Pakistan J. Zool., vol. 46(1), pp. 185-189, 2014.

Effect of Soybean Based Extenders on Sperm Parameters of Holstein-Friesian Bull During Liquid Storage at 4ºC

F.U. Rehman, M.S. Qureshi and R.U. Khan*
Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture, Peshawar, Pakistan.

Abstract.- Animal origin egg yolk extender has been associated with microbial contamination and interference with microscopic examination. Therefore, replacement of such extender with plant origin compound is inevitable without compromising the desired qualities of an ideal extender. In the current experiment, we compared the effect of soy-based extender (25% and 50%) of five adult Holstein-Friesian bulls semen preserved at 4ºC for 24 h with conventional egg yolk (control) and synthetic extender. Semen quality was assessed by measuring liveability, individual motility and membrane integrity after 24 h of extended semen stored at 4ºC. The results indicated that liveability, motility of sperm and membrane integrity decreased significantly ($P<0.05$) in soy 50% extender. The results of sperm liveability, individual motility and membrane integrity in 25% soy extender were comparable with the control. Therefore, we concluded that 25% soy milk could be used as a substitute of conventional egg yolk-based extender for bull semen stored at 4ºC.

Keywords: Bull semen, extenders, soybean based extender, egg yolk

INTRODUCTION

Egg yolk is a major constituent of extenders used for storage and cryopreservation of semen of domestic animals including bull, ram, goat and pig. The main advantage of egg yolk extender is the fraction of low density lipoprotein which protects the sperm phospholipids during cryopreservation (Amirat *et al*., 2005). However, wide variations in the constituents of egg yolk make the beneficial effect difficult to assess (Gil *et al*., 2003; Amirat *et al*., 2005). Moreover, the fertilizing capacity of spermatozoa is negatively affected by the risk of microbial contamination associated with egg yolk (Bousseau *et al*., 1998; Aires *et al*., 2003). It has also been reported that the fat globules of egg yolk makes the evaluation of sperm difficult (Singh *et al*., 2012). These circumstances demand for the partial or complete replacement of egg yolk with lecithin derived from plant source such as soybeans for the preservation of animal semen.

Currently, egg and milk based extenders are extensively used for semen extension and storage of different animal species (Kasimanickam *et al*., 2011; Khan *et al*., 2012). Also, vegetable origin extenders are in vogue which is considered to be alternative to milk or yolk based extenders (Gil *et al*., 2003). According to Aires *et al*. (2003) soy lecithin-based extender is superior to egg yolk-based extender for bovine and ram semen. Recently, El-Keraby *et al*. (2010) found that replacing whole soy-bean milk for traditional egg yolk increased sperm motility and decreased bacterial count in post-thawed bovine semen extender. So the search for non animal origin, well defined and contamination free medium for the extension of semen is highly desired (Ansari *et al.*, 2013).

Singh *et al*. (2012) observed that 25% soya based extender produced better motility, viability, membrane and acrosome integrity of bovine sperm at 5°C at different time interval. Zhang *et al*. (2009) documented that the extender supplemented with 6% soybean lecithin caused higher sperm motility and plasma membrane integrity in cryopreserved boar sperm. Kasimanickam *et al*. (2011) reported that DNA fragmentation index, mitochondrial membrane potential and sperm motility were especially improved in the soy based extender compared to the milk based extender during liquid storage.

According to the World Organization for Animal Health (2003) the semen processing compound should be free of any biological risk (Marco-Jimenez *et al*., 2004). Several commercial imported non-animal origin extenders are available. These extenders have shown promising results in

* Corresponding author: rifatullahkhhan@gmail.com
0030-9923/2014/0001-0185 $ 8.00/0

different species on various quality assessment parameters. However, the high cost of such extenders makes them difficult to use in developing countries. The vegetable origin chemical medium are favoured in assisted reproduction. Like egg yolk, soybean contains a high amount of low-density lipoprotein like lecithin which could successfully replace the current yolk based extender for the preservation of semen in species of domestic animals. Therefore, the current study was designed to compare the post-thawed sperm parameters of Holstein-Friesian bulls by replacing egg yolk with soybean milk in the extender.

