Indian Journal of Animal Sciences **83** (5): 498–501, May 2013/Article

Seminal plasma ascorbic acid level and its relationship to sperm characteristics in Murrah buffalo bulls

S K MAURYA[1], O P SINGH[2] and SUSHANT SRIVASTAVA[3]

Narendra Deva University of Agriculture and Technology, Kumarganj, Uttar Pradesh 224 229 India

Received: 16 August 2012; Accepted: 3 November 2012

ABSTRACT

Ascorbic acid levels were investigated in the seminal plasma of Murrah buffalo bulls and its relation was investigated with various parameters of sperm morphology. Semen samples were collected using bovine artificial vagina. Semen volume, sperm concentration, initial motility, livability, sperm abnormality, hypo osmotic swelling, acrosomal integrity and post thaw motility were assessed using standard procedures. Ascorbic acid contents in the seminal plasma were estimated by reverse phase high performance liquid chromatography (RP-HPLC) procedure using UV detection at 254 nm. Bulls were divided into 3 categories depending on the extent of sperm abnormality. The mean ± SEM values of seminal plasma ascorbic acid (mg/ dl) were 4.81 ± 0.08, 3.17 ± 0.09 and 1.79 ± 0.05 in good, medium and poor groups of Murrah buffalo bulls. The seminal plasma ascorbic acid differed significantly in all 3 groups. The seminal plasma ascorbic acid level was positively correlated with spermatozoa with volume/ ejaculate (r^2 = 0.710), conc./ ejaculate (r^2 = 0.915), initial motility (r^2 = 0.944), livability (r^2 = 0.933), acrosomal integrity (r^2 = 0.955), HOS reactiveness (r^2 = 0.946) and post-thaw motility (r^2 = 0.906); whereas it was negatively correlated with sperm abnormality (r^2 = –0.896).

Key words: Ascorbic acid, RP-HPLC, Seminal plasma, Sperm quality

Ascorbic acid is thought to act as an excellent reducing agent since it is also able to reduce catalytically active transition metal ions and free radicals; thus it acts as anti oxidant in the body (Buettner and Jurkiewicz 1991). Moreover, ascorbic acid concentrations in seminal plasma are also positively related to the percentage of morphologically normal spermatozoa in man, and it was suggested that ascorbic acid is a protective vitamin in the epididymis. In buffalo bulls, the role of ascorbic acid was studied by supplementing ascorbic acid (Singh *et al.* 1996, Shukla and Mishra 2005) but there is no study on direct estimation of ascorbic acid in seminal plasma and its relationship with semen characteristics.Thus the present investigation was done to evaluate the semen quality of buffalo bulls in relation to the levels of seminal plasma ascorbic acid.

MATERIALS AND METHODS

Animals, semen collection and evaluation: Semen samples were collected from Murrah buffalo bulls (20) maintained at Network Project on Buffalo, N. D. University of Agriculture and Technology, Kumarganj, Faizabad using a bovine artificial vagina. Immediately after collection, the ejaculate was placed in a 37° C water bath and the volume was recorded. The percentage of progressively motile spermatozoa was estimated by microscopic examination, and a subjective assessment of the progressive status was recorded according to Ax *et al.* (2000). Sperm concentration was measured using standard hemocytometer methods, the percentage of viable spermatozoa was estimated by viewing 200 spermatozoa under 400× magnification using eosin-aniline blue staining (Ax *et al.* 2000).

Depending upon the sperm abnormality in tail region, the buffalo bulls were divided into groups a, good; b, medium; and c, poor. Bulls showing tail abnormality of less than 20% were placed in the category of good bulls and those having tail abnormality of 20 to 40% were categorized as medium; whereas the bulls having tail abnormality more than 40% were placed in poor group. Of the 20 bulls, 8 were of good quality and 6 each in medium and poor groups.

Freezing and thawing: After initial evaluation, semen was extended in Tris-fructose-yolk-glycerol diluter (Davis *et al.* 1963) at 35°C to an extent that each ml of diluted semen contained 50 million motile spermatozoa. The diluted semen was filled in 0.25 ml Mini French straws and kept into a rack. The racks were then kept in the cold handling cabinet at the 4°C for 4 h. Further, the rack along with straw was

Present address: [1]Assistant Professor, Department of Veterinary Biochemistry (skmaurya@yahoo.com). [2]Reader, Department of Animal Husbandry, UP College, Varanasi (opsingh.upcoll @gmail.com). [3]Assistant Professor, Department of Animal Reproduction (sushantvet@gmail.com).

