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STUDIES ON THE FREEZABILITY OF KUNDHI BUFFALO BULL SEMEN

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ABSTRACT

This study was conducted to assess the post thaw quality of Kundhi buffalo semen diluted in tris based diluents. After collection the ejaculates were pooled and checked for color, volume, pH, mass activity, progressive linear motility% (PLM), and intactness of cell membrane. The semen qualifying these tests was divided into four aliquots A, B, C and D, diluted with tris based diluents; the last three diluents containing sugar types glucose, fructose and lactose respectively. Aliquot A was without sugar and severed as control. These were frozen, stored for 24 hrs, thawed and incubated at 35^0C for 5 hrs. The incubated semen aliquots were examined for PLM and membrane integrity of sperm cells with phase-contrast microscopy and HOST procedures. It was observed that all the ejaculates were creamy white in color. The mean (±SEM) volume, mass activity, sperm concentration, PLM, and intactness of cell membrane were 1.70 (±0.09) ml, 3.22 (±0.074), 1.58 (±0.136) x 10^9/ml, 64.53 (±0.757) % and 55.63 (±0.945)% respectively for fresh semen. A significant difference was observed in pH values of the fresh semen samples among the bulls. There was a significant decrease in the number of motile cells and the cells with intact membrane after thawing and incubation of the semen at 35^0C. Addition of fructose in the diluents was found to be superior in maintaining higher PLM and intactness of membrane after thawing and incubation. The objective method, Osmotic resistance test (ORT) was found to be the useful parameter for assessment of in-vitro fertility of Kundhi buffalo bull semen.

Key words: Semen Freezing, Sperm membrane integrity, Progressive linear motility, Kundhi buffalo.

INTRODUCTION

The livestock sector in Pakistan is still in a developing phase. Despite of remarkable contribution of buffaloes, there is always shortage of scientific information on this animal especially in the field of reproduction. Most of the animals are low producers having longer age at calving and long calving interval with low lifetime production. This is mainly due to lake of availability of superior breeding bulls or availability of superior germ plasma. Although artificial insemination (AI) is being widely used in nearly all cattle / buffalo breeding countries, but the number of breeding bulls has greatly reduced and consequently the quality of the bulls has become a matter of vital importance (Ax *et al*., 2000).

The contribution of a bull, either through natural or artificial insemination in terms of reproductive efficiency, production of meat, milk or work is of great importance, because each bull or its semen represents half of its genetic composition of its progeny. For extensive use of out standing sires, careful studies of breeding bulls are imperative. The knowledge of sexual behavior and semen evaluation are valuable tools to estimate the reproductive efficiency of a breeding bull (Brohi, 1993).

In Pakistan the andrological research activities are aimed at improving semen evaluation and processing techniques, development of suitable extenders for semen (Usmani, 1996). The life span of unprocessed spermatozoa in whole warm semen is limited to a few hours, high temperature has been found unsuitable and the rate of deterioration is rapid due to increased metabolic processes. Cooling can extend its life for several days, however if the temperature becomes too low crystallization of water within the cells occurs and causes damage to the cells. Therefore, it needs to extend the semen in a suitable medium, which has little adverse effect on the sperm during freezing process (Ahmad *et al*., 1986).

A lot of work has been done on sexual performance and semen picture of various cattle and buffalo breeds in different parts of the world (Brohi, 1993). For male germ cells or any other cell of the living organisms to survive freezing, they need to be extended in diluents that contain substance to protect them against cold shock and deleterious consequence of freezing (Arthur, 2003). For semen evaluation procedures a comprehensive and more reliable study is still needs to be undertaken to overcome the low quality post-thaw semen.

Objective type of semen assessment techniques (Revell and Mrode, 1994) now-a-days is considered to be reliable marker for semen assessment before and after freezing. Hypo osmotic swelling test (HOST) has been shown to be useful in detecting suitable change in the sperm membrane of bulls (Correa *et al*., 1997) and for buffalo bull semen (Kakar *et al.*, 1998). The current study was therefore proposed to investigate quality characteristics of Kundhi buffalo semen using different

compositions of Tris based medium for freezing in order to establish a suitable extending medium for freezing Kundhi buffalo bull semen and to investigate a suitable sugar type in the freezing medium.

