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Effect of Bovine Serum Albumin on Motility, Plasmalemma, Viability and Chromatin Integrity of Buffalo Bull Spermatozoa

Shamim Akhter,[1]* Bushra Allah Rakha,[2] Razia Iqbal[3] and Muhammad Sajjad Ansari[3]
[1]*Animal Physiology Laboratory, Department of Zoology, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi 46300, Pakistan*
[2]*Department of Wildlife Management, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi 46300, Pakistan*
[3]*Department of Zoology, University of Gujrat, Hafiz Hayat Campus, Gujrat 50700, Pakistan*

Abstract.- The present work was designed to study the effect of different concentrations of bovine serum albumin (BSA) in extender on post-thaw quality of buffalo spermatozoa. Semen was collected from three buffalo bulls with artificial vagina (42°C) for three weeks (replicates). Qualifying semen ejaculate from each bull was split into five aliquots and diluted in *tris*-citric egg yolk extender containing either 0.0 (control) or 1.0 or 2.0 or 3.0 or 4.0 mg/ml BSA. Diluted semen was cooled to 4°C in 2 h and equilibrated for 4 h at the same temperature. Cooled semen was filled in 0.5 ml plastic straws at 4°C, kept over liquid nitrogen vapours (5cm) for 10 min and then plunged in the liquid nitrogen for storage. Frozen straws were thawed at 37°C for 30 seconds in duplicate for the assessment of sperm motility, plasma membrane integrity, viability and chromatin damage at 0, 2 and 4 h post-thaw. Sperm motility and chromatin damage remained similar in all experimental extenders at 0, 2 and 4 h post-thaw. However, higher ($P>0.05$) values of sperm chromatin damage were observed in extender without BSA supplementation. Sperm plasma membrane integrity and viability were observed higher ($P<0.05$) in extenders containing BSA compared to control at 0 h post-thaw. However, aforementioned parameters were similar in all extenders at 2 and 4 h post-thaw. The results of this study suggest protective role of BSA in cryopreservation of buffalo semen and needs further investigations.

Keywords: Buffalo semen, bovine serum albumin, sperm viability, chromatin damage.

INTRODUCTION

Cryopreservation of the buffalo semen is associated with oxidative stress that reduces the viability of semen upto 50% (Andrabi, 2009). Freeze-thawing process accelerates the production of reactive oxygen species (ROS) molecules (Baumber *et al*., 2005) due to plasma membrane lipid peroxidation (Kankofer *et al*., 2005). These excessive ROS molecules can deteriorate the quality (motility, plasma membrane and acrosomal integrity and DNA integrity) of buffalo semen at higher rate compared to cattle bull spermatozoa (Nair *et al*., 2006; Kumaresan *et al*., 2005; 2006; Garg *et al*., 2008) which may be due to higher contents of polyunsaturated phospholipids present in buffalo sperm membrane (Sansone *et al*., 2000; Andrabi, 2009).

Buffalo semen has natural defensive system against oxidative stress consisting of enzymatic and non-enzymatic compounds with antioxidant activity which protects the sperm from ROS molecules (Andrabi, 2009). It is known that indigenous antioxidant system of semen is insufficient (Baumber *et al*., 2005; Nichi *et al*., 2006) to protect the sperm from *in vitro* oxidative stress induced lipid peroxidation in buffalo semen (Nair *et al*., 2006). Freeze-thawing cycles also reduce the levels of indigenous antioxidants in bovine semen (Bilodeau *et al*., 2000; Stradaioli *et al*., 2007). This is the reason extra antioxidant supplements are required in semen extender to protect the motility, plasma membrane integrity, viability and DNA integrity of buffalo spermatozoa (Andrabi, 2009; Ansari *et al*., 2010, 2011; Kumar *et al*., 2011).

