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Effects of cysteamine supplementation on cryopreserved buffalo bull semen quality parameters

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ABSTRACT

This work aimed to determine the effect of cysteamine (25, 50, 100 and 200 μM) incorporated during dilution on frozen thawed buffalo semen quality. Semen was collected twice weekly for 7 consecutive weeks from three Egyptian buffalo bulls using an artificial vagina. Semen samples were pooled and extended with a Tris-based extender, cooled, equilibrated and finally frozen in liquid nitrogen. The diluted semen was evaluated for motility, viability, morphology, plasma membrane and DNA integrity, in addition to oxidative stress and in vitro fertilizing capability. The post thaw motility and velocity parameters noticeably increased with different concentrations of cysteamine (mainly 100 μM) during different incubation periods. The post thaw sperm viability and normality significantly ($p < 0.05$) improved with concentrations of 50 and 100 μM. Plasma membrane integrity substantially increased at all concentrations of cysteamine. Cysteamine reduced alanine aminotransferase (at all concentrations), aspartate aminotransferase (at 25–100 μM), and creatine kinase (at 100 and 200 μM). Cysteamine at a concentration of 100 μM noticeably enhanced the total antioxidant capacity and glutathione peroxidase and decreased nitric oxide production. Cysteamine, at concentrations of 100 and 200 μM, increased the DNA intensity in the comet head (%) and decreased the DNA % in the comet tail. The comet tail length and moment substantially decreased at concentrations of 50–200 μM. Cysteamine did not affect the in vitro fertilizing capability of sperm. In conclusion, cysteamine incorporation (mainly at a concentration of 100 μM) in buffalo semen extender showed varying protective effects on different sperm parameters against cryo-damage; however, it did not affect the in vitro fertilizing capacity of sperm.

1. Introduction

Cooling and/or freezing are milestones in semen processing. It is well known that these processes are associated with the production of reactive oxygen species (ROS), and an imbalance between the produced ROS and the intracellular detoxification mechanism results in oxidative stress damage [1]. The oxidative stress defense mechanism is generally poor in buffalo spermatozoa; therefore, lipid peroxidation in the plasma membrane is tremendously high [2]. Spermatozoa exposed to ROS suffer from depressed fertility potential as a consequence of decreased viability, post thaw motility, and intracellular enzymatic activity [3]. This fact encourages researchers to implement supplements, which have antioxidant properties, in semen extenders to overcome the detrimental effect of cryopreservation [4]. Different biological (natural) and chemical (synthetic) antioxidants that attack lipid peroxidation and ROS [5] have been used to provide cryoprotective effects to sperm, thus improving semen parameters, including sperm motility and membrane integrity, after thawing [4].

Natural or enzymatic antioxidants include superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, which also cause the reduction of hydrogen peroxides to water and

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alcohol [1]. Synthetic or nonenzymatic antioxidants include vitamins and minerals such as vitamin C, vitamin E, zinc, taurine, hypotaurine and glutathione [1].

Cysteamine (NH2-CH2-CH2-SH, β-mercaptoethylamine) is a naturally existing low molecular-weight thiol compound that modulates the endocrine and metabolic status of the animals [6]. It plays a crucial role in the defensive mechanism against ROS by improving the synthesis of glutathione (GSH) [7–9]. Cysteamine increases the rate of cellular cysteine uptake through the formation of mixed disulfides with cysteine. These mixed disulfides enter cells via transport system and reduced intracellularly to release the two thiol compounds [10]. The cysteine is then utilized in GSH synthesis [9]. Glutathione is a large class of antioxidants agents, able to react directly with many ROS by mean of its sulfhydryl group reducing power and as co-factor for antioxidant enzymes [11,12].

The addition of cysteamine to the in vitro maturation/culture media improved the rate of embryo development in cattle [13] and buffalo [14]. Moreover, cysteamine incorporation in semen extender before cryopreservation has different effects on the post-thawing semen quality parameters in different species including ram [15], buck [16], bull [3] and buffalo bull [17].

The aim of the present study was to investigate the effect of cysteamine on cryopreserved buffalo semen characteristics, sperm-free enzymatic and antioxidant levels and the in vitro fertilizing capacity of cysteamine-treated sperm.

