The Pharma Innovation Journal 2021; 10(7): 1563-1567

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; 10(7): 1563-1567

www.thepharmajournal.com
Received: 05-05-2021
Accepted: 08-06-2021

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# Effects of ascorbic acid incorporation on Cyto-morphological attributes on Cryopreservability (-196$^0$C) of Murrah bull Semen

**Saurabh, Sushant Srivastava, Bhoopendra Singh, Kabir Alam, Rabindra Kumar, Rajesh Kumar and Mayank Kumar Dubey**

## Abstract
The present study was carried out at Deep Frozen Semen Laboratory of C.V.Sc. & A.H., A.N.D.U.A. & T., Kumarganj, Ayodhya (U.P.) with the aim to evaluate the effect of ascorbic acid on cryopreservation of Murrah bull semen. Six ejaculate were collected from each 8 mature Murrah bull at frozen semen laboratory, were used to evaluate the effect of antioxidant at post-dilution and at post thaw stage. The semen sample was extended with Tris-Egg-Yolk-Citric-acid-Fructose-Glycerol (TEYCAFG) extender and was divided into three groups: Group 1 without any additive/ control ($T_0$), group second contain dilutor with ascorbic acid (.20mg/ml) treatment group ($T_2$) and third group's semen contain dilutor with ascorbic acid (.50mg/ml) treatment group ($T_3$). Significant differences ($p$<0.05) was observed between the treatments group and control of post diluted and post thaw semen. Progressive motility, sperm viability, sperm abnormality, acrosomal integrity and hypo-osmotic swelling test (HOST) was evaluated at both post-dilution and post-thaw stage. Ascorbic acid fortified groups ($T_1$ & $T_2$), recorded significant ($p$<0.05) improvement in progressive motility, live spermatozoa, acrosomal integrity and HOST positive spermatozoa, while significant ($p$<0.05) decreased sperm abnormalities in post-thawed semen.

**Keywords:** murrah bulls, semen, ascorbic acid, acrosomal integrity, HOST

## Introduction
The livestock industry is an important source of livelihood and income to majority of the population worldwide including developing countries like India. There is interest rise in the field of reproduction and management in buffalo species due to their high adaptive ability to tropical and subtropical climatic conditions and their capacity to survive in areas unsuitable for cattle and other domestic animals (Patricia *et al.*, 2013) [10]. There is enough probability for the genetic improvement of livestock through implementing various reproductive technologies such as artificial insemination (AI), multiple ovulation & embryo transfer (MOET), and *in vitro* embryo production. When compared to natural mating or other assisted reproductive technologies, AI is more successful, economical, and simple technique (Vishwanath, 2003; Mohanty *et al.*, 2018) [13, 32]. Success of artificial insemination technique is depending upon the development of cryopreservation protocol for bull spermatozoa.

Semen cryopreservation has detrimental effects on spermatozoa, including cell membranes, mitochondria, and DNA due to the production of reactive oxygen species (ROS). Production of reactive oxygen species (ROS) is also increases during Freezing-thawing of spermatozoa in semen that can damage motility, plasmalemma functionality, viability, acrosome and induce sperm chromatin damage (Aitken *et al.*, 1998) [17]. Sikka (1996) [11] observed the most common ROS such as superoxide anion, hydrogen peroxide, peroxyl radicals, hydroxyl radicals, nitric oxide and peroxynitrite anion. Therefore, new procedures and molecules have been tested to improve the overall semen quality, following freezing and thawing (Srivastaava, 2011) [12]. The addition of antioxidant compounds to the semen dilutors before bull semen cryopreservation can reduce the production ROS radicals and their detrimental effects on sperm (Bilodeau *et al.*, 2001) [24]. Ascorbic acid is a very efficient non-enzymatic antioxidant and a scavenger of oxygen free radicals which are toxic products of many metabolic processes (Dawson *et al.*, 1992) [32]. Vitamin C is non-enzymatic antioxidant has been presented as electron donor for some trans plasma membrane redox systems.

