Original Article

The effects of cysteine and L-carnitine on the DNA integrity of post-thaw sperm of frozen buck semen

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Abstract

Background: The addition of antioxidants to semen extenders is fundamental to limit oxidative changes resulting from cryopreservation. **Aims:** This experiment was designed to clarify the effects of cysteine and L-carnitine (LC) on post-thaw sperm criteria. **Methods:** Semen samples were collected once weekly from five mature Zaraibi bucks for 10 successive weeks. Fresh semen samples were evaluated for basic semen characteristics, and the accepted samples were pooled and divided into seven aliquots: control (Tris egg yolk extender), cysteine (2.5, 5 and 10 mM) and LC (2.5, 5 and 7.5 mM). Aliquots were diluted and subjected to cryopreservation procedures. After thawing, sperm criteria were evaluated regarding motility, viability, plasma membrane (hypo-osmotic swelling test, HOST) and acrosome integrity, total antioxidant capacity (TAC), level of malondialdehyde (MDA), and DNA comet assay. **Results:** Addition of both 10 mM cysteine and 7.5 mM LC significantly increased post-thaw sperm motility, viability, HOST, and acrosome integrity. Comet assay revealed significant decreases in DNA damage with best results at 2.5 mM cysteine and 7.5 mM LC. Besides, 10 mM cysteine and 7.5 mM LC exhibited significant diminish in MDA levels. Cysteine was positively correlated with sperm viability, acrosome integrity, and comet, but negatively correlated with MDA and tail length. Positive correlations were declared between LC and progressive motility, viability, HOST and TAC, while negative correlations were found with MDA, comet, tail DNA, tail moment, and olive tail moment. **Conclusion:** Supplementing cysteine and LC to frozen buck semen improved sperm criteria and decreased DNA damage, possibly due to their effectiveness in the suppression of MDA formation.

Key words: Buck, Cysteine, Frozen semen, L-carnitine

Introduction

Cryopreservation is an effective technique extensively used in livestock for long-term storage of semen. Artificial insemination of cryopreserved semen instigates rapid genetic improvement, disease control and economic gain (Mia *et al*., 2013). However, during cryopreservation and thawing processes, sperm cells are exposed to physical and chemical stress. The resulted cryo-damage adversely affects sperm quality, leading to reductions in sperm motility, deterioration of membrane integrity, DNA damage and apoptosis (Shaygannia *et al*., 2020; Jia *et al*., 2021); consequently decreasing the fertilizing capability of semen after the freezing-thawing process (Alcay *et al*., 2016). Cryopreservation-induced cryo-damage has been attributed to oxidative stress, intracellular ice crystal formation and cryoprotectant toxicity or combinations of these conditions (Peris-Frau *et al*., 2020). Oxidative stress, which results from an imbalance between ROS production and seminal antioxidant defense capacity (Gadea *et al*., 2013), is considered a major contributing factor affecting semen quality after the cryopreservation and thawing processes (Sánchez-Rubio *et al*., 2020). *In vivo*, ROS at basal levels is essential for maintaining the capacitation/ acrosome reaction and fertilizing ability of the sperm. Nevertheless, it is believed that during cryopreservation stages, an excessive generation of ROS occurs as well as weakening of the seminal natural antioxidant capacity (Bucak *et al*., 2010). Therefore, an imbalance results between the production and elimination of ROS, causing oxidative stress. Excessive ROS production during cryopreservation has been associated with reduced post-thaw motility, viability, membrane integrity, sperm function and fertility (Balamurugan *et al*., 2018). Accordingly, goat semen is more prone to cryo-damage. Therefore, the addition of antioxidants is an essential demand to balance ROS that ameliorates sperm criteria and the subsequent fertilizing ability of cryopreserved goat semen (Naijian *et al*., 2013).

A wide variety of antioxidants have been tested to minimize the oxidative damage caused by cooling and the freezing-thawing processes (Shafiei *et al*., 2015; Rostami *et al*., 2020). The amino acid cysteine, is composed of thiol groups that acts as a glutathione precursor which provides antioxidant properties

(Adekunle *et al*., 2022) to sperm cells via its easy ability to penetrate the plasma membrane (Alomar, 2021) and augment the biosynthesis of intracellular glutathione (Zhu *et al*., 2022).

