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L-Cysteine Addition in Tris-Citric Acid Extender Enhances Functional Characteristics of Post-Thaw Spermatozoa in Achai and Holstein Friesian Bulls under Subtropical Environment

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ZU, HK, and YM designed the study. AG, ZU and MSK extracted samples. HAU, MTT, IMK and NK analysed the data statistically and wrote the article.

Key words
L-cysteine, Achai, Holstein Friesian, Bull semen, Post thaw.

ABSTRACT

Freezing and thawing processes have the potential to adversely affect post-thaw quality of semen. In the current study, we endeavored to ascertain the most favorable application of L-cysteine for cryopreservation of Achai and Holstein Friesian bull semen under the subtropical conditions of Peshawar region of Pakistan. Semen of six adult bulls, three Achai (the indigenous breed) and three Holstein Friesian (exotic breed) amount to the experimental material at Government Cattle Breeding and Dairy Farm Harichand, Charsadda. Separate artificial vagina was utilized for semen collection from either breed, maintained at 42°C temperature. Gross and microscopic examination was carried out subsequent to the semen collection. Semen specimen with more than 70% motile characteristics were used for further processing. The semen was split into four aliquots in which *tris*-citric acid extender (TCA) was supplemented with various concentrations of L-cysteine 0 mM, 0.5 mM, 1.0 mM and 1.5 mM. Cooling and equilibration of the diluted semen was done at 4°C for 4 h subsequently filled in straws at the same temperature and were cooled in liquid nitrogen vapors for 10 min. followed by storage at -196°C for assessment. Progressive motility and viability of spermatozoa were significantly higher in 1.5 mM and 1.0 mM L-cysteine supplemented groups in comparison with 0.5 mM or 0 mM. The number of supra-vital hypo-osmotic swelling test positive spermatozoa at 1.5 mM and 1.0 mM was greater in comparison with lower concentration of 0.5 or 0 mM L-cysteine. Furthermore, significant improvement for acrosomal integrity was recorded in semen samples treated with 1.5 mM L-cysteine followed by 1mM, 0.5mM. It is concluded that 1.5 mM is the most favorable concentration of L-cysteine to be added to the TCA extender for improving the post-thaw quality of semen of Achai and Holstein Friesian bulls under the existing environmental conditions.

INTRODUCTION

Artificial insemination (AI) is a cosmopolitan technology widely used in livestock industry for utmost use of genetically superior sire to enhance animal productivity to cope with the increasing demand of meat and milk in the under developed countries of the world. Cryopreservation is an essential feature in AI programme to maintain the quality of semen, but this procedure may adversely affect the post-thaw quality of semen (Aboagla and Terada, 2007). Production of reactive oxygen species (ROS) occurs during freezing and thawing of semen associated with a reduction in sperm membrane fluidity and decrease in sperm function following cryopreservation (Chatterjee and Gagnon, 2001). Lipid profile of plasma membrane and acrosome are important sites in spermatozoa which are adversely affected by cryopreservation process (McGetrick *et al*., 2014). Cryo-preservation does affect lipid composition and organiza-tion of sperm plasma membranes in rams (Hinkovska-Galcheva *et al.,* 1989) and boars (Buhr *et al.*, 1994). Cryopreservation also disrupted trans-bilayer-phospholipid asymmetry in ram

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sperm plasma membranes (Hin-kovska-Galcheva *et al.,* 1989; Muller *et al.,* 1999). Extenders containing cysteine improved the glutathione peroxidase activity and glutatione content of spermatozoa, while reduced the ROS, DNA oxidative alterations and lipid peroxidation level, enabling spermatozoa avoid ROS to attack DNA, the plasma membrane and mitochondria (Zhu *et al*., 2017).

