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Supplementation of lake extender with cysteamine preserves quality parameters and fertility potential of post-thawed rooster sperm during cryopreservation process

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Abstract

Background In the cryopreservation process, rooster spermatozoa are vastly sensitive to reactive oxygen species (ROS). This study aimed to investigate the effects of Lake extender supplemented via Cysteamine (CYS) on the quality and fertility characteristics of rooster semen during the cryopreservation process.

Methods Semen samples were collected from 10 proved Ross-308 roosters, diluted and cryopreserved in the Lake extender which contained 0, 1, 2, 4, and 8 mM of CYS (C-0, C-1, C-2, C-4, and C-8, respectively). Motility parameters, membrane integrity, abnormal morphology, mitochondrial activity, acrosome integrity, viability, apoptosis status, lipid peroxidation, DNA fragmentation, ROS concentration, as well as fertility potential were evaluated after thawing.

Results total motility and progressive motility were higher ($P \leq 0.05$) in C-1 and C-2 compared to the other groups. The C-1 showed higher ($P \leq 0.05$) membrane integrity, mitochondrial activity, acrosome integrity, viability, and lower ($P \leq 0.05$) late apoptosis, lipid peroxidation, DNA fragmentation, and ROS concentration compared to the other groups. During fertility evaluation, C-1 presented a higher fertility rate than the control group. In cases of velocity parameters, abnormal morphology, early apoptosis, necrosis, and hatching rate, no significant difference ($P > 0.05$) was found.

Conclusion using the optimal concentration of CYS (1 mM) in the Lake extender is useful for protecting rooster sperm during the cryopreservation process and it could be used for reproductive programs.

Keywords Apoptosis status, Cryopreservation, Cysteamine, Fertility, Rooster

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Background

Researchers have been trying to optimize the cryopreservation media to improve reproductive performance in the breeding poultry flocks; However, the processes like freeze-thaw process induce structural and biochemical shocks on sperm, mostly because of the reduction in sperm quality after the freezing-thawing process [1]. Reactive oxygen species (ROS) reduce the fertility potential of post-thawed sperm via decreasing sperm motility and fertilization capacity [2]. To overcome this issue, antioxidant supplementation has been reported to show an effective approach to protect sperm quality against destructive shocks during the cryopreservation process [1].

Cysteamine (CYS), known as 2-mercaptoethylamine or amino ethanethiol, is the decarboxylated derivative of the amino acid cysteine. In cells, the amino Thiol is derived from the degradation of coenzyme A, which in turn, is obtained by cysteine and vitamin B5 (Pantothenate). Coenzyme A degradation yields pantetheine, which is hydrolyzed via pantetheinase or vanin, generating CYS and pantothenic acid. The CYS is converted to hypo-taurine through enzyme activity Cysteamine decarboxylase. Cysteine, a component of coenzyme A, is performed on by cysteine dioxygenase to form cysteine sulfonate that is decarboxylated by cysteine sulfonate decarboxylase to form hypo-taurine [3]. Both CYS and its oxidized form, Cystamine, have been reported to show protective effects in cells and tissues such as antioxidant effects on increasing cysteine and glutathione concentration [3].

No research has yet been done to investigate the impact of different concentrations of CYS in Lake extender on the quality parameters of rooster's sperm after the freeze-thaw process. Therefore, this research aimed to evaluate the effect of supplementing the Lake extender with the different concentrations of CYS on the motility parameters, abnormal morphology, membrane integrity, mitochondrial activity, viability, lipid peroxidation, acrosome integrity, apoptosis status, DNA fragmentation, ROS concentration as well as fertility potential of rooster's sperm during the freeze-thaw process.

Materials and methods

Chemicals, and ethics approval

Chemicals were provided by Sigma (St. Louis, Missouri, USA) and Merck (Frankfurter Str. 250, Darmstadt, Germany) companies. Research Ethics Committees in the Animal Science Research Institute of Iran (ASRI) approved all of the methods that were applied in this study (IR. ASRI. REC.2019.1019).

Preparation of semen samples

Ten 32-week-old commercial strains of Ross broiler breeder roosters *(Gallus domesticus*) were randomly selected from the research breeder flock of the Department of Animal Sciences, Tarbiat Modares University. Experimental roosters were individually housed in cages (70 × 60 × 75 cm) at a temperature range between 20 and 23 °C, under a photoperiod of 15-hour (h) light: 9 h dark with lights on at 07:00 and lights off at 22:00, food and water provided *ad libitum*. The roosters' semen samples were obtained twice weekly via abdominal massage. Then, the obtained samples were placed in the water bath (37 °C) and transported to the laboratory for the preliminary investigation. Samples with the following characteristics were assigned for the next steps of this study: >0.2 mL volume, $>3\times10^9$ sperm cells/mL concentration, >80% total motility, and >85% normal morphology. Selected samples were finally pooled for omitting the male individual differences.