2. Materials and methods

2.1. Semen collection and processing

Three proven fertility Egyptian buffalo bulls aged 4–5 years housed at the Teaching Farm, Faculty of Veterinary Medicine, Moshtohor, Qalyubia Governorate, Egypt, were used in the current study. Semen samples were collected twice per week from the three buffalo bulls for 7 consecutive weeks (n = 42 total collections) with an artificial vagina maintained at 42–45 °C. Spermatozoa activity, concentration and morphology were assessed microscopically after holding the semen at 37 °C for 10 min. Samples that had motility, livability and normality ≥70% and concentration ≥800 × 10^6/mL motility were pooled and used as semen sources. Pooled semen samples were diluted with Tris-based extenders (Tris 254 mM, citric acid 78 mM, fructose 70 mM, egg yolk 14% (v/v), glycerol 6% (v/v), pH 6.8 with gentamycin sulfate, 500 μg/ml). In details: pooled samples were evaluated for the total sperm concentration, and the volume the added extender was calculated to obtain a final concentration of 60 × 10^6 spermatozoa/ml. Semen was diluted using one step dilution method according to Del Sorbo et al. [18]. Diluted semen was divided into 5 experimental groups supplemented with cysteamine hydrochloride (M6500, Sigma–Aldrich Co.) at different concentrations, including 0 (control), 25, 50, 100 and 200 μM. Extended semen was slowly cooled to 5 °C within 2 h, packed into polyvinyl midi-straws (Minitub, Germany) and equilibrated for 2 h. After equilibration, semen underwent a manual freezing according to Singh et al. [19] using isotherm box. The straws were suspended in horizontal position on a rack on liquid nitrogen vapor (5.5 cm above liquid nitrogen, N2) for 10 min after which the straws were plugged in LN2 tank until thawing. Frozen semen straws (n = 4/group each time) were thawed at 37 °C for 40 s for the different evaluations.

2.2. Semen quality evaluation

2.2.1. Assessment of sperm motility and velocity parameters

After thawing, semen was incubated at 37 °C, and the motility and velocity parameters were evaluated at different incubation periods, including 0, 1 and 2 h of incubation, using a computer-assisted sperm analyzer (CASA; Hamilton Thorne, Inc., Beverly, MA, USA) with a 10 × objective at 37 °C and the SETUP specific for buffalo bull semen according to Kumar et al. [20] (frame rate 60 Hz, frames acquired 30, minimum contrast 35, minimum cell size 5 pixels, cell size 9 pixels, cell intensity 110 pixels, average path velocity (VAP) 50 μm/s, straightness (STR) 70%, VAP cutoff 30 μ/s and straight linear velocity (VSL) cutoff 15 μ/s). Ten microliters of diluted semen specimen were placed in a prewarmed Makler chamber and evaluated. Motility values, including total, progressive and rapid motility, were recorded as percentages. The velocity parameters, including VSL μm/s, VAP μm/s, curvilinear velocity μm/s (VCL), beat cross frequency Hz (BCF), amplitude of lateral head displacement, μm (ALH), linearity (LIN, [VSL/VCL] × 100) and STR ([VSL/VAP] × 100), were assessed. The sperm motilities were calculated with speed standards set as fast >80 μm/s, medium >60 μm/s, slow >20 μm/s and static. Eight microscopic fields were analyzed for each evaluation.

2.2.2. Semen viability and morphology

Sperm viability and morphology were assessed microscopically (200/400X Olympus DP12) using eosin-nigrosin staining according to Beheshti et al. [21]. Briefly, a thin smear on a prewarmed slide was prepared by mixing 10 μL frozen–thawed semen with a 10 μl drop of the supravital stain [1% (w/v) eosin B, 5% (w/v) nigrosin in 3% solution of trisodium citrate dehydrate]. Two hundred spermatozoa were counted and classified as live (unstained heads) or dead (stained/partial stained heads) spermatozoa. In addition, normal sperm morphology was assessed.

2.2.3. Sperm plasma membrane integrity

Plasma membrane integrity was evaluated using the hypo-osmotic swelling test (HOS) according to Akhter et al. [22]. The HOS solution was prepared from sodium citrate 0.735 g and fructose 1.351 g (Merck KGaA, Germany) in 100 ml distilled water, osmotic pressure ~ 190 mOsm/kg. Fifty microliters of semen sample were mixed with 500 μl of prewarmed HOS solution and incubated at 37 °C for 60 min. After incubation, a drop of semen sample was examined using a phase contrast microscope (400X). Two hundred cells were counted per smear, and the percentage of HOS-positive sperm (swollen and/or curled tails indicating an intact plasma membrane) was calculated.

2.3. Extracellular enzymatic activity measurement

Frozen thawed semen samples were centrifuged at 3000 rpm for 20 min. The supernatant fluid was collected and kept at −20 °C until being assayed for aspartate aminotransferase (AST, AS 10 61, Biodiagnostic, Egypt), alanine aminotransferase (ALT, AL 10 31, Biodiagnostic, Egypt) and creatine kinase (CK, Cat No. #K777-100, Biovision, USA) enzyme activity using colorimetric assays as described previously [23,24].

2.4. Semen lipid peroxidation measurement

Glutathione peroxidase (GP 2524, Biodiagnostic, Egypt), nitric oxide (NO 25 33, Biodiagnostic, Egypt) and total antioxidant capacity (TAC, TA 25 13, Biodiagnostic, Egypt) were determined colorimetrically using commercial kits according to the methods given in previous publications [20,25,26].

2.5. Comet assay (single-cell electrophoresis assay)

Spermatozoa DNA damage in triplicate samples per trial was assessed using the comet assay as previously described [27]. A

minimum of 100–200 sperm cell nuclei per sample were assessed using the image analysis software Tn. TekCometScore™ freeware (Ver. 1.5) for % of DNA in the head, % of DNA in the tail, tail length and tail moment length. Head DNA (%) is the intensity of the head compared with the intensity of the whole comet. Tail DNA (%) is the intensity of the tail compared with the intensity of the whole comet. Tail length is the distance of DNA migration from the center of the edge of the head to the end of the tail. Tail moment length is the distance from the center of the head to the center of the tail and calculated as tail DNA % × length of tail.

