Pakistan J. Zool., vol. 47(6), pp. 1645-1648, 2015.

Effect of Milk Based Extenders on Motility and Acrosomal Integrity of Buffalo Bull (*Bubalus bubalis*) Spermatozoa at 5°C

Shamim Akhter,[1]* Muhammad Sajjad Ansari,[2] Bushra Allah Rakha,[3] Tahir Aziz,[2] Syed Murtaza Hassan Andrabi,[4] Nemat Ullah,[4] Muhammad Anwar[4] and Mazhar Qayyum[1]

[1]Department of Zoology, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan
[2]Department of Zoology, University of Gujrat, Pakistan
[3]Department of Wildlife Management, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan
[4]Animal Reproduction Programme, Animal Sciences Institute, National Agricultural Research Centre, Islamabad, Pakistan

Abstract.- This study was designed to compare six different milk based extenders for the liquid storage of Nili-Ravi buffalo bull semen. Semen was collected from three Nili-Ravi buffalo bulls with artificial vagina (42°C) at weekly intervals (n=5). Semen was diluted (at 37°C) with six milk based extenders; homogenized milk (HOM), whole buffalo milk (BUF), whole cow milk (COW), whole camel milk (CAM), modified homogenized milk (MOD) and skimmed milk (SKIM). After dilution, semen was cooled to 5°C in 2 h and then it was refrigerated at 5°C for 96 h. Sperm motility (%) and normal acrosomal ridge (NAR; %) were recorded at 24 h intervals till 96 h. Sperm motility did not differ up to 72 h of storage in all experimental extenders, while higher ($P<0.05$) motility was observed in SKIM after 96 h of storage, that remained similar to sperm motility in HOM extender. Overall least square mean of sperm motility for SKIM was higher ($P < 0.05$) compared to HOM, BUF, COW, CAM and MOD after 96 h of storage period. Percentage of sperm with NAR remained similar ($P>0.05$) in SKIM, COW, BUF and HOM extenders at 24 h of storage. However, at 72 and 96 h of storage, values for NAR were higher ($P<0.05$) in SKIM and COW compared to HOM, BUF, CAM and MOD extenders. The values of NAR in terms of least square means remained higher ($P<0.05$) for SKIM and COW extenders compared to other extenders. In conclusion, skimmed milk based extender was found superior for chilled buffalo semen stored at 5°C.

Keywords: Milk based extenders, semen preservation, buffalo.

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. Semen of farm animals has been stored either in the liquid or cryopreserved state (Shannon, 1978; Ansari *et al*., 2011). Cryopreservation of bovine semen is practiced worldwide for many reasons but the use of liquid semen for AI has its own advantages. Major benefits of liquid semen are better conceptions (Anzar *et al*., 2003; Sharma and Sahni, 1988) with relatively lower number of spermatozoa.

Buffalo is the main dairy animal in most of the developing countries of the world particularly in Asia, where facilities for artificial breeding of animals are limited (Anzar *et al*., 2003). These countries produce buffalo bull semen mainly for their own AI use. There is consequently no need to store nationally produced semen for a long period. Therefore, the use of liquid semen stored at refrigerated temperature is the most appropriate preposition for AI in these countries. Use of chilled semen also makes AI programme independent from the use of liquid nitrogen, which is expensive and difficult to keep regular supplies in most of the buffalo rearing countries (Akhter *et al*., 2008).

Milk based extenders have been successful in maintaining sperm fertility and gave results comparable to egg yolk-citrate diluents for cow bull semen (Foote, 1978). A few scattered reports on milk-based extenders for liquid buffalo semen are available (Kumar *et al*., 1993; Pramanik and Raina, 1998), but no comprehensive attempt has been made to evaluate appropriate milk-based extenders for buffalo semen preservation. Hence, a comprehensive study was conducted to evaluate pertinent milk based extenders for preservation for buffalo semen at 5°C up to 96 h of storage.

* Corresponding author: sashraf1993@gmail.com
0030-9923/2015/0006-1645 $ 8.00/0
Copyright 2015 Zoological Society of Pakistan

MATERIALS AND METHODS

The study was conducted at Animal Reproduction Programme, National Agricultural Research Centre, Islamabad, Pakistan.

