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Original Article

Effect of Cystamine on Sperm and Antioxidant Parameters of Ram Semen Stored at 4 °C for 50 Hours

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Abstract

Physical and chemical changes caused by oxidative stress in the spermatozoa membrane can reduce spermatozoa function and even lead to death. Cystamine (NH2-CH2-CH2-SH, β-mercaptoethylamine) is a natural substance that modulates the endocrine and metabolic status of animals. This substance has antioxidant and anti-apoptotic effects by inducing intracellular cysteine accumulation. Cystamine is used to treat many diseases despite its many side effects. Sheep semen is sensitive to the stressful condition of chilling storage, which restricts semen storage for artificial insemination in commercial herds. The effect of cystamine on spermatogenesis is not yet fully understood. The present study aimed to investigate the effect of cysteamine addition to the sheep sperm extender during cooling storage on semen quality parameters. Sperm samples were collected from six Edilbayevskaya rams (2 and 3 years old, 70-85 kg). The samples were diluted by extender and supplemented with different concentrations of cysteamine (0, 1, 2, 5, and 10 mM) and cooled to 4°C for 50 h. Motility parameters, membrane integrity, viability, lipid peroxidation, and mitochondrial activity of cooled semen were evaluated at 0, 25, and 50 h of cooling storage. Although cysteamine failed to affect semen quality at start time (0 hrs), extender supplementation with cysteamine improved sperm total motility, progressive motility, and mitochondrial membrane potential during storage periods ($P \leq 0.01$). Moreover, using 1 and 2 mM cysteamine functionally and viably improved ($P \leq 0.01$) sperm membrane compared to other treatments. Antioxidant potential (AOP), lipid peroxidation (LPO), and total glutathione (tGSH) (except AOP at 50 h) were significantly different after semen storage at 4 °C. Therefore, levels of AOP and tGSH were significantly increased by using cysteamine. Cysteamine supplementation (1 and 2 mM cysteamine) leads to lower levels of LPO ($p<0.01$) at 0, 25, and 50 h. Therefore, finding and using the best concentrations of cysteamine in a cooling extender could be effective in saving sheep semen against damages of the cooling storage process.

Keywords: Cooling, Cystamin, Semen, Sheep

1. Introduction

Physical and chemical changes caused by oxidative stress in the spermatozoa membrane can reduce spermatozoa function and even lead to cell death. Cold and osmotic shocks during sperm cooling, freezing, and thawing of sperms, especially at temperatures below zero degrees Celsius, are stressors on the spermatozoa membrane (1-3). Changes in the spermatozoa membrane reduce spermatozoa motility and fertility (4). The approved quality of sperm/oocytes during cold storage is acceptable for only a few days (5). However, long-term storage of these cells as gametes by freezing in liquid nitrogen or on dry ice causes damages and significantly increases cell death, and reduces spermatozoa counts (6). Species, race, individual characteristics, seasonal variations (7), acrosome sensitivity to temperature, and ice crystal formation affect spermatozoa quality (8). Some researchers have studied the destructive effects of oxidative stress on spermatozoa (9-11). Studies on sheep sperm storage at temperatures between 0 and 15 ° C have shown that the fertility of ram spermatozoa decreases if stored for more than 24 hours (12).

Materials used as sperm expanders must have the appropriate pH (13), buffer capacity (14), and osmolality (15). Milk, egg yolk, and soy lecithin are used to increase cold tolerance for sperm (16, 17), and glycerol and dimethyl sulfoxide are used to protect against forming intracellular ice crystals (16). Tris buffers (18), sugars (19), citric acid salts (20), and antibiotics are also used to dilute sperm (21).

Preservatives protect the spermatozoa membrane from overflowing by increasing dehydration and forming intracellular ice crystals to keep sperm at a low temperature (22). These protectors prevent damage to the spermatozoa membrane and reduce extracellular ice formation by lowering the freezing point of the environment (23).

