Pakistan J. Zool., vol. 47(6), pp. 1571-1577, 2015.

Ascorbic Acid Inclusion in Semen Extender Improves the Post-Thawed Semen Quality of Sahiwal Cattle (*Bos indicus*)

M.U. Sohail,[1] A. H. Shahzad,[2*] S. Iqbal,[3] M.Z. Shabbir,[4] Z. Iqbal,[5] S. Abbas,[2] M. Younus,[6] D. Nak,[6] Y. Nak[6] and T. Arshaad[7]

[1]*Department of Physiology, Government College University, Faisalabad, Pakistan*
[2]*College of Veterinary and Animal Sciences, Jhang-35200, Pakistan*
[3]*Semen Production Unit, Qadirabad, Distt. Sahiwal 57000, Pakistan*
[4]*Quality Operations Laboratory, University of Veterinary and Animal Sciences, Lahore-54000*
[5]*Livestock and Dairy Development Department, Punjab*
[6]*Department of Obstetrics & Gynecology, Faculty of Veterinary Medicine, Uludag University, Bursa, Turkey*
[7]*VETMED 22-A Shahra-e-Fatima Jinnah, Lahore-54000*

Abstract.- The antioxidant effects of ascorbic acid at different inclusion rates were evaluated upon semen (n=150) from mature Sahiwal cattle bulls (*Bos indicus*, n = 6) in summer season. The semen was diluted in Tris-citrate egg yolk extender containing different levels (0.0, 1.0, 2.0, 3.0, and 4.0 mg/mL) of ascorbic acid at 37.0ºC. Semen was cryopreserved at -196ºC in 0.5 mL French straws. Semen straws were thawed at 37ºC to assess the spermatozoa indices in terms of motility, viability, plasma membrane and acrosomal integrity under phase-contrast microscope. Supravital staining, hypo-osmotic swelling test and normal acrosomal reaction analysis tests were also performed for viability, plasma membrane and acrosomal integrity, respectively. The data were subjected to one way ANOVA. The results revealed significant improvement ($P< 0.05$) in post thaw sperm quality in terms of motility, vitality, acrosomal and plasma membrane integrity by the increasing concentrations of ascorbic acid (1.0, 2.0, 3.0, 4.0 mg/mL) in semen extender. Quality parameters were higher ($P< 0.05$) when 3.0 mg/mL of ascorbic acid was added in semen extender followed by 2.0 and 4.0 mg/mL. In conclusion the addition of ascorbic acid at the rate of 3.0 mg/mL in the semen extender may improve the semen quality of Sahiwal cattle bull.

Key words: Ascorbic acid, cryopreservation, spermatozoa motility, Sahiwal bulls.

INTRODUCTION

Artificial insemination (AI) is getting significant importance in modern livestock farming all over the world. Genetically superior males are being used to improve production potential of domestic animals. Livestock farmers are rapidly changing their breeding practices from traditional breeding to modern AI; however, lower pregnancy rate with cryopreserved semen is a major obstacle in getting maximum benefits from this technique (Vishwanath, 2003). AI is not frequently practiced in developing countries because the conception rate of cryopreserved semen is generally lower than that of fresh semen. Cryopreservation technique is being used to preserve the genetic material over extended periods of time, as it slowdowns the sperm cell metabolism and maintains its viability for future use. However, of the many constraints in AI with cryopreserved semen, one is oxidative damage of sperms from reactive oxygen species (ROS), ultimately resulting in decreased ability of sperm to fertilize the ovum (Alvarez and Storey, 1993; Chatterjee and Gagnon, 2001; Ball, 2008; Ijaz *et al.*, 2009).

Bovine spermatozoa carry a little reservoir of indigenous antioxidant such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and small molecular antioxidants such as ascorbate, reduced glutathione, rate, vitamin E and β-carotene (Sikka, 2004; Aitken and Fisher, 1994) derived from seminal secretions.

