

Addition of L-Cysteine and Vitamin E to Semen Diluent Enhances Freeze-thawed Spermatozoa Characteristics in Crossbred Cattle Bulls under Subtropical Environment

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ABSTRACT

Vitamins and amino acids are important trace nutrients that are essential for body growth and reproductive soundness, whereas the lack of these nutrients is directly related to reproductive performance. Hence, the antioxidants agents (L-cysteine and vitamin E) were added into semen diluent and assessed the freeze-thawed semen quality parameters. The ejaculates were collected from two crossbred groups (*Sahiwal* × *Holstein-Friesian*), and (*Achai* × *Jersey*) and, split into seven aliquots while extended with three different concentration (5, 7.5 and 10 mmol) of L-cysteine diluents and followed by other three concentrations (1.2, 2.4 and 4.8 mmol) of vitamin E diluents and, the last aliquot of semen was extended with control (no additives) to semen diluent. The spermatozoa freeze-thawed quality were assessed and found that total motility, progressive motility, membrane functionality, acrosomal integrity, and viability percentage were significantly higher ($P < 0.05$) in the diluent groups treated with 7.5 mmol of L-cysteine or 4.8 mmol of vitamin E. Correspondingly the DNA integrity and mitochondrial membrane potential percentage were found significantly higher ($P < 0.05$) in diluent groups treated with 7.5 mmol L-cysteine or 4.8 mmol vitamin E. The juvenile results indicated that L-cysteine and vitamin E improves the freeze-thawed spermatozoa characteristics in crossbred bulls.

INTRODUCTION

The idea behind cross breeding is to generate crossbred offspring with early maturity, superior dairy characteristics and adaptability to harsh local environmental condition. However, little attention has been devoted to improving the quality of breeding programs (Sattar and Mirza, 2009). Over 50% of crossbred juvenile bulls used for semen collection were reported to have complications related to the quality of semen, cryo-biology and libido

(Mukhopadhyay *et al.*, 2010; Mandal and Tyagi, 2004). Compared to crossbred bulls, indigenous or purebred bulls were reported to have sperm with higher freezing ability and fertility. Due to poor (initial) semen quality nearly 50% to 55% of ejaculates collected from crossbred bulls were inappropriate for freezing (Sudheer and Xavier, 2000). Various efforts have been made to determine the etiology of poor quality semen production in crossbred bulls in the seminological, andrological, genetical and probable an ecological stage with varied success (Gulia *et al.*, 2010; Mandal *et al.*, 2008). Crossbred progeny have been reported to be more prone to reproductive problems than purebred offspring (Bitew and Prasad, 2011). One important factor of sub-fertility in cattle production is the

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Authors' Contribution

IMK and YZ presented the concept of the study. IMK and AK processed the data. SUR, JZA and DX analysed the data. YZ, ZC and HL did funding acquisition. IMK and MAR defined methodology. YZ administrated the project. SUR provided technical support. ZC and IMK wrote the manuscript.

Key words

Crossbred bulls, Cryopreservation, Freeze-thawed semen, L-cysteine, Vitamin E

use of substandard semen in artificial insemination (AI) (Gnoth *et al.*, 2005). Sub-fertility may occur as a result of disorders in semen production including reduced or loss of sperm motility (Dirami *et al.*, 2013).

Cryopreservation is the key technique for artificial insemination and providing a platform to distribute the elite genetic material worldwide but the freezing-thawing cycle can have detrimental effect on sperm biology, causing damage and modifications that eventually lead to the death of sperm cell, thereby reducing fertility (Trachootham *et al.*, 2009). Cryopreservation can generate reactive oxygen species (ROS) which interact with sperm cell plasma membrane leading to trans-omics disturbance and suffer the fertility. When ROS exceed the normal level and spermatozoa defence mechanisms are decreased not only the plasma structure but also DNA can be damaged. This damage can reduce spermatozoa fertility and the capability to develop a healthy embryo (Pons-Rejraji *et al.*, 2009). Another study found that low ROS production is positively correlated with spermatozoa motility whereas high ROS production was negatively correlated with sperm motility. This ROS-induced decline in spermatozoa motility, is primarily attributed to decreased ATP production (Armstrong *et al.*, 1999).