2.6. In vitro embryo production

2.6.1. In vitro maturation (IVM) of bovine oocytes

According to Longobardi et al. [28], heterologous in vitro fertilization (IVF) was used to assess the fertilizing capability of buffalo sperm treated with 100 μM cysteamine. Bovine ovaries were collected post slaughtering and transported in a thermos filled with a warm saline solution (38 °C) to the laboratory. Ovaries were individually rinsed with saline solution and dried with gauze. Follicles 2 and 8 mm in diameter were aspirated using a 10 ml syringe with an 18-gauge needle. Only cumulus oophorous cells (COCs) with an intact compact cumulus were selected and transferred into a petri dish containing the washing medium (Sigma Tissue culture Medium; TCM-199 HEPES supplemented with 10% fetal calf serum). The COCs were washed 3 times with the washing medium and 3 times with the in vitro maturation medium, as follows. According to the method of Elkhawagah et al. [29], oocytes were matured in TCM-199 Earle's salt medium supplemented with 10% Sigma fetal calf serum (FCS), 5 μg/ml follicle-stimulating hormone (FSH) (Folltropin, Bioniche Animal Health USA, Inc.), 5 μg/ml luteinizing hormone (LH) (Lutropin, Bioniche Animal Health USA, Inc.), 0.2 mM sodium pyruvate, 10 μg/ml gentamycin and 1 mg/ml estradiol 17β. The oocytes were cultured in 70 μl droplets of IVM medium (20 oocytes per droplet) covered by paraffin oil at 38.5 °C in 5% CO_2 for 24 h.

2.6.2. In vitro fertilization (IVF) of bovine oocytes

TALP Medium (Sigma) supplemented with 250 mM epinephrine, 1 mM hypotaurine, 20 μg/ml heparin, 0.2 mM pyruvate, 2 mM penicillamine, 10 μg/ml gentamicin and 6 mg/ml BSA was used for in vitro fertilization [30]. Frozen-thawed buffalo sperm were prepared by centrifugation (30 min at 300 g) on a Percoll discontinuous gradient (45 and 90%). Semen (with or without 100 μM cysteamine) was added at a final concentration of 1×10^6 spermatozoa/ml to the IVF droplets containing the oocytes. The IVF dish was incubated for 18h, after which the cumulus cells were completely removed and the zygotes were washed three times and cultured for 6 days in synthetic oviductal fluid (SOF; [31]) at 38.5 °C with 5% CO_2 and 5% O_2 for 7 days.

2.6.3. Embryo fixing and quality assessment after Hoechst staining

After 7 days of incubation, embryos were fixed with 2% paraformaldehyde, mounted on a microscope slide, stained with Hoechst 33258 and covered with a coverslip. Using a Nikon Eclipse TE 2000-S fluorescence microscope equipped with a B2A (346 nm excitation/460 nm emission) filter, embryos were evaluated for cleavage, morula (contained more than 32 cells without an organized outer ring of cells) and blastocyst (contained more than 50 cells with an outer ring of cells around a blastocoel cavity) rates [32].

3. Statistical analysis

The data were analyzed and presented as the mean ± SE with one-way analysis of variance (ANOVA) using SPSS (Ver. 25). Multiple comparisons of the means were performed using general linear model (GLM) multivariate followed by Dunnett's post hoc test. The chi-square test was used to compare the proportions of matured oocytes, cleaved embryos, morulae and blastocysts. The P value was set at $P < 0.05$ to define statistical significance.

4. Results

4.1. Effect of cysteamine on motility parameters

The effect of different concentrations of cysteamine on sperm motility parameters is presented in Table 1. The total motility was decreased with cysteamine treatment at concentrations 50–200 μM after thawing, 200 μM after 1 h and 50 and 200 μM after 2 h of incubation ($P < 0.01$) compared to the control. The progressive motility was improved directly after thawing in samples treated with 100 μM cysteamine ($P < 0.01$) compared with controls.

4.2. Effect of cysteamine on velocity parameters

The effect of cysteamine treatment on different sperm velocity parameters is shown in Table 2. Cysteamine incorporation in extender medium before freezing had a variable effect on sperm velocity parameters after thawing and incubation for up to 2 h at 37 °C. Cysteamine significantly ($P < 0.01$) improved the sperm VSL value in samples treated with 100 μM and decreased the VCL value in samples treated with 200 μM directly after thawing compared to the control. The ALH values decreased significantly ($P < 0.01$) after thawing in response to cysteamine treatment at concentrations of 100 and 200 μM and after 1 h at a concentration of 200 μM, while they increased significantly ($P < 0.01$) after 2 h at concentrations of 100 and 200 μM compared to the control. The BCF values significantly increased in samples treated with 50, 100 and 200 μM after thawing ($P < 0.01$), 100 and 200 μM after 1 h ($P < 0.01$) and 50 μM cysteamine after 2 h ($P < 0.01$) compared to the control. The STR values increased significantly ($P < 0.01$) after thawing in all cysteamine-treated samples and after 1 h in samples treated with 200 μM, while after 2 h, they decreased significantly ($P < 0.01$) with cysteamine treatment at 100 μM compared to the control. The LIN values increased significantly ($P < 0.01$) after thawing at all concentrations of cysteamine and after 1 h by 50 and 200 μM cysteamine addition. However, after 2 h, it decreased significantly ($P < 0.01$) in samples treated with 100 μM cysteamine compared to the control.

