (ET-0), 4 (ET-4) or 8 (ET-8) hours. Post-thawed motility and velocity parameters, apoptotic like changes, plasma membrane functionality, and mitochondrial membrane potential (MMP) were evaluated. RESULTS: Post-thawed total motility is improved in the GSH-1 compared to the GSH-0 group (P<0.10). Total motility responded quadratically to increasing levels of ET such that the highest value is recorded at ET-0. Although progressive motility (PM) is not affected by GSH or ET, the highest PM is obtained in the GSH-1×ET-0 group (P<0.05). The VAP and STR is improved in the GSH-1 compared to GSH-0 group; however, VAP decreases quadratically, and STR decreases linearly as ET is advanced (P<0.05). The interactive effect of GSH by ET tends to affect the wobble coefficient (WOB), such that the highest value is recorded in the GSH-1×ET-0 group. Within both GSH supplemented and control groups, the amplitude of lateral head displacement (ALH) is highest (P<0.05) in the ET-0 group. The percentage of live spermatozoa quadratically decreases and the percentage of dead sperm quadratically increases in response to graded levels of ET (P<0.01). The highest plasma membrane functionality is also noted in the GSH-1×ET-0 group (P<0.05). Mitochondrial membrane potential quadratically decreases in response to increasing levels of ET (P<0.05). CONCLUSION: Generally, GSH supplementation to rooster sperm extender has some beneficial effects on post-thawed sperm motion characteristics, but does not positively interact with ET.

Keywords: cryopreservation; glutathione; rooster, semen extender.
INTRODUCTION

Unsaturated lipids play a critical role in membrane fluidity and integrity, as well as the biological functions of cells. Avian sperm plasma membrane is a poly-unsaturated fatty acids-enriched construct that is susceptible to lipid peroxidation (LPO). The antioxidants in semen protects the sperm plasma membrane against reactive oxygen species (ROS) originating from metabolism in active spermatozoa. During semen cryopreservation, however, LPO is intensified as ROS production overwhelms the defensive antioxidant system. The increased LPO can reduce sperm quality and fertility (4, 12).

The importance of antioxidant system on ROS scavenging and neutralization is more prominent in cryopreserved sperm. There is a close relationship between the compromised function of poultry sperm and the LPO level produced during in vitro storage (10). Ogbuewu et al. (28) demonstrated that by increasing Ca$^{2+}$ leakage, the sperm trigger a cellular cascade which in turn stimulates LPO and apoptosis during cooling. Several antioxidants such as catalase, glutathione (GSH), glutathione peroxidase and superoxide dismutase (SOD) are involved in the defense mechanism against LPO to keep sperm functionality (8).

The addition of natural herbal antioxidants (1, 21) or synthetic antioxidant (33, 34) to the semen extender improves the quality of the fresh or frozen avian sperm. Glutathione is a tripeptide with antioxidant property of its thiol groups. GSH involves in cellular processes including metabolism, cell division, protein metabolism and catabolism, gene expression and extra cellular matrix synthesis (22). Effects of GSH on protection of cellular components against free radicals, peroxides, and heavy metals have been documented (29). Anel-López et al. (2) reported that supplementation of 1- or 5-mM GSH to the cryopreservation medium for 2 h improves the post-thawed physiological quality and kinematics of red deer spermatozoa. The higher quality of sperm extended by medium supplemented with GSH was also reported in boar (16) and human (17). More recently, Shamiah et al. (32) suggested that 0.2 mM GSH in the extender ameliorates the adverse effect of rooster sperm storage and increased the fertility rate. These findings have demonstrated GSH as an effective antioxidant to the sperm extender. Although the addition of 0.1-1 mM GSH to the thawing medium has shown to decrease the motility and fertility in rooster (35), Masoudi et al. (24) reported the beneficial and adverse effects of GSH to freezing medium on post-thawed rooster sperm quality indices at concentrations of 1 and 4 mM, respectively.