Sperm membrane structures, plasma membranes, and acrosome are sensitive to frostbite. Non-diffuse carbohydrates such as sucrose, lactose, raffinose, or trehalose are used in spermatozoa membranes to dehydrate cells and reduce the formation of intracellular ice (24). In tris-containing developers, monosaccharides have a stronger protective effect than disaccharides. High trehalose provides the greatest protection in terms of the maintenance of spermatozoa motility, recovery rate, and acrosomal integrity by increasing membrane fluidity (25).

Fusion changes and fusion between plasma and acrosomal membranes occur due to increased membrane permeability and calcium uptake (26). One of the determinants of spermatozoa sensitivity to cold is probably the shape and size of the sperm head. Therefore, another influential factor is animal genetics. Wild boar, cows, rams, and horses spermatozoa are very sensitive, dog and cat sperm are somewhat sensitive and human and rooster spermatozoa are the least sensitive to low temperatures (27).

The health of sperm DNA is one of the essential factors for fertilization, endogenous and exogenous factors can lead to serious damages and endanger reproductive performance (28). The most important cause of spermatozoa DNA damage is increasing oxidative stress level, which has detrimental effects on most types of infertility (29). Oxidative stress has been reported to ultimately damaged spermatozoa DNA by incubating spermatozoa at high oxygen pressures which reduced sperm viability and motility (30). Oxidative stress reduces the health of spermatozoa DNA and the fertilization potential of these cells through lateral damage to proteins and fats in the spermatozoa plasma membrane. Increased oxidative stress in the semen of infertile animals indicates that it plays an important role in the structural disorders and functional capacities of spermatozoa through various mechanisms. The fact that increased ROS levels are associated with impaired spermatozoa count, motility, shape, and DNA health suggests an important role. Also, despite the various clinical evidence, there is a growing concern that an underlying spermatozoa DNA damage could affect the embryo, pregnancy, and offspring (30).

Free radicals are highly reactive and unstable molecules that have one or more unpaired electrons in

their outer orbit, so they react with other adjacent atoms and molecules to become stable. Free radicals are produced by natural physiological processes and metabolisms of the body and tend to obtain electrons from biological macromolecules such as fatty acids, proteins, and nucleic acids (30).

Cystamine (NH2-CH2-CH2-SH, β-mercaptoethylamine) is one of the natural substances that modulate the endocrine and metabolic status of animals (31). However, the concentration of cystamine is very low in animals and humans. Synthesis and oxidation of fatty acids, oxidation of pyruvate in the citric acid cycle, and depletion of tissue somatostatin are the main tasks of cystamine. Cystinosis is a lysosomal storage disorder caused by a mutation in the cystinosin-lysosomal cystine transporter (CTNS) on chromosome 17p3 and cystamine is used to treat it (32). Cystamine extends a patient's life by decreasing cellular cysteine levels. This substance has antioxidant and anti-apoptotic effects by inducing intracellular cysteine accumulation. Researchers have reported that cystamine added to sperm enhancers positively affects sperm motility and morphology (33). The present study aimed to elucidate the protective effects of different doses of cystamine on sperm parameters and oxidative stress during storage of ram sperm fluid at 4 °C.

2. Material and Methods

2.1. Animals Semen Collection

Sperm samples were collected from six Edilbayevskaya rams (2 and 3 years old, 70-85 kg). The rams were kept in the same feeding, shelter, and lighting conditions. A total of 40 ejaculations were obtained from rams using artificial vaginas during autumn to early winter. Ejaculations were processed in a volume of 0.5 to 2 ml, minimum spermatozoa concentration of 2×10^9 sperm per ml, and motility> 80. In this study, eight ejaculates collected from each ram were stored at 37 ° C and divided into equal parts, and diluted with a Tris-based enhancer.

2.2. Semen Processing

In this experiment, semen samples were diluted by the extender and supplemented with different concentrations of cystamine. Each accumulated ejaculate was divided into four equal aliquots and diluted (37 ºC) with the based extender, including 0 (control), 1, 2, 5, and 10 mM cystamine, at a final concentration of about 400×10^6 spermatozoa/ml (single-step dilution). Diluted samples were stored in glass tubes and cooled from 37 ºC to 4 ºC for 50 h in a cold cabinet, and maintained at 4 ºC. Sperm and oxidative stress parameters were determined at 4 ºC for 0, 24, 48, and 72 h of liquid storage. Motility parameters, membrane integrity, viability, lipid peroxidation (LPO), and mitochondrial activity of cooled semen were evaluated at 0, 25, and 50 h of cooling storage.

