EFFECT OF CYSTEIN HYDROCHLORIDE AND VITAMIN E SUPPLEMENTATION ON QUALITY OF CRYOPRESERVED HARIANA BULL SEMEN

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ABSTRACT

The present study was aimed to evaluate the beneficial effects of supplementation of cystein hydrochloride and Vitamin E on seminal attributes during different stages of semen cryopreservation in Hariana bull. Thirty two ejaculates from four Hariana bulls were collected after initial examination the qualified ejaculates were divided into three aliquots; one aliquot was extended with glycerolated egg yolk tris citrate (GEYT) extender (Control), second aliquot was extended with GEYT supplemented with Cystein hydrochloride @ 5.0 mM (T1) and the third aliquot was extended with GEYT supplemented with VitaminE @ 2.5 mM (T2). All semen samples were equilibrated and cryopreserved. The effect of cystein hydrochloride and vitamin E was determined by assessment of sperm viability, progressive motility, hypo-osmotic response, and acrosomal integrity at three stages i.e. after dilution, pre-freeze and post –thaw. It was observed thatsupplementation of cysteine hydrochloride (5mM) and vitamin-E (2.5 mM) caused significant ($P<0.05$) increase in all sperm attributes at post thaw semen.

KEYWORDS: Bull, cryopreservation, cystein hydrochloride, vitamin c, semen

INTRODUCTION

Artificial insemination(AI) is one the assisted reproductive technologiesinimproving genetic makeup of animal. Cryopreservation of sperm is integral part of AI program, but in spite of development in cryopreservation protocols, spermatozoa suffer from irreversible damages both at structural and functional level leading to a reduction in viability, motility and fertility (Thomas *et al.,* 2006).Mammalian spermatozoa are highly sensitive to oxidative damage due to presence of high lipid bi-layer of plasma membrane.The extenderused is considered as important factorwith ambient PH and buffering capacity followed by appropriate osmolality to protect spermatozoa from cryogenic injury. Oxidative damage of bull and buffalo spermatozoa during preservation could be hindered by addition of suitable additives to semen extenders (Ansari *et al*., 2011).Cysteine, a sulphur-containing amino acid with thiol group is an important component of sperm nucleic acid and maintains the integrity of DNA. Cysteine is also a component of glutathione that acts as an antioxidant(Bilodeau *et al*. 2000). Vitamin E is a hydrophobic antioxidant that stop chain reaction of peroxidation by scavenging peroxyl radicals in the plasma membrane (Niki, 1987) and maintain sperm DNA integrity (Hughes *et al*., 1998). Vitamin E isprincipal constituent of antioxidant system of spermatozoa and is one of the major membrane protecting reactive oxygen species and lipid per-oxidation attack (Yousuf *et al.,* 2003).

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Antioxidants supplementation has been emerged as one of the most powerful way to overcome the excessive production of free radicals during the process of cryopreservation. Taking into account the role of cystein hydrochloride and vitamin E as antioxidants, research was designed for comparative evaluation of damages during the process of cryopreservation of Hariana bull semen.

MATERIALS AND METHODS

The present study was conducted on fourHariana bulls of 4.5 to 5.5 years age and weighing between 430 to 490 Kg, reared under uniform managemental conditionsat Instruction Livestock Farm Complex, College of Veterinary & Animal Husbandry, Mathura, Uttar Pradesh, India. Thirty two ejaculates from four Hariana bulls were collected and transferred to laboratory and placed in water bath at 34 ^{0}C for physico-morphological studies.