Previous studies have found that the antioxidant property of vitamin E can improve the freeze-thawed traits of spermatozoa and contribute to the management of male infertility (Keskes-Ammar *et al.*, 2003). Vitamin E and beta-carotene have been shown to have the antioxidant property that support cells against ROS damage (Almbro *et al.*, 2011). Vitamin E is considered to be the most active agent in the antioxidant system of spermatozoa, where it protects the cell membrane against attack from ROS and lipid peroxidation (LPO) (Yousef *et al.*, 2003). The administration of antioxidant agents to semen diluent has been improving the indicators of spermatozoa motion (e.g. spermatozoa longevity, individual motility, progressive motility and viability) in *Ghezel* rams (Aminipour *et al.*, 2013). However, cattle bull semen has natural guard system against the ROS and, that are considered insufficient, when the spermatozoa going through the critical phase of freeze-thawed imposed stress (Nichi *et al.*, 2006).

Therefore, it is essential to strengthen the semen diluent with appropriate antioxidant supplements to reduce ROS damages during the freeze-thawing of bull semen. Cysteine is another active agent, also known to act as an intracellular antioxidant and guard's cells against oxidative stress and damage (Meister and Andersson, 1983). L-cysteine comprises an important thiol group that works as a non-enzymatic antioxidant and easily penetrates into sperm cells. The addition of cysteine to semen diluent has been reported to improve the freeze-thawed motility

and viability of boar (Kaeoket, 2010), buck (Kulaksiz and Daskin, 2010) and bull semen (Sariözkan *et al.*, 2009).

This study assessed the effect of L-cysteine and vitamin E administration to semen diluent on freeze-thawed spermatozoa characteristics. The ideal concentrations of antioxidant supplements for the cryopreservation media of crossbred bull semen was also determined that resulting in the good post-thawed semen characteristics. We perceived that addition of L-cysteine and vitamin E to semen diluent would improve the freeze-thawed semen qualities (e.g., spermatozoa motility, membrane functionality, acrosomal integrity, DNA integrity, and spermatozoa viability ratio and mitochondrial membrane potential) of crossbred bull.

MATERIALS AND METHODS

Animals and rearing system

Six physically and andrologically healthy mature bulls of two crossbred groups, (*Sahiwal* × *Holstein-Friesian*) and (*Achai* × *Jersey*), were selected from the National Agriculture Research Center (NARC) in Islamabad. The average age of the bulls were 3.0 ± 0.14 years. All experimental bulls were reared under the same feeding regime and housing facilities. Seasonal green fodder *ad-libitum* and formulated feed (e.g., maize gluten 30 of 15%, cotton seed cake 18%, maize grain broken 14%, canola meal 10%, molasses 16%, wheat bran 25%, and mineral mixture 2%) were being offered.

Semen collection

According to standard artificial insemination procedure, total of sixteen ejaculates were collected from each bull. The following criteria were considered for collected ejaculates: volume of 2–6 mL, concentration $\geq 60 \times 10^6$ spermatozoa mL⁻¹ and total motility of 70% for conducting the post-thaw experiments (Tuncer *et al.*, 2010). The volume of collected semen was measured while using a graduated tube and the initial spermatozoa total motility was determined microscopically (magnification 400X; Olympus BX20, Tokyo, Japan). The fresh spermatozoa concentration was evaluated using a digital photometer (L'Aiglon; IMV, Cedex, France) and pooled ejaculates were used for further assessment.

Addition of L-cystiene and vitamin E in semen diluent

The stock diluent comprised of 1.56% citric acid (Fisher Scientific, UK), 3.0% tris-(hydroxymethyl)-aminomethane (Research Organics, USA), 0.2% glucose (Scharlau, Spain), 7% glycerol (Riedel-deHaen, Germany), 20% egg yolk in distilled water, at pH 6.8 and standard osmotic pressure of 320 mOsmol kg⁻¹ for cattle bulls was used (Beheshti *et al.*, 2011). The semen samples 60×10^6

spermatozoa / mL concentration were used for dilution and then divided into seven different experimental fractions. The 1st, 2nd and 3rd fractions were diluted containing Cysteine (C8786; Sigma–Aldrich Co., St. Louis, MO, USA) at concentrations of 5 mmol (L-cysteine-1), 7.5 mmol (L-cysteine-2) and 10 mmol (L-cysteine-3); the 4th, 5th and 6th fractions were diluted containing Vitamin E (Sigma–Aldrich Co., St. Louis, MO, USA) at concentrations of 1.2 mmol (vitamin-E-1), 2.4 mmol (vitamin-E-2) and 4.8 mmol (vitamin-E-3) whereas the 7th (control fraction) was without any addition of additives to the diluent. Vitamin E was dissolved in absolute ethanol of (0.05% v/v) to prepare a stock solution of 10 mM before it was added to the diluent (Motemani *et al.*, 2017).