2.3. Determination of Motility and Viability Parameters

Spermatozoa motility was determined using a phase-contrast microscope (magnification 200°). First, the normal semen step was evaluated at 37 °C, and spermatozoa motility was estimated under several microscopic fields for each semen sample. The average of the collected data was recorded as the final mobility rate. Spermatozoa viability was assessed by staining with a standard kit (SYBR-14 / PI Molecular Probe: L 7011 Invitrogen) and staining protocol of Garner and Johnson (34). Live spermatozoa with a healthy membrane do not penetrate the dye during staining and remain colorless, but the dye material penetrates dead spermatozoa from their damaged membrane and turns pink. The procedure was to place 5 µl of semen sample on a preheated slide (37° C) and was analyzed by CASA after covering with a slide. The evaluation was based on counting at least 200 sperm.

2.4. Determination of Mitochondrial Activity

Rhodamine 123 dye was used to evaluate mitochondrial activity. Rhodamine 123 is a cationic fluorescent probe that attaches to the inner membrane of the mitochondria. Its bonding depends on the potential between the membranes. Rhodamine actively

enters the mitochondrial respiratory chain and its accumulation in the mitochondria produces green fluorescence. Lack of fluorescence indicates a defect in mitochondrial membrane potential. In this method, the samples were thawed and then centrifuged. The sperm pellet was then mixed with 500 ml of Tris buffer. Then, 10 µl of rhodamine was added to the sample in a dark place and placed at room temperature for 20 minutes. Next, 10 µl of PI was added to the sample. Then the mitochondrial activity of the samples was measured using a flow cytometer. Rhodamine-positive and iodide-negative propidium samples are active with mitochondria, and rhodamine-positive and propidium-positive samples are inactive with mitochondria.

2.5. Sperm DNA Integrity

Sperm DNA damage was examined by single-cell gel electrophoresis, which was performed under neutral conditions. The developed sperm cells were washed with Ca^{2+} and Mg-free solution. Each pre-cleaned slide was pre-coated with a layer of 1% normal melting point of agarose in PBS and then dried at room temperature.

2.6. Determination of Lipid Peroxidation (LPO)

LPO was measured according to the method of Ohkawa, Ohishi (35). The incubation time of the samples was three hours in this method and finally, the production of reactive bodies with Thiobarbituric acid (Thiobarbituric Acid Reactive Substances: TBARS) including malondialdehyde (MDA), which accounted for about 50% of the value of TBARS was recorded in the medium containing the spermatozoa sample. The concentration of complex MDA+TBA was measured at 532 nm. The amount of light absorption of the reaction mixture was measured by a spectrophotometer after centrifugation.

2.7. Determination of Total Antioxidant Activity

The samples were immediately centrifuged at 14000 g for 7 minutes at 4 °C after semen liquefaction at 37 °C to measure total antioxidant activity. The supernatant was removed from the precipitate and diluted 10 times with distilled water and immediately used to measure antioxidants. Standard solution of ferrous sulfate (at concentrations of 125, 250, 500, and 1000 µM) and FRAP solution were used to measure TAC. All materials were purchased from Sigma Company (36).

2.8. Determination of Total Glutathione

It was used to measure total glutathione based on the formation of a chromophoric tone. Tris (2-carboxyethyl) phosphine buffer was used to reduce glutathione oxide. The staining method was performed using the Randox kit (Germany) to measure the activity of the glutathione peroxidase enzyme in seminal plasma.

2.9. Statistical Analysis

SPSS software (version 19) was used for data analysis. Data were statistically analyzed by a one-way ANOVA test. Tukey test was used to compare the means and the difference between the means was considered significant at the level of $p<0.05$. The data were expressed as mean±standard deviation and the graphs were plotted by Excel.