## Materials and Methods

### Experimental animals

The study was carried out during January 2020 to March 2021. Present study was conducted on eight Murrah bulls of the age group between 4 – 8 years old age and those were managed at Deep Frozen Semen Lab, College of Veterinary Sciences & Animal Husbandry, A.N.D.U.A.T. Kumarganj, Ayodhya of Uttar Pradesh. All bulls was maintained similar environment and feeding management system.

### Source of semen

Semen samples were collected early in the morning, before feeding, from trained buffalo bulls by using an artificial vagina maintained at temperature between 40 and 42$^0$C. Semen was collected from buffalo bull two times in a day / twice in a week/ alternate day / as per need for quality and fertlity analysis. Forty eight ejaculates (6 from each bull) will be collected, processed, frozen and evaluated for various seminal characteristics.

### Semen evaluation

Each semen sample was examined for routine semen parameters like volume, colour, % live count, total sperm concentration, abnormal sperm as per standard methods. The selected ejaculates (having 65% visual motility) was divide into three parts; part one was used for dilution with untreated ($T_0$) control and other two part semen was fortified with different concentration of ascorbic acid @ 0.20mg/ml ($T_1$) and @ 0.50 mg/ ml ($T_2$).

### Trypan Blue Viability Test

Trypan blue viability test was used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dye, such as trypan blue whereas dead cells do not. In this viability test, sperm cells are simply mixed with dye and then visually examined to determine whether spermatozoa take up or exclude dye. During microscopic evaluation, a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm.

### Normal acrosomes

The percent acrosomal integrity of sperm was determined in fresh semen by preparing smears stained with Giemsa, Two hundred spermatozoa was counted with a phase contrast microscope (100X) for their normal apical ridge (NAR).

### Hypo-osmotic swelling test

HOS solution (osmotic pressure 100m Osmol/kg) was maintained at 37 $^0$C for 5 minutes before use. Hundred micro liters of each semen sample were mix with 1000 microliters of HOS solution and incubated at 37 $^0$C for 30 minutes. After incubation, a semen sample drop was examined under a high power magnification of phase-contrast microscope (40 X) for different swelling pattern.

### Post-thaw Evaluation

Immediately after thawing post-thaw seminal attributes were assessed as per the methods described earlier. Parts of post-thaw semen from all treatments were used to assess *in vitro* fertility tests (HOST) as per the methods described earlier.

### Statistical Analysis

Data will be presented as mean and standard error of the mean (SEM). Analysis of variance (ANOVA) was used to assess differences among the bulls and treatments. When the F ratio is significant ($P < 0.05$), Tukey's HSD test will used to compare treatment means (SYSTAT, 1996).

## Results and Discussion

### Initial progressive motility

The percent (means±S.E) individual motility of spermatozoa in post diluted semen were recorded as 70.33±0.23, 72.71±0.25 and 72.31 ± 0.25 whereas post thaw spermatozoa motility was recorded as 45.50±0.34, 58.13±0.37, 58.77±0.40 respectively in $T_0$, $T_1$ & $T_2$ treated groups (Table-01). The mean value of per cent initial motility was in agreement with observation of Senger and Sharma Sandeep *et al.* (2015) [33] and Patel *et al.* (2016) [2] where as mean progressive motility was reported higher than observation of Srivastava (2011) [12], Sandeep *et al.* (2015) [33], Isnaini *et al.* (2020) [28], and lower than that finding of Dutta and Deka (1993) [1], Maurya *et al.* (2013) [4] and Walid S. EL. Nattat *et al.* (2016) [3]. The variation in the initial motility has been attributed to factor like degree of sexual excitement, method of semen collection and variation in the development of sperm cell in the seminiferous tubules during spermatogenesis, variation in the secretory component of semniferous tubules and epididymal epithelium (Cupps and Briggs, 1965) [30] and the accessory sex glands contributing to seminal plasma (Galloway, 1964) [14]. The mean per cent of post thaw motility was agreement with observation of Mittal *et al.* (2014) [31], Patel *et al.* (2016) [2] and Doidar *et al.* (2018) [6] and whereas higher than the mean per cent of post thaw motility in our study was reported higher than observation of Srivastava (2011) [12], El-Nattat *et al.* (2016) [3] whereas lower than that finding of Gokhale *et al.* (2002). The main excuse for the deterioration in the percent post thaw progressive motility might be due to freezing damage, ROS production and damage caused due to formation of ice crystal formation in mitochondria and Axomemes during cryopreservation that impairs sperm motility. Increase post thaw motility of ascorbic acid fortified group is comparable to unfortified group might be due antioxidant property of ascorbic acid prevent toxic effects of reactive oxygen species like hydrogen peroxide release during cryopreservation (Mittal *et al.,* 2014) [31].