L-cysteine can absorb into cells and can be converted into taurine which in combination with fatty acids of cell membrane is transformed into acyl-taurine, improving osmoregulation and surfactant distinctiveness of sperm cells (Kaeoket and Kaeoket, 2010). It has been reported that as an antioxidant cysteine is a rate-limiting factor for intracellular glutathione biosynthesis in cells which is involved in protection against ROS-mediated oxidative stress. Glutathione (l-g-glutamyl-l-cysteinylglycine) is a tripeptide thiol complex associated with gamete cell fertilization, production of protein and DNA (Nasr-Esfahani *et al*., 1992). Additionally L-cysteine, being a forerunner of intracellular glutathione, has been demonstrated to infiltrate the cell membrane rapidly, improving the intracellular glutathione content (GSH) production both *in vivo* and *in vitro* which are associated with protection of lipid and protein content of spermatozoa membrane due to indirect radical searching characteristics. L-cysteine prevents ROS-mediated deleterious effects on sperm membrane integrity, motility, viability during sperm liquid storage and frozen thawed bull semen (Bilodeau *et al.,* 2001). The assessment of spermatozoa functional characteristics such as sperm motility, viability, membrane and acrosomal integrity are fundamental criteria in the appraisal of cryopreserved semen (Salamon and Maxell, 2000). However cryopreservation is associated with ROS-mediated oxidative stress which may impair spermatozoa functional characteristics. Cryopreservation process leads to lipid peroxidation and oxidative stress, resulting in irreversible damage to sperm morphology associated with decreased survivability and fertilization ability (Jafaroghli *et al*., 2011).

Toxic effects of Reactive oxygen species (ROS) are naturally prevented by various antioxidants in bovine semen under normal physiological circumstances (Nissen and Kreysel, 1983). On the other hand, subsequent to the freeze-thawing event, this natural defense capacity shuts down to protect spermatozoa against cryo-damages (Chatterjee *et al*., 2011; Gadea *et al*., 2004). Because of these facts, to diminish the adverse consequences of ROS during cryopreservation, a variety of antioxidants are utilized in semen storage media having capability to make better the post-thaw semen functional characteristics in bulls (Bucak *et al*., 2010).

Though research investigation on semen cryopreservation associated with inclusion of L-cysteine in semen storage media in diverse animal species have been carried out in different region of the world (Ansari *et al*., 2011). The indigenous breeds of cattle like Sahiwal show better semen quality characteristics than exotic breeds (Khan et al., 2018). On the other hand, to our knowledge, no study has been reported hitherto-in subtropical environment of Peshawar, Pakistan, to establish the best concentration of L-cysteine in the semen extenders of bulls, despite the fact that the dairy cattle enterprise has been the integral contributor in the domestic economy. Consequently, herein, in the current study, we have attempted to establish the most favorable concentration of L-cysteine in semen cryopreservation of Achai (the local indigenous breed) and Holstein Friesian (exotic breed) bulls under the subtropical environmental conditions of Peshawar to guard spermatozoa from damages caused by free radicals and to reduce the oxidative stress occurring during the process of cryopreservation.

MATERIALS AND METHODS

The present research project was conducted in accordance with guidelines of the ethical committee of Faculty of Animal Husbandry and Veterinary Science, The University of Agriculture, Peshawar.

Experimental animals

Six andrologically healthy bulls, 3 each from Holstein Friesian and Achai breeds were used as experimental animals. These animals were reared at the Government Cattle and Breeding Farm Harichand as semen producer for government AI programme for the last two years. They were maintained under consistent feeding, accommodation and lighting conditions.

Semen assortment and dealing out

A total of 96 Semen samples from the six experimental bulls were collected with the help of artificial vagina maintained at 42°C. Breed wise processing of semen was separately performed. Two consecutive ejaculates were collected from each bull at weekly interval for 3 weeks in April- May, 2014. Gross and microscopic examination was carried out subsequent to the semen collection. Semen motility was measured by standard motility analysis technique. Briefly a drop of semen was spread over glass slide and 100 sperms were counted under the microscope, in which the proportion of motile sperm was determined. Semen specimens with more than 70% motile characteristics were used for further processing. The semen was split into four aliquots in which tris-citric acid extender (TCA) was supplemented with various

concentrations of L-cysteine (Sigma, ST Louis USA No. C-8755) *i.e.* 0 mM (control), 0.5 mM, 1.0 mM and 1.5 mM. In the current experiment, Tri-citric egg yolk extender (pH 7.0; osmotic pressure ~320 mOsmol kg–1) was used for preparation of experimental extenders that consisted of 1.56% citric acid (Fisher Scientific,loughborough, Leicestershire, UK), 3.0% tris-hydroxymethyl-aminomethane (Research Organics, Cleveland, USA) were dissolved in water . Fructose (Scharlau, Spain) 0.2%, 7% glycerol (Merck Darmstadt, Germany) and 20% egg yolk were supplemented to extender. Antibiotics streptomycin sulfate 1mg/ml, procaine penicillin 300 IU/ml and benzyl penicillin were added to the extender. Diluted semen was cooled at 4°C for 2 h, equilibrated for 2 h at 4°C, filled in French straws (0.5 ml) at 4°C, kept in liquid nitrogen vapours for 10 min and then stored in the liquid nitrogen (-196°C) for assessment. Thawing of frozen semen straws was carried out at 37°C for 30 seconds in a hot-water bath.

