

Glutathione Addition in Tris-citric Egg Yolk Extender Improves the Quality of Cooled Buffalo (*Bubalus bubalis*) Bull Semen

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Abstract.- The present study was designed to see whether adding glutathione to tris-citric egg yolk extender improves the quality of buffalo bull spermatozoa stored at 5°C for five days. Semen collected from Nili-Ravi buffalo (*Bubalus bubalis*) bulls (n = 5) was diluted (37°C; 10 x 10⁶ motile spermatozoa per ml) with extender having glutathione (0.0, 0.5, 1.0, 2.0 and 3.0mM). The extended semen was cooled from 37°C to 5°C in 2 hours and stored at 5°C for five days. Post-extension spermatozoal quality viz; motility, viability, plasma membrane integrity and normal acrosomes were studied at 1st, 3rd and 5th day of storage. There was no difference (P > 0.05) in motility (%), viability (%), plasma membrane and acrosomal integrity (%) of sperm due to glutathione at 1st day after dilution. Highest (P < 0.05) sperm motility, viability and plasma membrane integrity was observed in extender containing glutathione 0.5 and 1.0mM at 3rd and 5th day of storage. Moreover, the percentage of spermatozoa with normal apical ridge was higher (P < 0.05) in extender containing glutathione 0.5mM at 3rd and 5th day of storage. It is concluded that glutathione (0.5-1.0mM) in tris-citric egg yolk extender improves the quality of buffalo bull spermatozoa stored at 5°C for five days of storage.

Keywords: Buffalo bull semen, antioxidant, glutathione, liquid storage.

INTRODUCTION

Artificial insemination (AI) is an assisted reproductive technique used to improve the genetic potential of livestock breeds and exploiting the germplasm from superior sires (Vishwanath and Shannon, 1997). AI is practiced in farm animals by the use of semen in the liquid or frozen state (Weitze, 1991; Maxwell and Watson 1996; Vishwanath and Shannon, 2000). Although frozen bovine semen has been widely used all over the world, the use of liquid semen for AI has its own advantages. AI with liquid semen has resulted in higher fertility rates (Anzar *et al.*, 2003; Sharma and Sahni, 1988; Shannon and Vishwanath, 1995) with lower numbers of spermatozoa (Vishwanath *et al.*, 1996).

Commonly, tris, citrate and milk based buffers are used to preserve buffalo semen at chilling

(approximately 5°C) temperatures and are capable of maintaining the quality and fertility of stored semen up to three days (Sansone *et al.*, 2000). Oxidative stress during liquid storage is a major limiting factor which degrades the sperm quality (Baumber *et al.*, 2000; Vishwanath and Shannon, 2000; Sikka, 2004; El-Sissy *et al.*, 2007) and fertility (Maxwell and Salamon, 1993; Vishwanath and Shannon, 1997) through production of reactive oxygen species molecules (ROS; superoxide, hydroxyl, hydrogen peroxide, nitric oxide, peroxynitrite) (Baumber *et al.*, 2000). These products increase the lipid peroxidation (LPO) levels of unsaturated fatty acids in the plasma membrane (Kadirvel *et al.*, 2008; El-Sissy *et al.*, 2007). Respiration for energy production and the presence of dead/damaged spermatozoa are major sources of ROS production (Vishwanath and Shannon, 1997) during liquid storage at ambient temperature. It is well documented that during the liquid storage of buffalo semen, oxidative stress accelerates the over production of ROS, resulting in higher LPO levels in the cell membrane (Nair *et al.*, 2006; El-Sissy *et al.*, 2007; Kadirvel *et al.*, 2008).

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The resulting oxidative stress is likely to cause mitochondrial dysfunction and deterioration of sperm motility, viability, plasmalemma integrity and sperm morphology (Nair *et al.*, 2006; El-Sissy *et al.*, 2007; Garg *et al.*, 2008; Kadirvel *et al.*, 2008). There are neither protections nor repair systems for sperm integrity and therefore the addition of exogenous protectants is required to reduce this ROS-mediated damage (Vishwanath and Shannon, 1997).