Besides, cysteine acts as a ROS scavenger, protecting sperm proteins, DNA and membrane lipids from oxidative damage (Atessahin *et al*., 2008).

L-carnitine (LC), a vitamin-like compound, is biosynthesized from two essential amino acids; lysine and methionine (Gülçin, 2006). LC is a powerful antioxidant that increases the activities of antioxidant enzymes such as GSH-Px and SOD (Setyawan *et al*., 2009). Moreover, LC plays an important role in mammalian sperm maturation and metabolism (Abd-Allah *et al*., 2021). It has been suggested that the LC in the epididymal fluid stabilizes the sperm plasma membrane and functional metabolic pathways and increases motility (Longobardi *et al*., 2017).

No previous studies have compared the effects of different concentrations of cysteine and LC supplementation on cryopreserved buck semen. To this end, the study was designed to clarify the effects of cysteine and LC on post-thaw sperm parameters including sperm motility, CASA assessed kinetic sperm parameters, sperm viability, plasma membrane and acrosome integrity, total antioxidant capacity (TAC), lipid peroxidation indicated by the level of malondialdehyde (MDA) and comet assayed sperm DNA integrity. Correlations between the previously mentioned parameters and frozen semen supplemented with various doses of cysteine and LC were also assessed.

Materials and Methods

Location and animals

This study was carried out in the Animal Reproduction Research Institute (ARRI), Agriculture Research Centre (ARC), Giza, Egypt. Five mature Zaraibi bucks (2-4 years old and 50-60 kg weight) were used in the present study. The animals were housed in a clean wide semi-open shed stall and received the same management in the experimental farm of ARRI. All bucks were clinically healthy with apparently normal external genitalia. A proper general management schedule for disease prevention and hoof trimming was applied. Bucks received the essential daily requirements of ration according to the National Research Council (NRC, 2007), and were fed twice daily on a basal diet consisting of a concentrated mixture adequate in protein, energy, minerals and vitamins. Clean water was provided *ad libitum*. All experimental procedures followed the guidelines of the Ethics and Animal Experimentation Committee of Suez Canal University with the registration number (2017001).

Semen collection and evaluation

Before the beginning of the study, bucks were trained for semen collection using an artificial vagina (IMV, L'Aigle, France) adjusted to the proper temperature (42-43°C) and lubricated by sterile vaseline. Semen collection began three weeks prior to the initiation of the experiment. Throughout the study period, semen samples were collected once weekly for 10 successive weeks during the breeding season. Semen collection was performed early in the morning using a doe as a teaser. Two successive ejaculates were obtained from each buck.

Immediately after collection, fresh semen samples were evaluated at 37°C within 15 min. Semen characteristics of each buck were examined separately including volume, mass activity, sperm progressive motility percentage and sperm cell concentration. Ejaculates (volume ≥0.5 ml, mass motility ≥3, progressive motility ≥75% and sperm concentration ≥2.5 × 10^9/ml) of the bucks were pooled to obtain the suitable volume used for dilution and processing. The pooled semen was centrifuged (Harmonic Series, German) at 1800 × g for 15 min. to remove seminal plasma (Tabarez *et al*., 2017).

Semen processing and cryopreservation

Pooled ejaculates were divided into 7 equal aliquots corresponding to different treatments; Tris egg yolk-based extender (control; TBE) and 6 antioxidant concentrations added to TBE. Tris egg yolk-based extender was composed of the following ingredient: tris (300 mM, Loba Chemie PVT, India), glucose (28 mM, ADWIC, Egypt), citric acid (95 mM, Alpha Chemika Mumbai, India), 5% glycerol (Fisher Scientific, USA), 15% egg yolk, penicillin G sodium (1000 IU/ml), Streptomycin sulfate (1000 µg/ml) and distilled water to 1000 ml, pH was adjusted to 6.8 (Küçük *et al*., 2014). The antioxidant groups included cysteine (Oxford Lab Chem, India) at concentrations 2.5, 5 and 10 mM (Sarıözkan *et al*., 2014) and LC (Titan Biotech, India) at concentrations 2.5, 5, and 7.5 mM (Longobardi *et al*., 2017). Each aliquot was diluted to reach a final concentration of approximately 400 × 10^6 sperm/ml, then cooled and equilibrated within 150 min. at 5°C. After that, the cooled extended semen was aspirated into a 0.25 ml straw, thermally sealed and frozen using liquid nitrogen (LN) vapour, then immersed in an LN tank (-196°C) and kept for storage (Naijian *et al*., 2013).