Freezing and thawing processes

In this study, the Lake extender was used for semen cryopreservation, which contained D- Potassium citrate (0.25 g/50 mL), Glycine (0.187 g/50 mL), Magnesium acetate (0.035 g/50 mL), fructose (0.4 g/50 mL), Polyvinylpyrrolidone (0.15 g/50 mL), Sodium glutamate (0.96 g/50 mL), and Glycerol (3% v/v) [4]. Osmolarity and PH were adjusted to 7.1 and 310 mOsm/kg, respectively. The different concentrations of CYS were supplemented to the Lake extenders as follows: without CYS (control), 1 mM (C-1), 2 mM C-2), 4 mM (C-4), and 8 mM (C-8) CYS, respectively. Sperm samples were diluted with the freezing medium according to the experimental groups and then aspirated into 0.25 ml French straws (IMV, L'Aigle, France) to obtain a final concentration of 100×10^6 spermatozoa/straw. Straws were subsequently sealed with polyvinyl alcohol powder and equilibrated at 5 °C for a period of 3 h. After equilibration, the straws were frozen in liquid nitrogen vapor, 5 cm above the liquid nitrogen for 12 min in a 40 × 20 × 20 cm cryo-box contained 8000 cm^3 liquid nitrogen. The straws were subsequently plunged into liquid nitrogen for the storage. The frozen straws were individually thawed at 37 °C for 30 s in a water bath before the evaluation.

Evaluation of sperm quality

Sperm motility

Sperm class analysis software (Version 5.1; Microptic, Barcelona, Spain) was used to investigate sperm motility characteristics. For this purpose, roosters' semen samples, which were pooled, were diluted to 20×10^6 sperm/mL with PBS buffer, then 10 µL of semen was placed into a pre-warmed chamber slide (Leja 4; 20 mm height; 38 °C, Leja Products, Luzernestraat B.V., Holland), and roosters' sperm motion traits were determined. At least, six fields which contained a minimum of 400 sperm cells, were investigated for all samples at a 5-second average

time to read each sample. The following parameters, which were recorded, included: total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, m/s), straight-line velocity (VSL, m/s), linearity (LIN, %), and curvilinear velocity (VCL, m/s) [5].

Sperm membrane integrity

Hypo-osmotic swelling test (HOST) was performed according to Revell and Mrode's method to evaluate sperm cells with intact membrane [6]. Twenty µL of semen were added to 200 µL of the hypo-osmotic solution (100 mOsm/L, 55.5 mM fructose, and 19.2 mM sodium citrate). An inverted light microscope (×400 magnification) was used to assay sperm cells after 45 min incubating at room temperature. 200 spermatozoa were recorded in 5 various microscopic fields. Afterward, a number of sperm cells with swollen and non-swollen tails were recorded as intact and damaged membrane, respectively and the percentage was determined.

Sperm abnormalities

Sperm morphology was evaluated using Hancock solution [7]. In this regard, Fifty µl of each sample were added to Eppendorf tubes containing 1 mL of Hancock solution (62.5 mL formalin (37%), 150 mL sodium saline solution composed of 9.01 g NaCl in 500 mL double-distilled water, and 150 mL PBS buffer). 10 µL of mixture was put on a slide and covered with a cover slip. The abnormal acrosome rate (abnormal mid-pieces, tail defects, acrosome and cap abnormalities, and detached heads) was obtained by calculating 200 sperm cells under a phase-contrast microscope (×1000 magnification, oil immersion).

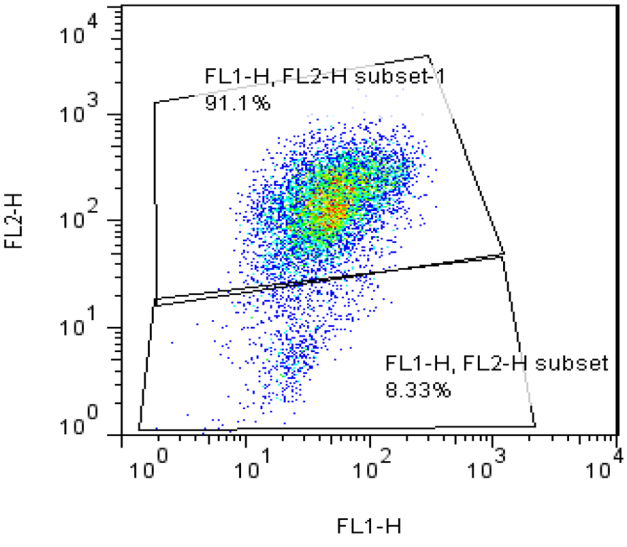

Fig. 1 Evaluation of mitochondrial activity via Rhodamin-123 (Rh123) and PI. Sperm cells with positive signal for Rh123 and negative signal for PI ($Rh123^+/PI^-$) were recorded as spermatozoa with active mitochondria