Bovine serum albumin (BSA), a highly soluble protein naturally occurs in mammalian semen that can protect the cell from harmful effects of free radicals in oxidative stress (Fukuzawa *et al*., 2005; Roche *et al*., 2008). Therefore, it was hypothesized that addition of BSA in extender may improve the motility, plasma membrane integrity,

* Corresponding author: shamim@uaar.edu.pk
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viability and DNA integrity of buffalo semen. This study was designed to study the effect of BSA in extender on post-thaw quality of buffalo bull spermatozoa.

MATERIALS AND METHODS

Extender preparation

The stock extender was prepared using *tris*-citric acid as a buffer, consisted of 1.56% citric acid, 3.0% *tris*–(hydroxymethyl)-amino methane 0.2% fructose, 7% glycerol and 20% egg yolk in distilled water (Akhter *et al*., 2010). BSA was added to stock extenders at the rate of 1, 2, 3 and 4mg/ml to make experimental extenders, while extender without any supplement was kept as control.

Semen collection and initial evaluation

Two consecutive ejaculates were collected from three Nili-Ravi buffalo bulls with artificial vagina for a period of three weeks (replicate). After collection, semen was immediately transferred to laboratory for initial evaluation. Sperm progressive motility was assessed microscopically (200X) at 37°C and concentration was determined with bovine photometer ACCUCELL (IMV, France) at 530nm wavelength. The semen samples having volume >1ml, concentration >0.5 billion/ml and motility ≥60% were used for further processing (Akhter *et al*., 2008).

Semen processing

Semen aliquots were diluted with one of the experimental and control extenders at the rate 50 × 10^6 motile spermatozoa/ml approximately. Extended semen were cooled to 4°C in 2 h, equilibrated for 4 h at 4°C. Semen was then filled in 0.5ml French straws with suction pump at 4°C in the cold cabinet unit, kept on liquid nitrogen (5cm) vapours for 10 min (Andrabi *et al*., 2008) and then plunged in liquid nitrogen. For each extender, semen straws were thawed in duplicate at 37°C for 30 seconds for assessment of post-thaw semen quality at 0, 2 and 4 h of incubation at 37°C.

Sperm progressive motility

A drop of semen was placed on pre-warmed glass slide and cover slipped. Visual motility (%) was assessed as described in earlier section.

Sperm plasma membrane integrity

Sperm plasma membrane integrity was evaluated using supravital hypo-osmotic swelling test. In brief, hypo-osmotic swelling (HOS) assay was performed as described by Jeyendran *et al*. (1984). After the incubation of HOS, equal drop of HOS solution and eosin [0.5% (w/v), sodium citrate 2.92%] was placed on a warm slide, mixed for 10 seconds and cover slipped before the evaluation for plasma membrane integrity under phase contrast microscope at 400X. A total of one hundred spermatozoa were observed in at least five different fields. Clear heads and tails and swollen tails were considered intact with biochemically active sperm membranes, while pink heads and tails and unswollen tails were considered disrupted, inactive sperm membranes.

Sperm viability

Sperm viability (live sperm with intact acrosome) was assessed by dual staining procedure as described by Kovacs and Foote (1992). Supravital stain Trypan-blue was used to distinguish live and dead spermatozoa while Giemsa stain was used to evaluate the integrity of the acrosome membrane. Equal drops of trypan-blue and semen were placed on glass slide, mixed quickly and air-dried before the fixation in formaldehyde-neutral red for 5 min. Then slides were rinsed in running distilled water before applying Giemsa stain (7.5%) for 4 h. The slides were rinsed, air-dried and mounted with Canada Balsam. Trypan-blue penetrated non-viable, dead spermatozoa with disrupted membrane, which appeared stained in blue, whereas live and intact spermatozoa appeared unstained. Giemsa stain accumulated in spermatozoa with an intact acrosome (staining the acrosome region in purple). Two hundred spermatozoa were evaluated in at least five different fields in each smear under phase contrast microscope at 1000X.