4.3. Effect of cysteamine on sperm viability, morphology and membrane integrity

As shown in Table 3, sperm viability and normality were improved by cysteamine 50 and 100 μM treatment compared to the control ($p < 0.05$). The sperm plasma membrane integrity improved with all concentrations of cysteamine, and the highest effect was recorded at 100 μM cysteamine.

4.4. Effect of cysteamine on extracellular enzymatic leakage and oxidative stress biomarkers

The measurements of extracellular enzymatic activity in extended semen revealed substantial differences after inclusion of cysteamine in buffalo semen extender (Fig. 1). A noticeable decrease in the extracellular leaked AST ($P < 0.05$), ALT ($p < 0.005$) and CK ($p < 0.01$) was found in the cysteamine groups in comparison to the control. Cysteamine supplementation, at all

Table 1
Effect of cysteamine on motility parameters of buffalo semen.

Parameter	Group	Post-thaw	1 h	2 h
Total motility (%)	Control	87.78 ± 1.15[a]	76.00 ± 0.99[a]	60.96 ± 2.87[a]
	Cysteamine 25 μM	81.83 ± 1.47	75.13 ± 1.32	52.83 ± 1.77
	Cysteamine 50 μM	79.50 ± 1.58[b]	69.83 ± 0.58	43.92 ± 1.21[b]
	Cysteamine 100 μM	79.21 ± 1.70[b]	75.67 ± 2.81	63.00 ± 4.27
	Cysteamine 200 μM	73.04 ± 2.77b	60.67 ± 3.00[b]	49.83 ± 3.93[b]
	Sig.	0.01	0.01	0.01
Progressive motility (%)	Control	34.04 ± 0.42[a]	28.08 ± 1.01	19.58 ± 2.62
	Cysteamine 25 μM	36.79 ± 0.52	29.79 ± 1.60	14.25 ± 1.48
	Cysteamine 50 μM	36.00 ± 1.54	28.29 ± 0.72	13.46 ± 1.47
	Cysteamine 100 μM	40.38 ± 0.92[b]	29.50 ± 1.57	17.83 ± 2.38
	Cysteamine 200 μM	36.00 ± 1.99	25.04 ± 1.70	18.71 ± 2.52
	Sig.	0.01	NS	NS
Rapid motility	Control	45.87 ± 0.81	35.46 ± 1.41	23.83 ± 3.04
	Cysteamine 25 μM	46.88 ± 0.89	37.75 ± 2.15	17.21 ± 1.87
	Cysteamine 50 μM	45.29 ± 1.95	35.29 ± 0.93	15.50 ± 1.70
	Cysteamine 100 μM	48.50 ± 1.40	37.13 ± 2.27	23.79 ± 3.40
	Cysteamine 200 μM	42.13 ± 02.72	29.29 ± 2.16	21.67 ± 2.97
	Sig.	NS	NS	NS

Values are presented as the mean ± SE.

concentrations, reduced ALT leakage, while concentrations lower than 200 μM decreased AST outflow. Cysteamine treatment at 100 and 200 μM reduced CK in the sperm-free extender.

Assessment of oxidative stress biomarkers demonstrated that TAC levels showed a tendency ($p = 0.08$) to differ after cysteamine supplementation. This was accompanied by marked changes in

Table 2
Effect of cysteamine on velocity parameters of buffalo semen.