* Corresponding author: ahshahzad@uvas.edu.pk
0030-9923/2015/0006-1571 $ 8.00/0

Authors' Contributions: AHS, MUS and ZI conceived and designed the project. SI, AHS and TA executed the experiments and analyzed the samples. MZS, SA, MY, DN and YN analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

These antioxidants concentrations are further diluted by addition of semen extender (Bilodeau *et al*., 2000). Normally, these antioxidants neutralize ROS; however, during the process of freeze and thaw, excessively produced species are not properly neutralized by indigenous antioxidants. Therefore, oxidative damage of spermatozoa declines progressive motility, vitality and fertilizing ability of the semen (Chatterjee and Gagnon, 2001; Aitken and Fisher, 1994; Ball, 2008).

Keeping in view the increasing demand of AI in domestic animals, evidence of potential damage of sperm cells by excessively produced ROS during the process of freeze and thaw, efforts are on to find suitable antioxidants for inclusion in semen. The addition of exogenous antioxidants in semen has been practiced for several decades with significant improvement in fertility (Ciereszko and Dabrowski, 1995; Foote *et al*., 2002; Purdy *et al*., 2004; Sönmez *et al*., 2005; Boonsorn *et al*., 2010). Though exact mode of action of ascorbic acid as a natural antioxidant is yet unknown, still it is considered for enhancing the fertility and reproductive efficiency of the males (Ciereszko and Dabrowski, 1995; Hsu *et al*., 1998). Foote *et al.* (2002) found that addition of ascorbic acid in cattle bull semen improved sperm motility and fertilizing ability. Similarly, numerous other studies are available which tested the impact of addition of ascorbic acid in *in-vitro* or *in-vivo* trials concerning bovine, boar, humans, rabbit, canine and stallion semen with controversial efficacy and usefulness (Beconi *et al*., 1993; Aurich *et al*., 1997; Donnelly *et al*., 1999; Pena *et al*., 2003; Michael *et al*., 2007). Semen quality is reduced in summer in the form of progressive motility (Marai *et al*., 2002), DNA as well as plasma membrane and acrosomal integrity (Roca *et al*., 2005). It has been well documented that elevated temperature results in increased ROS production which are detrimental to sperm cell. Summer stress is global issue as 60% world dairy animals are affected by it (Wolfenson *et al*., 2000).

No studies are available on using specific quantity of ascorbic acid as an antioxidant in Sahiwal cattle frozen semen. The aim of this study was therefore, to elucidate the post-thaw quality of Sahiwal cattle bull semen extended with diluents containing different ascorbic acid concentrations during summer season.

MATERIALS AND METHODS

Semen collected aseptically from 4-6 year old Sahiwal cattle bulls (n=6) maintained at Semen Production Unit, Qadirabad, Punjab was used in the present study during the month of August. The bulls were routinely used for semen collection, and their frozen semen was supplied to all concerned quarters of Punjab province under the set-up controlled by Livestock Breed Improvement Department, Government of Punjab. The bulls were kept under standard management and nutrition plan with access to clean drinking water *ad libitum*. The bulls had uniform sperm motility to access the effect of treatment, if any. Each bull was maintained on seasonally available fodder and concentrate ration to meet or exceed daily nutritional requirement. The bulls were scored for body condition on individual basis in accordance to the criterion of Ministry of Agriculture, Fisheries and Food (MAFF, 1986).

Semen collection

Semen collection was carried out aseptically by artificial vagina (IMV Technologies, Normandy, France). Two ejaculates obtained from each bull twice a week were pooled together for processing. Each bull was given sexual stimulation for adequate time before semen collection. Immediately after collection, semen was transferred to a water bath at 37ºC. Sperm concentrations and initial percentage of motility was recorded. Spermatozoa concentration was calculated using spectrophotometer (Spectronic 20, Beckman Instruments, Fullerton, CA). Percentage motility was estimated at 37˚C under light microscopy. Semen samples showing good motility (>70%), morphology and concentration were pooled and extended in a Tris–citrate egg yolk extender with 20% (v/v) egg yolk and 7% (v/v) glycerol at 37ºC within 10 min. Experimental extenders were added with different levels (0.0, 1.0, 2.0, 3.0, and 4.0 mg/mL) of ascorbic acid. Semen was cryopreserved at -196ºC using 0.5 mL French straws. For evaluation of post-thaw parameters, five straws were used separately for each treatment and mean value was taken for comparison.