The semen sample which fulfilled the criteria for cryopreservation (e"3.5 mass motility, e"70% progressive motility and e"80%viable sperm and e"500 million spermatozoa/ml)were divided into three aliquots.One aliquot was extended with glycerolated egg yolk tris citrate (GEYT) extender (Control), second aliquot was extended with GEYT supplemented with Cystein hydrochloride @ 5.0 mM (T1) and third aliquot was extended with GEYT supplemented with Vitamin E @ 2.5 mM (T2). After extension of semen, Control, T1& T2samples were equilibrated at 4-5^{0}C for 4 h in cold handling cabinet and processed for cryopreservation under vapour of liquid

nitrogen using biological freezer (IMV, Technologies France) by lowering the temperature from 4^{0}C to -10^{0}C@ 5^{0}C/min, -10^{0}C to -100^{0}C @40^{0}C/min, -100^{0}C to -140^{0}C @ 20^{0}C/min in a total period of 7minutes and 5 seconds. All the semen samples were evaluated for sperm viability (Hancock (1951), progressive motility (Ahmad, 1994), plasma membrane integrity (HOST) (Jayendran et al., 1984)and acrosomal Integrity (Hancock, 1952)at three stages i.e. after dilution with extender, at pre-freeze (after equilibration at 4-5 ^{0}C for 4 h) and at post-thaw (37^{0}C for 45seconds).

Statistical analysiswas performed by using Statistical Package for Social Science (SPSS Version 20 for Windows, SPSS Inc., Chicago, USA). Effect of different inclusion of cysteine and vitamin E were analyzed by using oneway analysis of variance and significance was tested at 5% level (pÂ0.05). Duncan's multiple range test was used to compare the treatment means for various sperm attributes.

RESULTS AND DISCUSSION

Viability of spermatozoa following freeze-thaw is always a challenging step as 30 to 40% of reduction in viability of sperm is seen. Inthe present study, mean percentage of live spermatozoa was significantly (pÂÙ0.05) reduced in T1 and T2 as compared to control group during all stages i.e., at after dilution, pre-freeze and post thaw (Table1). Decreased live sperms at each step of cryopreservation may be attributed to injuries occur due to formation of ice crystals in the extra and intracellular environment of spermatozoa and increasing solute concentration (Mazur, 1984), sperm susceptibility for freezing and thawing temperature, ROS production and lipid peroxidation (Patel *et al.,* 2016). The present finding is in agreement with the finding of various other reports in different breeds (Patel *et.al.,* 2015). El-Sheshtawy *et al.* (2008) and Ansari *et al*. (2010)also observed a higher percentage of viable sperm in extender containing cysteine 1.0 mM and 2.0 mM compared to that containing 0.5 mM and control group.While,Bucak*et al.* (2008)noticed increased catalase activity after the addition of cysteine to semen extender. Similarly,higher percentage of viable spermatozoa in the semen extended with a synthetic analogue of vitamin E (Patel *et.al.,* 2016).

Progressive motility serves as index of fertilization and serves as significant determinant of sperm capacity for bringing out fertilization. In the present study, the mean percentage of progressively motile spermatozoa was significantly (pÂÙ0.05) higher in T1 and T2 as compared to control groupat after dilution, pre-freeze and post thaw stages (Table1) with a significant (P<0.05) increase after thawing indicatingcysteine and Vitamin E role in maintaining higher sperm motility. The lower values of progressive motile sperms in control group might be due to excessive generation of ROS by dead, immature and abnormal spermatozoa during sperm processing (e.g., extending, freezing, thawing process), accompanied by low scavenging and antioxidant concentrations in seminal plasma and semen extender inducing oxidative stress (Sikka, 2004)might have caused deleteriouseffects on the sperm plasma membrane systemby causing lipid per oxidation of the membrane (Bucak *et al.,* 2008). While the higher values obtained in T1 and T2 indicated that cysteine and vitamin Emight have improved the motility by reducing oxidative stress through scavenging of ROS molecules (Alvarez and Storey, 1983).Present findingsare in agreement with finding of earlier studies in different breeds (Anghel *et al.*,2010 and Ansari *et al.,* 2010).