Acrosome integrity

Sperm acrosome integrity was evaluated via Pisum Sativum Agglutinin (PSA) in accordance with the method of Thys et al. 2009 [8]. According to this method, 5 µL of the semen sample experimental rooster was added to 100 µL ethanol. After 20 min, 10 µL of the suspended sample was added to 30 µL PSA (50 µg/mL) on a glass slide. 200 spermatozoa were then observed on the slide by a fluorescent microscope (BX51, Olympus) which was equipped with a FITC filter (×400 magnification) and fluorescence illumination. Sperm cells with green heads were recognized as the intact acrosome while those lacking green heads were revealed as the disrupted or damaged acrosome.

Flowcytometric assessment of mitochondrial activity, apoptosis status, DNA fragmentation, and ROS concentration

ROS concentration, DNA fragmentation mitochondrial activity, and apoptotic-like changes were evaluated by FACSCalibur (Becton Dickinson, San Khosoz, CA, USA) flow cytometer set [9]. At least, 10,000 spermatozoa were investigated per measurement at a flow rate of 100 cells/s. The sperm population was obtained through a forward-angle and 90° light scatter to eliminate aggregates and debris. The wavelength of excitation was 488 nm which was supplied through an argon laser at 250 mW. Green fluorescence (Rh123, Annexin V, and DCF) was detected on FL1 detector with a 530/30 nm band-pass filter, and red fluorescence (PI) was detected on FL2 detector with a 585/42 nm band-pass filter. Single-stranded DNA, as a sign of fragmentation which emits a red fluorescence, was identified at a 670-band pass filter (FL3). Obtained data were analyzed via FlowJo software (Treestar, Inc., San Carlos, CA).

To assess mitochondrial activity, a sperm suspension was made using 10 µL of Rhodamin-123 solution (Rh123, 0.01 µg/mL; Invitrogen TM, Eugene, OR, USA) which was added to 300 µL diluted semen and subsequently incubated in the dark room for 20 min. sperm suspension was then centrifuged at 500×g for 3 min and re-suspended, again, using 500 µL Tris buffer. Next, 10 µL of propidium iodide (PI, 1.0 µg/mL) was supplemented to the sperm suspension. Sperm cells with a positive signal for Rh123 and a negative signal for PI (Rh123+/PI-) were recorded as spermatozoa with active mitochondria (Fig. 1) [10].

Annexin V-FITC kit (IQP, Groningen, The Netherlands) and PI evaluated the externalization of phosphatidyl serine as an index of apoptotic-like changes in sperm cells [11]. The samples were washed in calcium buffer and adjusted to the concentration of 1×10^6 spermatozoa/mL followed by the addition of 10 µL Annexin V–FITC (0.01 µg/mL) to 100 µL of the sperm suspension, which were then incubated in a room with 20 min temperature

22 °C. Afterward, 10 µL of PI was supplemented to this suspension and at least incubated for 10 min in the room 22 °C. suspension which was subsequently evaluated by flow cytometer and sperm subpopulations, was classified into four groups: [1] viable cells were negative for both Annexin V and PI (A-/PI-); [2] early apoptotic cells were positive for Annexin V but negative for PI (A+/PI-); [3] late apoptotic cells were positive for both Annexin V and PI (A+/PI+); and [4] necrotic cells were negative for Annexin V but positive for PI (A-/PI+) (Fig. 2).

The Sperm Chromatin Structure Assay (SCSA) evaluated sperm DNA damage [12]. An aliquot of washed spermatozoa in PBS was diluted to a concentration of 1×10^6 spermatozoa/mL. This cell suspension was treated by an acid detergent solution which contained 0.15 mol/L NaCl, 0.1%, 0.08 M HCl, and Triton X-100 for 40 s, and was then stained via a phosphate-citrate buffer contained 6 µg/mL purified acridine orange. This purified compound, when associated with single-stranded DNA, emits a red fluorescence which is detected at a 670-band pass filter (Fl-3). The percentage of abnormal spermatozoa was calculated by the DFI frequency dot plot which was obtained via the ratio between the red and total (red plus green) fluorescence intensity (Fig. 3) [13].

