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Effect of Fructose Addition in Skim Milk Extender on the Quality of Liquid Nili-Ravi Buffalo (*Bubalus bubalis*) Semen

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Abstract.- This study was designed to identify the effect of fructose addition in skim milk extender on quality of buffalo bull spermatozoa (motility, *in vitro* longevity, plasma membrane integrity, normal apical ridge and abnormalities) stored at 5°C for seven days. For this purpose, semen from five Nili-Ravi buffalo bulls was collected and diluted at 37°C with a final concentration of 10 x 10^6 motile spermatozoa per ml in skim milk extender containing 5mM or 10mM fructose or without fructose (control). The extended semen was cooled from 37°C to 5°C in 2 hours and stored at 5°C for seven days. Sperm motility, *in vitro* longevity and plasma membrane integrity did not differ (P>0.05) in all three experimental extenders at 1st day of storage. Sperm motility, *in vitro* longevity, plasma membrane integrity on 3rd, 5th and 7th day of storage was significantly (P<0.05) higher in extender containing fructose 5mM and 10mM as compared to control. Sperm morphology *viz*; normal acrosomes; head, mid piece and tail abnormalities remained similar (P>0.05) in all three experimental extenders upto seven days of storage at 5°C. In conclusion, addition of 5mM and 10mM fructose in skim milk extender improved the preservability of buffalo bull semen stored at 5°C.

Keywords: fructose, semen quality, liquid semen, buffalo bull spermatozoa

INTRODUCTION

The development of artificial insemination (AI) technique has allowed the rapid dissemination of genetic material from a small number of superior sires to a large number of females (Vishwanath and Shannon, 1997). Buffalo bull semen is routinely preserved in liquid or frozen state according to suitability for AI (Anzar *et al*., 2003).

Commonly, tris, citrate and milk based extenders are used for the storage of buffalo semen at 4-5°C temperatures (Andrabi, 2009; Sansone *et al*., 2000). Skim milk extender has been recognized as an appropriate storage medium in liquid state for buffalo bull spermatozoa because of its higher ability to conserve semen quality, availability and economic suitability (Akhter, 2006). Storage of spermatozoa in liquid state requires a suitable energy supplement in extender (Sansone *et al*., 2000). Fructose is a major naturally occurring energy source for buffalo spermatozoa in the seminal plasma for metabolic processes (Sansone *et al*., 2000). Dilution of the semen for freezing purpose decreases the available source of energy for spermatozoa (Akhter, 2006). Therefore, addition of energy source in semen extender is required for proper functioning of the spermatozoa. We hypothesized that addition of fructose as energy source in skim milk extender can improve its ability to maintain the quality (motility, *in vitro* longevity, plasma membrane integrity, normal apical ridge and abnormalities) of Nili-Ravi buffalo bull semen stored at 5°C.

Therefore, present experiment was designed to study the effect of 5mM and 10mM fructose addition in skim milk extender on motility, *in vitro* longevity, plasma membrane integrity, normal apical ridge and abnormalities of buffalo bull spermatozoa stored at 5°C.

MATERIALS AND METHODS

Extender preparation

Powdered skimmed milk (SKIMZ®; CANDIA) 10% (w/v) without the addition of fructose was used for preparation of extender as control. Second and third extenders were prepared

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by adding 5mM and 10mM fructose to control extender, respectively. Antibiotics (Streptomycin sulphate @ 1 mg/ml and Benzyl Penicillin @ 1000 i.u./ml) were added in each extender at room temperature.

Semen collection and initial evaluation

Semen from five Nili-Ravi buffalo bulls of similar age group and known fertility maintained at Semen Production Unit, Qadirabad, Sahiwal, Pakistan was colleted with artificial vagina maintained at 42°C, for a period of three weeks (replicates). Semen collection was performed once a week and two ejaculates were collected per collection. After collection, semen was transferred to laboratory for initial evaluation. Visual progressive sperm motility was assessed microscopically at 400X. Sperm concentration was assessed by Neubauer haemocytometer. Qualifying semen ejaculates were pooled to get sufficient semen for a replicate having motility >60% and split into three aliquots for further processing.

Semen processing

Semen aliquots were diluted at 37^{o}C with skim milk extender without fructose (control) or with extenders containing 5mM or 10mM fructose. The extended semen was cooled from 37^{o}C to 5^{o}C in 2 hours and stored at 5°C for seven days. All the semen quality assays were performed at 1st, 3rd, 5th and 7th day of storage.