The membrane integrity plays a significant role in fertilization (Jeyendran *et al.,* 1984). In this study, the mean percentage of HOS reactive spermatozoa was significantly (pÂÙ0.05) higher in T1 and T2 as compared to control groupat after dilution, pre-freeze and post thaw stages and there wasno significant difference between T1 & T2 groups (Table 1). The mean percentage HOS reactive spermatozoa among different bulls differ between control andT1 or T2 at the start and end of equilibration period.However, differentiation was pronounced and differed significantly (P<0.05) after thawing. Similarly, significantly (P<0.05) higher percentage of post-thawed sperm with intact plasm membrane in Sahiwal bull extended semen containing cysteine @ 1.0 mM and 2.0 mM than containing 0.5 mM and control group (Ansari *et al.,* 2010)indicating protective ability of cysteine (Corton *et al.,*1989).Further, vitamin E acts both as a membrane-stabilizer and a potent antioxidant molecule protecting cell membrane against lipid peroxidation and ROS attacks (Niki and Noguchi, 2004).

Acrosome integrity is one of the determinant factors for the success of fertilization (Celeghini *et.al.,* 2010) and its mean percentage was significantly (pÂÙ0.05) higher in T1 and T2 as compared to control group at post thaw stage but there was no significant difference amongst the groups at after dilution and pre-freeze stages (Table1). Acrosomal damage is a common phenomenon at cryopreservation (Hossain et al., 2011) due to decreased antioxidant potential of semen and increased lipid peroxidation levels and ROS molecules (Kadirvel *et al.*, 2009)and also at freeze-thawing (Gangwar *et.al.,* 2018).While cysteine maintains acrosomal integrity by scavenging ROS molecules through glutathione mediated pathway by increasing antioxidant activity in the semen-extender complex. A non-significant difference in acrosomal integrity of spermatozoa among different bulls except bull 4was observed between control and T1 or T2 at after dilution and at pre-freeze stage, however at post thaw period same was significantly (P<0.05) higher

compared to control group.These findingsare is an accordance with the earlier studies in different breeds (El-Sheshtawy *et al.*, 2008 and Ansari *et al.,* 2010)

The present study revealedthat of 5mM cystein hydrochloride and 2.5 mM vitamin E in the extender are suitable for freezing bull spermatozoa in GEYT extender having 20% egg yolk and 7% glycerol with 80 million /ml sperm concentration in extended semen.

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Table 1: Sperm viability, progressive motility, HOS reactive spermatozoa and acrosomal integrity of Hariana bull semen at cryopreservation extended with GYET, GYET+CysteinHcl and GYET+Vit E (Mean±SEM, n=8)