MATERIALS AND METHODS

Present study was conducted in the Department of Animal Reproduction, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University, Tandojam, Pakistan. Thirty-two semen samples were collected from four Kundhi bulls twice a week over a period of eight weeks and were assessed for fresh and frozen characteristics. Before collection, bulls were properly cleaned, especially the perpetual area with antiseptic luke warm water. An intact bull was used as a dummy. The donor bulls were allowed one or two false mounts before each collection and the samples were collected early in the morning twice a week, using artificial (AV) vagina maintaining inner temperature 40-45^{0}C. Sterilization of all items was maintained before the day of collection and kept in an incubator at 50^{0}C. The temperature of semen processing room was maintained at 20-25^{0}C during the whole study hours. Immediately after collection, each sample was transferred to laboratory and placed in a water bath at 35^{0}C.

Each fresh semen sample was examined through necked eyes. Samples clear and free from any contamination like blood, pus and dirt were used for further processing. The volume of each sample was recorded through the graduated collection tube. Semen sample color was also checked and the normal color range was recorded. Mass activity was observed by placing an undiluted semen drop on glass slide under warm stage of Phase Contrast Microscope (10X) and the grading was recorded as ; 0 = no mass motility, + = less than 20 percent of sperms showing progressive motion, ++ = 40 to 60 percent showing progressive motion with slow wave, +++ = 60 to 80 percent showing progressive movement with wave more intense and ++++ = 80 to 100 percent showing progressive movement with rapid wave waking eddies, (+++ or more are recommended for A.I. purpose).

For determination of sperm motility, a drop of diluted semen was examined under cover slip at magnification of 20X. The number of sperms moving straight in forward direction in the field of microscope was recoded and expressed in percentage. Sperms having at least 60% motility or more were used for freezing.

Sperm concentration was measured using Hemocytometer by using fixing solution (3% sodium chloride) as described by Henery, (1991), Sodium chloride, 30gms, Formal dehyde (37%), 4 ml and distilled water, 1000 ml. 0.1 ml semen was added in 9.99 ml of fixing solution, and the suspension was allowed for 5 minutes for fixation. A small drop of the suspension was placed on both chambers of hemocytometer, covered with cover slide and observed under light microscope at 40X magnification. Sperm were counted in 5 squares, one in middle and four corner squares. The Sperms concentration was calculated by using the formula;

Sperm per ml = n × 5 × df × 1000. (1)

Where N is the No of sperms counted, 5 is the No of chambers counted on hemocytometer and df is the degree of freedom that is equal to 100. pH was checked using digital pH meter. Assessment of membrane integrity was made by processing the fresh semen sample for hypo osmotic swelling (HOST) test (Revell and Mrode, 1994) using 150 mOsm/kg (Osmotic resistance test (ORT) solution (Fructose, 1.4g, Tri sodium citrate, 0.74g and distilled water, 100ml). The semen was diluted with 1:4 in the test solution, incubated at 35^{0}C for 30 minutes. The sperms, which swelled in the reaction of test solution, were recorded as test results. These were considered as the cells having intact membrane and were expressed in percentages.

Tris based diluent was used for freezing the semen. The pooled semen samples from four bulls divided into four aliquots was diluted with tris 3.3g and citric acid 1.68g in each sample. Aliquot A was without any sugar, B containing glucose 1.25g, C containing fructose 1.25g and D containing lactose 1.25g. Egg yolk 25%, glycerol 8%, penicillin 1000 I.U./ml and streptomycin 1000 µg/ml were added to each diluents. Distilled water was added to make 100 ml of each diluents. One-step dilution of the semen was adopted. The samples after dilution were placed in cold cabinet to bring the temperature at 4-5^{o}C in 5-6 hours. Dilution rate was adjusted to 20 million spermatozoa per 0.50 ml medium straws and six hours equilibration time was observed in cold handling cabinet (4 ^{o}C).

Filling of the straws was completed keeping the equilibrated semen in the cold cabinet at 4-5^{o}C using manual suction pump. The open end of straw was sealed with polyvinyl chloride powder and freezed semen by holding the loaded straws in liquid nitrogen vapors above the surface of liquid nitrogen for 8 minutes and were plunged in to the liquid nitrogen and kept for 24 hours or till assessment.

Finally evaluation of the frozen semen was made. The frozen semen was taken out from the container and thawing was carried out at 37^{0}C for 9 seconds. The thawed semen aliquots were transferred into dry, clean sterilized glass vials and stored in an incubator at 35^{0}C for 5 hrs. The livability of the spermatozoa was noted for all the extenders by recording the percentage of motility at hourly intervals for 5 hours. Hourly membrane integrity was checked using ORT method, using Osmotic resistance test (ORT) solution as, Fructose, 0.09g, Tri sodium citrate, 0.5g and Distilled water, 100ml.