Glutathione, a naturally occurring tri-peptide in semen plays an important role in scavenging reactive oxygen intermediates and other radicals with the help of the glutathione reductase/peroxidase cycle (Meister and Anderson, 1983). Glutathione addition to semen extender is known to improve the quality of bovine (Foote *et al.*, 2002; Munsu *et al.*, 2007), ovine (Bucak and Tekin, 2007) and swine semen (Funahashi and Sano, 2005) during liquid storage. To our knowledge, no information is available on the effect of glutathione on the quality of dairy buffalo bull semen during liquid storage at 5°C for five days. The present study was, therefore, planned to evaluate the effect of glutathione in tris-citric egg yolk extender on the quality (sperm motility, viability, plasma membrane integrity and normal apical ridge) of cooled buffalo (*Bubalus bubalis*) bull semen.

MATERIALS AND METHODS

Preparation of extenders

Stock extender consisted of tris-citric egg yolk buffer (1.56 g citric acid (Fisher Scientific, UK) and 3.0 g tris-(hydroxymethyl)-aminomethane (Research Organics, USA); distilled water 80 ml; fructose (Scharlau, Spain) 0.2% w/v; egg yolk 20%). Antibiotic, gentamycin sulphate (Reckitt Benckiser, Pakistan) at the rate of 500 µg/ml, tylosin tartrate (VMD, Belgium) at the rate of 100 µg/ml, lincomycin hydrochloride (Pharmacia & Upjohn, Belgium) at the rate of 300 µg/ml, and spectinomycin hydrochloride (Pharmacia & Upjohn, Belgium) at the rate of 600 µg/ml were added to extender. Stock extender was divided into five aliquots and supplemented with glutathione (GSH) @ 0.0, 0.5, 1.0, 2.0 or 3.0mM to prepare five

experimental extenders as control, 0.5mM GSH, 1.0mM GSH, 2.0mM GSH and 3.0mM, respectively.

Semen collection and initial evaluation

Semen was collected with an artificial vagina at 42°C from five adult Nili-Ravi buffalo bulls (*Bubalus bubalis*) at Semen Production Unit Qadirabad, Sahiwal, Pakistan at weekly intervals for 3 weeks (replicates). Semen samples were transferred to the laboratory immediately for initial examination (volume, motility, concentration). Sperm motility (%) was assessed (X 200) using phase contrast microscopy. Sperm concentration was determined using a Neubauer haemocytometer. Semen ejaculates were split into five aliquots for dilution in different experimental extenders.

Semen processing

Semen aliquots were diluted to 10×10^6 motile spermatozoa ml⁻¹ at 37°C with each of the five experimental extenders upto 20 ml. Diluted semen was cooled to 5°C in 2 hrs at 0.275°C min⁻¹ and stored for five days at 5°C.

Sperm functional assays

To assess the functional status of the sperm, assays were performed at 1st, 3rd and 5th day of storage (Akhter *et al.*, 2008).

Sperm motility

A drop of semen sample (5 µl) was placed on pre-warmed (37°C) glass slide and progressive motility was assessed using phase contrast microscopy at X 400; 37°C (Akhter *et al.*, 2008).

Sperm viability

Viability of buffalo sperm (live/dead) was assessed with 0.4% trypan blue stain as described by Brito *et al.* (2003). Semen samples (5µl) and equal amount of trypan blue solution was mixed with the cover slip edge on glass slide and air dried for 10 min for fixation. The fixed slides were examined using phase contrast microscopy (X 1000; oil immersion). Spermatozoa stained blue were categorized as dead while unstained were regarded as live. A total of hundred spermatozoa per extender/replicate were counted.

Sperm plasma membrane intactness

Plasma membrane integrity (PMI) of buffalo bull spermatozoa was determined by the hypo-osmotic swelling (HOS) test (Jeyendran *et al.*, 1984). The HOS test solution contained sodium citrate (0.73 g) and fructose (1.35 g) in 100 ml distilled water; osmotic pressure ~ 190 mOsmol Kg⁻¹). For evaluation of buffalo sperm tail plasmalemma integrity, each sample (50 μ l) was mixed with HOS solution (500 μ l) and incubated for 30-40 min at 37°C. Samples of diluted spermatozoa (5 μ l) were placed on glass slides and examined by phase contrast microscopy (X 400) for the assessment of sperm PMI. A total of one hundred spermatozoa were classified as having intact or damaged plasma membranes as indicated by the occurrence of tail swelling and coiling (Andrabi *et al.*, 2008).