Parameter	Group	Post-thaw	1 h	2 h
VAP (μm/s)	control	64.40 ± 0.86	59.13 ± 0.31	52.48 ± 1.63
	Cysteamine 25 μM	64.49 ± 0.62	60.18 ± 0.70	50.10 ± 1.32
	Cysteamine 50 μM	64.30 ± 0.12	61.32 ± 0.49	50.72 ± 1.20
	Cysteamine 100 μM	65.14 ± 0.46	60.25 ± 1.01	53.93 ± 1.89
	Cysteamine 200 μM	62.77 ± 0.59	58.87 ± 1.40	54.18 ± 2.07
	Sig.	NS	NS	NS
VSL (μm/s)	control	54.13 ± 0.61[a]	50.62 ± 0.13	45.58 ± 1.40
	Cysteamine 25 μM	55.44 ± 0.72	51.56 ± 0.47	43.34 ± 1.11
	Cysteamine 50 μM	55.31 ± 0.23	52.83 ± 0.40	44.37 ± 1.04
	Cysteamine 100 μM	56.92 ± 0.52[b]	51.58 ± 0.63	45.70 ± 1.46
	Cysteamine 200 μM	55.45 ± 0.42	51.90 ± 1.31	47.53 ± 1.79
	Sig.	0.01	NS	NS
VCL (μm/s)	control	101.50 ± 1.45[a]	96.08 ± 0.51	85.24 ± 2.27
	Cysteamine 25 μM	100.23 ± 1.31	95.77 ± 1.00	83.31 ± 1.78
	Cysteamine 50 μM	99.23 ± 0.50	97.96 ± 0.86	83.85 ± 1.34
	Cysteamine 100 μM	99.45 ± 1.03	96.92 ± 1.58	89.73 ± 2.54
	Cysteamine 200 μM	93.97 ± 0.86[b]	93.18 ± 1.76	90.33 ± 2.51
	Sig.	0.01	NS	NS
ALH (μm)	control	4.60 ± 0.07[a]	4.54 ± 0.03[a]	4.26 ± 0.05[a]
	Cysteamine 25 μM	4.47 ± 0.07	4.54 ± 0.06	4.50 ± 0.06
	Cysteamine 50 μM	4.41 ± 0.05	4.45 ± 0.06	4.25 ± 0.05
	Cysteamine 100 μM	4.30 ± 0.06[b]	4.56 ± 0.09	4.49 ± 0.07[b]
	Cysteamine 200 μM	3.99 ± 0.06[b]	4.26 ± 0.05[b]	4.52 ± 0.03[b]
	Sig.	0.01	0.01	0.01
BCF (Hz)	control	29.26 ± 0.26[a]	28.35 ± 0.24[a]	29.27 ± 0.26[a]
	Cysteamine 25 μM	30.04 ± 0.23	28.90 ± 0.30	28.76 ± 0.17
	Cysteamine 50 μM	30.49 ± 0.30[b]	28.88 ± 0.27	30.30 ± 0.29[b]
	Cysteamine 100 μM	31.32 ± 0.23[b]	29.45 ± 0.19[b]	29.09 ± 0.17
	Cysteamine 200 μM	32.81 ± 0.26[b]	30.20 ± 0.19[b]	30.03 ± 0.36
	Sig.	0.01	0.01	0.05
STR (%)	control	84.39 ± 0.39[a]	86.13 ± 0.26[a]	87.21 ± 0.25[a]
	Cysteamine 25 μM	86.17 ± 0.28[b]	86.08 ± 0.29	87.00 ± 0.18
	Cysteamine 50 μM	86.29 ± 0.27[b]	86.21 ± 0.13	87.46 ± 0.18
	Cysteamine 100 μM	87.08 ± 0.22[b]	85.54 ± 0.41	85.25 ± 0.44[b]
	Cysteamine 200 μM	88.08 ± 0.18[b]	88.04 ± 0.17[b]	87.83 ± 0.16
	Sig.	0.01	0.01	0.01
LIN (%)	control	55.61 ± 0.21[a]	54.42 ± 0.20[a]	54.63 ± 0.27[a]
	Cysteamine 25 μM	57.29 ± 0.09[b]	55.88 ± 0.38	53.75 ± 0.31
	Cysteamine 50 μM	57.83 ± 0.29[b]	55.54 ± 0.37[b]	53.96 ± 0.59
	Cysteamine 100 μM	58.83 ± 0.27[b]	54.38 ± 0.20	52.21 ± 0.39[b]
	Cysteamine 200 μM	60.13 ± 0.26[b]	56.38 ± 0.41[b]	53.29 ± 0.62
	Sig.	0.01	0.01	0.01

Values are presented as the mean ± SE. Sig: significance VAP: Average path velocity (μm/s). VSL: Straight linear velocity (μm/s). VCL: Curvilinear velocity (μm/s). ALH: Amplitude of lateral head displacement (μm). BCF: Beat cross frequency (Hz). STR: straightness ([VSL/VAP] × 100). LIN: Linearity ([VSL/VCL] × 100).

Table 3
Effect of cysteamine on cryopreserved buffalo semen viability, normality and plasma membrane integrity.

Cysteamine (μm)	Viability (%)	Normality (%)	Membrane integrity (%)
0	47.75 ± 0.63	43.33 ± 1.20	52.50 ± 2.10
25 μM	50.00 ± 2.71	48.27 ± 1.22	62.25 ± 1.11*
50 μM	62.67 ± 1.67*	57.27 ± 1.41*	65.67 ± 2.40**
100 μM	64.25 ± 1.32*	59.67 ± 3.18*	70.80 ± 1.65***
200 μM	44.00 ± 0.82	47.20 ± 2.13	60.40 ± 0.93*

Data are presented as the mean ± SE with *, ** and *** superscripts differing significantly at $p < 0.05$, 0.01 and 0.001, respectively, compared with the control.

glutathione peroxidase activity ($p < 0.001$) and NO production ($P < 0.01$). Cysteamine treatment at 100 μM increased TAC ($p < 0.05$) and glutathione peroxidase ($p < 0.01$) and decreased NO production ($p < 0.01$). High concentrations of cysteamine (200 μM) markedly ($p < 0.01$) increased NO levels.