Post-thaw semen evaluation

Frozen straws were thawed in a water bath adjusted at 37°C for 30 s. The thawed semen was assessed for sperm motility, dynamic sperm motility parameters, viability, plasma membrane and acrosome integrity, TAC, lipid peroxidation and sperm DNA integrity judged by comet assay.

Sperm motility

Motility was assessed using Computer-Assisted Sperm Analysis [(CASA), instrument SpermVision™ software minitube Hauptstraße 41. 84184 Tiefenbach, Germany]. Pre-adjusted CASA for goat sperm analysis was used to evaluate sperm motility and motility parameters. On a pre-warmed slide at 37°C, 10 µL of thawed semen sample was placed and covered with a

coverslip to be examined at ×100 magnification. At least 200 sperms were counted in six fields for each treatment of the study as described by Cirit *et al*. (2013).

Dynamic sperm motility parameters were recorded by CASA including: progressive motility (%), velocity straight line (VSL, µm/s), velocity curved line (VCL, µm/s), velocity average path (VAP, µm/s), linearity index (LIN = (VSL/VCL) × 100), straightness (STR = (VSL/VAP) × 100) and amplitude of lateral head displacement (ALH, µm).

Sperm viability

Sperm viability was determined using an eosin-nigrosine stain (Evans and Maxwell, 1987). The stain was prepared as 1.67 g eosin-Y (Riedel-deHaën Co., Germany), 10 g nigrosin (Winlab Co., UK) and 2.9 g sodium citrate dissolved in 100 ml distilled water. A total of 200 sperm cells were counted under X400. A sperm with an unstained head was considered as a viable sperm, while those displaying a partial or complete purple colour were considered as dead (Shafiei *et al*., 2015).

Sperm plasma membrane integrity

The hypo-osmotic swelling test (HOST) was done to evaluate the functional integrity of the sperm membrane as appointed by Jeyendran *et al*. (1984). The HOST solution of 75 mOsm/kg osmolarity was prepared by dissolving 0.367 g sodium citrate and 0.675 g fructose separately in 50 ml distilled water. The final HOST solution was prepared by mixing equal volumes of sodium citrate and fructose solutions (Vasquez *et al*., 2013). Briefly, 150 µL of HOST solution was mixed with 10 µL of thawed semen. The mixture was incubated for 30 min at 37°C. The incubated sample (10 µL) was placed on a clean slide and covered to be examined immediately under ×400. A total of 200 spermatozoa were counted in different microscopic fields to assess the percentage of positively reacting spermatozoa that showed varying degrees of tail curling (Anghel *et al*., 2010).

Acrosome integrity

Acrosome integrity was determined using Giemsa stain 7.5% (Egyptian Diagnostic Media, Egypt) dissolved in distilled water and prepared freshly before use according to Watson (1975) and Chowdhury *et al*. (2014). A total of 200 sperm cells were examined randomly for each sample under a microscope (×400). An intact acrosome was characterized by the presence of a normal apical ridge, while sperms with damaged acrosomes did not show the apical ridge (Shahzad *et al*., 2016).

Total antioxidant capacity

TAC levels were assessed by colourimetric determination of H_2O_2 in the thawed semen samples using a spectrophotometer (at 37°C, λ= 505 nm) according to Koracevic *et al*. (2001).

Lipid peroxidation levels

Malondialdehyde (MDA) level was estimated in each semen sample as an LPO product and a thiobarbituric acid (TBA) determinant (da Silva Maia *et al*., 2010). MDA concentrations were measured by a spectrophotometer (λ= 534 nm) as described by Ohkawa *et al*. (1979).

Sperm DNA integrity

The neutral comet assay (single-cell gel electrophoresis) was applied to detect the sperm DNA integrity in post-thawed buck spermatozoa as explained by Bucak *et al*. (2010). Spermatozoa with fragmented DNA showed extensive migration of DNA from the sperm head, causing a "comet" pattern, while a whole sperm head without a comet had intact DNA (Sarıözkan *et al*., 2014).