Cryopreservation

All diluted samples were cooled at 4 °C over 2 h in cooled cabinet and after cooling the samples were exposed to equilibration time (e.g., total refrigeration time 4 h at 4 °C) (Leite *et al.*, 2010). At the end of cooling and equilibration period the extended samples were added to 0.25 mL French straws and sealed with a filling and sealing device (IMV, L'Aigle, France). The semen straws were cryo-preserved through programmable bio-freezer (Minitub, Tiefenbach, Germany) at -20°C/min to -100°C and at -10°C/min from -100°C to -140°C and finally plunged into liquid nitrogen. After a week, the frozen straws were thawed for freeze-thawed assessment.

Post-thawed semen evaluation

The following parameters were evaluated in the freeze-thawed semen of bulls whereas all the parameters were tested for three times consecutively.

Spermatozoa total motility and progressive motility

The spermatozoa total motility and progressive motility parameters were assessed by computer assisted sperm motion analysis (CASA, Version 5.1; Microptic Barcelona, Spain). A total of 10 µL of freeze-thawed semen was placed in pre-warmed Makler chamber (Sperm Meter; Sperm Processor Pvt. Ltd, Aurangabad, India) and examined. We randomly selected three microscopic fields each scanned five times. The mean of these fifteen scans was used for spermatozoa motility analysis.

Spermatozoa membrane functionality (Hypo-osmotic swelling test)

Spermatozoa plasma membrane functionality was determined via hypo-osmotic swelling tests (Pons-Rejraji *et al.*, 2009). A total of 0.1 mL freeze-thawed semen was mixed with 1 mL HOST solution consisting of 0.5 mL D-fructose (1.47%), sodium citrate (2.7%) and eosin

0.5% (w/v). Following incubation at 37°C for 40 min, 5 µL of sample was removed with micropipette, transferred to a pre-warmed glass slide while covered with cover slip and placed under the compound microscope at 40X for observation. A total of 200 spermatozoa were counted in five different fields for each sample. The HOST solution provided hypotonic media for the spermatozoa, resulting in coiled and swollen tails due to the different osmotic potential. Intact plasma membrane functionality was indicated by swollen, coiled tails and unstained heads, whereas un-swollen, uncoiled tails, and pink heads indicated disruptive spermatozoa membrane functionality.

Spermatozoa viability

To determine the ratio of viable spermatozoa, eosin (1%) and nigrosin (5%) stain was mixed in 3% sodium citrate dehydrates solution. Nigrosin stain provides a background for spermatozoa visualization while eosin enters into the dead spermatozoa membrane and produces a pink color, allowed dead spermatozoa identification. A small drop of freeze-thawed semen was placed on clean pre-warmed slide. A comparatively large drop of stain was added and mixed for 30 s using an applicator stick. A thin smear was then made and air dried for 1 to 3 min. After drying, the slide was observed under the compound microscope at 100X. The spermatozoa with unstained heads were considered live while those with stained or partially stained heads were considered dead. For each sample, the percentage of spermatozoa viability was calculated based on a total of 200 spermatozoa counted in 5 different microscopic fields.

Spermatozoa acrosomal integrity

Trypan-blue and giemsa staining were conducted for spermatozoa acrosomal integrity in bull (Kovacs and Foote, 1992). In this protocol, trypan-blue was used to differentiate live and dead sperms, and giemsa stain was used to evaluate sperm acrosomal integrity. One drop of trypan-blue (2.0%) was mixed with one drop of freeze-thawed semen on a clean pre-warmed slide and mixed using the edge of a clean cover slip. After drying in air, the slide was fixated with formaldehyde-neutral red solution [86 mL IM HCl + 14 mL formaldehyde (Merck, Darmstadt, Germany) + 0.2g neutral red (MP Biomedicals, Eschwege, Germany)] for 5 min. Subsequently the slide was washed with distilled water and dipped in (7.5) giemsa stain (MP Biomedicals, Eschwege, Germany) for 4 h. After staining with giemsa stain, the slide was again rinsed with distilled water and dried in air. After covering with cover slip, the slide was covered with Canada balsam (Merck, Darmstadt, Germany). For each smear, five different fields were evaluated under a phase-contrast microscope at 1000X

(LABOMED LX400), in resulting analysis of total 200 spermatozoa. As trypan-blue stain is permeable to none viable sperm and reflect blue appearance, whilst color of viable spermatozoa with intact membrane remained unchanged. In contrast, spermatozoa with intact acrosomes are permeable to giemsa stain, which turns the acrosomal part purple (Tartaglione and Ritta, 2004).