### Viability

Trypan blue viability test was used to determine the number of viable cells present in a cell suspension. In our study mean viability of post diluted semen was observed as 82.79±0.29, 83.85±0.23 and 83.79±0.21 whereas viability of post thaw spermatozoa were 64.27±0.41, 72.06±0.41 and 71.27±0.49 of $T_0$, $T_1$ and $T_2$, treatment respectively (table no-01). It differed significantly among the control and ascorbic acid treatment groups of post diluted and post thaw semen. The finding of post diluted semen was similar to the observation was recorded by Felipe-Perez *et al.* (2008) whereas post thaw viability was higher than the finding of Felipe-Perez *et al.* (2008). In post diluted semen viability of spermatozoa was not differed among treatment and control group whereas higher viability was observed ascorbic acid fortified groups in compared to control. Higher post thaw viability was observed might be due to addition of antioxidant compounds to the semen dilutors before bull semen cryopreservation can reduce the production ROS radicals and their detrimental effects and improves the liveability and quality of thawed spermatozoa (Bilodeau *et al.,* 2001; Bansal & Bilaspuri, 2011) [24, 20].

**Sperm abnormality**

The over mean (±SE) percent sperm abnormality of Murrah buffalo semen in post diluted semen of was reported 8.08±0.18, 8.04±0.12 & 8.04±0.10 whereas in post thaw semen was recorded 16.27±0.27, 11.58±0.16 &12.83±0.22 as $T_0$, $T_1$ and $T_2$ treatment respectively (Table-01). Sperm abnormality of post diluted semen did not differed significantly ($P$<0.05) among the treatments and control but in post thaw semen differed significantly ($P$<0.05) among the treatments and control. The mean abnormal sperm count in present study was comparable with the finding of Baruti *et al.* (2018) [21], Almadaly *et al.* (2019) [17] whereas higher finding was observed by Tomar and Singh (1996) [15], Pathak *et al.* (2018) [9] and lower than that of Kumar *et al.* (1993) [29], Srivastva (2011) [12], Maurya *et al.* (2013) [4] and mean abnormal sperm count of post thaw was in tuned with the finding of Mukesh Kumar (2015) and El-Sheshtawy and El-Nattat (2020) [8] whereas higher than the finding was recorded by Mittal *et al.* (2014) [31] and Pathak *et al.* (2018) [9] but lower than that of Doidar *et al*., (2018) [6]. Post thaw abnormality was higher than post diluted semen might be due to cryo damages during cryopreservation. Lower post thaw sperm abnormality was recorded in ascorbic acid incorporated groups compared to untreated control might be due to antioxidant effects of ascorbic acid in dilutor.

**Per cent intact acrosome**

The mean percent of intact acrosome of post diluted semen was recorded as 90.31±0.18, 90.13±0.19 & 90.31 ± 0.20 and post thaw was observed as 71.23±0.33, 80.33±0.44 & 81.42 ±0.44 of $T_0$, $T_1$ and $T_2$ treatment groups of post diluted and post thaw respectively (Table-01). The mean per cent intact acrosome of post diluted semen was in agreement with finding of Maurya *et al.* (2013) [4] and was higher than that recorded by Andrabi *et al.* (2008) [18], Doidar *et al.* (2018) [6] and whereas lower than that reported by Chaudhary *et al.* (2017) [25]. Non-significantly ($P$<0.05) differed among the treatments groups of post diluted semen whereas significantly differed between treatments and control of post thaw semen. Post thaw observation of intact acrosome were in tuned with the previous report of Srivastava (2011) [12], Mittal *et al.* (2014) [31] and Doidar *et al.* (2018) [6] whereas higher values than that of Sandeep *et al.* (2015) [33] and lower than that recorded by Andrabhi *et al.* (2008) [18]. A higher percent normal acrosome in semen is desirable, as it plays an important role in the process of fertilization. A significant improvement was observed in the post thaw acrosomal integrity of spermatozoa that was preserved in the dilutor fortified with additive like ascorbic acid (0.2mg/ml & 0.5mg/ml) as compared to control. This clearly indicate that ascorbic acid offered better protection of acrosome and acrosomal membrane. Similarly Sandeep *et al.* (2015) [33] also reported that supplementation of vitamin C increased percent of intact acrosome during pre or post thawing stage of buffalo spermatozoa however increase was statistically significantly ($p$<0.05) only during post thaw stage.