Dichlorofluorescin diacetate (DCFH-DA) was used to assess the intracellular H_2O_2 content that was introduced as ROS concentration in this study [14]. Semen samples were washed with PBS to adjust the concentration of $3–5 \times 10^6$ spermatozoa/ml. 25 µM DCFH-DA was supplemented to sperm suspension and then incubated for 40 min at 25 °C. The suspension was then re-washed to remove the supernatant. PBS was supplemented to readjust the same sperm concentration ($3–5 \times 10^6$ spermatozoa/mL) at 25 °C (1000 rpm for 8 min) and 2 µL of PI (PI, 1.0 µg/mL) was added to the semen before analyzing with flow cytometry (Fig. 4).

Lipid peroxidation

Malondialdehyde (MDA), an index of sperm lipid peroxidation, was measured using thiobarbituric acid reaction [15]. Briefly, 1 mL of the diluted semen (250×10^6 sperm/mL) was mixed with 1 mL of cold 20% (w/v) Tricholoro acetic acid to precipitate protein. The precipitate was pelleted by centrifuging (960×g for 15 min), and 1 mL of the supernatant was incubated with 1 mL of 0.67% (w/v) Thiobarbituric acid in a boiling water bath at 100 ºC for 10 min. After cooling step, a spectrophotometer (UV-1200, Shima- dzu, Japan) was used to determine the absorbance at 532 nm. Each MDA concentration was indicated as nmol/ml.

The fertility performance

Fertility efficiency was evaluated by artificial insemination. Like roosters, broiler breeder hens were randomly

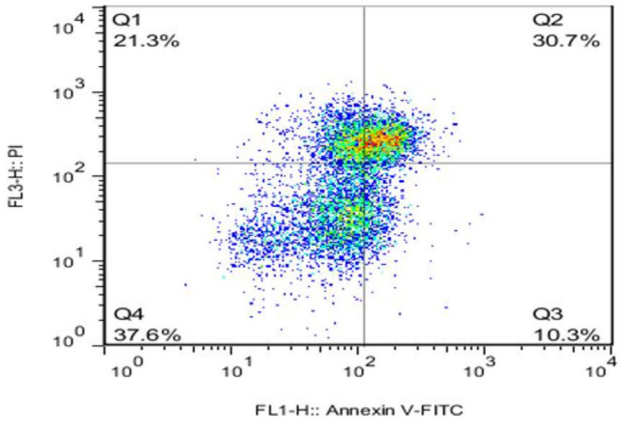

Fig. 2 Annexin V-FITC kit and PI were used to assess phosphatidyl serine externalization as an index of apoptosis in spermatozoa. Sperm subpopulations were classified into four groups: (Q1) live cells negative for both Annexin V and PI (A^-/PI^-); (Q2) cells with early apoptosis positive for Annexin V but negative for PI (A^+/PI^-); (Q3) cells with late apoptosis positive for both Annexin V and PI (A^+/PI^+); and (Q4) necrotic cells negative for Annexin V but positive for PI (A^-/PI^+)

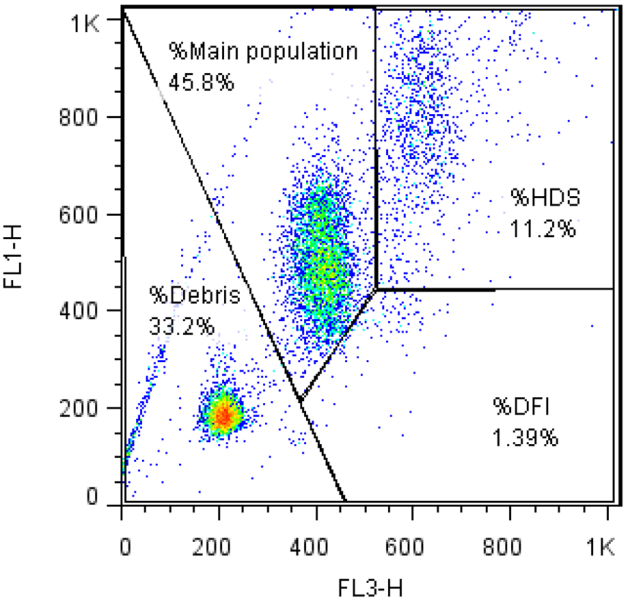

Fig. 3 Evaluation of sperm DNA fragmentation via Sperm Chromatin Structure Assay (SCSA) based on green (FL1) and red (FL3) fluorescence. Main population includes sperm without DNA fragmentation. The percentage of abnormal spermatozoa was calculated from the DFI frequency dot plot obtained from the ratio between the red and total (red plus green) fluorescence intensity

selected from the research breeder flock of poultry farm, Tarbiat Modares University, and housed at this farm. Breeder hens were divided into three groups (20 hens in each group). These groups were artificially inseminated with fresh semen and semen samples contained 0 and 1 mM CYS, respectively. The first group was artificially inseminated with fresh semen. The second group was artificially inseminated with post-thawed semen samples