Table 1. Mean ± SEM of various parameters of sperm morphology and seminal ascorbic acid contents in all three group of Murrah buffalo bulls (N=90)

Parameter/ Animal	Good (n=30)	Medium (n=30)	Poor (n=30)
Volume (ml)	4.98 [a] ± 0.4	3.09 [b] ± 0.09	2.66[c] ± 0.10
Total sperm /ejaculate (10^6)	6817 [a] ± 179.1	4728 [b] ± 160.1	3756 [c] ± 110.1
Initial motility (%)	79.4 [a] ± 0.57	61.63 [b] ± 1.17	31.00 [c] ± 1.38
Livability (%)	85.8 [a] ± 0.64	71.70 [b] ± 1.0	40.27 [c] ± 1.31
Tail abnormality (%)	14.20 [a] ± 0.64	28.30 [b] ± 1.0	58.73 [c] ± 1.42
Acrosomal integrity (%)	90.40 [a] ± 0.55	73.07 [b] ± 1.02	48.00 [c] ± 1.17
HOS reactive spermatozoa (%)	61.60 [a] ± 0.47	36.20 [b] ± 0.71	17.43 [c] ± 0.52
Post thaw motility (%)	54.50 [a] ± 0.77	44.7 [b] ± 0.57	19.53 [c] ± 0.78
Seminal plasma ascorbic acid (mg/ dl)	4.81[a] ± 0.08	3.17[b] ± 0.09	1.79[c] ± 0.05

Values with different superscripts in a row differ significantly (P<0.05).

kept in liquid nitrogen vapours till the temperature of the straws reached –100 to –120°C. At this temperature straws were held for 9–10 min and then plunged into liquid nitrogen (–196°C) and stored for 24 h. After 24 h storage in liquid nitrogen the straws were thawed at 37°C for 30 sec for post thaw evaluation.

Preparation of seminal plasma for HPLC: For preparation of seminal plasma, fresh semen was centrifuged at 5 000 rpm for 10 min. The supernatants were collected and re-centrifuged at same rpm for 10 min to eliminate any cells. Nine volumes of cold ethanol (–20°C) were added to the seminal plasma and left with constant stirring for 90 min at 4°C to precipitate the proteins; and then centrifuged at 10 000 rpm for 10 min. The supernatants were collected and stored at –20°C until a 20 µl aliquot was injected onto the HPLC system.

HPLC system: The HPLC was performed on HPLC system that comprised isocratic pumps and UV-VIS detector; a rheodyne injector (20 µl sample loop) and phenomenex C18 column (5 µm particle size). For data acquisition, Winchrome software was used. The experiments were performed in isocratic mode. The mobile phase consisted of 2.5% glacial acetic acid (HPLC grade) and 80% methanol (HPLC grade) in HPLC grade water. The flow rate was maintained at 1 ml/min and spectrophotometric detection at 254 nm was employed. The isocratic chromatographic system was conditioned by passing the eluent through the column until a stable base line was observed. Then, repeatable retention times were obtained for three subsequent injections.

Statistical analysis: An independent t test was considered to compare the scores of each of the measures and some of the parameters data among the groups. The ANOVA model was utilized for statistical analyses of elements concentration between all groups. The Pearson correlation and linear regression model was utilized to examine the relation between seminal ascorbic acid and sperm parameters quality. P <0.05 was considered statistically significant. Data were analyzed using Graphpad Prism version 5.00 software.

RESULTS AND DISCUSSION

Mean ± SEM of various parameters of sperm morphology and seminal ascorbic acid contents in all 3 groups of Murrah buffalo bulls are presented in Table 1.

All seminal characteristics like ejaculate volume, initial and post-thaw motility, livability, sperm abnormality, acrosomal integrity and HOS reactiveness of sperms differed significantly (P<0.05) in all 3 groups of good, medium and poor buffalo bulls.

The mean ± SEM values of seminal plasma ascorbic acid (mg/ dl) were 4.81 ± 0.08, 3.17 ± 0.09 and 1.79 ± 0.05 in good, medium and poor groups of Murrah buffalo bulls. The seminal plasma ascorbic acid differed significantly (P<0.05) in all 3 groups. The values of ascorbic acid in seminal plasma were higher than that reported by Banerjee and Ganguly (1973). This may be due the difference in sample preparation, as in the present investigation, seminal plasma was purified.