Post-thawed semen evaluation

Sperm motility

A drop of thawed semen was put on a pre-warmed glass slide and was covered with a cover slip. Percentage motility for each straw was assessed in three microscopic fields under a phase-contrast microscope at 40X. The mean of three microscopic fields was considered as a single data point subjectively, with ±5% margin of error (Ijaz *et al*., 2009).

Sperm cell vitality

The vitality of spermatozoa in each sample was observed by staining the air dried sample with supra vital dye. A small droplet of semen was mixed properly with a drop of dye and spread over clean and grease free glass slide. Two hundred spermatozoa were counted under bright field microscopy (40X). Complete or incomplete stained spermatozoa (head) were counted as dead and sperm cells having no stain penetration were considered as live (Ijaz *et al*., 2009).

Acrosome integrity

Normal acrosomal ridge (NAR) integrity of sperm cells was assessed by examining the acrosomal membrane integrity.Three drops of frozen–thawed semen sample were mixed with 50 µL of 1% formaldehyde citrate in 2.9% (w/v) tri-sodium citrate dihydrate. Two hundred sperm acrosomeswere observed under oil immersion lens of a phase-contrast microscope. Abnormalities like absent, ruffled and swollen acrosomes were indexed as described previously (Ijaz *et al*., 2009).

Plasma membrane integrity

Sperm plasma membrane integrity was assessed using hypo-osmotic swelling test (HOST) as described earlier (Ijaz *et al*., 2009). Briefly, a hypo-osmotic solution (190 mOsm/L) was prepared by dissolving 0.735 g of tri-sodium citrate dihydrate and 1.351 g D-fructose in 100 mL of de-ionized water. The test was performed by first mixing 500 µL of hypo-osmotic solution with 50 µL of each frozen–thawed semen sample and incubated at 37ºC for 45 min. After incubation and mixing a drop of the sample, it was mounted on a pre-warmed slide and examined under a phase-contrast microscope (40X). Two hundred spermatozoa per slide were counted, and the percentage of spermatozoa exhibiting tail curling was determined. The mean of three observations was considered as a single data point.

Statistical analysis

Data were presented as mean ± S.D and subjected to ANOVA using SPSS- 13.0, a statistical package (SPSS Inc., Chicago, IL). Statistical differences among means ($P < 0.05$) were identified using Duncan's multiple range tests.

RESULTS

Spermatozoa quality

All parameters of post thaw spermatozoa quality; motility, vitality, HOST response and NAR % were improved ($P < 0.05$) by the addition of increasing concentrations of ascorbic acid (1.0, 2.0, 3.0, 4.0 mg/mL) in semen extender. The quality parameters were highest when 3.0 mg/mL of ascorbic acid was added in semen extender followed by 2.0 and 4.0 mg/mL (Table 1). The minimum motility, HOST response and NAR % was found in semen extender with 0.0 mg/mL inclusion level. The higher concentration of ascorbic acid (4.0 mg/mL) inclusion reduced the motility, vitality, NAR % and hypo-osmotic swelling response of the spermatozoa compared to 3.0 and 2.0 mg/mL.

Sperm motility

Spermatozoal motility is the most frequently observed characteristic, however owing to acrosomal damage sperm cell could be highly motile but not fertile. After thawing the frozen semen straw, motility rate differences were significant among treatment groups ($p>0.05$). Motility of T3 group containing 3.0 mg/ml ascorbic acid was significant higher (64.97±3.27) than that of T0 (50.63±3.50) and T1 (53.67±4.12) having 0.0 mg/ml and 1.0 mg/ml of ascorbic acid ($p<0.05$). In a similar fashion T4 containing 4.0 mg/ml of ascorbic acid resulted in decreased progressive motility (62.73±2.80). This indicates that ascorbic acid provides beneficial effects up to a certain inclusion level and beyond this it has a negative impact on motility percentage.

Table I. - Effect of ascorbic acid addition on post-thawed Sahiwal cattle bull semen quality cryopreserved in a Tris-egg yolk based extender.