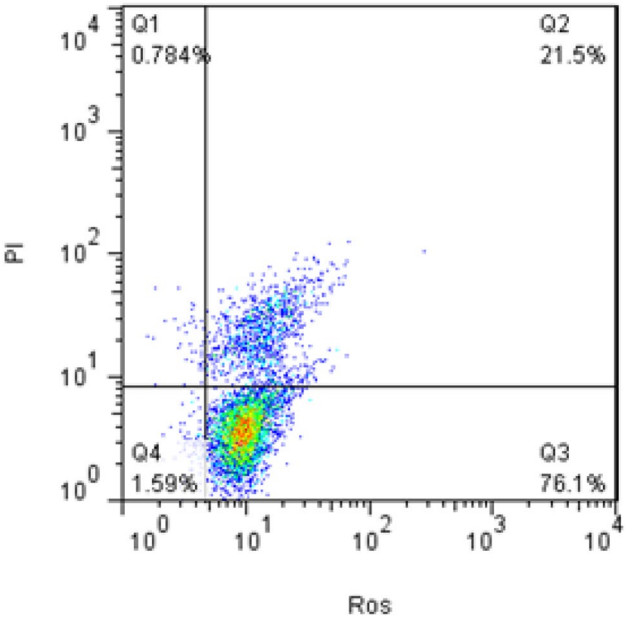

Fig. 4 Dichlorofluorescin diacetate (DCFH-DA) assessed the intracellular H_2O_2 content that introduced as ROS concentration based on fluorescence of $H_2O_2^-$ and PI. ROS^-/PI^- quadrant contains live cells without $H_2O_2^-$; ROS^+/PI^- quadrant contains live cells with $H_2O_2^-$; ROS^+/PI^+ quadrant contains dead cells with $H_2O_2^-$; and ROS^-/PI^+ quadrant contains dead cells without $H_2O_2^-$. The percentage of cells sperm with ROS^+ and PI^+ were detected in Q2

containing 0 mM CYS and the third group was artificially inseminated with post-thawed semen samples containing 1 mM CYS. Each straw (1/4 mL) contained 100×10^6 spermatozoa and artificial insemination was done twice weekly for one month. Approximately, 5 days following the last artificial insemination, the eggs (384 eggs in each group) were collected and selected to incubate. The fertility hatching rates were evaluated through eggs candling, 7 and 21 days after the beginning of incubation, respectively.

Statistical analysis

Levene's and Shapiro-Wilk's tests were used to check data normal distribution and homogeneity of variances, respectively. According to general linear model (GLM) Proc, data were analyzed and compared by Tukey's multiple range test using SAS software (SAS Institute, version 9.1, 2002, Cary, NC, USA). The analyzed results were presented as mean in addition to their standard error of the mean (SEM) for each group. Throughout the paper, results were considered significant when $P \leq 0.05$ and non-significant otherwise. GENMOD procedure using Chi-Square was applied to analyze fertility and hatching rates.

The used statistical model equation: $Y_{ij} = \mu + T_i + e_{ij}$.

Where: Y_{ij} is the jth observation of the ith treatment, μ is the population mean, T_i is the treatment effect of the ith treatment, and e_{ij} is the random error.

Results

Sperm motility and velocity parameters

Table 1 indicates the effects of supplementing the Lake extender with CYS on velocity and motility characteristics in the post-thawed rooster sperm. The TM and PM were significantly higher in the C-1 and C-2 treatments compared to the other treatments ($P \leq 0.05$). Different treatments significantly showed no difference in VCL, VAP, VSL, and LIN ($P > 0.05$).

Sperm membrane integrity, mitochondrial activity, abnormal morphology, and acrosome integrity

Table 2 indicates the effect of supplementing the Lake extender with CYS on membrane integrity, abnormal morphology, mitochondrial activity, and acrosome integrity of frozen-thawed rooster sperm. Membrane integrity was significantly found higher in C-1 when compared to the other groups ($P \leq 0.05$). The C-0 and C-2 treatments presented higher membrane integrity than the C-4 and C-8 treatments ($P \leq 0.05$). Cryopreservation medium, supplemented via different concentrations of CYS, was not significant on the rate of sperm cells with abnormal

Table 1 CYS effect ons sperm motility and velocity parameters after freezing-thawing process