The data were analyzed using analysis of variance methods. The difference between the means was calculated applying LSD test where appropriate.

RESULTS

In the present study the color of all semen samples was white, creamy with normal appearance. The mean (±SEM) of ejaculate volume of the semen was found to be 1.70 (±0.09) ml ranging from 0.70 – 3ml. (Table 1) Analysis of variance showed no significant (P>0.05) difference between the bulls for ejaculate volume. The mean (±SEM) pH value of fresh semen was 5.81(± 0.06) with the range of 5.15-6.53 (Table 1). A significant (P<0.05) difference was observed between the bulls for pH values. For mass activity swirling movement was observed. All the sample appeared to have score of +++ to ++++. For statistical interpretation these were given numerical values.

The mean (±SEM) numerical value was found to be 3.22 (± .07), which ranged from 3 to 4 (Table 2). Analysis of variance showed no significant (P>0.05) difference between the bulls for mass activity. The mean (±SEM) motility percentage of Kundhi buffalo bull semen was 64.55 (±0.76), which ranged between 60-75%. (Table 2). No significant (P>0.05) difference was for PLM among the bulls. The mean (±SEM) sperm concentration of semen was found to be $1.58x10^9$(±0.14) per ml, which ranged from 0.83-4.9 billion/ml (Table-3). Analysis of variance showed no significant (P>0.05) difference for semen concentration between the bulls.

Table 1 Mean (± SEM) Volume and pH of Kundhi buffalo bull semen

Bull No.	No. of collections	Volume (ml)		pH*	
		Mean	Range	Mean	Range
1	08	1.787	1.4 – 3	5.9	5.24 – 6.53
2	08	1.637	0.7 – 2	5.9	5.69 – 6.21
3	08	1.487	1.1 – 2	5.5	5.15 – 5.90
4	08	1.887	1.3 – 3.3	5.7	5.25 – 6.02
All bulls	32	1.70 (±0.09)	0.7-3.3	5.81 (±0.06)	5.15 – 6.53

*The values for this parameter were significantly different from each other within the column (P<0.05)

Table 2 Mean (± SEM) Mass activity and PLM% of Kundhi buffalo bull semen.

Bull No.	No. of collections	Mass Activity*		PLM (%)*	
		Mean	Range	Mean	Range
1	08	3.1	3 – 4	57.7	60 – 75
2	08	3.3	3 – 4	58.2	60 – 70
3	08	3.1	3 – 4	63.1	60 – 70
4	08	3.7	3 – 4	64.3	60 – 70
All bulls	32	5.81(±0.06)	64.53(±0.76)	64.53(±0.76)	60 – 75

*The values for this parameter were not different from each other within the column (P>0.05)

Table 3 Mean (± SEM) sperm concentration (billion/ml) and percent intact spermatozoa of Kundhi buffalo bull semen.

Bull No.	No. of collections	Sperm concentration*		Intact spermatozoa*	
		Mean	Range	Mean	Range
1	08	1.3	1 – 1.77	53.12	45 – 60
2	08	1.9	1.16 – 4.9	48.12	40 – 60
3	08	1.5	0.83 – 2.5	55.62	50 – 60
4	08	1.5	1.12 – 2.9	58.12	55 – 60
All bulls	32	1.58(±0.14)	0.83 – 4.9	55.63(±0.94)	40 – 60

*The values for this parameter were not different from each other within the column (P>0.05).

The mean (±SEM) of the sperm cells having intact cell membrane as assessed by applying ORT was found to be 55.63(±0.94)% which ranged from 40-60% (Table-3). Analysis of variance showed no significant (P>0.05) difference between the bulls. The semen from four Kundhi bulls qualifying the fresh evaluation was pooled, frozen, stored for 24 hours, thawed and incubated at 30ºC for 5 hours. This was evaluated for PLM and membrane intactness after every hour allowing the semen in the incubator. Motility % after thawing at hourly interval was

recorded in all the samples. The mean motility percentage of the cells after 5-hrs incubation was found to be 13.67 (Table-4). Statistical analysis showed a significant ($P<0.05$) difference between the diluents for progressive linear motility %. The mean (±SEM) percentage of the cells with intact membrane after 5 hrs incubation was found to be 9.1 (Table-5). Statistical analysis showed a significant ($P<0.05$) difference between the diluents for maintaining intactness of the membrane. The diluent C (fructose) was found to be superior to A (no sugar) and B (glucose). However diluent D (lactose) was not different from diluent C as indicated from LSD values. A significant ($P<0.05$) difference and progressive decrease in the cells having intact membrane under incubation was observed for the diluents.