Treatment Groups		Semen quality indices			
	Ascorbic acid concentrations (mg/mL)	**Motility**	**Vitality**	**HOST**	**NAR**
T0	0.0	50.63±3.50^{d}	61.27±6.15^{cd}	49.97±3.62^{c}	30.77±3.15^{c}
T1	1.0	53.67±4.12^{c}	60.43±3.17^{d}	51.37±3.98^{c}	36.20±3.53^{b}
T2	2.0	63.93±3.87^{a}	65.27±2.97^{b}	58.27±4.48^{a}	40.73±3.33^{a}
T3	3.0	64.97±3.27^{a}	69.10±3.76^{a}	60.10±3.35^{a}	41.57±1.99^{a}
T4	4.0	62.73±2.80^{ab}	62.73±2.80^{c}	54.20±3.68^{b}	37.60±1.57^{b}

$^{a-d}$Means ± SD within a column lacking a common superscript differ ($P< 0.05$).
Hypo-osmotic swelling test: HOST, Normal acrosomal ridge: NAR

Sperm viability

After thawing semen sample were stained by eosin-nigrosin stain and evaluated for viable sperm cells. Sperm viability percent differences were significant among treated and control groups ($p>0.05$). Staining of frozen semen after thawing has shown that the sperm viability of control group was significantly lower than that of T2 and T 4 groups. Table I shows that among treated groups T3 has significantly enhanced percentage of viable sperm cells. This viability reduced on further increase in inclusion level of ascorbic acid in the semen extender (T4). In this way sperm viability of T3 (69.10±3.76) was higher ($p<0.05$) than that of T0 (61.27±6.15), T1 (60.43±3.17), T2 (65.27±2.97) and T4 (62.73±2.80) groups (Table I).

Sperm cell membrane integrity

Ascorbic acid addition in Tris egg yolk extender also improved sperm cell membrane integrity of sahiwal cattle bulls shown in Table I. Membrane integrity was improved in treated groups (1.0 mg per ml, 2.0 mg per ml, 3.0 mg per ml) as compared to control group(T0) having no inclusion of ascorbic acid($p<0.05$). Addition of ascorbic acid beyond this concentration in Tris egg yolk extender showed decreased integrity. Sperm cell membrane integrity results indicate that among treated groups, group T2 (58.27±4.48) and T3 (60.10±3.35) were insignificant between each other but significant among other treated and control group. Overall mean of T3 was higher to any other group including controlled one.

Acrosomal integrity

The damage to acrosome may occur during dilution, cooling, freezing and thawing process. Intact apical ridge is essential for fertilizing capacity of spermatozoa and for functional efficiency of the acrosome. The pooled mean percentages of sperms with intact acrosome in Tris yolk extender with corresponding inclusion levels of ascorbic acid (0, 1.0mg/ml, 2.0mg/ml, 3.0mg/ml and 4.0 mg/ml) differed significantly in terms of normal acrosomal integrity (30.77±3.15, 36.20±3.53, 40.73±3.33, 41.57±1.99 and 37.60±1.57 respectively). The post-thaw mean percentage of sperms with maximal intact acrosome was recorded as 41.57±1.99in T3 group containing 3.0 mg/ml of ascorbic acid. The intact acrosomal integrity per cent was significantly higher in T3 group containing 3.0mg/ml as compared to other treatment groups including controlled group.

DISCUSSION

Bovine sperm plasma membrane is predominantly affluent in polyunsaturated fatty acids (PUFA). This high proportion of polyunsaturated fatty acids makes spermatozoa highly prone to oxidation-reduction related cell injuries during processing of frozen semen. The results obtained in this study emphasize that ascorbic acid can significantly improve post-thaw semen quality of cattle bulls. Ascorbic acid is well known antioxidant that protects the spermatozoa vitality by preventing oxidative damage of DNA and membranes (Padayatty *et al*., 2003). It is believed that ascorbic acid inhibits oxidative species and protects cell membrane and acrosome of sperm cells. Aitken *et al*. (1994) demonstrated that the functional competence of spermatozoa and the

integrity of their DNA are affected by ROS at different rates, in different ways. Spermatozoa are vulnerable to ROS damage due to their high polyunsaturated fatty acid contents. All steps involved in cryopreservation of semen favors ROS generation that eventually reduces fertilizing capacity of the sperms (O'Flaherty *et al*., 1999). Further, intracellular built-in antioxidant competency in spermatozoa declines after cryopreservation. Use of antioxidants has been investigated in a many species (Bilodeau *et al*., 2000; Bucak *et al*., 2010; Neagu *et al*., 2010; Tuncer *et al*., 2010b). In bovines use of antioxidants has resulted in improved semen quality (Beconi *et al*., 1993; Foote *et al*., 2002; Andrabi, 2008).