	Treatments					
Parameters	**C-0**	**-1**	**C-2**	**-4**	**C-8**	**SEM**
TM (%)	42.3[b]	48.6[a]	46.5[a]	40.7[bc]	38.5[c]	1.1
PM (%)	19.3[b]	24.9[a]	22.4[a]	18.5[bc]	16.2[c]	1.3
VAP (µm/s)	40.5	42.2	41.5	40.8	39.9	2.0
VSL (µm/s)	35.4	36.3	34.8	33.9	34.0	1.8
VCL (µm/s)	50.1	51.3	51.6	49.4	48.7	1.5
LIN (%)	70.6	70.7	67.4	68.6	69.8	1.7

Different letters within the same row show significant differences among the groups ($P \leq 0.05$)

Treatment: C-0 = Lake extender without CYS, C-1 = Lake extender plus 1 mM CYS, C-2 = Lake extender plus 2 mM CYS, C-4 = Lake extender plus 4 mM CYS, C-8 = Lake extender plus 8 mM CYS

TM = total motility, PM = progressive motility, VAP = average path velocity, VSL = straight line path velocity, VCL = curvilinear path velocity, LIN = linearity

Table 2 CYS effects on membrane integrity, abnormal morphology, mitochondrial activity and acrosome integrity after freezing-thawing process

	Treatments					
Parameters	**C-0**	**-1**	**C-2**	**-4**	**C-8**	**SEM**
MI (%)	44.2^{b}	50.3^{a}	45.0^{b}	40.0^{c}	37.4^{c}	1.2
AM (%)	19.2	19.0	18.1	17.5	17.1	1.6
MA (%)	50.5^{b}	55.7^{a}	51.6^{b}	49.2^{bc}	46.1^{c}	2.0
AI (%)	64.5^{bc}	68.0^{a}	65.1^{b}	63.9^{bc}	62.8^{c}	1.0

Different letters within the same row show significant differences among the groups ($P \leq 0.05$)

Treatments: C-0 = Lake extender without CYS, C-1 = Lake extender plus 1 mM CYS, C-2 = Lake extender plus 2 mM CYS, C-4 = Lake extender plus 4 mM CYS, C-8 = Lake extender plus 8 mM CYS

MI = membrane integrity, AM = abnormal morphology, MA = mitochondrial activity, AI = acrosome integrity

Table 3 CYS effects on sperm viability, early apoptosis, late apoptosis and necrosis after freezing-thawing process

	Treatments					
Parameters	**C-0**	**-1**	**C-2**	**-4**	**C-8**	**SEM**
Viability (%)	45.5^{b}	50.0^{a}	46.0^{b}	41.5^{c}	34.3^{d}	1.2
Early apoptosis (%)	5.5	4.5	5.0	5.0	6.4	1.0
Late apoptosis (%)	41.8^{b}	39.0^{a}	42.5^{b}	46.0^{c}	51.1^{d}	1.1
Necrosis (%)	7.2	6.5	6.5	7.5	8.2	0.9

Different letters within the same row show significant differences among the groups ($P \leq 0.05$)

Treatment: C-0 = Lake extender without CYS, C-1 = Lake extender plus 1 mM CYS, C-2 = Lake extender plus 2 mM CYS, C-4 = Lake extender plus 4 mM CYS, C-8 = Lake extender plus 8 mM CYS

Table 4 CYS effects on MDA concentration, DNA fragmentation, and ROS concentration after freezing-thawing process

	Treatments					
Parameters	**C-0**	**-1**	**C-2**	**-4**	**C-8**	**SEM**
MDA (nmol/ml)	4.50^{b}	3.85^{a}	4.30^{b}	4.55^{b}	5.0^{c}	0.21
DF (%)	15.7^{b}	11.3^{a}	14.5^{b}	16.0^{c}	18.5^{d}	1.0
ROS (%)	26.4^{b}	24.2^{a}	26.0^{b}	28.5^{c}	31.0^{d}	0.8

Different letters within the same row show significant differences among the groups ($P \leq 0.05$)

Treatment: C-0 = Lake extender without CYS, C-1 = Lake extender plus 1 mM CYS, C-2 = Lake extender plus 2 mM CYS, C-4 = Lake extender plus 4 mM CYS, C-8 = Lake extender plus 8 mM CYS. DF = DNA fragmentation

morphology ($P > 0.05$). Mitochondrial activity and acrosome integrity were found more in C-1 compared to the other groups ($P \leq 0.05$).

Viability and apoptosis status

Viability rate and apoptosis status in post-thawed samples have been presented in Table 3. The C-1 group significantly illustrated the higher rate of viable cells and lower rate of late apoptotic cells when compared to the other groups ($P \leq 0.05$). The C-0 and C-2 groups considerably presented higher viable and lower late apoptotic cells than the C-4 and C-8 groups ($P \leq 0.05$). Moreover, the lowest viable and the highest late apoptotic cells were found in the C-8 group ($P \leq 0.05$). The extender supplemented via the different concentrations of CYS showed no effect on the early apoptosis rate and necrosis rate ($P > 0.05$).