MATERIALS AND METHODS

Semen collection and initial evaluation

This study was performed using five adult Holstein Friesian bulls of 6-7 year of age from Semen Production Unit, Jamrud, Khyber Agency, Federally Administrative Tribal Area (FATA), Pakistan. Two ejaculates per week were collected from each bull for a period of four weeks with the help of artificial vagina at 25°C. A total of 40 ejaculates were assessed in this experiment. Immediately after collection, semen samples were processed to determine sperm viability and motility at 37ºC using phase contrast microscope with heated stage. Ejaculates having greater than 80% motility were used in this study. A final concentration of 70 × 10^6 motile sperms per ml was achieved by diluting with either soya extender, egg yolk-based or commercial extender (AndroMed® Minitube, Germany). Extended semen samples were gradually cooled at 4ºC by transferring them to refrigerator temperature and stored for 24 h. The extended semen samples were immediately assessed for quality parameters.

Preparation of soybean milk

The soybean milk was prepared according to the method described by El-Keraby *et al*. (2010). Briefly, a total of 10 grams of soybean were washed and soaked in 100 ml distilled water and then boiled for 30 min. After boiling, the water was discarded and the whole soybean grains were washed again and finally cooled down with 50 ml distilled water containing 0.25% $NaHCO_3$. The grains were then grounded for 5 min and then the slurry was cooled. Soymilk was extracted by filtration through a clean cotton cloth, centrifuged and then boiled again for 10 min. The slurry was then allowed again to cool down.

Extender preparation

The soy extenders were prepared with different concentrations of soy milk in Tris glucose buffer (Tris 274mM, citric acid 87 mM, glucose 43 mM, benzyl pencillin 100 mg/l, streptomycin 100 mg/l) as described by Singh *et al*. (2012). The control extender was composed of 20% egg yolk prepared in Tris glucose buffer. The commercial extender (AndroMed®) contained phosphate, Tris, citric acid, sugars, antioxidants, buffer, glycerol, distilled water and antibiotics (Penicillin and Streptomycin)

Sperm quality assays

Sperm concentration was determined by the improved Neumbauer haemocytometer slide (GmbH & Co., Brandstwiete 4, 2000 Hamburg 11, Germany). The percentages (%) of motile sperm were estimated by visual examination under low-power magnification (× 10) using a phase-contrast microscope with heated stage. Assessment of live spermatozoa was performed using an eosin–nigrosin blue staining mixture (Blom, 1950; Singh *et al*., 2012). Sperm plasma membrane integrity was assessed by hypo-osmotic swelling test (HOST) as described by Jeyendran *et al*. (1984).

Statistical analysis

Data were statistically analyzed using a statistical software (SPSS, version 12.0). One-way analysis of variance was used to test the significance of extenders on the studied traits (Steel *et al*., 1997). Means of the significantly affected traits were separated by Duncan Multiple Range Test (Duncan, 1955). P-value less than 0.5 was considered to be statistically significant.

RESULTS

The results indicated that semen preserved after 24 h showed significantly high ($P<0.05$) sperm liveability % in control, 25% soy and synthetic

(AND) extenders (Fig. 1). The percentage of live sperms was significantly higher in 25% soy than 50% soy extender. No significant change was observed in control, soy 25% and AND in term of sperm motility percentage (Fig. 2). The HOST % was higher ($P<0.05$) in soy 50% extender (Fig. 3). We could also infer from the results that compared to control and commercial extenders, the soy 25% extender maintained the quality of sperm.