Preparation of extenders

For the preparation of buffalo (BUF), cow (COW) and camel (CAM) milk extenders, buffalo, cow and camel milk was boiled, as per domestic consumption and cooled by keeping in refrigerator over night. The fat layer was removed, and milk was filtered through cotton wool. Filtered fresh milk (from buffalo, cow and camel), homogenized milk (Milk Pack®) and skim milk (SKIMZ®-Candia; 10 %) was warmed in a double boiler at 92-95°C for 10-12 min, and then cooled to room temperature. The milk was slowly poured leaving the surface scum of albumin on the sides of boiler. Modified homogenized milk extender (MOD) was prepared with addition of egg yolk (20% v/v) and glycerol (5% v/v). 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

Two consecutive ejaculates per week were collected from three Nili-Ravi buffalo bulls with artificial vagina (42°C) for a period of 4 weeks. After collection, semen was immediately transferred to laboratory. Visual motility was assessed microscopically (x400; Olympus BX20) with closed circuit television (Graham *et al.*, 1970). Sperm concentration was assessed by digital-photometer (Dr. Lange LP 300 SDM; Germany) at 560 nm. The neat semen samples with more than 60% motile spermatozoa were used for further dilution.

Semen processing

Semen collected from Nili-Ravi buffalo (*Bubalus bubalis*) bulls (n=3) was pooled and diluted (37°C; 10 x 10^6 motile spermatozoa per ml) with experimental extenders. Liquid semen was stored at 5°C for five days.

Semen assays

The quality of semen extended with experimental extenders was assessed using semen quality assays *viz.*, progressive motility (%) and normal acrosomal ridge (NAR). All the mentioned assays were performed at 0, 24, 48, 72 and 96 h of storage.

Sperm motility

A drop of semen sample was placed on a pre warmed glass slide and covered with a cover slip. The slide was examined immediately at 400 X with closed circuit television (Graham *et al.*, 1970) at 37°C and progressive sperm motility was recorded as percentage.

Sperm acrosomal morphology

Semen sample **(**500 µl) was fixed by 50 µl of 1% formal citrate (Hancock, 1959) which was prepared by adding 1 ml of 37% commercial formaldehyde in 99 mL of 2.9% (w/v) sodium citrate. Acrosomal integrity characterized by normal apical ridge was assessed under oil immersion (1000X) using a phase-contrast microscope (X1000; Olympus BX20). One hundred spermatozoa were counted to determine the percentage of intact acrosome.

Statistical analysis

Data on semen quality were analyzed using Analysis of variance (ANOVA) in two factor factorial (Time x Extender). When F-ratio was found significant, LSD test was applied to compare the treatment and interval means (MSTAT-C, Ver.1.42).

RESULTS

Progressive motility (%) of buffalo bull spermatozoa for five days of storage

The data on progressive motility of buffalo bull spermatozoa in six milk based extenders for five days of storage are given in Table I. The percent motility was significantly ($P<0.05$) affected by extender and preservation interval but extender interval interaction effect was not significant ($P>0.05$). Sperm motility did not differ ($P>0.05$) due to extenders up to 72 h of storage, however, at 96 h of storage, sperm motility was better conserved ($P<0.05$) in SKIM compared to BUF, COW, CAM and MOD. The least square means indicate that SKIM extender was better ($P<0.05$) to conserve sperm motility compared to all experimental extenders.

Table I.- Motility (%) of buffalo bull spermatozoa in different extenders stored at 5°C.

Extenders	Hours of storage					
	0	24	48	72	96	LSM ± SE
HOM	^{w}60.0 ± 0.0	^{w}60.0 ± 0.0	^{wx}52.5 ± 5.0	^{xy}42.5 ± 8.7	^{y}38.8 ± 11^{ab}	50.8 ± 4.3^{b}
BUF	^{w}60.0 ± 4.1	^{wx}53.8 ± 4.8	^{xy}46.3 ± 4.8	^{y}37.5 ± 6.5	^{z}27.5 ± 9.6^{c}	45.0 ± 5.7^{c}
COW	^{w}60.0 ± 4.1	^{wx}52.5 ± 9.6	^{x}46.3 ± 7.5	^{y}35.0 ± 5 .8	^{y}30.0 ± 4.1^{bc}	44.7 ± 5.4^{c}
CAM	^{w}57.5 ± 5.0	^{wx}51.3 ± 10	^{xy}45.0 ± 10	^{yz}37.5 ± 6.5	^{z}31.3 ± 6.3^{bc}	44.5 ± 4.6^{c}
MOD	^{w}61.3 ± 2.5	^{w}53.8 ± 4.8	^{w}46.3 ± 7.5	^{x}38.8 ± 6.3	^{x}33.8 ± 4.8^{bc}	46.8 ± 4.9^{c}
SKIM	^{w}65.0 ± 5.8	^{x}61.3 ± 2.5	^{y}52.5 ± 5.0	^{z}47.5 ± 2.9	^{z}46.3 ± 2.5^{a}	54.5 ± 3.7^{a}
LSM ± SE	^{w}60.6±1.0	^{wx}55.4 ± 1.6	^{x}48.5 ± 1.6	^{y}39.7 ± 1.8	^{y}34.5 ± 2.8	

The values in the same column and row with different superscripts differed significantly (P<0.05)
w, x, y… along the row; a, b, c…down the column.
For abbreviations: BUF, whole buffalo milk; CAM, whole camel milk; COW, whole cow milk; HOM, homogenized milk; LSM, least square means; MOD, modified homogenized milk; SKIM, skimmed milk.