4.5. Effect of cysteamine on DNA integrity of frozen-thawed buffalo sperm

The data in Fig. 2 indicate the comet tail analysis of spermatozoa DNA intactness. This assessment revealed that the DNA intensity (%) in the comet head decreased at 25 μM ($p < 0.01$) but increased at 100 μM ($p < 0.001$) and 200 μM ($p < 0.05$) cysteamine. On the other hand, the DNA intensity in the comet tail (%) increased with cysteamine 25 μM ($p < 0.01$) but decreased at 100 μM ($p < 0.001$) and 200 μM ($p < 0.05$). The comet tail length and moment substantially ($p < 0.001$) decreased at 50–200 μM cysteamine.

4.6. Effect of cysteamine-treated semen on the developmental potential of in vitro-produced embryos

Depending on semen quality assessment results, a cysteamine concentration of 100 μM was selected to test the effect of cysteamine-treated semen on the developmental potential of in vitro-produced embryos. A total of 235 cumulus-oocyte complexes (COCs) were selected for in vitro culture. Of these, 115 were fertilized with cysteamine-treated semen, whereas 120 formed the control group. As presented in Table 4, no difference was found between the 2 experimental groups in the different parameters of in vitro-produced embryos.

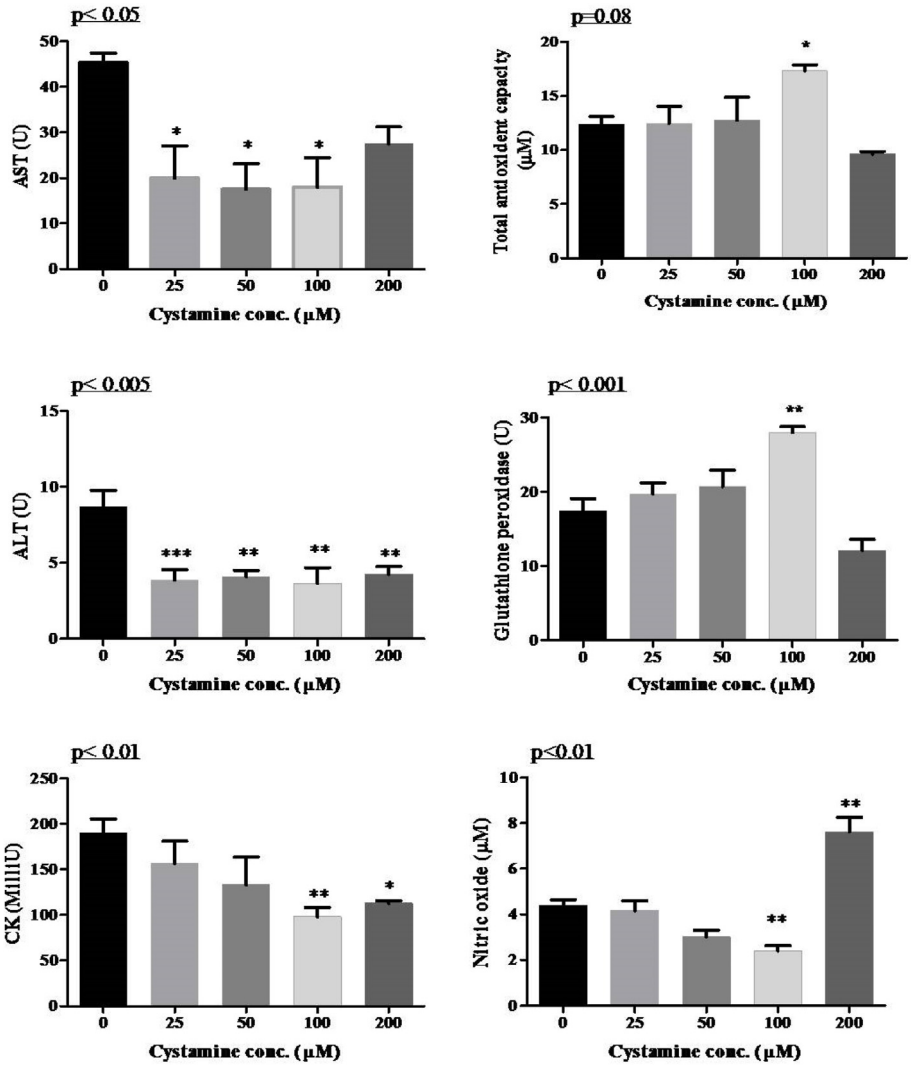

Fig. 1. Influence of cysteamine supplementation on buffalo semen biochemical characteristics; aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), nitric oxide, glutathione peroxidase and total antioxidant capacity. Data are presented as the mean ± SEM. *, ** and *** indicate significant differences at $p < 0.05$, 0.01 and 0.001, respectively, compared with the control.