**Hypo-osmotic swelling reactive spermatozoa (%)**

The average per cent HOS reactive spermatozoa in post diluted semen of Murrah buffalo bull under experimental condition was observed 50.67±0.27, 52.77±0.28 and 52.63±0.24 of $T_0$, $T_1$ and $T_2$ treatment respectively (Table-01). The finding of HOS reactive spermatozoa percentage in present study was fairly comparable with the finding of Srivastava (2011) [12], Bhakat *et al.* (2015) [23] and whereas higher than the finding of Mukesh Kumar (2015) but lower than the observations recorded by Pathak *et al.* (2018) [9]. These variations may be due to differences in viability of sperm among the bulls, species, season of semen collection and age of the bull which are known to affect sperm viability (Saxena and Tripathi, 1983; Srivastava, 2011) [7, 12].

The overall mean (±SE) per cent Post-thaw HOS reactive sperm recorded as 32.98±0.45, 41.27±0.33 and 40.46±0.39 respectively in control ($T_0$) and ascorbic acid fortified extenders ($T_1$ and $T_2$) were given in table 01. The present observation was in agreement with the finding of Arboud *et al.* (2020) [19] and whereas lower than those of El-Nattat *et al.* (2016) [3]. It differed significantly among the experimental bulls as well as among the treatments. In our study post thaw HOS reactive percentage of spermatozoa was improved higher in ascorbic acid fortified dilutors in comparison to control. Improvement in HOS positive spermatozoa in ascorbic acid treatment groups were higher as compared to control might be due to antioxidants property of control. Ascorbic acid is a very effective non-enzymatic antioxidant and a scavenger of oxygen free radicals which are toxic products of many metabolic processes (Dawson *et al.*, 1992) [26].

**Conclusion**

Incorporation of additives such as ascorbic acid in semen extender significantly improved the post-thaw semen quality as well as functional and structural intigrity of spermatozoa. However, ascorbic acid provide better protect to spermatozoa during post dilution, freezing and thawing stresses than control.

**Acknowledgement**

Authors thank to Dean, College of Veterinary Science & Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj, Ayodhya, Uttar Pradesh India for providing necessary facilities and support.

**Table 01:** Effect of Ascorbic acid incorporation in Tris dilutor on seminal attributes and *in vitro* fertility test (means±S.E) in post diluted semen of Murrah Bulls (Pool)

| Treatment | Stage | Seminal attributes | | | | *In Vitro* fertility Test |
|---|---|---|---|---|---|---|
| | | Motility, (%) | Viability-DS, (%) | Abnormality, (%) | Acrosomal Integrity, (%) | HOS reactive sperm, (%) |
| Control (T0) | Post dilution | $70.33^{a}$ ±0.23 | $82.79^{a}$±0.29 | 8.08±0.11 | $90.31^{a}$±0.18 | $50.60^{a}$±0.27 |
| Ascorbic acid 0.20 mg/ml ($T_1$) | Post dilution | $72.71^{b}$ ±0.25 | $83.85^{b}$±0.23 | 8.04±0.12 | $90.13^{ab}$±0.19 | $52.77^{b}$±0.28 |
| Ascorbic acid 0.50 mg/ml ($T_2$) | Post -dilution | $72.31^{bc}$±0.25 | $83.79^{bc}$±0.21 | 8.04±0.10 | $90.31^{abc}$±0.20 | $52.63^{bc}$±0.24 |
| Control (T0) | Post- thaw | $45.50^{a}$±0.34 | $64.27^{a}$±0.41 | $16.27^{a}$±0.27 | $71.23^{a}$±0.33 | $32.98^{a}$±0.45 |
| Ascorbic acid 0.20 mg/ml ($T_1$) | Pos-thaw | $58.77^{b}$±0.40 | $72.06^{b}$±0.41 | $11.58^{b}$±0.16 | $81.42^{b}$±0.44 | $41.27^{b}$±0.33 |
| Ascorbic acid 0.50 mg/ml ($T_2$) | Post- thaw | $58.13^{bc}$±0.37 | $71.27^{bc}$±0.49 | $12.83^{c}$±0.22 | $80.33^{bc}$±0.44 | $40.46^{c}$±0.39 |