Sperm chromatin damage

Sperm chromatin damage was assessed using toluidine blue stain as described by Mello (1982) at 0, 2 and 4 h post thaw incubation at 37°C. For this,

semen was air dried on glass slide and fixed in freshly made 96% ethanol-acetone (1:1) at 4°C for 30 min, hydrolyzed in 4.0N HCl at 25°C for 30 min. Then slides were rinsed three times in distilled water for 2 min each and smears were stained with 0.05% toluidine blue for 10 min. The staining buffer consisted of 50% citrate phosphate (Mcllvain buffer, pH 3.5). Two hundred spermatozoa were evaluated in at least five different fields using light microscopy (1000X) in each smear. Sperm heads with intact chromatin stained light blue and sperm with damaged chromatin stained violet or purple.

Statistical analysis

Effect of BSA on different semen quality parameters *viz*; motility, plasma membrane integrity, viability and chromatin damage were analyzed by the analysis of variance (ANOVA) in randomized complete block design. When the F-ratio was significant ($P<0.05$), LSD test was used to compare the treatment means (MSTAT-C, Version 1.42).

RESULTS AND DISCUSSION

The study was conducted to determine the effect of different concentrations *viz.*, 1.0, 2.0, 3.0 and 4.0 mg/ml of BSA in extender on post-thaw quality of buffalo bull spermatozoa.

Sperm progressive motility

The data on effect of BSA in extender on progressive motility of buffalo bull spermatozoa at 0, 2 and 4 h of incubation at 37°C post-thaw are presented in Figure 1A. Sperm progressive motility did not differ ($P>0.05$) in all experimental extenders at 0, 2 and 4 h post-thaw at 37°C. Similarly, supplementation of BSA in extender did not improve the kinetics parameters of equine semen during liquid storage (Ball *et al.*, 2001). Contrary, studies on bovine (Uysal *et al.*, 2007; caprine (Amidi *et al.*, 2010; Anghel *et al.*, 2010), equine (Kreider *et al.*, 1985; Klem *et al.*, 1986), ovine (Uysal *et al.*, 2007) and lagomorph semen (Alvarez and Storey, 1983) reported improvement in sperm motility and motion characteristics through BSA inclusion in extenders. However, the exact mechanism through which it stimulates the motility