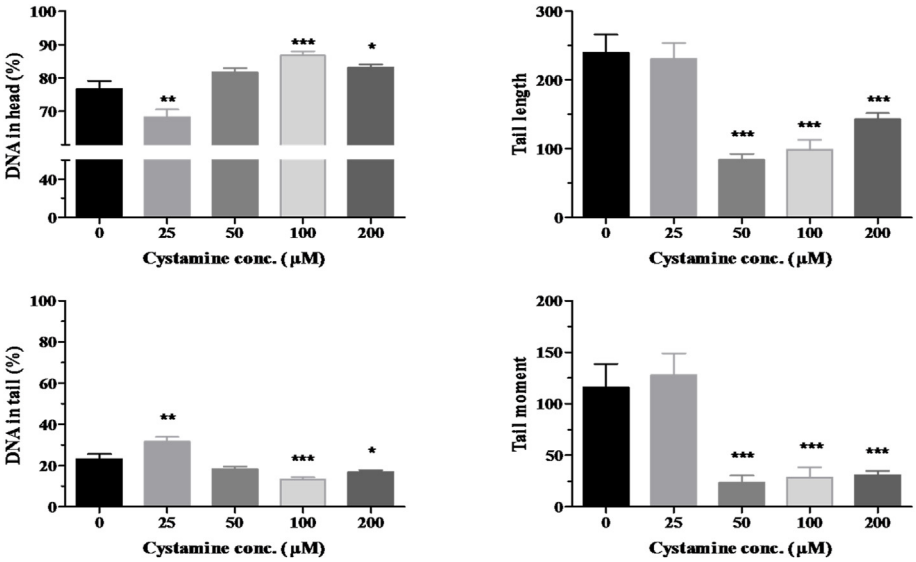

Fig. 2. Influence of cysteamine supplementation on DNA integrity of buffalo frozen-thawed spermatozoa examined with comet assays and evaluated using image analysis for % of DNA in head, % of DNA in tail, tail length and tail moment length. Data are presented as the mean ± SE. *, ** and *** indicate significant differences at $p < 0.05$, 0.01 and 0.001, respectively, compared with the control.

5. Discussion

Semen cryopreservation procedures result in sperm damage, which negatively affects sperm fertility and survival in the female reproductive system [33]. Due to the generation of reactive species (ROS) and lipid peroxidation during semen cryopreservation, oxidative stress has been recognized as a major factor affecting sperm quality [34]. Therefore, adding antioxidants to frozen sperm before thawing can improve its quality to a certain extent [35].

In the present study, we used different concentrations of cysteamine to verify its ameliorative effects against spermatozoa damage during cryopreservation. The concentrations of cysteamine used in the present study were lower than those used in any previous studies [17,36,37].

Sperm CASA motility analysis is one of the most important tools for assessing the fertilizing potential of spermatozoa [38]. A positive correlation has been recorded between the sperm motility and kinetics analyzed by CASA and pregnancy rate in buffalo [39], as well as non-return rate [40], pregnancy rate [41,42] and in vitro fertilization rate in cattle [43,44]. In addition, sperm progressive motility has been suggested to be used for fertility estimation of frozen-thawed semen during low and peak breeding seasons in swamp buffalo [45].

It has been stated that cryopreservation process deleteriously affects the sperm motility and morphological and functional integrity [16,46]. In the present study, cysteamine supplementation, especially at a concentration of 100 μM, improved the sperm progressive motility directly after thawing, while in the meantime decreased the total sperm motility compared to the control. It has been stated that the sperm total motility includes both progressive and non-progressive motility [47]. Our hypothesis is that, the control group may contain high percentage of non-progressive motile sperm compared to the different cysteamine groups. In addition, we recorded an increase in the sperm kinetic values including VSL, BCF, STR and LIN with all cysteamine groups and the prominent increase was with cysteamine 100 μM. Our results are in agreement with those recorded in goat by Bucak et al. [16], and bull by Sariözkan et al. [3] and Güngör et al. [48], who verified a stimulatory effect of cysteamine on frozen thawed sperm motility and morphology. In contrast, a negative effect of cysteamine on cryopreserved sperm motility was noted at concentrations of 4 mM [36] and 5 mM [49] in bulls and at 0.75, 1.25, 2.5 and 5 mM in buffalo [17]. This negative effect might be attributed to the high concentrations of cysteamine in the diluting media compared to our concentrations. Cysteamine has been reported to have a cryoprotective effect on the axosoma and mitochondrial integrity, by increasing the glutathione synthesis resulting in improvement of post-thawing sperm motility as well as morphological integrity [16,50].

The post thawing sperm viability and normality percentage decreased in all experimental groups compared to the fresh semen. Cysteamine with the concentrations 50 and 100 μM improved the sperm viability and normal morphology compared to the control. Similar to our results, Bucak et al. [16], recorded an improving effect on sperm morphology by cysteamine incorporation in goat semen extender. However, Swami et al. [17] found a negative effect of different concentrations of cysteamine in frozen thawed buffalo sperm. Sperm cryoinjury occurs during cryopreservation due to the changes in temperature and osmotic pressure, ice crystal damage and oxidative damage from ROS production. The cryoinjury of sperm leads to morphological changes and decreased viability and motility [51]. The incorporation of cysteamine into semen

Table 4
Effect of cysteamine (100 μM)-treated semen before cryopreservation on the developmental potential of in vitro-produced embryos.

	Evaluated COCs	Cleavage (%)	Morula (%)	Blastocyst (%)
Control	120	29 (24.2)	24 (20)	13 (10.8)
Cysteamine 100 μM	115	31 (26.9)	23 (20)	11 (9.5)

Chi square test: not significant COCs: Cumulus-oocyte complex.

extenders increases the production of GSH, a crucial part of cellular defenses against ROS (9), which successfully shields the semen from ROS attack and improves sperm viability and normality.