Table 4 Effect of post-thaw incubation on progressive linear motility (%) (PLM) and sperm membrane integrity (INT) of Kundhi buffalo semen

Diluents	PLM at various Incubation Times (Hours)					INT during various Incubation Time (Hours)				
	1	2	3	4	5	1	2	3	4	5
A	21.2^{D}	13.7^{D}	7.5^{D}	0.0	0.0	8.7^{D}	3.1^{D}	3.1^{D}	0.0	0.0
B	24.3^{CD}	18.8^{C}	13.1^{C}	0.0	0.0	18.1^{C}	13.8^{C}	7.8^{C}	0.0	0.0
C	40.6^{A}	34.3^{A}	22.2^{A}	0.0	0.0	35.3^{A}	21.6^{A}	14.3^{A}	0.0	0.0
D	32.5^{AB}	26.2^{AB}	18.5^{AB}	0.0	0.0	25.0^{AB}	18.2^{AB}	12.6^{AB}	0.0	0.0

Figure with different superscripts in same columns were significantly different ($P<0.01$).

DISCUSSION

Normal color of buffalo semen is white to creamy white (Mc Gown *et al.* 1995). The current study findings are also in agreement to Brohi, (1993) in Kundhi buffalo bulls and Kumar, (1993) in Indian buffalo bull semen. The volume in the current study (0.7 – 3.3 ml) falls in the range (1.5 – 3.3 ml) reported by Sansone, (2000). In current study all the bulls were of the age and young therefore variation in the ejaculate was not expected. However, the higher values (3.6 ml and 4.14 ml) have been reported by others (Brohi, 1993) in Kundhi Buffalo. The variation might be due to the difference in age, as the bulls used by Brohi were fully mature. The mean pH value (5.81 ±0.06) found in the current study is slightly lower than the mean (6.16) reported by Brohi, (1993) in Kundhi buffalo and Younis, (1996) in the Nili Ravi buffalo (6.04 - 6.93). This might have been due to concentration of semen, season and hygienic conditions (Alvi-Shoushtari and Babazadeha-Hebashi, 2006). Decrease in the pH level as was observed during the current study might have seen due to sperm cells more active producing more lactic acid during hot climatic conditions (Aghangari, 1992). However none of the pH level recorded in the present study falls in the lethal level for sperm cells (Mann and Mann, 1988). Duration after collection and individual aliquot also influence the level of pH in fresh semen as was the case in present study.

Mass activity found (3.22 ±0.074) in the current study was slightly higher than the reported values (2.65 ±1.14) in Nili Ravi bulls (Javed *et al.,* 2000. and Heuer *et al.*, (1982) and in Indian bulls (2.54) Vyawanare *et al.,* (1989). The desirability can be attributed to the effect of warm climatic conditions, in which sperm might be more rigors due to high temperature. The motility percentage (60-75%) found in the current study in different bulls agreed with the reported (63%) values by Brohi *et al.,* (1993) in Kundhi buffalo bulls and Jainuden *et al.,* (1982) in swamp buffalo. Higher values than the current figures have been reported in Murrah buffalo bull by Jainuden *et al.,* (1982). The deviation in PLM is might be due to the young age of bulls and also high temperature during semen collection the cells were utilize energy some at faster rate leaving defect drying quality alleging less number of progeny while sperms. The sperm concentration of the ejaculates found in the current study (1.58×10^9 / ml) in Kundhi buffalo falls in the range ($1-4 \times 10^9$ / ml) reported in other breeds of buffalo (Arthur, 2003; Ghokhale *et al.,* 2002; Aguiar *et al.,* 1994). This indicates that the number of cells required for maintaining fertility level of the semen from Kundhi bulls was acceptable to be used for AI programme even in stressful situation such as hot climatic condition as was during the current study period.

The diluent –C (fructose) was found to be superior to A (no sugar) and B (glucose). However diluent D (lactose) was not different from diluent C as indicated from LSD values. A significant ($P<0.05$) difference and progressive decrease in the motility percentage under incubation was observed in all the diluents. All the cells were found dead after 4 hrs incubation time. This indicates that sperms have limited life after thawing and should be deposited in female tract as soon as possible. It was observed that the diluent containing fructose (monosaccharide) was superior to all other sugars used in the current study. It maintained significantly higher PLM (40.6%) value than other sugars. The sugar component was found essential to provide energy to the cells for their motility as poorest value (21.2%) was recorded for sugar free diluents. There was progressive decrease in the number of motile cells after incubation of the semen at 35^0C. Although the

parameter is of subjective nature (Rodry and Martry nez, 2000), the findings recorded in the current study were comparable to others (Revell and Mrode, 1994) in cattle.