Spermatozoa DNA integrity

TNE buffer (200 μ L) was mixed with 20 μ L of freeze-thawed semen at 37 °C to obtain a final concentration of 2×10^6 spermatozoa / mL. Clean glass slides were used to prepare three smears from each semen aliquot. After drying in air, the smears were dipped overnight in Carnoy's solution [methanol and glacial acetic acid (Merck, Darmstadt, Germany) in a 3:1 ratio] for fixation. The slides were then allowed to dry in air and shifted to tampon solution [80 mMol citric acid (Merck, Darmstadt, Germany)] and 15 mmol sodium phosphate (Sigma, NY, USA), pH 2.5) for 5 min in a water bath at 75 °C. After incubation in tampon solution acridine orange (0.2 mg / mL, pH 2.5; Sigma, NY, USA) was applied to the slides. The slides were then covered with cover slips while they were still soaking and evaluated under fluorescent microscope (LABOMED LX400). Acridine orange is a fluorescent cationic dye with affinity for nucleic acids and permeable to sperm cells. Acridine orange interacts with single or double-strand DNA via intercalation or electrostatic attraction respectively. For each smear, five different fields were evaluated to count a total of 200 sperms. Intact sperm DNA with indicated by a green appearance while disrupted DNA appeared yellowish green to red (Evenson *et al.*, 2002).

Mitochondria membrane potential

JC-1 is a lipophilic cationic dye and sensitive marker for mitochondrial membrane potential (MMP) (T4069; Sigma –Aldrich Co.), was used for assessment of sperm MMP. In spermatozoa with high MMP, thereby the JC-1 marker forms multiplexes, known as J-aggregates, and releases orange fluorescence, while spermatozoa with low MMP then the JC-1 leftovers in monomeric form and releases green fluorescence. The thawed ejaculate samples were centrifuged for 5 min at 500(x) g and supernatant fluid was then discarded. The residual spermatozoa were diluted in phosphate buffer saline at a concentration of (1×10^6) spermatozoa per mL, afterward 1 mL of the suspension was stained with 1 μ L of JC-1 stock solution (200 μ Mol dissolved in DMSO) and incubated at 38°C for 40 min, and eventually examined by flow cytometry. Red and orange fluorescence of JC-1 dye was noticed (530 nm) with FL1 and (585 nm) FL2 detectors, correspondingly

(Minervini *et al.*, 2013).

Statistical analysis

The obtained data were statistically analyzed using SPSS version 16, SPSS Inc., Chicago, IL, USA. The Kolmogorov Smirnov test was employed to test the normal distribution of all the collected data. The results of this study are presented as (Mean \pm SEM). The total motility, sperm viability, membrane functionality, acrosomal integrity, DNA integrity and mitochondria membrane activity percentage of freeze-thawed spermatozoa of crossbred bulls were comparing between the different experimental diluent groups using one-way analysis of variance (ANOVA). Duncan multiple range test to determine significant difference; the value of ($P < 0.05$) was considered statistically significant.