Semen quality assays

Progressive sperm motility was assessed using phase contrast microscope as described earlier. Sperm *in vitro* longevity of each extended buffalo bull semen sample was assessed for six hours at 37°C, after two hours interval (Akhter *et al*., 2008). Sperm plasma membrane integrity was determined by hypo-osmotic swelling assay (HOS). The HOS solution (osmotic pressure ~ 190mOsm/ kg) contained sodium citrate 0.735g and fructose 1.351g, dissolved in 100ml distilled water. To perform assay, 50µl semen sample was mixed with 500µl pre-warmed (37°C) HOS solution and incubated at 37°C for 30 to 40 minutes. After incubation, a drop of semen sample was examined using phase contrast microscope (400X). One hundred spermatozoa were counted and the percentage of cells with curled tails (swollen/intact plasma membrane) was recorded as HOS positive (Akhter *et al*., 2008). Sperm morphology was assessed by mixing 500 µl of semen sample with 50µl of 1% formal citrate. A drop of semen sample was examined under phase-contrast microscope (1000X). One hundred spermatozoa were counted to determine the percentage of intact acrosomes, and head, mid piece and tail abnormalities (Andrabi *et al.*, 2008).

Statistical analysis

The data are presented as Mean (± SE). The data on semen quality parameters were analyzed using Analysis of Variance (ANOVA). When F-ratio was found significant, the means were compared using LSD test. A 5% ($P<0.05$) level was used to determine statistical significance.

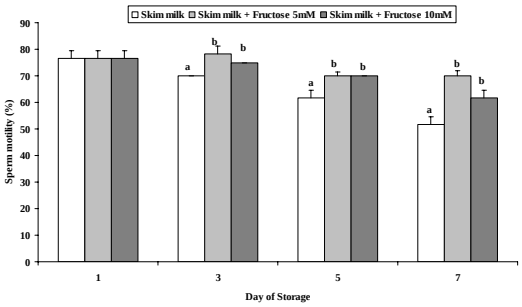
RESULTS

Motility, in vitro *longevity and plasma membrane integrity of semen*

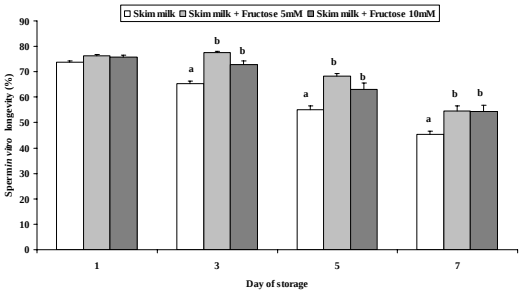
The data on the effect of fructose addition in semen extenders on motility, *in vitro* longevity and plasma membrane integrity of buffalo spermatozoa are presented in Figure 1A-C. There was no difference ($P > 0.05$) in sperm motility, *in vitro* longevity and plasma membrane integrity on 1st day of storage in extender with fructose supplementations or control. However, sperm motility, *in vitro* longevity and plasma membrane integrity on 3rd, 5th and 7th day of storage was significantly better in extenders containing 5mM and 10mM fructose as compared to control.

Normal apical ridge of semen

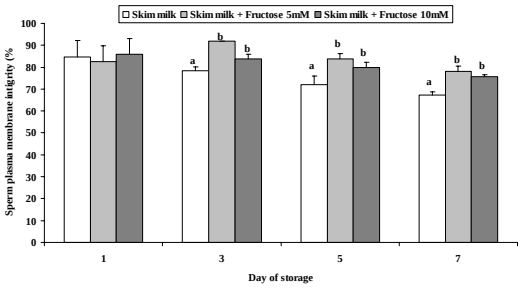
The data on the effect of fructose addition in semen extenders on normal apical ridge of buffalo spermatozoa are presented in Figure 1D. Percentage of sperm with normal apical ridge did not differ ($P>0.05$) in extenders containing 5mM and 10mM fructose as compared to control for seven days of storage at 5°C.



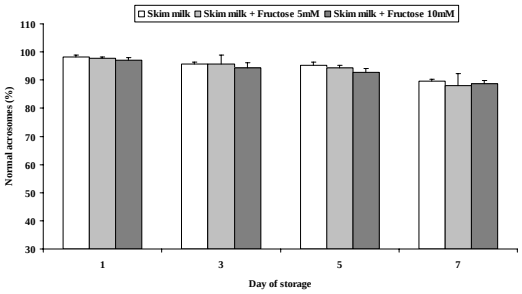
A: Motility



B: *In vitro* longevity



C: Plasma membrane integrity



D: Normal acrosome

Fig 1: Effect of fructose addition in semen extender on motility (A), *in vitro* longevity (B), plasma membrane integrity (C) and normal acrosome (D) of buffalo bull spermatozoa on different days of storage at 5°C. Bars with different letters differ significantly (P<0.05) at a given day.