Group	Stages	Bull 1	Bull 2	Bull 3	Bull 4
Mean percentage of live spermatozoa					
Control	After Dilution	83.50±0.90^{a}	83.00±0.86^{a}	82.38±0.49^{a}	81.75±0.79^{a}
	Pre-freeze	78.88±0.79^{a}	79.00±1.07^{a}	78.50±1.28^{a}	72.25±1.03^{a}
	Post- thaw	71.00±1.65^{a}	70.00±1.60^{a}	68.88±1.60^{a}	66.88±1.37^{a}
T1	After Dilution	86.37±0.86^{b}	85.88±0.76^{b}	84.50±0.46^{b}	83.88±0.72^{ab}
	Pre-freeze	81.88±0.66^{b}	82.00±1.05^{b}	81.38±1.21^{ab}	78.38±0.92^{b}
	Post- thaw	75.88±0.35^{b}	74.50±1.23^{b}	73.38±1.45^{b}	71.00±1.37^{b}
T2	After Dilution	87.38±1.00^{b}	87.25±0.77^{b}	85.63±0.49^{b}	84.88±0.69^{b}
	Pre-freeze	83.13±0.74^{b}	83.63±0.82^{b}	83.00±1.12^{b}	78.88±1.11^{b}
	Post- thaw	77.63±0.18^{b}	76.25±0.95^{b}	74.63±1.53^{b}	72.00±1.35^{b}
Mean percentage of progressively motile spermatozoa					
Control	After Dilution	70.88±0.61^{a}	70.13±0.93^{a}	68.75±0.90^{a}	67.25±0.88^{a}
	Pre-freeze	65.00±1.10^{a}	64.25±1.14^{a}	63.38±0.92^{a}	60.75±1.08^{a}
	Post- thaw	58.25±1.54^{a}	56.88±1.33^{a}	55.75±1.08^{a}	53.75±1.17^{a}
T1	After Dilution	73.75±0.52^{b}	72.63±0.90^{ab}	71.38±0.86^{ab}	69.63±0.75^{ab}
	Pre-freeze	68.38±1.16^{b}	67.75±1.28^{ab}	66.50±1.12^{ab}	64.50±1.29^{b}
	Post- thaw	62.75±1.37^{b}	61.25±1.51^{b}	59.50±1.05^{b}	57.88±1.04^{b}
T2	After Dilution	75.12±0.61^{b}	73.75±0.84^{b}	72.63±0.96^{b}	71.50±1.07^{b}
	Pre-freeze	70.00±0.98^{b}	69.00±1.21^{b}	68.00±1.13^{b}	65.38±1.07^{b}
	Post- thaw	64.75±1.23^{b}	62.50±1.53^{b}	60.88±1.22^{b}	58.88±1.15^{b}
Mean percentage of HOS reactive spermatozoa					
Control	After Dilution	80.25±0.98^{a}	80.12±0.76^{a}	79.00±0.62^{a}	77.63±0.78^{a}
	Pre-freeze	74.38±0.70^{a}	75.88±1.01^{a}	73.88±1.13^{a}	71.38±1.02^{a}
	Post- thaw	68.38±1.67^{a}	67.63±1.62^{a}	65.38±1.89^{a}	63.38±1.14^{a}
T1	After Dilution	82.75±0.98^{ab}	82.25±0.82^{ab}	81.25±0.62^{b}	79.88±0.76^{b}
	Pre-freeze	76.75±0.59^{b}	78.50±0.98^{ab}	76.13±1.23^{ab}	73.75±1.43^{ab}
	Post- thaw	72.88±0.39^{b}	71.38±0.90^{b}	69.88±1.14^{b}	67.38±0.94^{b}
T2	After Dilution	84.25±0.98^{b}	83.63±0.80^{b}	82.50±0.65^{b}	81.13±0.74^{b}
	Pre-freeze	78.50±0.73^{b}	79.63±1.10^{b}	78.25±1.15^{b}	76.88±1.32^{b}
	Post- thaw	74.25±0.49^{b}	73.13±0.93^{b}	70.63±1.20^{b}	68.88±1.12^{b}
Mean percentage of sperm acrosomal integrity					
Control	After Dilution	82.25±0.94	81.75±0.79	81.00±0.56	80.00±0.78
	Pre-freeze	77.13±0.91	77.50±1.05	76.75±1.36	72.88±1.19^{a}
	Post- thaw	69.00±1.84^{a}	68.38±1.55^{a}	65.00±1.72^{a}	64.38±1.20^{a}
T1	After Dilution	83.75±0.88	83.25±0.70	82.13±0.48	81.38±0.70
	Pre-freeze	78.50±0.90	79.50±1.05	78.00±1.36	74.75±1.06^{ab}
	Post- thaw	73.13±0.69^{b}	71.63±0.94^{b}	69.88±1.39^{b}	68.75±1.01^{b}
T2	After Dilution	84.75±0.79	84.13±0.81	83.00±0.80	81.75±0.70
	Pre-freeze	79.75±1.03	80.13±1.26	78.88±1.50	77.63±1.38
	Post- thaw	74.75±0.56^{b}	73.25±0.86^{b}	71.50±1.13^{b}	69.38±1.11^{b}