3. Results and Discussion

Sperm motility and viability, mitochondrial activity, and DNA integrity rates (%) of semen from Edilbayevskaya enriched with different concentrations of cysteamine are shown in table 1. The percentage of total motility for 0, 1, 2, 5, and 10 mM cysteamine was not significant at the start of sampling, but motility increased compared to that of the control group using cysteamine ($P<0.01$). Cysteamine levels did not significantly improve spermatozoa viability, mitochondrial activity, and DNA integrity rates at the start of the experiment compared to those of the control group. Cystamine supplementation at the dose of 1 mM increased mitochondrial activity, with maximum activity at 25 (77.75±0.96) and 50 (78.15±2.22) hours after storage of semen at 4 °C. Although cysteamine failed to affect semen quality at the start time (0 hrs), extender supplementation with cysteamine improved ($P\leq0.01$) spermatozoa total motility, progressive motility, and mitochondrial membrane potential during storage periods. Moreover, using 1, 2, 5, and 10 mM cysteamine improved ($P\leq0.01$) spermatozoa membrane

functionality and viability compared to other treatments. Figure 1 presents the decrease in spermatozoa motility and viability, mitochondrial activity, and DNA integrity rates with increasing storage time and using cysteamine significantly improves this trait.

Oxidative stress parameters are shown in table 2. Antioxidant potential (AOP), LPO, and total glutathione (tGSH) (except AOP at 50 h) were significantly different after semen storage at 4°C. So that the levels of AOP and tGSH increased with the use of cysteamine. Cystamine supplementation decreased LPO levels ($p<0.01$) at 0, 25, and 50 h (Table 2) while increasing cysteamine increased tGSH levels at 0, 25, and 50 h of the liquid storage ($p<0.01$). The highest level of AOP at 0 and 25 (hours) was related to the level of 1 mM of cysteamine, and the lowest level of LPO was related to the same level of 1 mM cysteamine. Cysteamine consumption of increased tGSH level compared to that of the control group. The highest amounts of tGSH were related to experimental groups 5 mM, 1mM, and 10 mM at 9379.00±729.00, 8945.30±70.70, and 8850.00±204.10, respectively.

Table 1. Mean (±SE) sperm motility, sperm viability, mitochondrial activity, and DNA integrity rates (%) of Edilbayevskayaram semen supplemented with different concentrations of cysteamine for different storage times at 4 ºC

Treatment	Motility (%)			Sperm viability (%)			Mitochondrial activity (%)			DNA integrity rates (%)		
	h[1] of cooling storage			h of cooling storage			h of cooling storage			h of cooling storage		
	0	25	50	0	25	50	0	25	50	0	25	50
0	82.65±1.15	66.58±1.34	57.75±1.26	73.33±0.67	59.78±1.75	53.00±2.16	81.65±1.40	67.25±4.86	64.28±1.74	80.65±1.40	66.75±2.06	58.25±2.36
1	83.90±1.33	79.33±2.12	71.75±2.06	72.28±1.48	68.00±2.94	59.00±1.83	82.25±1.71	77.75±0.96	78.15±2.22	79.75±1.71	73.75±1.50	72.75±2.06
2	82.65±2.70	77.50±2.38	69.00±2.94	70.50±1.73	68.50±3.70	58.00±2.16	82.00±1.83	76.00±1.41	76.50±3.11	78.75±3.30	73.50±4.12	71.50±2.38
5	84.65±2.15	75.75±1.71	66.50±1.29	73.25±1.26	71.00±4.08	56.00±1.83	81.73±1.47	76.50±1.29	76.00±3.47	79.25±3.20	72.25±3.78	70.50±2.38
10	82.08±3.05	74.70±3.50	67.50±1.29	70.50±2.38	69.75±1.71	56.50±1.29	82.00±2.45	76.25±2.36	77.00±1.83	78.25±2.63	73.00±2.45	71.38±2.75
P value	0.479	0.000	0.000	0.051	0.001	0.005	0.990	0.000	0.000	0.722	0.024	0.000
SD[2]	1.68	9.16	9.87	2.25	7.05	3.65	0.39	6.79	9.01	1.47	4.65	9.58
SEM[3]	0.47	2.19	2.36	0.63	1.97	1.02	0.11	1.90	2.52	0.41	1.30	2.68