RESULTS AND DISCUSSION

The impact of L-cysteine and vitamin E on freeze-thawed sperm motility in both of crossbred bulls are demonstrated in Table I, Figures 1 and 2. Addition of antioxidants (L-cysteine and vitamin E) to semen freezing diluent with 7.5 mmol of L-cysteine and 4.8 mmol of vitamin E improved motility of freeze-thawed spermatozoa as compared to the control group ($P < 0.05$) followed by 5 mmol of L-cysteine and 2.4 mmol of Vitamin E groups. Similarly, 7.5 mmol L-cysteine, and 4.8 mmol of vitamin E groups are yielded highest progressive motility and that was statistically significant as compared to control group ($P < 0.05$). The study design consisted of two crossbred groups e.g. Sahiwal \times Holstein-Friesian and Achai \times Jersey while parallel with different concentrations of both L-cysteine and vitamin E supplements. Both of crossbred groups were tested under different concentrations of L-cysteine and vitamin E to evaluate freeze-thawed spermatozoa traits. The results showed that motility parameters of freeze-thawed spermatozoa were significantly higher ($P < 0.05$) in group of L-cysteine-2 and vitamin-E-3 for both types of crossbred bulls. Spermatozoa motility is crucial to cross the female reproductive tract and hurdles around the oocyte for succeeding fertilization (Bucak *et al.*, 2012). As formerly described, spermatozoa motility and viability are usually affected features during freezing-thawing cycle and hence, these are the crucial causes for reduced fertility (Li *et al.*, 2010). Data about the sperm motility in our study are wholly in conformity with different studies in which addition of L-cysteine and vitamin E augmented motility of freeze-thawed bull (Amirat-Briand *et al.*, 2009), ram (Bucak *et al.*, 2009) and buck spermatozoa (Kulaksiz and Daskin, 2010).

Table I. The effect of different treated semen diluents on freeze-thawed spermatozoa total motility, progressive motility, membrane functionality, DNA integrity, acrosomal integrity, sperm viability and MMP (%) in (Sahiwal × Holstein-Friesian) bulls (Mean ± SEM).

Parameters	Control diluent	L-cysteine group			Vitamin E group		
		L-c-1 diluent	L-c-2 diluent	L-c-3 diluent	Vit-E-1 diluent	Vit-E-2 diluent	Vit-E-3 diluent
TM (%)	50.00±1.60 ^c	52.80±1.44 ^b	60.60±1.89 ^a	51.12±2.20 ^{bc}	51.90±1.66 ^{bc}	53.65±1.98 ^b	59.40±1.44 ^a
PM (%)	15.70±2.02 ^c	17.10±1.88 ^{bc}	21.77±1.67 ^a	15.88±2.10 ^c	16.43±1.33 ^{bc}	18.29±2.10 ^b	20.68±1.90 ^{ab}
MF (%)	42.29±1.63 ^c	45.56±1.77 ^b	54.48±1.74 ^a	43.82±1.62 ^{bc}	44.38±1.43 ^{bc}	46.45±1.89 ^b	53.12±1.77 ^a
DNAI (%)	92.83±1.56 ^b	92.20±2.24 ^c	96.62±1.44 ^a	92.72±2.45 ^b	93.45±1.44 ^{ab}	93.48±1.66 ^{ab}	97.77±1.85 ^a
AI (%)	40.83±1.56 ^c	43.41±1.60 ^b	54.11±2.33 ^a	40.15±1.45 ^c	41.00±1.40 ^{bc}	43.00±1.80 ^b	52.00±2.15 ^{ab}
MMP (%)	37.86±1.18 ^c	42.89±2.14 ^b	49.50±1.88 ^a	40.27±2.53 ^{bc}	41.33±1.88 ^{bc}	44.18±1.90 ^b	48.44±2.10 ^{ab}
SV (%)	49.87±1.50 ^c	52.66±1.44 ^b	66.35±1.65 ^a	50.50±1.73 ^{bc}	50.98±1.88 ^c	51.44±1.40 ^{bc}	62.66±1.60 ^a

*Control diluent containing no additives of L-cysteine and vitamin E; L-c-1 diluent containing 5 mmol of L-cysteine; L-c-2 diluent containing 7.5 mmol of L-cysteine; L-c-3 diluent containing 10 mmol of L-cysteine; Vit-E-1 diluent containing 1.2 mmol of vitamin E; Vit-E-2 diluent containing 2.4 mmol of vitamin E; Vit-E-3 diluent containing 4.8 mmol of vitamin E; Mean values bearing different superscripts in a rows differ significantly: a, ab, b, bc, c ($P < 0.05$); TM, total motility; PM, progressive motility; MF, membrane functionality; DNAI, DNA integrity; AI, acrosomal integrity; MMP, mitochondria membrane potential; SV, sperm viability.