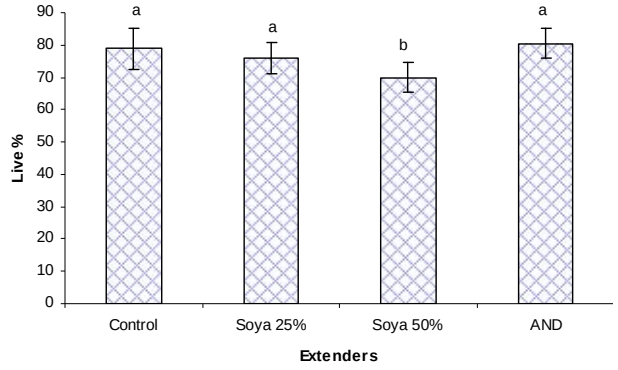

Fig. 1. Live sperm % in control, soya 25%, 50% and commercial extender of Holstein-Friesian bull semen; [ab]Values bearing different superscripts differ significantly $P<0.05$; Cooling temperature was 4°C and the duration of storage was 3 hrs.

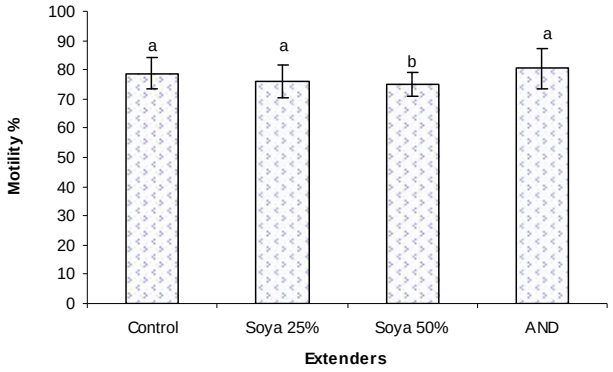

Fig. 2. Motility % in control, soya 25%, 50% and commercial extender of Holstein-Friesian bull semen; [ab]Values bearing different superscripts differ significantly $P<0.05$; Cooling temperature was 4°C and the duration of storage was 3 hrs.

DISCUSSION

The process of cryopreservation is associated with poor sperm quality and reduced conception rate. Chilling semen to low temperature is less harmful to the integrity of sperm than freezing and thawing which causes greater damage to the sperm (Singh *et al*., 2012). The former method is also cost effective, easy to prepare and transport. The most obvious advantage of sperm preservation at lower temperature is the dose of number of sperm cells. In cryopreservation, the number of sperms per insemination is 15-20 million per insemination, whereas, in liquid semen, the dose is just one million per insemination.

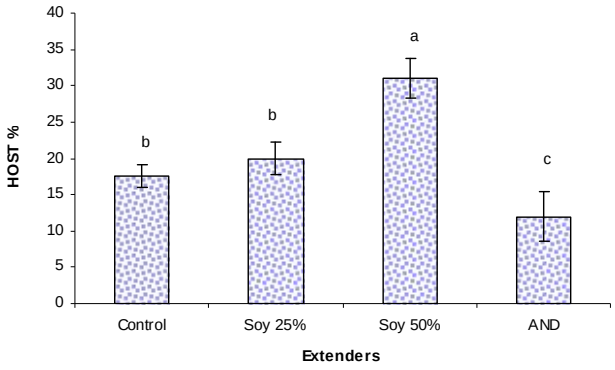

Fig. 3. Sperm membrane integrity in control, soya 25%, 50% and commercial extender of Holstein-Friesian bull semen; [abc]Values bearing different superscripts differ significantly $P<0.05$; Cooling temperature was 4°C and the duration of storage was 3 hrs.

Previous studies have demonstrated that inclusion of soybean in the extended semen improved post-thawed sperm characteristics (Singh *et al*., 2012). Papa *et al*. (2011) concluded that extender containing extender containing soybean source can be used alternative to conventional extender that contain skimmed milk and or egg yolk. Akhtar *et al*. (2011) reported that sperm motility and viability were better in soya lecithin based extender compared to milk, tris-citric egg yolk and egg yolk-citrate extender of buffalo sperms stored at 5ºC. In a recent study, de Paz *et al*. (2010) also suggested that lecithin soybean extender is equally effective in preserving the motility and viability of liquid ram sperm at 15 and 5ºC as an egg-yolk extender. Zhang *et al*. (2009) suggested that the enhanced improvement of sperm parameter in soybean added extender is due to its low viscosity

and less debris. In addition, in the presence of soybean in the extender, the phospholipids of the sperm membrane may be replaced which maintain its structure and function (Grahm and Foote, 1987; Trimeche *et al*., 1997). According to another hypothesis, phosopholipids from soybean may integrate with sperm membrane to form a protective film against the lethal factors (Zhang *et al*., 2009).