Viability and acrosome integrity

Dual staining procedure *i.e.* trypan-blue and Giemsa stains was used for appraisal of Sperm viability and Acrosome integrity of the spermatozoa in the semen (Kovacs and Foote, 1992). Differentiation between living and dead spermatozoa was carried out through the trypan-blue stain whereas acrosome integrity was appraised through Giemsa stain. In brief, equivalent sized drop of Trypan blue and semen were positioned on a slide and assorted rapidly. Smears were air-dried and fixed with formaldehyde-neutral red for 5 min. Subsequent to washing with distilled water, Giemsa stain (7.5%) was used for 4 hours. Consequently, the smears were washed, air-dried and escalated with Balsam of Canada. Try pan-blue gained access to non-viable spermatozoa with interrupted covering, that become visible stained in blue, whereas the alive spermatozoa emerged with no staining. Giemsa accumulate in spermatozoa with an intact acrosome, staining the acrosome section in violet. Two hundred spermatozoa were assessed in at least five diverse fields in each smear by a phase contrast microscope at 1000x (Anasari *et al*., 2011).

Plasma membrane integrity

Sperm plasma membrane integrity was appraised by supra-vital hypo-osmotic swelling test (HOS) test (Jeyendran *et al*., 1984). Following incubation phase, a 5µl aliquot of the suspension was located on a warm slide and a 5µl droplet of Eosin (0.5% w/v in sodium citrate 2.92%) was mixed for 10 seconds. A cover slip was kept on the mixture and phase contrast microscope at 400x was used for assessment. Five different fields were selected and a total of 200 spermatozoa were assessed. Sperms with unstained heads and tails but swollen tails designated biochemically dynamic and intact plasma membranes whereas red heads and tails but unswollen tails demonstrated inactive and disruptive sperm membrane.

Statistical analysis

The data were statistically analyzed using two-way analysis of variance (ANOVA) using SPSS version 16, Chicago, IL, USA. The mean differences among groups were compared by Duncan's Multiple Range Test.

RESULTS

Semen of both breeds was supplemented with L-cysteine and following post thaw quality parameters *i.e.* percentage of sperm motility, viability, acrosome integrity and spermatozoa plasma membrane integrity were evaluated. Findings of the present study established dose dependent response of L-cysteine in the semen extenders in both indigenous breed- the Achai and exotic breed-the Holstein Friesian (HF). Significant increase has been

Table I.- Post thaw quality parameters after supplementation of different level of L-cysteine in TCA semen extender.

Dose bull (mM)		Post thaw quality Indicators (Mean±S. D)				
		PM %	IM%	PMI%	AI%	Live/dead%
0.0	HF	21.16±0.40^{e}	46.33±0.61^{d}	41.83±0.40^{e}	56.83±0.90^{d}	62.0±0.68^{e}
	Achai	21.16±0.16^{d}	47.0±0.36^{d}	41. 33±0.61^{e}	58.83±0.74^{e}	63.16±0.79^{e}
0.50	HF	25.0±0.51^{d}	52.33±0.49^{c}	45.50±0. 34de	62.50±0.22^{c}	67.0±0.89^{d}
	Achai	26.66±0. 49^{c}	53.0±0.36^{c}	44.66±0. 33^{d}	61.83±0.40^{d}	66.83±0.60^{d}
1.0	HF	30.83±0.00^{b}	64.33±0.00^{b}	55.16±0.16^{b}	67.83±0.00^{b}	72.66±0.71^{c}
	Achai	4031.50±0.61^{b}	4965.66±0.33^{b}	57.0±0.25^{b}	4069.16±0.30^{b}	74.0±0.73^{b}
1.50	HF	36.33±0.50^{a}	70.83±0.60^{a}	60.83±0.40^{a}	72.0±0.51^{a}	75.50±0.67^{a}
	Achai	36.33±0.95^{a}	70.50±0.56^{a}	59.83±0.26^{a}	73.0±0.36^{a}	78.16±0.47^{a}