DNA fragmentation, lipid peroxidation, and ROS concentration

The data related to lipid peroxidation, DNA fragmentation, and ROS concentration have been illustrated in Table 4. C-1 significantly had less amount in lipid peroxidation and lower DNA fragmentation and ROS percentages than the other groups ($P \leq 0.05$). In addition, C-0 and C-2 treatments presented lower DNA fragmentation and ROS concentration than C-4 and C-8 treatments ($P \leq 0.05$). The C-8 treatment significantly illustrated the high lipid peroxidation, DNA fragmentation, and ROS concentration in comparison to the other groups ($P \leq 0.05$).

The fertility performance

For evaluation of post-thawed spermatozoa fertility potential, artificial insemination was performed using the best treatment group along with control and fresh semen groups, and the results which was presented in Table 5. The highest fertility rate was recorded in the fresh semen group ($P \leq 0.05$). Moreover, a significant improvement was observed in the fertility rate in the C-1 group, when compared to the control ($P \leq 0.05$). However, the hatching rate, which was calculated based on the fertilized eggs, was not significant ($P > 0.05$) among the experimental groups.

Table 5 CYS effects on fertility and hatching rate of rooster sperm

	Treatments		
Parameters	**Fresh**	**C-0**	**C-1**
Fertility rate (%)	91.6[a] (352/384)	67.9[c] (261/384)	76.8[b] (295/384)
Hatching rate (%)	71.1 (275/352)	67.4 (176/261)	72.8 (215/295)

Different letters within the same column show significant differences among the groups ($P \leq 0.05$)

Treatment: C-0 = Lake extender without CYS, C-1 = Lake extender plus 1 mM CYS, C-2 = Lake extender plus 2 mM CYS, C-4 = Lake extender plus 4 mM CYS, C-8 = Lake extender plus 8 mM CYS

Discussion

The high content of polyunsaturated fatty acids (PUFA) in the plasma membrane of sperm cell is the reason for its sensitivity to the freezing process, which reduces sperm viability and fertility performance [2]. In this study, the effect of different levels of CYS on sperm quality during the freeze-thaw process was evaluated and supplementation of the cryopreservation extender with 1 mM CYS resulted in higher sperm TM, PM, membrane integrity, mitochondrial activity, acrosome integrity, viability as well as fertility potential, and lower lipid peroxidation, late apoptotic, DNA fragmentation and ROS concentration in thawed rooster sperm.

The cryopreservation process results in an increase in the amount of ROS, which is one of the main reasons for disruptions in the plasma membrane structure (4), which affects the performance of antioxidant defense systems [16]. The antioxidant capacity of sperm may be insufficient against lipid peroxidation during cryopreservation [17], leading to ATP depletion and irreversible loss of sperm motility [18]. According to the results of various studies, some compounds act as antioxidants. These additives protect sperm from the harmful effects of ROS and improve sperm motility and membrane integrity [19]. Various studies have reported the different effects of CYS on semen quality in different animals. In roosters, CYS supplementation has been reported to increase TM, PM, membrane integrity, viability, mitochondrial activity, and reduced lipid peroxidation in chilled sperm [10]. In addition, CYS preserved the quality of frozen semen in the sheep [20]. whereas, CYS indicated no beneficial effect on buffaloes' sperm quality after thawing [21]. The difference in results may be due to differences in animal species and CYS concentrations.

The antioxidant role of CYS has been attributed to multiple mechanisms in cells [22]. The most likely of these in sperm is the improvement of reduced glutathione (GSH) levels. By using the CYS in an extender at optimal concentrations, the CYS could promote cysteine transport into cells, which can be further utilized to synthesize GSH (one of the most powerful intracellular antioxidants) and influence cell redox homeostasis [10]. As a cofactor of glutathione peroxidase (GPx), GSH plays an essential role in protecting male gametes from oxidative damage. The GPx prevents the lipid peroxidation cascade caused by H_2O_2 [23]. Therefore, GSH protects mitochondria from the oxidative stress that occurs inside the cells [24]. In addition, surface Thiol groups are involved in improving rooster sperm motility [25]. Cell aging is caused by oxidative substances that damage sperm membranes. The GSH neutralizes ROS and protects sperm from membrane damage by inhibiting lipid peroxidation [25]. Moreover, there is a positive relationship between mitochondrial activity and fertility. Mitochondria provide ATP for sperm to reach the fertilization site [26]. Therefore, mitochondrial activity of stored sperm is an effective indicator of fertility potential as it directly correlates with sperm fertility [27] and viability [28].