The egg yolk is still used as a major constituent of semen extenders in most of the domestic species such as goat, ram, bull, equine, boar and human being as well. Egg yolk is favoured because it contain large amount of low density lipoprotein called lecithin which help in protecting the membrane damage. However, bacterial contamination is the major drawback of using egg yolk for extending of semen. Therefore, the prevailing circumstance demand of developing a new extender which should have the same capacity to sustain the sperm features as the established extender. Soybean contain large amount of lipoprotein called soya lecithin similar to egg yolk lecithin which help in protecting membrane protecting effects against cold shock. This was obvious after storage of semen in soya based extender at 4ºC for 24 h in our result. The results indicated that soya 25% produced comparable results in comparison with the egg yolk extender. A fall in sperm quality (liveability, membrane integrity and motility) was observed when soya bean concentration was doubled. Our results are supported by the findings of Singh *et al*. (2012) who reported that sperm quality was negatively affected when soy bean concentration was increased beyond the 30%. Forouzanfar *et al*. (2010) attributed low sperm motility to the higher concentration of lecithin in the extender. Also the presence of higher soya concentration in semen extender decreased the sperm visibility under microscope (Singh *et al*., 2012).

In the current study, 50% soy produced significantly higher HOST values compared to the other extenders. The exact reason of this outcome could not be traced from the available literature. However, we infer that the sperm membrane integrity could not remain intact possibly due to decreased velocity and higher debris in the 50% soy extender. Secondly, 25% soy is the optimum concentration in which the sperm exhibited better membrane integrity.

CONCLUSION

On the basis of our results, we concluded that soy milk-based extender at the rate of 25% soy milk has the potential to maintain sperm quality parameters like liveability, motility and membrane integrity of Holstein-Friesian bull semen stored at 4ºC.

REFERENCES

AIRES, V.A., HINSCH K.D., SCHLOESSER, F.M., BOGNERA, K. AND SCHLOESSER, S.M., 2003. *In vitro* and *in vivo* comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. *Theriogenology*, **60**: 269–279.

AKHTER, S., ANSARI, M.S., RAKHA, B.A., ULLAH, N., ANDRABI, S.M.H. AND KHALID, M., 2011. *In vitro* evaluation of liquid-stored buffalo semen at 5c diluted in soya lecithin based extender (Bioxcell), tris-citric egg yolk, skim milk and egg yolk-citrate extenders. *Reprod. Dom. Anim.*, **46**: 45–49.

AMIRAT, L., ANTON, M., TAINTURIER, D., CHATAGNON, G., BATTUT, I. AND COURTENS, J.L., 2005. Odifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. *Anim. Reprod. Sci*., **129**: 535–543.

ANSARI, M.S., RAKHA, B.A., ULLAH, N., ANDRABI, S.M.H. AND AKHTAR, S., 2013. Glutathion addition in Tris-citric egg yolk extender improves the quality of cooled buffalo (*Bubalus bubalus*) bull semen. *Pakistan J. Zool*., **43**: 49-55

BLOM, E., 1950. A 1-min live–dead sperm stain by means of eosin–nigrosin. *J. Fertil. Steril*., **1**: 176–177.

BOUSSEAU, S., BRILLARD, J.P., MARQUANT-LE-GUIENNE, B., GUERIN, B., CAMUS, A. AND LECHAT, M., 1998. Comparison of bacteriological qualities of various egg yolk sources and the in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents. *Theriogenology*, **50**: 699–706.

DUNCAN, D.B., 1955. Multiple range and multiple F-test. *Biometrics*, **11**: 1-42.