The correlation coefficient (r^2, P<0.05) of seminal plasma ascorbic acid content and various parameters of semen characteristics are presented in Table 2.

The seminal plasma ascorbic acid level was significantly positively correlated (P<0.05) with spermatozoa with volume/ ejaculate (r^2 = 0.710), conc/ ejaculate (r^2 = 0.915), initial motility (r^2 = 0.944), livability (r^2 = 0.933), acrosomal integrity (r^2 = 0.955), HOS reactiveness (r^2 = 0.946) and post thaw motility (r^2 = 0.906); whereas it was significantly negatively correlated (P<0.05) with sperm abnormality (r^2 = –0.896).

The mechanism by which ascorbic acid in the seminal plasma of poor bulls is depleted has not been fully elucidated. It is possible that ascorbic acid deficiency in poor bulls may be due to an increase in oxidative damage induced by reactive oxygen species (ROS); as studies have indicated that seminal oxidative stress is associated with sperm motility and sperm quality (Abd-Almoaty *et al.* 2010). Thus, vitamin C supplementation gave the best seminal parameter including sperm concentration, sperm motility, progressive sperm motility, and normal sperm morphology in rats (Sonmez *et*

Table 2. Correlation coefficient (r^2, P<0.05) of seminal plasma ascorbic acid levels with various parameters of semen morphology (n=90)

	Ascorbic acid	Volume/ ejaculate	Conc/ ejaculate	Initial motility	Livability	Sperm abnormality	Acrosomal integrity	HOS reactiveness	Post thaw motility
Ascorbic acid		0.710	0.915	0.944	0.933	–0.896	0.955	0.946	0.906
Volume/ ejaculate	0.710		0.861	0.630	0.618	–0.565	0.645	0.637	0.593
Total sperm /ejaculate (10^6)	0.915	0.861		0.880	0.864	–0.782	0.897	0.852	0.792
Initial motility	0.944	0.630	0.880		0.995	–0.933	0.994	0.929	0.941
Livability	0.933	0.618	0.864	0.995		–0.937	0.989	0.914	0.949
Tail abnormality	–0.896	–0.565	–0.782	–0.933	–0.937		–0.926	–0.946	–0.989
Acrosomal integrity	0.955	0.645	0.897	0.994	0.989	–0.926		0.935	0.934
HOS reactiveness	0.946	0.637	0.852	0.929	0.914	–0.946	0.935		0.938
Post thaw motility	0.906	0.593	0.792	0.941	0.949	–0.989	0.934	0.938	

al. 2005, Krishnamoorthy *et al.* 2007), in rabbits (Yousef *et al.* 2005), in guinea pigs (Chinoy *et al.* 1986), in cows (Phillips *et al.* 1941, Dalvit *et al.* 1998), in stallions (Ralston *et al.* 1988), in rams (Fazeli *et al.* 2010).

Ascorbic acid is a strong antioxidant and decreases fatty acid peroxidation in the cell membrane. Ascorbic acid supplementation has been shown to decrease free radical formation in human seminal plasma (Dawson *et al*. 1992) and decrease the incidence of secondary sperm abnormalities by reducing free radical formation (Sikka 1992). Ascorbic acid is also involved in the synthesis of sex steroids such as testosterone, and peptide hormones; hydroxylation of steroids is especially vitamin C-dependent (Luck *et al*. 1995). Even ascorbic acid supplementation improved the total sperm output in rabbits during summer stress (El-Tohamy *et al.* 2012). These functions of ascorbic acid can help improve sperm viability and normality in buffalo bulls, as found in the present investigation.

Decrease of seminal plasma ascorbic acid is a risk factor for low normal morphology of spermatozoa and idiopathic male infertility. Measurement of seminal ascorbic acid in the seminal plasma of males with a history of subfertility or idiopathic infertility is necessary and can be helpful in fertility assessment (Colager and Marzony 2009).

In conclusion, our findings suggested that buffalo bulls with poor sperm characteristics have significantly low levels of ascorbic acid in their seminal plasma than bulls with good sperm characteristics. Association of seminal ascorbic acid with normal morphology of sperm may indicate that the decrease in seminal ascorbic acid content can be a risk factor for sperm abnormality and infertility in bulls.