Abnormalities of semen

The data on the effect of fructose addition in semen extenders on abnormalities (head, mid piece and tail) of buffalo spermatozoa are presented in Table I. There is no difference in sperm head, mid piece and tail abnormalities in extenders containing 5mM and 10mM fructose as compared to control upto 7 days of storage at 5°C.

DISCUSSION

Present experiment was designed to study the effect of fructose addition as energy source in skim milk extender on the quality (motility, *in vitro* longevity, plasma membrane integrity, normal apical ridge and abnormalities) of buffalo bull spermatozoa stored at 5 ºC.

Motility, *in vitro* longevity and plasma membrane integrity are the parameters used to determine the effect of experimental procedures on the storage of buffalo bull semen (Akhter *et al*., 2008; Andrabi *et al*., 2008). It is well recognized that sperm motility is affected by the properties of diluting media (Andrabi, 2009). *In vitro* longevity assessment is an indirect indicator of sperm liveability in female reproductive tract. Hypo-osmotic swelling test has been widely used to assess the functional intactness of the buffalo bull spermatozoa plasma membrane (Akhter *et al*., 2008; Andrabi *et al*., 2008). In our study, motility, *in vitro* longevity and plasma membrane integrity of buffalo bull spermatozoa on 1st day of storage remained similar in all three experimental extenders. However, sperm motility, *in vitro* longevity and plasma membrane integrity on 3rd, 5th and 7th day of storage was better ($P < 0.05$)) in extenders containing 5mM and 10mM fructose as compared to control. In similar studies, El-Azab *et al.* (1984) reported increase in motility of Egyptian buffalo bull spermatozoa after the addition of fructose and lactose than glucose in Tris based extender. Similarly, improvement in motility and percent liveability of Murrah buffalo bull spermatozoa was observed in milk based extender in frozen and liquid state after the addition of fructose, sucrose and raffinose in Tris and milk based extender (Kumar *et al.*, 1994a,b). Moreover, 2% glucose and 1% fructose were reported to provide

Table I.- Effect of fructose addition in semen extender on sperm abnormalities (head, mid piece and tail) of buffalo bull spermatozoa on different days of storage at 5°C.

Day of storage	Treatment	Sperm abnormalities (%)		
		Head	Mid piece	Tail
1	Skim milk	1.00 ± 0.0	0.30 ± 0.6	2.30 ± 1.2
	Skim milk + Fructose 5mM	1.00 ± 0.0	1.30 ± 0.6	3.30 ± 0.6
	Skim milk + Fructose 10mM	1.00 ± 1.0	1.00 ± 1.0	2.70 ± 0.6
3	Skim milk	2.30 ± 2.3	1.00 ± 1.0	3.70 ± 1.5
	Skim milk + Fructose 5mM	0.70 ± 1.2	1.00 ± 1.7	5.30 ± 0.6
	Skim milk + Fructose 10mM	1.70 ± 1.2	1.00 ± 1.0	4.30 ± 0.6
5	Skim milk	1.00 ± 0.0	1.00 ± 0.0	4.70 ± 1.2
	Skim milk + Fructose 5mM	1.00 ± 1.0	0.70 ± 1.2	9.70 ± 1.5
	Skim milk + Fructose 10mM	1.70 ± 1.5	0.00 ± 0.0	5.70 ± 0.6
7	Skim milk	1.70 ± 0.6	0.70 ± 1.2	5.70 ± 0.6
	Skim milk + Fructose 5mM	1.30 ± 1.2	1.00 ± 1.7	7.60 ± 1.0
	Skim milk + Fructose 10mM	1.30 ± 1.2	0.00 ± 0.0	6.30 ± 1.0

better motility of Murrah buffalo bull semen in Tris based extender at 5°C (Kumar *et al.,* 1992). An addition of 5mM and 10mM of fructose in skim milk extenders improved the survivability of buffalo bull spermatozoa in this study.