However assessment of mean percentage of motility using simple method is readily available and cost efficient and provides rapid means of semen evaluation in field conditions but objective type of semen assessments are still needed to be applied for precise analysis of semen sample (Molinia *et al.,* 1994). Membrane integrity of the spermatozoa before and after freezing is of paramount importance for achieving fertility targets in AI programme (Jainuden *et al.,* 1982; Revell and Mrode, 1994). Normally isotonic solutions are used for extension of the semen before freezing. The solution maintains equilibration with intra and extra cellular environment. In the current study hypoosomotic solution produced readable changes in the sperm cell. These changes include imbedded tail, swollen tail, mid piece and end piece, some of the cells were seen bent and some what round in shape. These cells have been found to have positive correlation in the fertility score in cattle and other species (Jainuden *et al.,* 1982; Jeyendran *et al.,* 1984; Revell and Mrode, 1994). The percentage (55.64 ±0.945) observed in the current study also fall in the range (40 - 60) reported by others (Jainuden *et al.,* 1982; Revell and Mrode, 1994). This indicates that the assessment test for fresh semen as used by others can also be applied in Kundhi buffalo semen assessment. Sperm membrane is vital and of paramount importance for maintaining cell integrity, which is essential for the maintenance of fertilizing ability of spermatozoa It has been found that the sperms having damaged cell membrane were still motile and not capable to fertilize the ovum (Samo*,* 1998; Jeyendran *et al.,* 1984; Revell and Mrode, 1994). The hyposomotic swelling tests developed by Jeyendran *et al.,* (1984) in human beings, Revell and Mrode, (1994) in cattle and Samo, (1998) in ram are considered to be objective and reliable methods of semen assessment.The freezing protocol developed and applied by Revell and Mrode, (1994) for cattle was applied in the current study in Kundhi buffalo semen and was found useful for this species. Various sugar components were added to the freezing medium and compared for maintaining membrane integrity after freezing and thawing. It was observed that although sugar type had no significant effect on the membrane integrity, the fructose was found to be superior to all other sugars used in the current study. It has been found that sugar component was essential to provide protection to the cell membrane (Mazur, 1970), which was true for sperm cells as found in the current study. The findings of the current study were comparable to the reports of others (Jeyendran *et al.,* 1984; Joshy *et al.,* 1990; Revell and Mrode, 1994; Samo, 1998). It has positive correlation with fertility score (Hammadeh *et al.,* 1999) and can be applied for semen assessment in Kundhi buffalo.

On the basis of current study it was concluded that, i. Kundhi buffalo bulls ejaculates well in the hot climatic conditions. ii. Kundhi buffalo bull semen freezes similar to other breeds of buffalo in hot season. iii.Fructose (monosaccharide) gives best results among other sugars for motility and intactness of sperm cell membrane after freezing and thawing. The semen after thawing was found to be limited fertile life.

REFERENCES

Ahmad Maqbool. K. M. Ahmad, A. Khan (1986). Cryopreservation of buffalo spermatozoa in Tris .Pakistan Vet. Journal: vol 6(1) p. 1-3

Aghangari, Y. T. (1992). Cryopreservation of ram semen for A.I. Ph.D. Thesis, Univ. Wales, Bangor.

Aguiar, P. H. P., V. J. Andrade, J. J. Abreu and N. B. N. Gomez., (1994). Physical and morphological semen characteristics of buffalo aged from four to eight year old. Proc. 4th Int. Buffalo Congr., Sao Paulo, Brazil. 3: 488-488.

Alvi, S. S. M. and B. Babazadeha-Hebashi. (2006). Seasonal variations in the characteristics of the Azerbaijani buffalo. Iran. J. of Vet. Res. 7:1-14.

Arther, (2003). Artificial Insemination. Arther's Veterinary Reproduction and Obstetrics 8th edi London, W.B. Sunder, Co. 751-758.

Ax, R. L., M. R. Dally, B. A. Didion, R. W. Lienz, C. C. Love, D. D. Varner, B. Hafez and M. E. Bellin. (2000). Artificial insemination in cattle. Reproduction in Farm Animals. E.S.E. Hafez and B. Hafez. 7th Ed. Pp 21 - 19.