1: Hour, 2: At least a significant difference, 3: Standard error of the mean

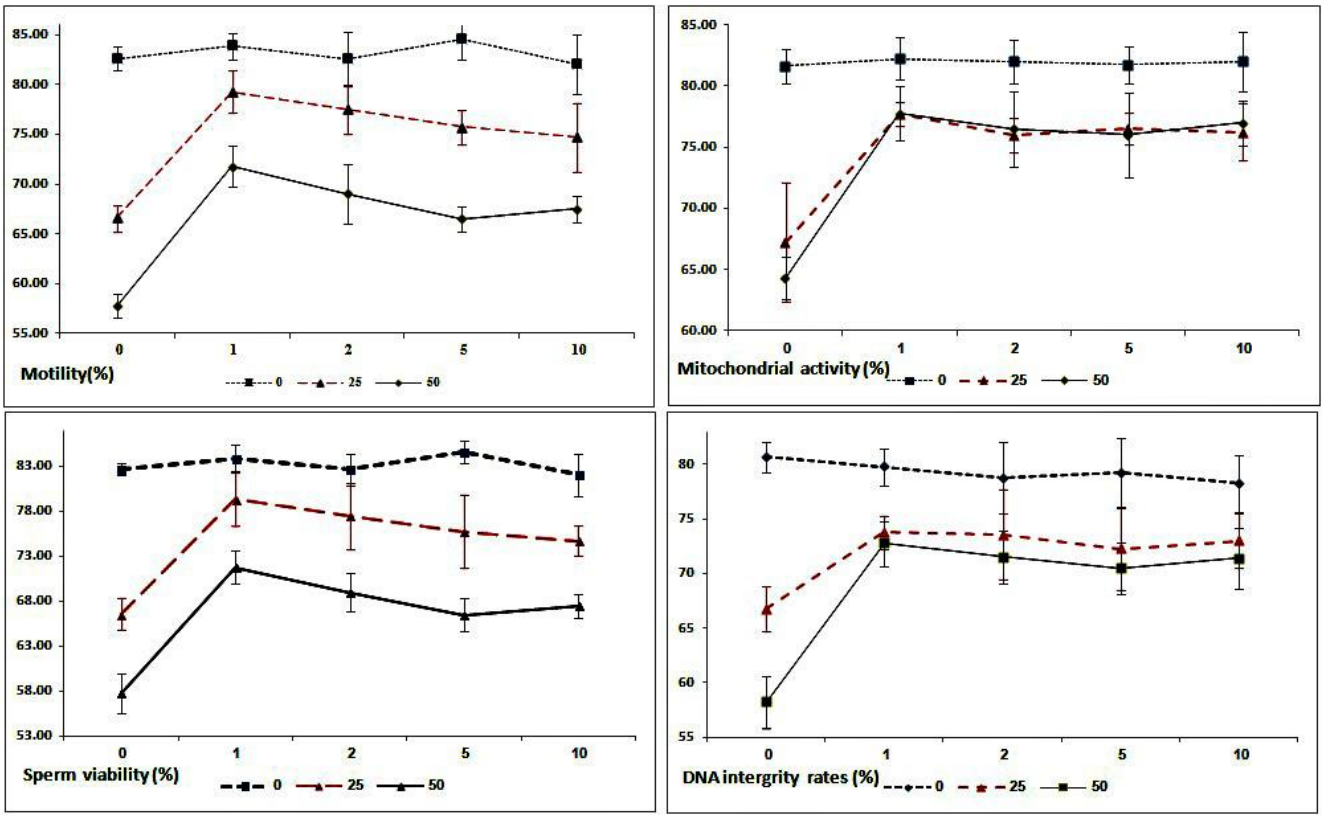

Figure 1. The trend of changes in sperm motility, viability, mitochondrial activity, and DNA integrity rates using different treatments at times 0, 25, and 50 (hours)