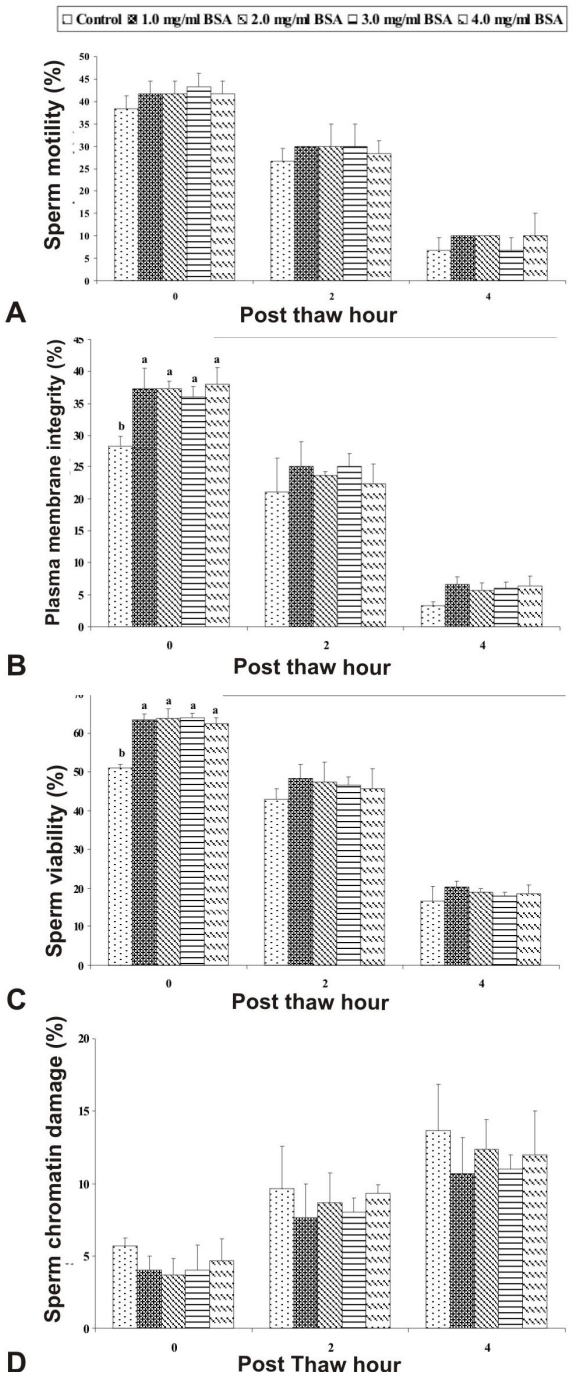

Fig. 1. Effect of bovine serum albumin in semen extender on progressive motility in % (A), plasma membrane integrity (B), viability (C), and chromatin damage (D) of buffalo bull spermatozoa at 0, 2 and 4 h post-thaw at 37°C. Different letters above the bars differ significantly ($P < 0.05$) at a given time.

is unknown (Klem *et al*., 1986; Harrison *et al*., 1982).

Sperm plasma membrane integrity

Sperm plasma membrane integrity is of particular importance due to its involvement in signaling and interaction with surrounding environment (Akhter *et al*., 2010). The data on effect of BSA in extender on plasma membrane integrity of buffalo bull spermatozoa at 0, 2 and 4 h of incubation at 37°C post-thaw is presented in Figure 1B. Percentage of sperm with intact plasma membrane were higher ($P<0.05$) in extender containing BSA 1.0 (37.3±3.2), 2.0 (37.3±1.2), 3.0 (36.0±1.7) and 4.0mg/ml (38.0±2.6) compared to control (28.3±1.5) at 0 h post-thaw. However, sperm plasma membrane integrity was recorded similar in all experimental extenders at 2 and 4 h post-thaw. Our findings are inline with the results for previous studies on bovine (Uysal *et al*., 2007) and caprine (Anghel *et al*., 1983) semen in which higher sperm plasma membrane integrity was reported after the addition of BSA in extender. It is believed that BSA addition reduce the lipid peroxidation of membrane system that resulted in higher membrane integrity (Lewis *et al*., 1997).

Sperm viability

The data on effect of BSA in extender on viability (live sperm with intact acrosome) of buffalo bull spermatozoa at 0, 2 and 4 h of incubation at 37°C post-thaw are presented in Figure 1C. Percentage of live sperm with intact acrosome was observed higher ($P<0.05$) in extender containing BSA 1.0 (63.3±1.5), 2.0 (63.7±2.5), 3.0 (64.0±1.0) and 4.0mg/ml (62.3±1.5) compared to control (51.0±1.0) at 0 h post-thaw. However, sperm plasma membrane integrity was recorded similar in all experimental extenders at 2 and 4 h post-thaw. It is known that oxidative stress during cryopreservation damages the acrosome functionality (Bailey *et al*., 2000). Higher percentages of viable sperm have been observed in bovine and caprine semen cryopreserved in extender containing BSA (Uysal and Bucak, 2007; Amidi *et al*., 2010; Anghel *et al*., 2010). It is suggested that improvement in sperm viability by BSA inclusion in extender is associated with reduction in free radicals produced from mitochondria, plasma membrane lipid peroxidation and dead/abnormal spermatozoa during freeze-thawing process (Cotran *et al*., 1989).

Sperm chromatin damage

Cryopreservation of buffalo spermatozoa resulted in reduced total antioxidant potential and higher DNA damage after thawing (Kumar *et al*., 2011). The data on effect of BSA in extender on chromatin damage of buffalo bull spermatozoa at 0, 2 and 4 h of incubation at 37°C post-thaw are presented in Figure 1D. Sperm chromatin damage did not differ ($P>0.05$) in all experimental extenders at 0, 2 and 4 h post-thaw at 37°C. However, higher values of chromatin damage were observed in control extender.