Sperm morphology (acrosome, head, mid piece and tail) is critical for the successful fertilization. The presence of normal acrosome on a spermatozoon is essential for the acrosomal reaction that is required at the proper time to facilitate fertilization (Thomas *et al*., 1997). It is believed that acrosomal integrity assessment can be an effective tool to predict the fertilizing ability of buffalo bull spermatozoa. A definite relationship has been established between fertility of preserved semen and percentage of sperm abnormalities (Soderquist *et al*., 1991). In our study, percentage of sperm with normal apical ridge and abnormalities (head, mid piece and tail abnormalities) did not differ in all three experimental extenders for seven days of storage at 5 °C. It is pertinent to mention that semen preservation does not necessarily increase the number of abnormal sperm in bovine (Revell *et al.*, 2003) and buffalo spermatozoa (Akhter *et al*., 2008).

It is concluded that addition of 5mM and 10mM fructose in skim milk extender used for preservation of buffalo bull spermatozoa at 5°C improved sperm motility, *in vitro* longevity and plasma membrane integrity. Therefore, fructose addition may be used for improving the preservability of liquid Nili-Ravi buffalo semen for artificial insemination programme.

REFERENCES

AKHTER, S., 2006. *Effect of milk based extenders containing different antibiotics on sperm motility, longevity, morphology, plasma membrane integrity, in vivo fertility and bacterial control of liquid semen from buffalo bulls.* Ph.D. thesis, Department of Zoology, University of Arid Agriculture, Rawalpindi, Pakistan.

AKHTER, S., ANSARI, M. S., ANDRABI, S. M. H., ULLAH, N. AND QAYYUM, M., 2008. Effect of antibiotics in extender on bacterial control and spermatozoal quality of cooled buffalo (*Bubalus bubalis*) bull semen. *Reprod. Domest. Anim.*, **43**: 272-278.

ANDRABI, S. M. H., 2009. Factors affecting the quality of cryopreserved buffalo (*Bubalus bubalis*) bull spermatozoa. *Reprod. Domest. Anim*., **44**: 552-569.

ANDRABI, S. M. H., ANSARI, M. S., ULLAH, N., ANWAR, M., MEHMOOD, A. AND AKHTER, A., 2008. Duck

egg yolk in extender improves the freezability of buffalo bull spermatozoa. *Anim. Reprod. Sci*., **104**: 427-433.

ANZAR, M., FAROOQ, U., MIRZA, M.A., SHAHAB, M. AND AHMED, N., 2003. Factors affecting the efficiency of artificial insemination in cattle and buffalo in Punjab, Pakistan. *Pakistan Vet. J*., **23**: 106-113.

EL-AZAB, E. A., EL-HARIRI, M. N., EL-SAWAF, E. A. AND HAZZA, A. M., 1984. Influence of buffer and sugar concentration, glycerol level, equilibration time on survival of buffalo spermatozoa frozen in straws. *10th International Congress on Animal Reproduction and Artificial Insemination*, June 10-14, 1994, University of Illinos at Urbana-Champaign, Illinois, USA. **2**: 192.

KUMAR, S., SAHNI, K. L. AND MOHAN, G., 1992. Effect of different sugars as a sole cryoprotectant in freezing of buffalo semen. *Buffalo J*., **3**: 305-309.

KUMAR, S., SAHNI, K. L. AND MOHAN, G., 1994a, Loss in motile and live sperm during freezing in milk, tris and citrate buffers containing various sugars. *Buffalo J*., **2**: 161-166.

KUMAR, S., SAHNI, K. L. AND MOHAN, G., 1994b. Effect of different sugars as a sole cryoprotectant during refrigeration preservation of buffalo semen. *Indian J. Anim. Sci*., **64**: 471-473.

REVELL, S.G., CRANFIELD, N. AND COOLEY, W., 2003. Sperm tail-stump defect in a Holstein bull. *Brit. Vet. Assoc*., **153**: 268-268.

SODERQUIST, L., JANSON, L., LARSSON, K. AND EINARSSON, S., 1991. Sperm morphology and fertility in A.I. bulls. *J. Vet. Med.*, **38**: 534–543.

SANSONE, G., NASTRI, M.J.F. AND FABBROCINI, A., 2000. Storage of buffalo (*Bubalus bubalis*) semen. *Anim. Reprod. Sci*., **62**: 55-76.

THOMAS, C. A., GARNER, D. L., DE-JARNETTE, J. M. AND MARSHALL, C. E., 1997. Fluorometric assessment of acrosomal integrity and viability in cryopreserved bovine spermatozoa. *Biol. Reprod*., **45**: 880–887.

VISHWANATH, R. AND SHANNON, P., 1997. Do sperm cells age? A review of the physiological changes in sperm duing storage at ambient temperature. *Reprod. Fertil. Develop*., **9**: 321-332.

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