In addition to the type of cryoprotectants, the equilibration time (ET) before freezing can affect the post-thawing sperm quality. There are evidences that optimal ET may improve the post-thawed quality of frozen spermatozoa via longer exposure to antioxidants. In bull, for instance, the optimal ET improved the efficacy of the antioxidant and cryoprotectants, leading to higher post-thawed plasma membrane integrity and viability (26). The optimal ET varies among species and extenders (7, 20, 23, 26). In roosters, however, the optimal ET is associated to the kind and proportion of cryoprotectants in the extender (31). In this regard, Moghbeli et al. (25) showed that in an 11% glycerol-base extender, a 2-h ET is associated with the best outcome, while the extenders containing 5% glycerol a 3-h ET is suggested (15). A recent study (3) used 60-min ET in presence of dimethyl sulfoxide for the freezing of Arian rooster spermatozoa. Another study showed that ET has no significant effect on the motility and acrosome integrity of Spanish poultry breeds spermatozoa when dimethyl-acetamide is used as cryoprotectant (31).

This study is conducted to examine whether the supplementation of the freezing medium with GSH would improve the post-thawed rooster spermatozoa quality indices.

MATERIALS AND METHODS

The Research Ethics Committee of Tehran University approved the present study. The study was conducted at Department of Animal Science, College of Agriculture and Natural Resources, University of Tehran, Iran.

Chemicals

Chemicals used in the present experiment were provided from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Birds and experimental design

Six 58-weeks-old broiler breeder roosters (Ross 308) are selected from a commercial flock.
and were individually caged (70×70×85 cm) under 14 h light (L):10 h dark (D) photoperiod and 22-24°C ambient temperature, during a 7-week period. Birds were fed with a standard diet containing 2754.57 Kcal ME kg⁻¹, 12% crude protein, 0.9% calcium and 0.45% available phosphate. Roosters were habituated to the conditions and abdominal massage (9) for two weeks. Thereafter, semen samples are then weekly collected from each individual rooster and placed in a water bath (37 °C) for primary quality assessment. The samples with the primary quality criteria (normal appearance, 0.3–0.6 mL ejaculation volume, at least 3 × 10⁹ spermatozoa mL⁻¹ and 80% motility) were pooled to eliminate individual differences between roosters. Pooled samples were then aliquoted into six sub-samples to evaluate effects of adding GSH (0 or 1 mM) in semen extender during the increasing levels of ET (0, 4 and 8 h) in six groups (a 2×3 factorial).

Extender preparation and cryopreservation

The modified poultry Beltsville semen extender (mPBSE) containing 8.67 g L⁻¹ sodium glutamate, 7.59 g L⁻¹ dipotassium phosphate, 3.2 g L⁻¹ sodium acetate, 5 g L⁻¹ fructose, 3.2 g L⁻¹ Tris, 0.64 g L⁻¹ potassium citrate, 0.34 g L⁻¹ magnesium chloride, 0.7 g L⁻¹ monopotassium phosphate is used. Soybean lecithin and DMSO were added to the basic medium at 0.5% (w/v) and 4% (v/v), respectively, with pH of 7.1 and osmolality of 310 mOsm kg⁻¹ (36). Samples were diluted with the freezing medium to meet the above mentioned experimental treatments. Diluted samples in a final concentration of 400×10⁶ spermatozoa mL⁻¹ were then aspirated into 0.25 mL French straws (IMV, L’ Aigle), sealed with polyvinyl alcohol powder and equilibrated at 5°C for different ET. In a 40×20×20cm cryobox containing liquid nitrogen (LN), straws were then frozen in LN vapour, 4 cm above the liquid nitrogen for 7 min. Thereafter, the straws were plunged into liquid nitrogen and stored until further analysis.

Evaluation of post-thawed sperm motions

A computer assisted semen analysis (CASA) fitted with the sperm class analyzer (SCA) software (Ver 5.1; Microptic, Barcelona, Spain) was used to analyze motion characteristics of the post-thawed sperm. Motility was evaluated using a preheated (37°C) Makler chamber with a phase-contrast microscope. Parameters measured included total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, μm s⁻¹), straight linear velocity (VSL, μm s⁻¹), average path velocity (VAP, μm s⁻¹), linearity (LIN, %), the amplitude of lateral head displacement (ALH, Mm), track straightness (STR, %), and wobble coefficient (WOB: VAP/VCL).

Membrane functionality

Hypotonic swelling test was carried out to evaluate plasma membrane functionality. Briefly, 5 μL semen with a 50 μL hypoosmotic solution (100 mOsm kg⁻¹, 57.6 mM fructose and 19.2 mM sodium citrate, pH=7) was incubated at 37 °C for 30 min. A sample was then prepared on a slide and the percentage of spermatozoa with a swollen “bubble” around the curled flagellum were considered as sperm with functional plasma membrane using a phase contrast microscope (1000x). A total of 200 spermatozoa per slide in five microscopic fields were observed to recorded sperm with functional membrane.