Cryopreservation has been indicated to induce several changes in the sperm acrosome and calcium channels, which is called cryoactivation [11]. However, increasing apoptotic factors such as phosphatidylserine externalizations during the freezing process can induce acrosome reactivity and apoptotic changes [29]. The usage of 1 mM CYS created a greater acrosome integrity and better viability which indicates the protective role of CYS on acrosome integrity and viability. It has been reported that the use of an optimal concentration of antioxidants in the freezing medium can effectively preserve sperm viability and acrosome integrity after the freeze-thawing process [2].

Oxidative stress increases DNA damage and apoptotic status [30]. In this regard, one on hand, the intense oxidative stress leads to mitochondrial fission, which causes mitochondrial dynamic response [31], aggregation [32], and dysfunction [33]. On the other hand, mitochondrial fission reduces ATP production [34], causing induced mitochondrial apoptosis, but optimal concentrations of CYS preserved sperm mitochondrial activity, and lower DNA fragmentation and apoptotic status were observed. Mitochondrial dysfunction also induces nuclear translocation of apoptosis factors as well as endonuclease G [35], which increases pore formation in the outer mitochondrial membrane and consequently mitochondrial permeability transition through rupture of the outer membrane due to matrix swelling [36], while the protective effect of 1 mM CYS preserved mitochondrial activity.

The different concentrations of CYS in the Lake freezing extender showed no effect on the abnormal morphology of sperm. This can be attributed to the fact that primary sperm abnormalities occur during spermatogenesis [37]. The results of this study were in agreement with Heidari et al. (2022) who reported extenders do not affect buck's sperm morphology [11].

High levels of antioxidants could be harmful to sperm functions [23]. Here, the present study indicated the optimal CYS concentration was 1 mM, and an increase

in CYS concentration (4 and 8 mM) led to reduce sperm quality. Therefore, sperm cells could lose their normal function if the extender contains high amounts of CYS. In accordance with the present results, high doses of antioxidants have been reported to be toxic to the physiological state of sperm [38]. In addition, high levels of CYS could destroy sperm mitochondria and lead to a reduction in sperm quality [23].

In female avian, inseminated spermatozoa are collected in sperm storage tubes (SST) and then gradually released to fertilize oocytes, but only high-quality inseminated sperm cells are able to reach the SST. Imposed shocks during the cryopreservation process reduce the post-thawed sperm quality [10], so the auxiliary effect of antioxidant addition to the cryopreservation extender has been demonstrated to improve sperm viability and then simplify the sperm transportation through the reproductive tract. Moreover, the optimum concentration of antioxidants positively enhances sperm functionality during passage through the reproductive tract [10]. In this study, hens that were inseminated via the fresh semen, showed a higher fertility rate because of the higher quality of the fresh semen, compared to post-thawed semen. In the post-thawed groups, the usage of 1 mM CYS showed higher fertility performance than the control group which could be related to the lower quality characteristics of the post-thawed semen which was collected from the control group. Nevertheless, the hatching rate was not different among experimental groups which shows the partial independence of embryo development from sperm quality. The obtained results in the present study agreed with other researchers who reported sperm quality did not affect hatching rate [4].

Conclusion

Supplementation of Lake freezing medium with 1 mM Cysteamine resulted in greater rates of total motility, progressive motility, membrane integrity, mitochondrial activity, acrosome integrity, viability as well as fertility potential, lower lipid peroxidation, apoptotic-like changes, DNA fragmentation, and ROS concentration. Therefore, Cysteamine has the sufficient potential to be used as an additive for cryopreservation of rooster's sperm in Lake extender because of its suitable performance in preserving the sperm quality after semen thawing.

Abbreviations

ROS	Reactive oxygen species
CYS	Cysteamine
TM	Total motility
PM	Progressive motility
VAP	Average path velocity
VSL	Straight-line velocity
VCL	Curvilinear velocity; LIN: linearity
PSA	Pisum sativum agglutinin
A/PI	Annexin V / Propidium Iodide
GSH	Glutathione
DCFH-DA	Dichlorofluorescin diacetate

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Author contributions

RM, SE, and MS: Investigation, Visualization, Conceptualization, Supervision, Validation, and Editing; M.H, AH, FZ, RN, and MRT: Writing- Reviewing, Methodology, Data curation, Conceptualization. All authors reviewed the results and approved the final version of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The current study was approved in the Research Ethics Committees of Animal Science Research Institute of Iran (IR. ASRI. REC. 1400. 000759). Broiler breeder roosters and hens were provided and housed by the poultry farm in the Department of Animal Sciences, Tarbiat Modares University under the technical supervision of the poultry farm's expert staffs, according to the husbandry guidelines, approved by this university and ASRI. Also, all in vivo experiments were performed in compliance with the ARRIVE guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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