REFERENCES

Abd-Almoaty M A, Saleh R, Sharma R and Agarwal A. 2010. Increased levels of oxidants and reduced antioxidants in semen of infertile men with varicocele. *Fertility and Sterility* **94**: 1531–34.

Ax R L, Dally M A, Lenz R W, Love C C, Varner D D, Hafez B and Bellin M E. 2000. Semen evaluation. *Reproduction in Farm Animals*. 7th edn, pp. 365–375. (Eds) Hafez B and Hafez E S E. Lippincott Williams and Wilkins, Philadelphia.

Banerjee A K and Ganguli N C. 1973. Some aspects of the composition of Buffalo semen. *Journal of Reproductive Fertility* **33**: 171–73.

Buettner G R and Jurkiewicz B A. 1991. Catalytic metals, ascorbate and free radicals: combination to avoid. *Radiation Research* **145**: 532–41.

Chinoy N J, Buchnee R P, Mehta R R, Seethalakshmi L, Sharma J D and Chinoy M R. 1986. Effects of vitamin C deficiency on physiology of male reproductive organs of guinea pigs. *International Journal of Fertilility* **31**: 232–39.

Colagar A H and Marzony E T. 2009. Ascorbic acid in human seminal plasma: determination and its relationship to sperm quality. *Journal of Clinical Biochemistry and Nutrition* **45**: 144–49.

Dalvit G C, P D Cetica and M T Beconi. 1998. Effect of á-tocopherol and ascorbic acid on bovine *in vitro* fertilization. *Theriogenology* **49**: 619–27.

Davis I S, R W Bratton and R H Foote. 1963. Livability of bovine spermatozoa at 5°C, 25°C and –85°C in tris buffered and citrate buffered yolk glycerol extenders. *Journal of Dairy Science* **46**: 333–36.

Dawson E B, Harris W A, Teter M C and Powell L C. 1992. Effect of ascorbic acid supplementation on the sperm quality of smokers. *Fertility and Sterility* **58**: 1034–39.

El- Tohamy M M, Kotp, El-Nattat and Mohamed A H. 2012. Semen characteristics and oxidative/antioxidati in semen and serum of male rabbits supplemented with antioxidants during heat stress. *Iranian Journal of Applied Animal Science* **2**: 175–83.

Fazeli P, M J Zamiri, A Farshad and B Khalili. 2010. Effects of vitamin C on testicular and seminal characteristics of Markhoz goats. *Iranian Journal of Veterinary Research* **11**: 267–72.

Krishnamoorthy G, Venkataraman P, Arunkumar A, Vignesh R C, Aruldhas M M and Arunakaran J. 2007. Ameliorative effect of vitamins (á-tocopherol and ascorbic acid) on PCB (Aroclor 1254) induced oxidative stress in rat epididymal sperm. *Reproductive Toxicology* **23**: 239–45.

Luck M R, Jeyaseelan I and Scholes R A. 1995. Ascorbic acid and fertility. *Biology of Reproduction* **52**: 262–66.

Phillips P H, Lardy H A, Boyer P D and Warner G M. 1941. The relationship of ascorbic acid to reproduction in the cow. *Journal of Dairy Science* **24**: 153–58.

Ralston S L, Barbacini S, Squires E L and Nockels C F. 1988. Ascorbic acid supplementation in stallions. *Journal of Equine Veterinary Science* **8**: 290–93.

Shukla M K and A K Misra. 2005. Effect of antioxidants alpha - tocopherol, ascorbic acid and n-propyl gallate on Murrah semen cryopreservation. *Buffalo Journal* **21**: 27–38.

Sikka S C. 1992. Oxidative stress and role of antioxidants in normal and abnormal sperm function. *Frontiers Bioscience* **1**:78–86.

Singh B, Chand D, Singh P and Yadav N K. 1996. Effect of vitamin C addition in the diluent on the quality of deep frozen Murrah buffalo bull (*Bubalus bubalis*) semen. *International Journal of Animal Science* **11**: 131–32.

Sonmez M, Turka G and Yuce A. 2005. The effect of ascorbic acid supplementation on sperm quality, lipid peroxidation and testosterone levels of male Wistar rats. *Theriogenology* **63**: 2063–72.

Yousef M I, A M A El-Morsy and M S Hassan. 2005. Aluminium-induced deterioration in reproductive performance and seminal plasma biochemistry of male rabbits: Protective role of ascorbic acid. *Toxicology* **215**: 97–107.