In the present study, cysteamine with all concentrations improved the sperm plasma membrane integrity, and its effect was prominent with the concentration 100 μM. A similar positive effect to cysteamine has been reported in cryopreserved bull [3] and buffalo [17] semen. The oxidative stress defense mechanism is generally poor in buffalo spermatozoa; therefore, lipid peroxidation in the plasma membrane with subsequent membrane damage is tremendously high [2]. Cysteamine enhances GSH which helps in maintaining the sperm acrosomal integrity and plasmalemma stability [51] by inhibiting the lipid peroxidation process [52].

The activity of transaminases (AST and ALT) and CK enzymes are good indicators of semen quality and are concerned with energy metabolism [53]. Transaminases play an important role in the catabolism of glutamate by bovine spermatozoa [53], while the CK shuttle is a source of extramitochondrial ATP and is responsible for transferring energy from mitochondria to the cytosol [54]. In the present study, the extracellular levels of AST, ALT, and CK were shown to be lower in cysteamine-treated groups, particularly at 100 M. It has been stated that good quality semen is characterized by lower seminal AST and ALT activities [55] which is consistent with our results. Spermatozoa are characterized by plasma membranes enriched with polyunsaturated fatty acids and cytoplasm poor in scavenging enzymes [56]. Therefore, they are highly susceptible to oxidative stress, which induces damage to their membranes, leading to extracellular escape of enzymes and other molecules. The addition of cysteamine markedly decreased the leakage of intracellular AST, ALT and CK enzymes; this is probably due to the maintenance of sperm plasma membrane intactness in response to cysteamine activity. The reduction in leakage of enzymes involved in energy metabolism might indicate an indirect role of cysteamine in improving sperm activity and/or motility.

Cryopreservation has been found to cause a NO level upsurge [57] and negatively impacts the viability and total motility of sperm cells [58]. In the present work, cysteamine (at a concentration of 100 μM) helped to increase TAC, enhance GPX activity and lower NO production. Meanwhile, high concentrations of cysteamine (200 μM) lowered GPX and increased the produced NO levels. The role of NO in mammalian sperm physiology seems paradoxical; low NO levels are beneficial, while high NO levels appear detrimental [59]. Elevated reactive oxygen species levels, including NO, in semen are produced from morphologically abnormal spermatozoa and seminal leukocytes [60]; therefore, they are negatively correlated with semen quality [61]. Our data related to the effect of cysteamine on GPX levels matches those reported in buffalo by Sarıözkan et al. [3] and Büyükleblebici et al. [36], who noticed an improvement in the level of GPX with the addition of cysteamine during the cryopreservation of bull semen. Additionally, Swami et al. [17] noted a decrease in TAC levels in the presence of cysteamine during cryopreservation of buffalo semen. These differences might be due to the high concentrations used in the aforementioned studies compared with our doses.

Cryopreservation's negative influences on spermatozoa are partially due to the induced oxidative stress. This attacks not only the fluidity of the spermatozoa plasma membrane but also the integrity of DNA in the sperm nucleus [62], leading to increased cellular apoptosis. Mitochondrial dysfunction and DNA damage are common consequences of oxidative stress [63]. In this study, the rate of DNA damage was reduced with 50 and 100 μM cysteamine but failed to diminish at levels below 25 μM. These results are in agreement with Sarıözkan et al. [3] and Tuncer et al. [49], who showed that cysteamine reduced the chromatin damage of spermatozoa compared with the control. On the other hand, our results are contrary to those shown in previous studies by Büyükleblebici et al. [36], who reported that cysteamine did not decrease bull semen chromatin damage during cryopreservation.

In the present study, cysteamine failed to improve the rate of in vitro-produced embryos, which may be attributed to the heterologous IVF technique used. Our results are in agreement with those reported by Sarıozkan et al. [64], who found a nonsignificant effect on the nonreturn rates of cattle inseminated by cysteine-treated sperm. However, Iqbal et al. [37] reported a significant effect of cysteine-treated buffalo sperm on improving the pregnancy rate of inseminated buffalo.

6. Conclusion

The addition of cysteamine (particularly at a concentration of 100 μM) to buffalo semen during dilution is beneficial in improving the post thaw motility, antioxidant properties and DNA intactness. These effects are primarily due to its enhancement of sperm vitality and normality, maintenance of membrane integrity, and prevention of the leakage of many important enzymes, such as AST, ALT and CK, which are involved in the energy metabolism that intensifies the spermatozoa resistance against the damaging effect of cryopreservation. Cysteamine failed to improve the rate of in vitro-produced embryos.

CRediT authorship contribution statement

Ahmed Reda Elkhawagah: Conceptualization, Writing – original draft, Methodology, Formal analysis, Methodology. **Nicola Antonio Martino:** Conceptualization, Writing – review & editing, Methodology. **Mohamed Mahmoud Moustafa Kandiel:** Investigation. **Karima Ghoneimy Mahmoud:** Investigation. **Alessandro Ricci:** Writing – review & editing, Resources, Investigation. **Leila Vincenti:** Conceptualization, Writing – review & editing, Supervision, Methodology, Funding acquisition, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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