Table II.- Normal acrosomal ridge (%) of buffalo bull spermatozoa in different extenders stored at 5 °C.

Extender	Hours of storage					
	0	24	48	72	96	LSM
HOM	^{w}68.5 ± 5.9^{a}	^{wx}64.3 ± 4.0^{ac}	^{xy}58.5 ± 4.4^{c}	^{y}56.0 ± 7.0^{b}	^{y}53.8 ± 5.2^{b}	60.2 ± 2.7^{c}
BUF	^{w}74.0 ± 0.8^{ab}	^{wx}67.3 ± 3.8^{abc}	^{xy}63.0 ± 6.8^{abc}	^{y}57.8 ± 7.0^{b}	^{y}55.5 ± 6.8^{b}	63.5 ± 3.3^{b}
COW	73.5 ± 7.2^{ab}	71.0 ± 4.3^{ab}	67.0 ± 8.4^{ab}	66.5 ± 5.1^{a}	63.5 ± 4.2^{a}	68.3 ± 1.7^{a}
CAM	^{w}66.0 ± 6.0^{a}	^{wx}60.3 ± 4.6^{c}	^{wx}59.5 ± 1.3^{bc}	^{x}57.5 ± 3.3^{b}	^{x}54.0 ± 5.2^{b}	59.4 ± 1.9^{c}
MOD	67.0 ± 8.0^{a}	60.8 ± 6.7^{c}	61.0 ± 4.5^{bc}	55.5 ± 4.9^{b}	54.3 ± 4.3^{b}	59.7 ± 2.2^{c}
SKIM	^{w}79.3 ± 4.1^{b}	^{wxy}74.3 ± 4.3^{a}	^{wx}71.0 ± 4.2^{a}	^{xy}66.5 ± 4.2^{a}	^{y}65.0 ± 4.8^{a}	71.2 ± 2.6^{a}
LSM	^{w}71.3 ± 2.0	^{x}66.2 ± 2.2	^{xy}63.3 ± 1.9	^{yz}59.9 ± 2.0	^{z}57.6 ± 2.1	

For abbreviations and statistical details see Table I.

Normal apical ridge (%) of buffalo bull spermatozoa for five days of storage

The data on the normal apical ridge of buffalo bull spermatozoa in six milk based extenders for five days of storage are given in Table II. Extenders and preservation interval affected (P<0.05) NAR but extender preservation interval interaction effect was not significant (P>0.05). Percent NAR remained similar (P>0.05) in SKIM, COW, BUF and HOM extenders at 24 h of storage. However, at 72 and 96 h of storage, percent NAR remained higher (P<0.05) in SKIM and COW extender compared to HOM, BUF, CAM and MOD extenders. The values of least mean square remained higher (P<0.05) for SKIM and COW extenders compared to other extenders.

DISCUSSION

Sperm progressive motility is routinely used parameter of semen quality and is assessed by visual estimate of the progressively motile sperm. Previous studies on Murrah buffalo bulls bred in India reported 60.8±1.5% to 69±4% of motile spermatozoa after dilution in milk extenders (Kumar *et al*., 1993). In present study, 60 % sperm motility was retained after 24 h storage of buffalo semen in HOM and SKIM extenders. After 96 h of storage, the SKIM and HOM extenders remained similar as reported earlier (Hussain, 1974; Ali, 1974), however, the least square means of sperm motility for SKIM was better (P<0.05) compared to HOM extender. Skim milk was earlier reported to be 3.2% better than homogenized whole milk for sperm motility at 37°C in bovine (Hussain, 1974). Presence of fat in homogenized milk may have resulted in reduced motility after storage of semen at refrigerated temperature (Kumar *et al*., 1993). Skim milk extender was also found efficient to conserve quality of ram semen at liquid storage compared to sodium citrate, Bioxcell® and Tris extenders (Kulaksiz *et al*., 2012).

Acrosomal cap on the sperm head is known to play a key role during fertilization as it contains the enzymes which enable the spermatozoon to penetrate through the zona pellucida of the oocyte.

Therefore, presence of intact acrosome is important criterion to assess the viability of spermatozoa and has been related to fertility of the cryopreserved bull semen (Saacke and White, 1972). In present study SKIM and COW extenders better conserved NAR expressed in terms of least square means compared to HOM, BUF, CAM and MOD extenders. Previously, more than 90% of spermatozoa with intact acrosome were observed in semen of buffalo bulls (Aguiar *et al*., 1994; Kumar *et al*., 1993). In a previous study on buffalo semen, egg yolk- citrate glucose, egg yolk tris, skim milk-egg yolk and citric acid whey conserved NAR with similar efficiency but minimum release of hyaluronoglucosaminidase was observed in skim milk egg yolk extender when cooled to 5°C (Kakar and Anand, 1984).