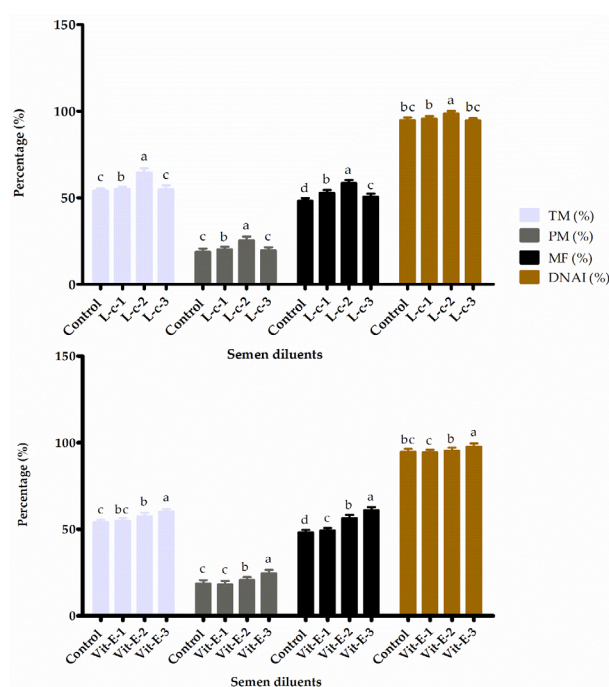


Fig. 1. The effect of L-cysteine and vitamin E supplemented diluents on freeze-thawed spermatozoa traits in Achai × Jersey bulls (Mean±SEM). Control diluent containing no additives; L-c-1 diluent containing 5 mmol of L-cysteine; L-c-2 diluent containing 7.5 mmol of L-cysteine; L-c-3 containing 10 mmol of L-cysteine; Vit-E-1 diluent containing 1.2 mmol of vitamin E; Vit-E-2 diluent containing 2.4 mmol of vitamin E; Vit-E-3 diluent containing 4.8 mmol of vitamin E; TM, total motility; PM, progressive motility; MF, membrane functionality; DNAI, DNA integrity; different

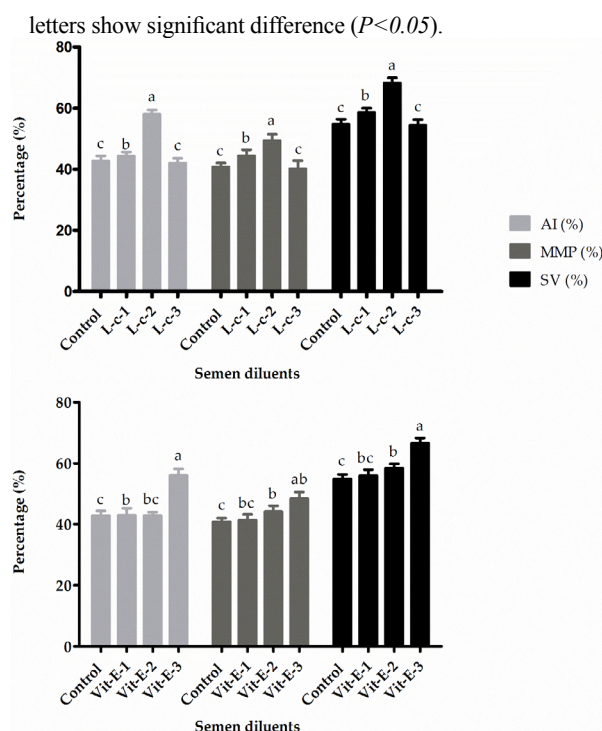


Fig. 2. The impact of L-cysteine and vitamin E supplemented diluents on freeze-thawed spermatozoa parameters in Achai × Jersey bulls (Mean±SEM). Control diluent containing no additives; L-c-1 diluent containing 5 mmol of L-cysteine; L-c-2 diluent containing 7.5 mmol of L-cysteine; L-c-3 containing 10 mmol of L-cysteine; Vit-E-1 containing 1.2 mmol of vitamin E; Vit-E-2 containing 2.4 mmol of vitamin E; Vit-E-3 containing 4.8 mmol of vitamin E. AI, acrosomal integrity;

MMP, mitochondria membrane potential; SV, sperm viability; different letters show significant difference ($P < 0.05$).