Table 2. Mean (±S.E.) AOP (mM×10^9), LPO (µmol, 10^9 cells/ml), and GSH (µmol, 10^9 cells/ml) levels of Edilbayevskayaram semen supplemented with different concentrations of cysteamine for different storage times at 4 ºC

Treatment	AOP[4]			LPO[5]			tGSH[6]		
	h[1] of cooling storage			h of cooling storage			h of cooling storage		
	0	25	50	0	25	50	0	25	50
0	73.25±1.50	47.83±0.99	40.70±6.31	254.93±6.64	235.48±9.44	162.75±4.86	8884.00±1438.00	5182.50±223.40	3396.80±476.30
1	74.75±0.96	54.75±1.71	41.53±1.31	128.28±6.24	152.25±5.32	133.50±5.51	8728.00±92.00	8945.30±70.70	5344.00±477.50
2	59.75±1.71	47.55±1.94	45.18±2.47	95.25±56.93	153.78±5.20	149.75±4.65	8467.00±447.00	7328.80±509.40	8030.00±174.00
5	47.73±2.06	46.05±4.35	46.53±6.87	78.58±8.28	163.25±5.38	112.50±11.45	9379.00±729.00	7292.30±285.50	8037.50±398.70
10	47.23±4.34	42.25±2.22	48.75±3.09	83.50±5.80	164.75±7.63	108.50±15.93	8973.00±1152.00	6803.80±539.20	8850.00±204.10
P value	0.000	0.000	0.119	0.000	0.000	0.000	0.000	0.000	0.000
SD[2]	21.21	7.22	5.40	117.34	55.68	37.34	536.21	2150.75	3651.77
SEM[3]	5.93	2.02	1.51	32.81	15.57	10.44	149.93	601.37	1021.07

1: Hour, 2: At least a significant difference, 3: Standard error of the mean, 4: Total antioxidant activity, 5: Lipid peroxidation levels, 6: Total glutathione

The plasma membrane of spermatozoa is rich in polyunsaturated fatty acids (PUFA). PUFA makes sperm highly susceptible to fat peroxidation due to ROS attacks. These changes eventually lead to sperm dysfunction through oxidative stress and the production of MDA (37). The experiment indicated that the process of semen storage at 4 °C damaged the desired traits. Cystamine has been used to treat cystinosis for more than three decades, and this combination can be used for many other purposes. Cystamine has many side effects in animals and humans, for example, it can cause lesions in diseased organs, ulcerative and growth, or even embryonic development problems (38). The use of Cystamine significantly affects sperm motility and viability, mitochondrial activity, DNA integrity rates, AOP, LPO, and tGSH.

Increased effect of cystamine on motility was obtained at 25 and 50 hours of fluid storage. Some researchers have shown that adding cystamine to semen has a positive effect on spermatozoa motility, morphology, and semen stored in low-temperature conditions (39). But Bucak, Ateşşahin (40) reported that high doses (5 and 10 mM) of cystamine had no positive effect on post-thaw sperm parameters other than motility and oxidative stress parameters. Cystamine increased tGSH levels throughout the experiment hours at doses of 1, 2, 5, and 10 mM. In the present research, a decrease in LPO levels was observed with cystamine. Cystamine levels were not adequate to lessen LPO levels as fluid retention time increased. The differences in the present study may be related to the different used doses. Akalin, Bucak (32) found that using cysteamine failed to reduce LPO levels which was consistent with the results of the present study.

Therefore, finding and using the best concentrations of cysteamine in the cooling extender could be effective in saving sheep semen against damages of the cooling storage process.

Authors' Contribution

Study concept and design: J. J and I. M.
Acquisition of data: S. A. and L. M.
Analysis and interpretation of data: D. Y. S. and M. D
Drafting of the manuscript: K. S.
Critical revision of the manuscript for important intellectual content: A. T. J.
Statistical analysis: S. M. S.
Administrative, technical, and material support: Y. F. M.

Ethics

All the procedures were approved by the Ethics Committee at the University of Tun Hussein Onn Malaysia, Johor, Malaysia under the project number of 2021-9548828-9.

Conflict of Interest

The authors declare that they have no conflict of interest.

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