CONCLUSION

It is concluded that BSA may have protective role in cryopreservation of buffalo semen for preserving plasma membrane, viability and chromatin integrity and needs further investigation.

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REFERENCES

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.

AKHTER, S., ANSARI, M.S., RAKHA, B.A., ANDRABI, S.M.H. AND ULLAH, N., 2010. Cryopreservation of buffalo (*Bubalus bubalis*) semen in Bioxcell® extender. *Theriogenology*, **74**: 951-955.

ALVAREZ, J.G. AND STOREY, BT., 1983. Taurine, hypotaurine, epinephrine and albumin inhibits lipid peroxidation in rabbit spermatozoa and protect against loss of motility. *Biol. Reprod.*, **29:** 548-555.

AMIDI, F., FARSHAD, A. AND KHOR, A.K., 2010. Effects of cholesterol-loaded cyclodextrin during freezing step of cryopreservation with TCGY extender containing bovine serum albumin on quality of goat spermatozoa. *Cryobiology*, **61:** 94-99.

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, S., 2008. Duck egg yolk in extender improves the freezability of buffalo (*Bubalus bubalis*) bull spermatozoa. *Anim. Reprod. Sci.*, **104**: 427-433.

ANGHEL, A., ZAMFIRESCU, S., DRAGOMIR, C., NADOLU, D., ELENA, S. AND FLORICA, B., 2010. The effects of antioxidants on the cytological parameters of cryopreserved buck semen. *Rom. Biotech. Lett.*, **15:** 26-32.

ANSARI, M.S., RAKHA, B.A., ULLAH, N., ANDRABI, S.M.H., IQBAL, S., KHALID, M. AND AKHTER, S., 2010. Effect of exogenous glutathione in extender on the freezability of Nili-Ravi buffalo (*Bubalus bubalis*) bull spermatozoa. *Anim. Sci. Pap. Rep.*, **28**: 235-244.

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

BAILEY, J.L., BILODEAU, J.F. AND CORMIER, N., 2000. Semen cryopreservation in domestic animals: A damaging and capacitating phenomena. *J. Androl.*, **22:** 508-515.

BALL, B.A., MEDINA, V., GRAVANCE, C.G. AND BAUMBER, J., 2001. Effect of antioxidants on preservation of motility, viability and acrosomal integrity of Equine spermatozoa during storage at 5^{o}C. *Theriogenology*, **56:** 577-589.

BAUMBER, J., BALL, B.A. AND LINFOR, J.J., 2005. Assessment of the cryopreservation of equine spermatozoa in the presence of enzyme scavengers and antioxidants. *Am. J. Vet. Res.*, **66**: 772-779.

BILODEAU, J.F., BLANCHETTE, S., GAGNON, C. AND SIRAD, M.A., 2000. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol. Reprod. Develop.*, **55**: 282-288.

COTRAN, R.S., KUMAR, V. AND ROBINS, S.L., 1989. *Pathologic basis of diseases*, 4th edn, W. B. Saunders Co. Philadelphia, 9-16.

FUKUZAWA, K., SAITOH, Y., AKAI, K., KOGURE, K., UENO, S., TOKUMURA, A., OTAGIRI, M. AND SHIBATA, A., 2005. Antioxidant effect of bovine serum albumin on membrane lipid peroxidation induced by iron chelate and superoxide. *Biochim. biophys. Acta*, **1668**: 145-155.

GARG, A., KUMARESAN, A. AND ANSARI, M.R., 2008. Effect of hydrogen peroxide on fresh and cryopreserved buffalo sperm functions during incubation at 37^{o}C *in vitro*. *Reprod. Domest. Anim.*, **10**: 1439-1445.

HARRISON, R.A., DOTT, H.M. AND FOSTER, G.C., 1982. Bovine serum albumin, sperm motility, and the 'dilution effect. *J. exp. Zool.*, **222**: 81–88.