Previous reports designated that addition of L-cysteine with concentration of 5 and 7.5 mmol in buffalo semen freezing extender significantly improved the motility of freeze-thawed spermatozoa (Beheshti *et al.*, 2011). The vitamin E is measured to be the main constituent of antioxidant system for spermatozoa (Lenzi *et al.*, 1996). In latest studies, the supplementation of vitamin E into freezing diluents has been amended the freeze-thawed spermatozoa functions of ram (Silva *et al.*, 2013), boar (Breininger *et al.*, 2005) and tom (Thuwanut *et al.*, 2008). Likewise, the addition of vitamin E into semen freezing extender resulting in optimistic effect on spermatozoa motility, integrity of membrane, DNA and MMP (Jeong *et al.*, 2009).

As shown in Table I and Figures 1 and 2 supplementation of crossbred bulls freezing diluent with 7.5 mmol of L-cysteine and 4.8 mmol of vitamin E are significantly improved membrane functionality of spermatozoa along with significant increase in acrosomal integrity as compared to control group ($P < 0.05$) and followed by the other groups. In this study, membrane functionality of freeze-thawed spermatozoa was significantly higher ($P < 0.05$) in groups of L-cysteine -2 and vitamin-E-3 compared with the control group. Plasma membrane is accountable for the conservation of cellular homeostasis and thereby the plasma membrane functionality applies a vital role on spermatozoa survival inside the female reproductive tract and, on conservation of spermatozoa fertilizing capability (Andrade *et al.*, 2007). Semen collection and processing for future uses requires proper freezing methods. Spermatozoa are prone to freezing, which is altered sperm membrane activity and the degree of spermatozoa membrane damages differs based on organization, membrane permeability, fluidity and the lipid composition of sperm membrane bilayer (Motemani *et al.*, 2017). Plasma membrane integrity in crossbred bull spermatozoa has been suggested to be significantly affected by cryopreservation compared to pure breeds (Hammerstedt *et al.*, 1990). Lipid peroxidation (LPO) of plasma membrane disturbs membrane construction through ROS production (Thomas *et al.*, 1998), while leading to deterioration in critical sperm properties, including membrane activity, motility and fertilization ability (Thippeswamy *et al.*, 2014). It has been recommended that vitamin E delivers a biological stability to the spermatozoa plasma membrane while as the increase nutritional intake of vitamin E content in resulting the semen was found less prone to LPO (Surai *et al.*, 2000; El-Sheshtawy *et al.*, 2008). El-Sheshtawy *et al.* (2008) found that adding L-cysteine (7.5 mmol) to tris-citric acid diluent increased the percent of intact membranes spermatozoa in Egyptian buffalo after

cryopreservation. Likewise, treating tris-based diluent with L-cysteine (7.5 and 10 mmol) resulted in enhanced percentages of sperm with efficient plasma membranes in bulls (Sariözkan *et al.*, 2009) and rams (Bucak *et al.*, 2010), in agreement with our findings.

In this conducted research, spermatozoa acrosomal integrity in both types of crossbred bulls was significantly higher ($P < 0.05$) in groups L-c-2 and vit-E-3 compared with control group. The percentage of intact acrosomes is positively related to fertility in freeze-thawed bull spermatozoa (Hafez, 2004). The intactness of acrosomes and their enzyme (acrosin and hyaluronidase) activities are indispensable for acrosomal reaction and spermatozoa-oocyte fusion (Sudheer and Xavier, 2000). The structural and functional integrity of the sperm acrosome is considered necessary to attain the high fertility however cryopreservation can damage the acrosomal layer, which diminishes the ability of spermatozoa to penetrate the zona pellucida (Yanagimachi, 1994). Some studies have suggested that vitamin E protects against LPO caused by ROS in bull and boar (Goovaerts *et al.*, 2006; Breininger *et al.*, 2005). In this study the groups treated with vitamin E had larger percentages of sperm with intact acrosome membranes compared to the control group. In addition, L-Cysteine has the aptitude to rapidly pass into the cells, where it is altered to taurine. The taurine is united with fatty acids in membrane and altered into acyl-taurine, improving the surfactant properties and osmoregulation of the spermatozoa membrane (Esteves *et al.*, 2007; Vazquez and Roldan, 1997).