Statistical analysis

The effects of different concentrations of antioxidants on the studied sperm parameters were tested by one-way analysis of variance (ANOVA) using the SPSS software (ver. 20, USA). Further comparisons between different treatment groups were performed by Duncan's test for multiple comparisons. Correlations between different concentrations of cysteine and LC and the values of progressive motilities, CASA kinetic parameters, sperm viability, HOST, acrosome integrity, TAC, MDA and parameters of comet assayed DNA were assessed by calculating Pearson correlation coefficients. The obtained data were expressed as mean±SEM. Significance was designated at a probability value of P<0.05.

Results

The effects of cysteine and LC on sperm motility and velocity parameters

The results showed that the addition of cysteine and LC to a Tris extender significantly (P<0.001) improved CASA assessed progressive motility as well as sperm kinetic characteristics (Table 1). Frozen buck spermatozoa supplemented with LC (5 and 7.5 mM) revealed a significant increase in progressive motility as compared to other groups. Regarding VSL, LIN, and STR, 2.5 and 7.5 mM LC resulted in the highest values compared to other treatments. Furthermore, ALH showed a significant decrease with the addition of LC (2.5 and 7.5 mM). Cysteine at 5 and 10 mM concentrations significantly (P<0.01) increased VCL. Control and cysteine (2.5 mM) showed significant (P<0.01) decreases in VAP as compared to the other antioxidant groups.

The effects of cysteine and LC on sperm viability

The use of a Tris egg yolk-based extender supplemented with cysteine and LC significantly (P<0.001) improved the post-thaw sperm viability

Table 1: Effect of different concentrations of cysteine and LC on motion characteristics of post-thaw buck semen assessed by CASA

Parameters	Control	Cysteine			L-carnitine			P-value
		2.5 mM	5 mM	10 mM	2.5 mM	5 mM	7.5 mM	
Progressive motility (%)	26.47 ±1.35^{d}	41.81 ±2.06^{c}	44.75 ±2.96^{c}	46.89 ±1.94^{bc}	46.10 ±1.03^{c}	51.72 ±0.97^{ab}	52.52 ±0.93^{a}	***
VSL (μm/sec)	39.68 ±1.06^{cd}	36.92 ±2.49^{d}	45.46 ±0.81^{ab}	40.45 ±1.61^{bcd}	48.68 ±2.59^{a}	44.47 ±0.93^{abc}	47.95 ±1.45^{a}	***
VCL (μm/s)	78.95 ±1.05^{c}	82.15 ±6.96^{c}	96.59 ±2.77^{ab}	98.38 ±3.83^{a}	86.26 ±2.99^{c}	87.19 ±1.91^{bc}	84.55 ±1.40^{c}	**
VAP (μm/s)	48.80 ±1.23^{b}	48.07 ±3.23^{b}	56.21 ±0.81^{a}	54.18 ±1.66^{a}	56.24 ±2.19^{a}	54.31 ±0.99^{a}	55.92 ±1.12^{a}	**
LIN (%)	49.50 ±0.96^{b}	45.30 ±1.35^{c}	47.09 ±1.98^{bc}	40.71 ±1.48^{d}	55.73 ±1.57^{a}	50.58 ±0.63^{b}	56.18 ±1.01^{a}	***
STR (%)	80.83 ±0.40^{b}	76.04 ±0.83^{c}	80.55 ±1.74^{b}	74.14 ±0.94^{c}	85.55 ±1.32^{a}	81.47 ±0.57^{b}	84.94 ±1.03^{a}	***
ALH (μm)	4.39 ±0.22^{b}	4.08 ±0.18^{bc}	3.78 ±0.29^{cd}	5.06 ±0.08^{a}	3.41 ±0.17^{d}	3.75 ±0.08^{cd}	3.37 ±0.11^{d}	***

VSL (μm/s): Velocity straight line, VCL (μm/s): Velocity curved line, VAP (μm/s): Velocity average path, LIN (%): Linearity index [LIN = (VSL/VCL) × 100], STR (%): Straightness [STR = (VSL/VAP) × 100], and ALH (μm): Amplitude of lateral head displacement. Different superscripts within the same row demonstrate significant differences (** P<0.01, and *** P<0.001)