JEYENDRAN, R.S., VAN DER VEN, H.H., PEREZ-PELAEZ, M., CRABO, B.G. AND ZANEVELD, L.J.D., 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J. Reprod. Fert.*, **70**: 219-228.

KANKOFER, M., KOLM, G. AND AURICH, J., 2005. Activity of glutathione peroxidase, superoxide dismutase and catalase and lipid peroxidation intensity in stallion semen during storage at 5^{o}C. *Theriogenology*, **63**: 1354-1365.

KLEM, Jr. M.E., KREIDER, J.L., PRUITT, J.B. AND POTTER, G.D., 1986. Motility and fertility of equine spermatozoa extended in bovine serum albumin and sucrose. *Theriogenology*, **26:** 569-576.

KOVACS, A. AND FOOTE, R.H., 1992. Viability and acrosome staining of bull, boar and rabbit spermatozoa. *Biotech. Histochem.*, **67**: 119-124.

KREIDER, J.L., TINDALL, W.C. AND POTTER, G.D., 1985. Inclusion of bovine serum albumin in semen extenders to enhance maintenance of stallion sperm viability. *Theriogenology*, **23**: 399-408.

KUMAR, R., MOHANARAO, G.J. AND ARVIND-ATREJA, S.J., 2011. Freeze-thaw induced genotoxicity in buffalo (*Bubalus bubalis*) spermatozoa in relation to total antioxidant status. *Mol. Biol. Rep.*, **38**: 1499-506

KUMARESAN, A., ANSARI, M.R. AND GARG, A., 2005. Modulation of post thaw sperm functions with oviductal proteins in buffaloes. *Anim. Reprod. Sci.*, **90**: 73-84.

KUMARESAN, A., ANSARI, M.R., GARG, A. AND KATARIA, M., 2006. Effect of oviductal proteins on sperm functions and lipid peroxidation levels during cryopreservation in buffaloes. *Anim. Reprod. Sci.*, **93**: 264-257.

LEWIS, S.E., STERLING, S., YOUNG, I.S. AND THOMPSON, W., 1997. Comparison of individual antioxidants and total antioxidant capacity of sperm and seminal plasma in fertile and infertile men. *Fertil. Steril.*, **67**: 142-147.

MELLO, M.L.S., 1982. Induced metachromasia in bull spermatozoa. *Histochem. Cell Biol.*, **3:** 387-392.

NAIR, S.J., BRAR, A.S., AHUJA, C.S., SANGHA, S.P. AND CHAUDHARY, K.C., 2006. Comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. *Anim. Reprod. Sci.*, **96**: 21-29.

NICHI, M., BOLS, P.E.J., ZUGE, R.M., BARNABE, V.H., GOOVAERTS, I.G.F., BARNABE, R.C. AND CORTADA, C.N.M., 2006. Seasonal variation in semen quality in *Bos indicus* and *Bos taurus* bulls raised under tropical conditions. *Theriogenology*, **66**: 822-828.

ROCHE, M., RONDEAU, P., SINGH, N.R., TARNUS, E.

AND BOURDON, E., 2008. The antioxidant properties of serum albumin. *FEBS Lett.*, **582**: 1783-1787.

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

STRADAIOLI, G., NORO, T., SYLLA, L. AND MONACI, M., 2007. Decrease in glutathione (GSH) content in bovine sperm after cryopreservation: Comparison between two extenders. *Theriogenology*, **67**: 1249-1255.

UYSAL, O. AND BUCAK, M.N., 2007. Effect of oxidized serum albumin, cysteine and lycopene on the quality of frozen thawed ram semen. *Acta Vet. Brno.*, **76**: 383-390.

UYSAL, O., BUCAK, M.N., YAVAS, I. AND VARISLI, O., 2007. Effect of various antioxidants on the quality of frozen-thawed bull semen. *J. Anim. Vet. Adv.*, **6:** 1362-1366.

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