*Values having different superscript in the columns indicates significant difference (P<0.05). PM, progressive motility; IM, individual motility; PMI, plasma membrane integrity; AI, Acrosome integrity; HF, Holstein Frisian.

recorded in the progressive motility and individual motility of spermatozoa in 1.5 mM or 1.0 mM L-cysteine supplemented groups in comparison with 0.5 mM or 0 mM in both breeds (Table I). Likewise current findings also indicated that the number of HOST positive spermatozoa at 1.5 or 1.0 mM was greater in comparison with lower concentration of 0.5 or 0 mM L-cysteine. Furthermore, significant response for acrosomal integrity was recorded in semen samples treated with 1.5 mM L-cysteine followed by 1mM, 0.5mM and control in both the breeds. Also live sperm percentage of semen samples of both breeds were found significantly higher ($P<0.05$) in semen extender containing L-cysteine 1.5 mM followed by 1mM (72.66± 0.71 and 78.16±0. 47), 0.5mM (67.0± 0.89 and 74.0± 0.73) and control (62.0± 0.68 and 63.16± 0.79), respectively.

DISCUSSION

The present study indicated the cryoprotective competence of L-cysteine in Tris–citric acid base semen extender. The inclusion of varied concentration of the L-cysteine in TCA extender demonstrated positive effects on the quality of cryoperserved semen indicators that includes motility, morphology, viability, membrane integrity, and acrosomal integrity in both local and exotic breed at subtropical environment of Peshawar. These findings are in agreement with previous studies who reported that L-cysteine has been associated with improvement in post-thawed sperm quality parameters that includes motility, morphology, and maintenance of viability, and membrane integrity in diverse mammalian species such as bovines, bucks and rams spermatozoa (Sarıozkan *et al*., 2014). The results showed that dead sperms in semen containing extender fortified with 1 and 1.5 % L-cysteine, reasonably reduced when compared with the control group. It is evident from review of literature that lower L-cysteine concentrations in semen extender has been strongly linked with enhancement of post-thawed sperm quality parameters (Ansari *et al*., 2011). The results are consistent with previous studies on Sahiwal bull, buffalo bull and ram under diverse environmental conditions, suggestive of the continuance of structural integrity subsequent to cryopreservation via a reduced concentration of L-cysteine in TCA semen extender (Ansari *et al*., 2011; Orin *et al*., 2015). According to Ansari *et al* (2011a, b) inclusion of L- cysteine at 1 or 2 mM in semen extender have exerted significantly positive response toward post thaw bull semen functional characteristics whereas above than that level such as 3.0 mM in semen extender worsen the post-thaw excellence of bull and buffalo semen. Likewise another study recently elucidated that addition of L-cysteine at 1 mg/ml in semen extender appreciably ($P<0.01$) improved the functional feature of post thaw spermatozoa that includes progressive sperm motility, viability, and plasma membrane integrity with reduced sperm/acrosome abnormalities in cryopreserved buffalo semen (Orin *et al*., 2015).

Considering all these earlier studies including our current findings, it seems reasonable that the enhancement in the qualities of frozen–thawed bull semen established in the current study, would be ascribed to *L*-cysteine addition in TCA semen extender since it is a predecessor of intracellular glutathione (Gadea, *et al*., 2002). Additionally, L-cysteine also has the potential to diminish lipid peroxidation that has been associated with production of free radicals during freezing-thawing process, and thus safeguard spermatozoa from reactive oxygen species (Chatterjee *et al*., 2011; Nissen and Kreysel 1983). Moreover another possible explanation for the current enhancement in the post thaw quality characteristics of bull spermatozoa might be ascribed to the Tris-citric acid base extender that has been utilized in the current experiment. According to Andrabi 2009, Tris–citric acid have been associated with provision of the most excellent buffering complex to enhance the post-thaw and motility of bovine spermatozoa.

CONCLUSIONS

In conclusion, this study established that the supplementation of L-cysteine in TCA semen extender enhances the functional characteristics of Achai and HF bull semen under subtropical conditions. Additionally, this study further demonstrated that optimal concentration of L-cysteine in the extended medium is 1.5mM for cryopreserved bull semen in the subtropical environment of Peshawar, Pakistan. Further elucidation using this concentration for A. I of the dairy cows in field condition under subtropical environment would certainly substantiate these in-vitro findings for increasing conception rates of local stock.

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Statement of conflict of interest

The authors declare that they have no conflict of interest.

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