Table 2: Effect of different concentrations of cysteine and LC on sperm viability, HOST, acrosomal integrity, TAC, and MDA of post-thaw buck semen

Parameters	Control	Cysteine			L-carnitine			P-value
		2.5 mM	5 mM	10 mM	2.5 mM	5 mM	7.5 mM	
Sperm viability (%)	38.50 ±2.69^{d}	51.75 ±2.56^{c}	52.50 ±3.07^{bc}	60.00 ±2.78^{ab}	58.40 ±1.47^{abc}	62.00 ±2.48^{a}	63.50 ±1.94^{a}	***
HOST (%)	36.50 ±2.84^{c}	46.83 ±2.88^{b}	50.13 ±1.25^{ab}	52.50 ±3.52^{ab}	51.67 ±1.36^{ab}	54.75 ±0.95^{a}	56.60 ±1.08^{a}	***
Acrosome integrity (%)	75.17 ±3.03^{b}	81.57 ±1.73^{ab}	85.33 ±2.28^{a}	87.6 ±2.93^{a}	88.00 ±2.05^{a}	87.40 ±2.89^{a}	89.00 ±2.41^{a}	**
TAC (mM/L)	0.86 ±0.07^{d}	2.73 ±0.37^{ab}	2.91 ±0.23^{ab}	3.27 ±0.36^{a}	1.55 ±0.25^{cd}	2.16 ±0.22^{bc}	2.09 ±0.36^{bc}	***
MDA (nmol/ml)	83.75 ±5.38^{a}	66.49 ±4.12^{b}	67.70 ±4.26^{b}	53.91 ±1.57^{c}	60.84 ±2.84^{bc}	62.61 ±3.34^{bc}	54.11 ±3.44^{c}	***

HOST: Hypo-osmotic swelling test, TAC (mM/L): Total antioxidant capacity, and MDA (nmol/ml): Malondialdehyde (product of lipid peroxidation). Different superscripts within the same row demonstrate significant differences (** P<0.01, and *** P<0.001)

percentage compared to the control group. The best results were obtained with cysteine (10 mM) and LC (5 mM and 7.5 mM) in comparison to the control and other treated groups (Table 2).

The effects of cysteine and LC on sperm membrane and acrosome integrity

The addition of cysteine and LC to the Tris extender significantly (P<0.001 and P<0.01, respectively) improved post-thaw HOST and acrosome integrity percentages of the frozen buck semen (Table 2). Cysteine (5 and 10 mM) and LC (5 and 7.5 mM) exhibited significant protective effects on sperm plasma membranes in post-thaw buck sperm. However, the addition of 2.5 mM cysteine did not cause any difference in acrosome integrity percentage when compared with the control.

The effects of cysteine and LC on levels of TAC and MDA

Cysteine and LC significantly (P<0.001) elevated the levels of TAC in extended buck semen. The highest levels of TAC were recorded in the cysteine group compared to the LC and control groups (Table 2). Moreover, MDA levels showed a significant (P<0.001) decrease in frozen buck semen supplemented with cysteine and LC. The lowest levels of MDA were noticed in cysteine (10 mM) and LC (7.5 mM) compared to the control and other groups (Table 2).

The effects of cysteine and LC on sperm DNA integrity

Neutral DNA comet assay of frozen buck semen showed a significant (P<0.001) decrease in the comet percentage of cysteine (2.5 and 5 mM) and LC (7.5 mM). Furthermore, the tail length significantly (P<0.05) decreased in cysteine (5 and 10 mM). However, cysteine (2.5 and 10 mM) and LC (7.5 mM) significantly (P<0.05) decreased the tail moment of frozen buck semen (Table 3 and Fig. 1).