The percentage of spermatozoa with high viability and increased DNA integrity in both of crossbred bulls were found when 7.5 mmol of L-cysteine and 4.8 mmol of vitamin E were added in freezing diluent ($P < 0.05$) compare to control group. Similarly, high MMP was observed when the freezing diluent treated with 7.5 mmol of L-cysteine and 4.8 mmol of vitamin E as compare to control group ($P < 0.05$) followed by the other treated groups as shown in Table I, Figures 1 and 2. In this study the DNA integrity of freeze-thawed spermatozoa was significantly higher ($P < 0.05$) in groups L-c-2 and vit-E-3 in both types of crossbred bulls. Cryopreservation method was found to damage the DNA integrity of buffalo spermatozoa, which affects the sperms functional credits and successful fetal development (Kumar *et al.*, 2011) and, can also decrease the antioxidant potential of sperm. Antioxidants contribute in protecting DNA integrity during freeze-thawing (Bucak *et al.*, 2010) by decreasing oxidative stress. Freeze-thawing induces adverse events such as ROS production and apoptosis-like changes; these events disturb the packaging of the sperm chromatin, in result a loss of DNA integrity (Topraggaleh *et al.*, 2014). L-cysteine is sulfur-containing amino acids

that are naturally found in seminal plasma and sperm nucleic acid. L-cysteine preserves DNA integrity and acts as an antioxidant directly and /or indirectly to defend cells against ROS (El-Sheshtawy *et al.*, 2008; O'Flaherty *et al.*, 1997), in agreement with the findings of the study. As vitamin E is a known inhibitor of LPO in cell membranes and acts as scavenger of ROS, thereby reducing oxidative damage during freeze-thawing of bull semen (Saacke and White, 1972).

Cryopreservation induced structural damages in mitochondria which altered the biochemical processes involved in ATP production, ultimately resulting in reduced sperm viability and motility (Figueroa *et al.*, 2017). Mitochondria membrane is the key site of ROS production due to oxidative phosphorylation (Thuwanut *et al.*, 2011). Kadirvel *et al.* (2009) found a negative relationship between mitochondrial membrane activity and ROS in freeze-thawed buffalo spermatozoa. Direct correlation exists among the MMP, sperm motility and fertilization capacity of freeze-thawed spermatozoa. This relationship confirmed that cessation or activation of motility and successful fertilization depends on the availability of ATP in the spermatozoa (Fraser and Strzezek, 2007). The significant growth in MMP of freeze-thawed spermatozoa supplemented with L-cysteine and vitamin E could be due to the high antioxidant potential of L-cysteine and vitamin E to eliminate intracellular ROS and afterward protect mitochondria membrane activity.

The percentage of viable spermatozoa was significantly higher ($P < 0.05$) in groups L-cysteine -2 and vitamin-E-3 compared to control group. Spermatozoa viability and motility are the most essential requirements for fertile spermatozoa (Hossain *et al.*, 2012). Freeze-thawing cycle results in substantial alterations in spermatozoa water content, creating considerable mechanical stress on the membrane and reducing spermatozoa viability (Hammerstedt *et al.*, 1990). The addition of L-cysteine (7.5 mmol) to tris-citric acid diluent was found to improve the percentage of viable ovine spermatozoa (Bucak *et al.*, 2008). Under field conditions, decrease in viable spermatozoa after freeze-thawing significantly reduces the fertility of bovine semen (Andrabi *et al.*, 2006). In similar studies on ovine semen the addition of L-cysteine to semen extender resulted in higher percentage of live spermatozoa in association with increased catalase activity (Thomas *et al.*, 1998; El-Sheshtawy *et al.*, 2008). Our results show that vitamin E exhibits cryo-protective effects through its ability to reduce ROS accumulation. Our findings agree with several studies in which the addition of vitamin E to diluents enhanced the rate of motility and viability of spermatozoa (O'Flaherty *et al.*, 1997; Hafez, 2004).

CONCLUSIONS

Addition of L-cysteine or vitamin E supplements at concentrations of 7.5 and 4.8 mmol to semen diluents respectively, providing more strength to MMP and afterward elevated the spermatozoa freeze-thawed motility parameters in both crossbred bulls compared with control. In addition our study confirmed that adding 7.5 mmol L-cysteine and 4.8 mmol of vitamin E in freezing diluents enhanced sperm membrane functionality and DNA integrity. Therefore, these supplements can be suggested as an additional component for freezing diluent while in semen preservation of crossbred bulls. Further studies are recommended, to assess in vivo and in vitro fertility rates of freeze-thawed crossbred semen preserved with L-cysteine and vitamin E.

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Statement conflicts of interest

The authors have declared no competing interest.

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