Phosphatidyl serine externalization

The externalization of phosphatidyl serine, an earliest indicator of apoptotic-like changes, was determined in the semen sample (19). Following washing semen samples in calcium buffer, the sperm concentration was readjusted to 1×10⁹ spermatozoa mL⁻¹ and a 10 mL Annexin V–FITC (0.01 mgmL⁻¹) was added to a 100 mL of the semen suspension. After incubation on ice for 20 min, 10 mL of propidium iodide (PI; 1 mg mL⁻¹) was then added to semen suspension and re-incubated on ice for additional 10 min. The apoptotic-like status was classified into three groups: A'/P⁺, viable non-apoptotic; A+/P⁺, apoptotic like; A'/P⁺ and A'/P⁺, dead.

Mitochondrial membrane potential

A fluorescent lipophilic cationic dye (JC-1; Invitrogen TM, Eugene, OR, and PI TM, Eugene, OR) was exercised to value sperm mitochondrial membrane potential. Concisely, 300 μL of diluted semen samples (containing 2×10⁶ spermatozoa) are supplemented with five μL of JC-1 solution and then incubated for 15 min in the dark (at 37°C). Afterwards, the achieved samples were washed and then evaluated by means of a flow cytometer device. JC-1-stained spermatozoa with green fluorescence (mitochondria with low membrane potential) were detected in FL1 channel and those with red
fluorescence (mitochondria with high membrane potential) were detected in FL2 channel (15).

**Flow cytometer procedure**
A FACS Calibur (Becton Dickinson, San Khosoz, CA, USA) flow cytometer was used. For each assay, 10,000 events were recorded at a flow rate of 100 cells s\(^{-1}\). Sperm population was gated using 90° and forward-angle light scatter to exclude to restrict the analysis to sperm and to eliminate small debris and other particles. The excitation wavelength was 488 nm supplied by an argon laser at 250mW; Green fluorescence (FL1) was measured using a 530/30 nm band pass filter and red fluorescence (PI, FL2) is measured using a 585/42 nm filter. The flow cytometry data was analyzed using FlowJo software (Treestar, Inc., San Carlos, CA).

**Statistical analysis**
Data were analyzed in a 2×3 factorial randomized design, using GLM procedure of SAS software. Before analysis, the normality of data was tested by UNIVARIATE procedure and Shapiro-Wilk. An orthogonal polynomial contrast was conducted to find linear and quadratic effects of increasing levels of ET on dependent variables (21, 27). Once that interaction effect of GSH×ET was significant, in addition to Tukey’s multiple comparison tests, an orthogonal polynomial contrast was separately conducted within each level of GSH to find the response of the dependent variable to graded levels of ET.

Data are reported as least squares mean and standard error (SEM), and significant differences and tendencies are declared at \(P<0.05\) and 0.05 \(<P<0.10\), respectively. The mathematical model used is \(Y_{ijk} = \mu + A_i + B_j + (A\times B)_j + e_{ijk}\), in which \(Y_{ijk}\) is the observations; \(\mu\) is the overall mean; \(A_i\) is the fixed effect of glutathione (GSH); \(B_j\) is the fixed effect of ET j; \((A\times B)_j\) is the two-way interaction of GSH i by ET j, and \(e_{ijk}\) is the residual random error.

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**Table 1.** Least squares mean for the effects of adding glutathione (GSH) to the freezing medium and equilibration time on the motility parameters of the rooster spermatozoa.

<table>
<thead>
<tr>
<th>GSH (mM)</th>
<th>Equilibration time (h)</th>
<th>TM (%)</th>
<th>PM (%)</th>
<th>VCL (μm s(^{-1}))</th>
<th>VSL (μm)</th>
<th>VAP (μm s(^{-1}))</th>
<th>LIN (μm s(^{-1}))</th>
<th>STR (%)</th>
<th>WOB (%)</th>
<th>ALH (μm s(^{-1}))</th>
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SEM: Standard error; TM: total motility; PM: progressive motility; VCL: curvilinear velocity; VSL: straight line velocity; VAP: average path velocity; LIN: linearity; STR: sperm track straightness; WOB: wobble coefficient (VAP/VCL); ALH: amplitude of lateral head displacement.