Correlations between sperm characteristics and frozen semen supplemented with cysteine and LC

Our data showed positive correlations between ALH (P<0.01), sperm viability (P<0.001), acrosome integrity (P<0.01) and comet percentage (P<0.001) and the cysteine supplemented extender. However, cysteine supplementation showed negative correlations with MDA (P<0.01) and the tail length (P<0.01) of buck

Table 3: Effect of different concentrations of cysteine and LC on DNA damage of post-thaw buck semen assessed by comet assay

Parameters	Control	Cysteine			L-carnitine			P-value
		2.5 mM	5 mM	10 mM	2.5 mM	5 mM	7.5 mM	
Comet (%)	7.50 $\pm 0.29^{a}$	4.50 $\pm 0.29^{d}$	5.43 $\pm 0.30^{c}$	7.70 $\pm 0.15^{a}$	6.03 $\pm 0.09^{bc}$	6.73 $\pm 0.15^{b}$	5.50 $\pm 0.29^{c}$	***
Head DNA (%)	90.45 $\pm 1.15^{e}$	97.53 $\pm 0.72^{a}$	93.93 $\pm 0.40^{d}$	96.40 $\pm 0.13^{abc}$	95.62 $\pm 0.43^{bcd}$	95.01 $\pm 0.15^{cd}$	97.31 $\pm 0.12^{ab}$	***
Tail DNA (%)	9.55 $\pm 1.15^{a}$	2.47 $\pm 0.72^{e}$	6.07 $\pm 0.40^{b}$	3.59 $\pm 0.13^{cde}$	4.38 $\pm 0.43^{bcd}$	4.99 $\pm 0.15^{bc}$	2.69 $\pm 0.12^{de}$	***
Tail length (PX)	7.96 $\pm 0.74^{abc}$	10.77 $\pm 1.87^{ab}$	5.00 $\pm 0.69^{c}$	6.06 $\pm 0.03^{bc}$	8.92 $\pm 0.51^{abc}$	11.88 $\pm 3.10^{a}$	9.98 $\pm 0.96^{ab}$	*
Tail moment	0.63 $\pm 0.05^{a}$	0.25 $\pm 0.01^{bc}$	0.36 $\pm 0.09^{bc}$	0.21 $\pm 0.00^{c}$	0.48 $\pm 0.01^{ab}$	0.46 $\pm 0.17^{abc}$	0.29 $\pm 0.05^{bc}$	*
Olive tail moment	1.19 $\pm 0.18^{ab}$	0.51 $\pm 0.14^{c}$	1.09 $\pm 0.02^{ab}$	0.64 $\pm 0.01^{c}$	0.93 $\pm 0.04^{b}$	1.27 $\pm 0.03^{a}$	0.62 $\pm 0.04^{c}$	***

Different superscripts within the same row demonstrate significant differences (* P<0.05, and *** P<0.001)

Table 4: Correlation between values for progressive motilities, CASA kinetic parameters, sperm viability, HOST, acrosome integrity, TAC, MDA, and DNA comet assay parameters of frozen buck semen supplemented with cysteine and L-carnitine

Sperm parameters	Cysteine		L-carnitine	
	r	P	r	P
Progressive motility	0.24	NS	0.49	0.001
VSL	0.15	NS	0.34	0.05
VCL	0.36	NS	-0.09	NS
VAP	0.28	NS	-0.00	NS
LIN	-0.34	NS	0.11	NS
STR	-0.23	Ns	0.01	NS
ALH	0.47	0.01	-0.08	NS
Sperm viability	0.65	0.001	0.47	0.001
HOST	0.37	NS	0.62	0.001
Acrosome integrity	0.51	0.01	0.10	NS
TAC	0.25	NS	0.35	0.05
MDA	-0.54	0.01	-0.32	0.05
Comet	-0.96	0.001	-0.46	0.01
Head DNA	-0.16	NS	0.66	0.001
Tail DNA	0.16	NS	-0.66	0.001
Tail length	-0.50	0.01	0.08	NS
Tail moment	-0.23	NS	-0.41	0.01
Olive tail moment	0.09	NS	-0.50	0.001

VSL (µm/s): Velocity straight line, VCL (µm/s): Velocity curved line, VAP (µm/s): Velocity average path, LIN (%): Linearity index [LIN = (VSL/VCL) × 100], STR (%): Straightness [STR = (VSL/VAP) × 100], ALH (µm): Amplitude of lateral head displacement, HOST: Hypo-osmotic swelling test, TAC (mM/L): Total antioxidant capacity, MDA (nmol/ml): Malondialdehyde (product of lipid peroxidation), and NS = Non-significant. Values of P<0.05 indicate differences