DE PAZ, P., ESTESO, M.C., ALVAREZ, M., MATA, M., CHAMORRO, C.A. AND ANEL, L., 2010. Development of extender based on soybean lecithin for its application in liquid ram semen. *Theriogenology*, **74**: 663–671.

EL-KERABY, F.E., OSMAN, K.T., GANAH, H.B. AND EL-

SIEFY, E.M., 2010. Soymilk-based extender for cryopreservation of bovine semen. *J. Anim. Poult. Prod*., **2**: 61 – 69.

FOROUZANFAR, M., SHARAFI, M., HOSSEINI, S.M., OSTADHOSSEINI, S., HAJIAN, M., HOSSEINI, L., ABEDI, P., NILI, N., RAHMANI, R.H. AND NASR-ESFAHANI, M.H., 2010. *In vitro* comparison of egg yolk–based and soybean lecithin–based extenders for cryopreservation of ram semen. *Theriogenology*, **73**: 480–487.

GIL, J., LUNDHEIN, N., SODERQUIST, L. AND MARTINES-RODRIGUEZ, H., 2003. Influence of extender, temperature, and addition glycerol on post-thaw sperm parameters in ram semen. *Theriogenology*, **59**: 1241–1255.

GRAHAM, J.K. AND FOOTE, R.H., 1987. Effect of several lipids, fatty acyl chain length and degree of unsaturation on the motility of bull spermatozoa after cold shock and freezing. *Cryobiology*, **24**: 42-52.

JEYENDRAN, R.S., VANDER-VEN, H.H., PEREZ-PELAEZ, M., CRABO, B.G. AND ZANEVLD, L.J.D., 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characters. *J. Repord. Fertil*., **70**: 219-228.

KASIMANICKAM, R., KASIMANICKAM, V., TIBRACY, A. AND PELZER, K., 2011. Effect of semen extenders on sperm parameters of ram semen during liquid storage at 4°C. *Small Rum. Res.*, **99**: 208-213.

KHAN, R.U., RAHMAN, Z.U., JAVED, I. AND MUHAMMAD, F., 2012. Effect of vitamins, probiotics and protein on semen traits in post-molt male broiler breeders. *Anim. Rep. Sci*., **135**:85-90.

MARCO-JIME´NEZ, F., PUCHADES, S., MOCE´, E., VIUDES-DE-CARTRO, M.P., VICENTE, J.S. AND RODRIGUEZ, M., 2004. Use of powdered egg yolk vs fresh egg yolk for the cryopreservation of ovine semen. *Reprod. Dom. Anim.*, **39**: 438–441

PAPA, F.O., FELÍCIO, G.B., MELO-O˜N, C.M., ALVARENGA, M.A., VITA, B.D., TRINQUE, C., PUOLI-FILHO, J.N.P. AND DELL'AQUA, Jr. J.A., 2011. Replacing egg yolk with soybean lecithin in the cryopreservation of stallion semen. *Anim. Reprod. Sci.*, **129**: 73-77.

SINGH, A.K., SINGH, V.K., NARWADE, B.M., MOHANTY, T.K. AND ATREJA, S.K., 2012. Comparative quality assessment of buffalo (*Bubalus bubalis*) semen chilled (5ºC) in egg yolk- and soya milk–based extenders. *Rep. Dom. Anim.*, **47**: 590-600.

STEEL, R.G.D., TORRIE, J.H. AND DIEKY, D.A., 1997. *Principles and procedures of statistics*. 3rd Ed. McGraw Hill Book Co. Inc., New York.

TRIMECHE, A., ANTON, M., RENARD, P., GANDEMER, G. AND TAINTURIER, D., 1997. Quail egg yolk: A novel cryoprotectant for the freeze preservation of Poitou Jackass sperm. *Cryobiology*, **34**: 385-393.

ZHANG, S.S., HU, J.H., LI, Q.W., JIANG, Z.L. AND XIAO-YING, Z., 2009. The croyoprotective effects of soybean lecithin on boar spermatozoa quality. *Afr. J. Biotechnol.*, **8**: 6476-6480.

(Received 16 August 2013, revised 11 September 2013)