Mean bearing different superscript (a, b, c) in a column differed significantly ($P$<0.05), separately for each attributes of post dilution and post thaw

**References**


1. Dutta GC, Dekka BC. Semen characteristics of Murrah buffalo bull. J Assam Vet. Council 1993;3:42-43.
2. Patel HA, Siddiquee GM, Chaudhari DV, Suthar VS. Effect of different antioxidant additives in semen diluent on cryopreservability (−196C) of buffalo semen. Veterinary world 2016;9(3):299.
3. El-Nattat WS, El-Sheshtawy RI, El-Batawy KA, Shahba MI, El-Seadawy IE. Preservability of buffalo bull semen in triscitrate extender enriched with bee's honey. Journal of Innovations in Pharmaceutical and Biological Sciences 2016;3(1):180-185.
4. Maurya K, Singh OP, Srivastava S. Seminal plasma ascorbic acid level and its relationship to sperm characteristics in Murrah buffalo bulls. Indian J Anim. Sci 2013;83(5):498-501.
5. Gokhale SB, Mushtaque M, Phadke NL, Dinodkar, Ambhore GS. Studies on the effect of hydrogen ion concentration of extender on semen characters of murrah buffalo bulls. Indian Journal of Animal Reproduction 2003;25:767-779.
6. Doidar YA, El-Nagar HA, Elrefy A, Mousbah AM. Cryopreservation and Quality Assessment of Buffalo Bull (Bubalus bubalis) Semen Using New Moringa Extender and Antioxidant Co-q10. Journal of Animal and Poultry Production 2018;9(9):375-381.
7. Saxena VB, Tripathi SS. Seasonal variation in abnormalities of spermatozoa of Jersey bulls. Indian J Anim. Sci 1983;53:193-194.
8. El-Sheshtawy RI, El-Nattat WS. Assessment of semen characteristics and *in vivo* conception rate of preserved buffalo bull semen extended in tris enhanced with Diospyros kaki. Bulletin of the National Research Centre 2020;44(1):1-6.
9. Pathak PK, Dhami AJ, Chaudhari DV. Seminal attributes, freezability and their interrelationships in zebu cattle and buffalo bulls from central gujarat. Indian J Vet Sci. Biotech 2018;14(2):01-08
10. Patricia AC, Da LUZ, Paulo Ramos SS, Cristiana A, Andre MJ, Antonio CAN. The Correlation between Age, Body Weight and Testicular Parameters in Murrah Buffalo Bulls Raised in Brazil. The Journal of Reproduction and Development 2013;59:14.
11. Sikka SC. Oxidative stress and role of antioxidants in normal and abnormal sperm function. Front. Biosci 1996;1:78-86.
12. Srivastava S. Effect of Amino acids incorporation of post thaw seminal attributes and *in vitro* fertility assay of Murrah bull spermatozoa. Thesis, Ph.D. Dr. Bhim Rao Ambedkar University, Agra, Uttra Pradesh, India 2011.
13. Vishwanath R. AI: the state of the art. Theriogenology 2003;59:571-84.
14. Galloway DB. A study of bulls with the clinical signs of seminal vesiculitis-clinical, bacterial and pathological aspects. Acta. Vet. Scand 1964;5(2):1-22.
15. Tomar SS, Singh SP. Studies on reaction time and some of the seminal seminal attributes and their inter-relationship in Murrah buffalo bulls. Indian J. Anim. Res 1996;30(1):49-54.
16. Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jenning Z, Irvine DS. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. Biol. Reprod 1998;59:1037-1046.
17. Almadaly EA, Tawfik FS, El-Kon II, Heleil BA, Fattouh EM. Effect of different cryoprotectants on the post-thaw sperm characteristics and *in vivo* fertility of buffalo (*Bubalus bubalis*) bull semen. Slov. Vet. Res 2019;56:541-551.