Skim milk which have all required nutrients for maintaining the liveability of spermatozoa (Jones and Foote, 1972) when used as extender for diluting buffalo semen (4-7°C) was found superior over Tris and EYC in linearity, lateral head displacement (ALH), average path velocity (VAP), and motility (Pramanik and Raina, 1998). In present study, skim milk extender was found superior in maintaining motility (%) and normal acrosomal ridge (%) over homogenized milk, whole buffalo milk, whole cow milk, whole camel milk and modified homogenized milk extenders.

The SKIM extender proved best in maintaining highest percentage of sperm motility, and optimum percentage of normal sperm acrosomes compared to HOM, BUF, COW, CAM and MOD extenders for Nili-Ravi Buffalo bulls semen preserved at 5°C.

REFERENCES

AGUIAR, P.H.P., ANDRADE, V.J., ABREU, J.J. AND GOMEZ, N.B.N., 1994. Physical and morphological semen characteristics of buffalo aged from four to eight years old. *Proc. 4th Int. Buffalo Congr., Sao Paulo, Brazil*, **3**: 486-488.

ANSARI, M.S., RAKHA, B.A., ULLAH, N., ANDRABI, S.M.H. AND AKHTER, S., 2011. Glutathione addition in tris-citric egg yolk extender improves the quality of cooled buffalo (*Bubalus bubalis*) bull semen. *Pakistan J. Zool.*, **43**: 46-55.

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

ALI, M., 1974. *Preservation of buffalo semen in modified milk diluents*. M.Sc. thesis. Univ. Agric. Faisalabad, pp. 35-37.

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

FOOTE, R. J., 1978. Extenders and extension of unfrozen semen. In: *Physiology of reproduction-artificial insemination of cattle* (eds. G. L. Salisbury., N. L. Van Demark and J. R. Lodge), Freeman, San Francisco, pp. 442-493.

GRAHAM, E.F., SCHMEHL, M.K.L. AND MAKI-LAURIA, M., 1970. Some physical and chemical methods of evaluating semen. In: *Proc. 3rd NAAB, Tech. Conf. Artif. Insemin. Reprod.*, National Association of Animal Breeders, Columbia, MO: 24.

HANCOCK, J.L., 1959. The morphological characteristics of spermatozoa and fertility. *Int. J. Fert*., **4**: 347 – 359.

HUSAIN, I., 1974. *Comparative study of different diluents for the preservation of cow bull spermatozoa at 37.5 °C and 4–5°C.* M.Sc. thesis. Univ. Agric. Faisalabad, pp. 49–50.

JONES, R.C. AND FOOTE, R.H., 1972. Nondialyzable skim milk in diluents for ram and bull semen. *J. Dairy Sci*., **55**: 856-861.

KAKAR, S.S. AND ANAND, S.R., 1984. Acrosomal damage and enzyme leakage during freeze preservation of buffalo spermatozoa. *Ind. J. exp. Biol*., **22**: 5-10.

KULAKSIZ, R., CEBI, C. AND AKCAY, E., 2012. The effect of different extenders on the motility and morphology of ram sperm frozen or stored at 4°C. *Turk. J. Vet. Anim. Sci*., **36**: 177-182.

KUMAR, S., SAHNI, K.L., BENJAMIN, B.R. AND MOHAN, G., 1993. Effect of various levels of yolk on deep-freezing and storage of buffalo semen in different dilutors without adding glycerol. *Buffalo J*., **1**: 79-81.

KUMAR, S., SAHNI, K.L. AND BISTHA, G.S. 1993. Cytomorphological characteristics of motile and static semen of buffalo bulls. *Buffalo J*., **2**: 117-127.

PRAMANIK, P.S. AND RAINA, V.S., 1998. Refrigerator (4-7 degrees C) preservation of buffalo semen in various extenders. *Indian J. Dairy Sci*., **51**: 375-379.

SAACKE, R.G. AND WHITE, J.M., 1972. Semen quality tests and their relationship to fertility. In: *Proc. 4th NAAB, Tech. Conf. Artif. Insemin. Reprod.*, National Association of Animal Breeders, Columbia, MO, pp. 22-27.

SHANNON, P., 1978. Factors affecting semen preservation and conception rates in cattle. *J. Reprod. Fert*., **54**: 519-527.

SHARMA, N.C. AND SAHNI, K.L., 1988. Fertility of frozen and liquid semen in buffaloes. *World Buffalo Congress*, 100.

(Received 7 October 2013, revised 4 September 2014)