*Orthogonal polynomial contrast (linear + quadratic) analysis is conducted for motion characteristic response to ET.

**The interactive effect of GSH×ET on PM and ALH are presented in Figures 1 and 2, respectively.
RESULTS

Table 1 shows the effects of GSH and ET on the post-thawed sperm motion and velocity characteristics. Glutathione supplementation improves (P<0.10) the post-thawed TM. Total motility responded quadratically to increasing levels of ET such that the highest value is recorded in ET-0 group. A significant interaction of GSH×ET (Fig. 1) shows that PM quadratically responds (P < 0.01) to graded levels of ET in the GSH-0 group, whereas the response (P < 0.01) is linear in GSH-1 group.

The highest PM is obtained in GSH-1×ET-0 group (P<0.05). The effect of GSH and its interaction with ET do not affect VCL and LIN (P>0.05), but VCL and LIN decreases linearly as ET increases (P<0.01). VAP and STR increases in the GSH-1 compared to the GSH-0 group; however, VAP decreases in a quadratic and STR decreases in a linear manner in response to graded levels of ET (P<0.05; Table 1). GSH and ET do not influence WOB (P>0.05), but the interactive effect of GSH and ET tends to affect WOB, such that the highest values is recorded in GSH-1×ET-0 group (P<0.08). The significant interaction between GSH and ET on ALH shows a quadratic reduction of ALH in response to the graded levels of ET within both GSH-1 and GSH-0 groups (Fig. 2). However, in comparison to the GSH-0 group, the ALH is higher in the GSH-1 group before ET of 4 h, but is lower onward.

Table 2 shows the effects of ET and GSH supplementation to the extender on the viability, apoptosis status, plasma membrane functionality (HOST), and MMP of rooster spermatozoa. Glutathione and its interaction with ET have no significant effect on the percentage of live and dead spermatozoa; however, the percentage of live spermatozoa decreases in a quadratic manner, and the percentage of dead spermatozoa increases in response to increasing ET (P<0.01). Glutathione and ET do not affect the percentage of apoptotic spermatozoa (P>0.05). There is a significant interaction between GSH and ET on plasma membrane functionality (P<0.01; Fig. 3) such that the highest membrane functionality is in the GSH-1×ET-0 group (P<0.05). Glutathione supplementation and its interaction with ET do not affect MMP (P>0.05), but it quadratically decreases as ET increases (P<0.05).
DISCUSSION

An increased ROS level in the sperm freeze-thaw process compromises sperm quality and fertilizing capacity. This study has investigated the potential effect of adding GSH to semen extender for different equilibration times on the post-thawed rooster semen quality.

The supplementation of GSH to the semen extender improves the post-thaw TM, VAP and STR parameters. The interactive effects of GSH and equilibration time reveals that PM, ALH, and plasma membrane function are improved in the GSH-1×ET-0 group. The beneficial effects of GSH on sperm functions were reported in avian and mammalian species before (14, 32), suggesting that GSH supplementation may compensate the decreased antioxidant capacity. Similar to the present study, the addition of 0.5 - 1.0 mM GSH to semen extender reduces DNA fragmentation of buffalo sperm (13) and improves fertility rate. Improved effect of GSH on the post-thaw TM, PM, membrane integrity and MMP of rooster [of 2- or 4- mM; (24)], and motility, viability, acrosome and membrane integrity of equine [2.5 or 5 mM GSH (11)] spermatozoa were also reported.

In comparison to GSH-0, GSH-1 shows higher PM, ALH and membrane functionality at ET-0. This results shows that longer exposure to GSH results in decreased sperm quality in some parameters. Although the discrepancy is not fully understood, an adverse effect of GSH supplementation in the thawing medium on rooster sperm motility and fertilizing ability has been reported before (35). It is suggested that the adverse effect could relate to low membrane stability at high GSH concentration when GSH in semen is in the normal range (6).