18. Andrabi S, Ansari M, Ullah N, Anwar M, Mehmood A, Akhter S. Duck egg yolk in extender improves the freezability of buffalo bull spermatozoa. Animal Reprod. Sci 2008;104:427-433.
19. Arboud MM, Waheeb RS, El-Sheshtawy RI, El-Amrawi GA. Assessment of Cattle Bull Semen Preservability Using Tris Extender Enriched with Turmeric Extract. Egypt. J Vet. Sci 2020;51(3):357-362.
20. Bansal AK, Bilaspuri GS. Impact of oxidative stress and antioxidants on semen function. Veterinary Medicine International 2011. Doi: 10.4061/2011/686137.
21. Baruti M, Deka BC, Bhuyan1 M, Tamuly S, Biswas RK, Sinha S. Biochemical Characterization of Swamp Buffalo (*Bubalus carabanesis*) Semen. Int. J Curr. Microbiol. App. Sci 2018;7(6):2618-2629.
22. Kumar M. Effect of Ascorbic Acid, Cysteine Hydrochloride and Prostagladin on Cryopreservability of Murrah Bull Spermatozoa. M.V. Sc. Thesis, C.V.Sc. &A.H., A.N.D.U.A.T., Kumarganj-Faizabad (U.P.) 2015.
23. Bhakat M, Mohanty TK, Singh S, Gupta AK, Chakravarty AK, Singh P. Influence of semen collector on semen characteristics of Murrah buffalo and Crossbred bulls. Adv. Anim. Vet. Sci 2015;3(4):253-258.
24. Bilodeau JF, Blanchette S, Gagnon C, Sirard MA. Thiols prevent $H_2O_2$ mediated loss of sperm motility in cryopreserved bull semen. Theriogenology 2001;56(2):275-86.
25. Chaudhary PJ, Dhami AJ, Chaudhari DV, Parmar SC. Freezability of cattle and buffalo semen and association of fresh and frozen-thawed sperm quality parameters. International journal of Current Microbiology and Applied Sciences 2017;6(12):1445-1454.
26. Dawson EB, Harris WA, Teter MC, Powell LC. Effect of ascorbic acid supplementation on the sperm quality of smokers. Fertil. Steril 1992;58:1034-1039.
27. Gil-Guzmán E, Ollero M, López MC, Sharma RK, Álvarez JG, Thomas Jr AJ *et al.* Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation. Hum Reprod 16, 1922-1930.
28. Isnaini N, Harsi T, Kurnianto SE. Individual Variation in Semen Characteristics of Murrah Buffalo Bulls. International Journal of Innovative Technology and Exploring Engineering 2020, 2001;9(3):58-60.
29. Kumar S, Sahni KL, Bistha GS. Cytomorphological characteristics of motile and static semen of buffalo bulls. Buffalo Journal 1993;9(2):117-127.
30. Cupps PT, Briggs JR. Changes in the epididymis associated with morphological changes in the spermatozoa. J. Dairy Sci 1965;48:1241-1244.
31. Mittal PK, Anand M, Madan AK, Yadav S, Kumar J. Antioxidative capacity of vitamin E, vitamin C and their combination in cryopreserved Bhadavari bull semen. Veterinary World 2014;7(12):1127-1131.
32. Mohanty TK, Lone SA, Kumaresan A, Bhakat M, Kumar R, Baithalu RK *et al.* Sperm dosage and site of insemination in relation to fertility in bovines. Asian Pacific Journal Reproduction 2018;7:1-5.
33. Sandeep, Singh P, Virmani M, Malik RK. Effect of vitamin C on the seminal and biochemical parameters of

murrah buffalo bull semen during different stages of freezing. Haryana Vet 2015;54(1):15-18.
34. Saxena VB, Tripathi SS. Seasonal variation in abnormalities of spermatozoa of Jersey bulls. Indian J Anim. Sci 1983;53:193-194.