Brohi, N. A. (1993). Sexual behaviour and semen characteristics of Kundhi buffalo bulls. 1993. M.Sc. Thesis. Department of Anim. Repro. Sindh Agriculture University Tandojam.

Correa, J. A., G. Heersch and P. M. Zavos. (1997). Sperm membrane functional integrity and response of frozen-thawed bovine spermatozoa during the swelling test incubation at varying temperatures. Theriogenology, 47:715-729.

Hammadeh, M. E., A. S. Askari, T. Georg, P. Rosembaum and W. Schmidt. (1999). Effect of freeze-thawing procedure on chromatin stability, morphological alteration and membrane integrity of human spermatozoa in fertile and subfertile men. Int. J. Androl. 22:155–62.

Henery J. B. (1991). Clinical Diagnosis & Lab. Management Methods (18th Edi.) Sunder Co. 499-502

Heuer, C. H. and M. A. Bader. Bajwa. (1982). Sperm morphology of the Nili-Ravi buffaloes. Pak. Vet. J. 2:155-160.

Jainuddin, M. R., T. A, Bangso and S. Dass. (1982). Semen characteristics of the swamp buffalo

(*babulus babulis*) . Anim. Repro. Sci. 4: 213-217

Javed, M. T., A. Khan and R. Kausar. (2000). Effect of age and season on some semen parameters of Nili-Ravi buffalo (*Bubalus bubalis*) bulls. Vet. Arhiv. 70, 83-94.

Jeyendran R. S., H. H. Van Der Ven, M. Perez-Pelaes, B. G. Crabo and L. J. D. Zaneveld. (1984). Development of an assay to asses the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J. Repro. Fertil. 70:219–28.

Joshi, A., A. K. Mathur, R. S. Srivastava and D. B. Kalra. (1990). Factors affecting metabolic behaviour of ram spermatozoa during cryopreservation. Ind.J. of Anim. Sci. 60:1336-1337.

Kakar, M. A., M. Anzar and M. Arslan, (1998). Assessment of post-thaw semen quality of buffalo and Sahiwal bulls using new semen assays. Pak. Vet. J. 18:74-80.

Kumar, S., K. L. Shani and G. Mohan. (1993). Effect of various levels of glycerol and yolk on freezing and storage of buffalo in milk tris and sodium citrate buffer. Buffalo, J, v. 63(5) p.499-504.

Mann, T. and L.W. Mann. 1988. Male reproductive function and semen. Themes and trends in physiology. Biochemistry and investigative Andrology. Spring-Velog, Berlin, New York 71-79.

Mazur, P., S. P. Leibo, J. Ferrant, E. H. Y. Chu, M. G. Hanna and L. H. Smith. (1970). Interactions of cooling rate, warming rate and protective additive on the survival of frozen mammalian cells. In: Wolstenholme, G.E.W., O'Connor, M.Eds., The Frozen Cell, Churchill, London. 69-88.

Molinia, F. C., G. Evans, P. I. Quintana Casares and W. M. C. Maxwell. (1994). Depart of Anim. Science. The University of Sydney, Sydemy NSW 2006. Australia Dept. of Ani. Science. Waite Agri. Res. Institute, Glen Osmond. SA 5064 Australia.

Revel, S. G. and R. A. Mrode. (1994). An Osmotic Resistance test for bovine semen. J. of Anim. Repro. Sci. vol 1 issue 36 p 77-86.

Rodrý´guez-Martý´nez H. (2000). Evaluation of frozen semen: traditional and new approaches. In: Chenoweth P.J, editor. Topics in bull fertility. Ithaca NY: International Veterinary Information Service. pp 502– 600.

Samo, M. U. (1998). Studies on the Preservation of Ram Semen Ph.D. Thesis School of biological Science Universities of Wales Bangor UK. Pp 433

Sansone, G., M. J. F. Nastri and A. Fabbricini.(2000). Storage of buffalo (*bubalus bubalis*) semen. Anim. Repro. Sci. pp 55-76

Usmani, R. H. (1996). Animal Institute, National Agricultural Research Center Islamabad Pakistan.pp

Vyawanare, R., R. A. S. Chauhan, S. P. Nema and M. L. Poswal. (1989). Studies on seminal attributes, enzyme leakage and preservability of buffalo semen, Ind. Vet. J. 66:1128-1132.

Younis, M. (1996). Studies on semen quality, freezability and fertility of buffalo bulls during low and peak breeding seasons. Ph.D. Dissertatiot of Anim. Repro. University of Agriculture, Faisalabad, Pakistan.