The improved membrane function in the GSH-1 group is explained by GSH antioxidant property and the resulting lower ROS production after freeze-thaw. Similar outcomes have been reported by other researchers working on avian and mammalian species (2, 17, 18, 24). Masoudi et al (33) showed that the 2-4 mM GSH addition had an effect on the rooster’s sperm mitochondria, while Gangwar et al (26) showed that GSH decreases the deleterious effect of ROS on mitochondrial activity of bull sperm. The inconsistency seen in different studies is probably associated with GSH concentrations. As MMP is not affected by GSH, it is postulated that the enhanced velocity parameters is a consequence of the membrane-protecting effect of GSH. In agreement with the present results, GSH addition to the extender improves the motility of thawed spermatozoa of boar, stallion and human and in vitro ability of sperms’ oocyte penetration (16, 17, 18, 37).

Although GSH addition does not change significantly the apoptotic level of spermatozoa

| Table 2. Least squares mean for the effects of adding glutathione (GSH) to the freezing medium and equilibration time (ET) on roosters’ spermatozoa viability, apoptosis status, plasma membrane functionality (HOST), and mitochondrial membrane potential (MMP). |

<table>
<thead>
<tr>
<th>Main effects</th>
<th>Parameter</th>
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<td>GSH (mM) ET (h)</td>
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<td>Quadratic</td>
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<tr>
<td>GSH×ET</td>
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</table>

SEM: Standard error; MMP: Mitochondria membrane potential; HOST: hypo-osmotic swelling test.

*Orthogonal polynomial contrast (linear + quadratic) analysis was conducted for variables response to ET.

**The interactive effect of GSH×ET on HOST is presented in Figure 3.
when assessed immediately after thawing, there is a possibility that GSH supplementation helps to maintain quality during the post-thaw period (16-18). Several studies show that the addition of different antioxidants to semen may exert an effect more efficiently during this period (16, 17, 18). However, these findings are in contrast with the results of (24) who reported a decrease in post-thawed rooster’s apoptotic spermatozoa by adding 2 mM GSH to the extender. They stated that the supplementation of rooster semen extender with GSH decreased the percentage of apoptotic cells due to its ability to protect the sperm membrane against deleterious ROS effects.

In agreement with the results of Shamiah et al. (32) who reported no interaction between GSH supplementation and storage period on the freshly stored cockerel semen, many of the evaluated parameters in the present study are not affected. Nevertheless, the findings indicate that ET is negatively associated with the post-thaw sperm quality as previously reported in stallion (37). In several species, the optimal ET is associated with the improved post-thaw sperm quality characteristics (7, 23, 26, 31). It is a species-specific feature and depends on the type of cryoprotectant and extender. Belala et al. (7) showed that increasing pre-freezing ET from 30 min to 6 h improves post-thawed TM, PM, VAP and VSL, and decreases ALH and VCL of canine spermatozoa. This effect is assumed to be a consequence of changes in spermatozoa membrane cryotolerance or their adaptation to low temperatures (30). By increasing ET up to 5 h, Igna et al. (20) demonstrated that canine spermatozoa motion parameters decreased due to the production of toxic substances or the toxicity of the extender components, especially cryoprotectants (7). In accordance, the results of the present study reveal that prolonged GSH exposure in the pre-freezing period not only has no positive interaction on post-thawed sperm quality, but also fails to ameliorate adverse effects of pre-freezing storage damage. Sperm quality decreases almost quadratically as ET increases. This is probably due to detrimental effects of storage as ET is advanced. Sperm is metabolically highly active and produces remarkable levels of ROS during storage (12). Therefore, the progressive production of ROS during pre-freezing time of sperm storage may overwhelm the antioxidant protective defense of semen even in the GSH-supplemented group. In addition, there are evidences that shows sperm quality parameters are non-linearly changed in response to GSH supplementation in freshly stored semen, where respective supplementation of GSH higher than 0.2 and 1.2 mM to rooster (32) and bull (14) semen extender decreases sperm quality via possible GSH toxicity. In this regard, Oliveira et al. (11) showed that the addition of more than 2.5 mM GSH reduces post-thawed total motility of frozen equine sperm, whereas lower doses of GSH enhanced progressive motility. It is documented that high GSH level has no beneficial effect on equine (5), rooster (24) and boar (16) spermatozoa. Longer exposure of spermatozoa to GSH during pre-refreezing period may result in a lower quality of sperm in GSH-supplemented groups with increasing ET.

In conclusion, the addition of GSH to the rooster semen extender is associated with some improved post-thawed sperm quality indices. The pre-freezing exposure of GSH not only has no positive interaction on the post-thawed sperm quality, but also could not ameliorate the pre-freezing adverse effect of semen storage.

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