The sperm motility, the capability to undertake the acrosome reaction, the ability to fuse with the vitelline membrane of the egg, and the DNA integrity; all are susceptible to oxidative damage (De-Lamirande and Gagnon, 1992). Sperm motility is dependent on the integrity of the plasma and mitochondrial membranes, which are composed of phospholipids. If fatty acids in the phospholipids are oxidized by ROS, sperm may be damaged and their motility gets impaired (Alvarez and Storey, 1993; De-Lamirande and Gagnon, 1992). DNA integrity is considered more vital importance in semen evaluation. Some authors are of the opinion that genomic status is more objective indicator of sperm's functional status. Sperm cell DNA is prone to injury if its acrosomal integrity is damaged. Sperm can penetrate the cumulus cells surrounding the ova by means of the enzyme hyaluronidase released from its acrosome. These acrosomal enzymes play a key role during sperm penetration in zona pellucida. Therefore, addition of exogenous antioxidants may improve the antioxidant status of spermatozoa. It has been concluded that that fragmentation of DNA can be checked by antioxidant and ascorbic acid can be used efficiently to control the destructive effects of ROS.

Another study, in chicken semen, was conducted to observe the effects of addition of ascorbic acid and beneficial results were documented by using 1% of this compound. Improved semen quality was observed after 24 h storage of chicken semen. Negative results were found when increased concentration of ascorbic acid was used. At 3% inclusion ascorbic acid not only resulted in reduced sperm motility but also increased morphological abnormalities. On the basis of this finding it was concluded that Ascorbic acid has beneficial effects on fresh semen. These results indicate that effect of ascorbic acid as an antioxidant may be specie specific in nature. We previously showed that ROS caused a decrease in sperm motility, associated with an increase in lipid peroxidation, and loss of membrane fatty acid (Ijaz *et al*., 2009; Adeel *et al*., 2009). However, ascorbic acid inclusion overturns non-specific sperm auto-agglutination and improves physical characteristics of sperm, particularly vitality and motility (Lindholmer, 1974). Ascorbic acid is a strong electron donor and this property makes it an excellent antioxidant. Indeed, ascorbic acid is found in aqueous phase and interrupts the chain reactions of lipid peroxidation by scavenging ROS generated during cellular metabolism (Padayatty *et al*., 2003). In another investigation (Batool *et al*., 2012) found that ascorbic acid inclusion was unable to prove beneficial impact on post thaw semen quality in bovine. In current study improved semen quality was noticed when ascorbic acid was used in summer season.

CONCLUSION

In conclusion, concentration of indigenously produced antioxidants declines during the freeze and thaw processes (Foote *et al*., 2002). Therefore, natural and synthetic antioxidants are frequently added in semen extender to inhibit peroxidation of sperm cell phospholipids, particularly of the polyunsaturated fatty acids. This study revealed that addition of ascorbic acid inTris–citrate egg yolk semen extender of concentrations ranging between 2.0 and 3.0 mg/mL are optimal for cryopreservation of Sahiwal cattle (*Bos indicus*) bull semen. Moreover, increased inclusion rate of ascorbic acid may result into altered plasma membrane fluidity which makes them to more susceptible to be damaged. Results of this study show that ascorbic acid is a useful antioxidant which has marvelous properties for frozen semen in summer season. Further studies are under process in investigating the effects of ascorbic acid inclusion on the

oxidants/antioxidant ratio and fertility rate of cattle.

ACKNOWLEDGEMENT

Authors are grateful to the Scientific and Technological Research Council of Turkey (TÜBİTAK) for the award of fellowship to Mr. Abid Hussain Shahzad under 2216 program.

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(Received 13 March 2015, revised 25 April 2015)