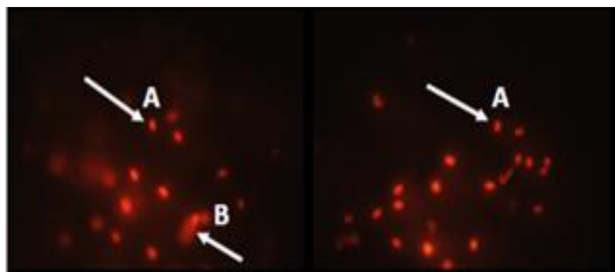

Fig. 1: Comet assay shows DNA damage in extended buck sperms (A) Intact DNA without comet tail B) Damaged DNA with B) Damaged DNA with long comet tail

semen. Positive correlations were noticeable between LC supplementation and progressive motility (P<0.001), VSL (P<0.05), sperm viability (P<0.001), HOST (P<0.001), TAC (P<0.05), and head DNA (P<0.001), while LC supplementation exhibited negative correlations with MDA (P<0.05), comet (P<0.01), tail DNA (P<0.001), tail moment (P<0.01), and olive tail moment (P<0.001) of buck sperm (Table 4).

Discussion

Semen cryopreservation is an effective technique, extensively used in livestock to upsurge male fertility (Elsayed *et al*., 2019). However, this technology-induced method causes physical and chemical sperm deteriorations that consequently perturbe the fertilizing capacity of sperms after the freezing-thawing process (Lv *et al*., 2019). Therefore, the current research aimed to study the supplementation of buck semen extender with cysteine and LC and its effect on cryopreserved buck spermatozoa.

Our results demonstrated that LC addition to egg yolk-based Tris enhanced the CASA kinetic parameters of frozen buck semen more than that of control and cysteine groups. However, the cysteine group showed significant improvement of CASA kinetic parameters over the control that showed less capability with LC. The current findings were in agreement with the study carried out by Iqbal *et al*. (2016). On the other hand, Bucak *et al*. (2010) in *Capra hircus* bucks and de Souza *et al*. (2019) reported that LC did not affect the dynamics of sperm motility in rams. These different results may be attributed to the differences between LC and cysteine concentrations. They may also be specific to semen from different species and breeds. Significant positive correlations were found between LC and post-thaw progressive motility as well as VSL of buck semen that confirmed the superior effect of LC on frozen buck semen compared to cysteine, whereas, cysteine was only found to have a significant positive correlation with ALH.

The concentrations of 10 mM cysteine and LC, especially 7.5 mM, revealed a significant increase in

sperm viability that was assured by the positive significant correlation. The findings of Anghel *et al*. (2010) and Memon *et al*. (2012) in bucks and Sharafi *et al*. (2015) in rams supported the previous finding. However, Bucak *et al*. (2008) observed no significant effect of cysteine on rams' post-thaw sperm viability. Few studies were available regarding the influence of LC on buck sperm viability. Longobardi *et al*. (2017) found that LC did not improve post-thawing sperm viability in buffalo bulls. The positive effect of LC on post-thaw sperm motility and viability could be explained by its ability to shuttle acetyl and acyl groups which pass the inner membrane of the mitochondria, serving as a buffer and trapping excess mitochondrial acetyl-CoA as acetyl-LC, thus protecting the activity of pyruvate dehydrogenase, which is the key enzyme for mitochondrial respiration (Jeulin and Lewin, 1996). Therefore, LC improves mitochondrial functions and ATP generation of the sperm (Longobardi *et al*., 2017).

In the present study, the addition of cysteine and LC to buck frozen semen significantly improved the plasma membrane integrity. Besides, adding LC to the Tris extender showed a significant positive correlation with HOST. Similar results were obtained by Anghel *et al.* (2010) and Memon et al. (2012) for cysteine, and Bucak *et al*. (2010) for LC. On the other hand, Atessahin *et al.* (2008) and de Souza *et al*. (2019) demonstrated no significant effect on HOST percentages after the addition of cysteine and LC to the semen extender. The correlation results show that while LC improved HOST for post-thaw frozen bucks' sperms, cysteine did not significantly correlate with HOST for post-thaw buck sperm.

In the current study, cysteine and LC significantly enhanced the percentage of acrosome integrities of frozen buck semen. Cysteine showed a higher positive correlation with sperm acrosome integrity percentage and therefore better efficiency in improving acrosome integrity compared to LC. Our results agreed with those of Memon *et al*. (2012) and Toker *et al*. (2016) for cysteine as well as those from Bucak *et al*. (2010) for LC. However, Atessahin *et al*. (2008) reported contradictory results in bucks that may be due to the differences in doses of cysteine and the breeds of bucks.

The positive effect of cysteine in sperm post-thaw motility, viability, plasma membrane and acrosome integrities may be due to the fact that cysteine is an amino acid and a precursor for intracellular glutathione that can inactivate the ROS and catalyze hydrogen or other superoxide detoxification (Atessahin *et al*., 2008). Also, cysteine acts as a glutathione peroxidase cofactor which destroys H_2O_2 (Anghel *et al*., 2010). Furthermore, enhancement in sperm motility may be due to the cryoprotective effect of cysteine on the functional integrity of mitochondria and axosome after the thawing process (Memon *et al*., 2012). The actual role of LC in improving plasma membranes as well as acrosome integrities is not fully elucidated. However, the increased TAC and decreased ROS concentrations during LC addition may suggest the beneficial effect resulted from its protection of plasma membranes against ROS damage (Longobardi *et al*., 2017).

Concerning antioxidant activities, the current study showed that 10 mM cysteine and LC (5 and 7.5 mM) treated-extenders increased TAC levels. The superiority of LC as an antioxidant was proven by its positive correlation with TAC, which was not achieved by cysteine. These findings were in agreement with those of Longobardi *et al*. (2017) for LC. However, Pradieé *et al*. (2016) observed no elevation in the oxidative capacity of ram semen supplemented with cysteine.

With respect to MDA concentration, cysteine and LC resulted in significantly diminished MDA levels of buck frozen-thawed semen. Moreover, lipid peroxidation was negatively correlated with cysteine and LC. This gives an idea about the protective effects of cysteine and LC on sperm membrane integrity of buck semen as MDA levels constitute a biomarker for lipid peroxidation that is harmful to sperm integrity. Therefore, optimizing low levels of MDA in post-thawed spermatozoa is considered a target for a successful cryopreserving agent (Sharafi *et al*., 2015). Increased MDA levels were the real cause for aggravation of oxidative stress whereby TAC depletion harms the sperm membrane integrity (Alahmar, 2019). Our results were similar to TAC results denoting the antioxidant properties of both cysteine and LC that positively influenced post thawing sperm motility, viability and acrosome integrity. Other authors confirmed the anti-lipid peroxidation effect of cysteine (Memon *et al*., 2012). Contradictory to these results, Bucak *et al*. (2008), Coyan *et al*. (2011), and Sharafi *et al*. (2015) found that cysteine did not influence MDA concentrations after the freezing-thawing process. Bucak *et al*. (2010) did not find any effect of LC on the post-thawing MDA levels either.

Consequent to the antioxidant potential of cysteine and LC, the later additives significantly improved DNA integrity compared to the control. This was demonstrated by decreased comet percentage, tail length and tail moment. These results were similar to the findings of Bucak *et al*. (2010) who stated that LC lowered the percentages of sperm with damaged DNA. Additionally, Toker *et al*. (2016) noted that cysteine has a protective effect to sperm DNA. The observed negative correlation of LC with comet percentage and tail parameters showed the superior beneficial effects of LC compared to cysteine on the improvement of sperm DNA integrity of post-thaw buck semen. Elevated lipid peroxidation as well as increased oxidative stress, manifested by increased MDA and reduced TAC, due to freezing and thawing of semen were causal factors for impaired sperm membrane integrity. Moreover, free radicals and peroxides have been found to impair DNA integrity causing damage (Peris *et al*., 2007). The negative and positive correlations of LC with MDA and TAC levels denoted a considerable antioxidant potential that mitigated freezing- and thawing-induced lipid peroxidation and oxidative stress that subsequently retained sperm membrane integrity.

The usage of cysteine (5 and 10 mM) and LC (5 and

7.5 mM) as supplements for egg yolk extenders could efficiently improve sperm post-thaw properties. This was achieved by the alleviation of TAC depletion and MDA induced DNA damage that improved DNA criteria in the comet assay.

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Conflict of interest

The authors confirmed the absence of conflict of interest.

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