Normal apical ridge

Samples of diluted spermatozoa (100 μ l) were fixed in 500 μ l of 1% formal citrate (2.9 g tri-sodium citrate dehydrate, 1 ml of 37% solution of formaldehyde, dissolved in 100 ml of distilled water). Normal acrosomes characterized by the presence of normal apical ridge (NAR) were recorded by counting one hundred spermatozoa using phase contrast microscopy (X 1000) (Andrabi *et al.*, 2008).

Data analysis

The data are presented as means \pm SD. Effects of glutathione in tris-citric egg yolk extender on different parameters were analyzed by the analysis of variance (ANOVA). When the F-ratio was found to be significant ($P < 0.05$), Duncan multiple range test was used; to compare different treatment means (MINITAB[®] Release 12.22, 1998).

RESULTS

Effect of glutathione on motility (%) of buffalo bull spermatozoa

The data on sperm motility of buffalo bull spermatozoa are summarized in Figure 1. Sperm motility did not differ in all experimental extenders at 1st day of storage. Higher ($P < 0.05$) sperm motility (%) was observed at 3rd and 5th day of storage in extender containing glutathione 0.5mM (56.7 \pm 2.9, 46.7 \pm 2.9) and 1.0mM (55.0 \pm 0.0,

46.7 \pm 2.9) as compared to extender containing glutathione 3.0mM (48.3 \pm 2.9, 33.3 \pm 7.6) and control (48.3 \pm 2.9, 35.0 \pm 5.0).

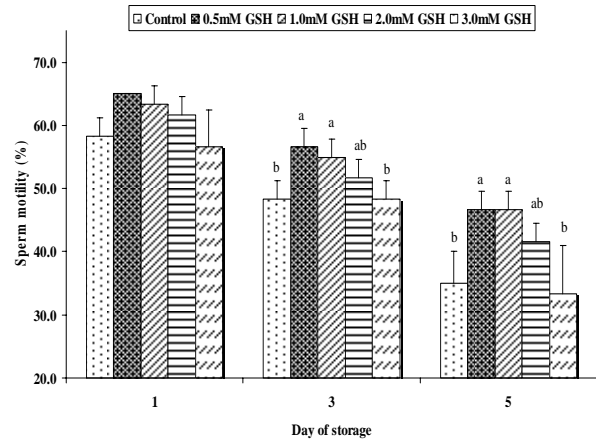


Fig. 1. Effect of glutathione (GSH) in tris-citric egg yolk extender on the motility of buffalo bull spermatozoa at 1st, 3rd, and 5th day of storage at 5°C. Bars with different letters differ ($P < 0.05$) at a given day.

Effect of glutathione on viability (%) of buffalo bull spermatozoa

The data on the viability of buffalo bull spermatozoa is presented in Figure 2. In all the five experimental extenders viability of buffalo bull spermatozoa was similar at 1st day of storage. Percentage of viable sperm was higher ($P < 0.05$) at 3rd and 5th day of storage in extender containing glutathione 0.5 mM (76.7 \pm 2.9, 66.7 \pm 2.1) and 1.0mM (75.0 \pm 0.0, 66.7 \pm 2.1) as compared to extender containing glutathione 3.0mM (68.3 \pm 3.1, 55.0 \pm 6.6) and control (68.3 \pm 2.1, 58.3 \pm 1.5).

Effect of glutathione on plasma membrane integrity (%) of buffalo bull spermatozoa

The data on sperm plasma membrane integrity is presented in Figure 3. Sperm plasma membrane integrity of buffalo spermatozoa did not differ due to glutathione in extenders at 1st day of storage. Sperm with intact plasma membrane was higher ($P < 0.05$) at 3rd and 5th day of storage in extender containing glutathione 0.5 mM (71.7 \pm 2.9, 61.7 \pm 2.9) and 1.0mM (70.0 \pm 0.0, 61.7 \pm 2.9) as compared to extender containing glutathione 3.0mM (63.3 \pm 2.9, 48.3 \pm 7.6) and control (63.3 \pm 2.9, 50.0 \pm 5.0).

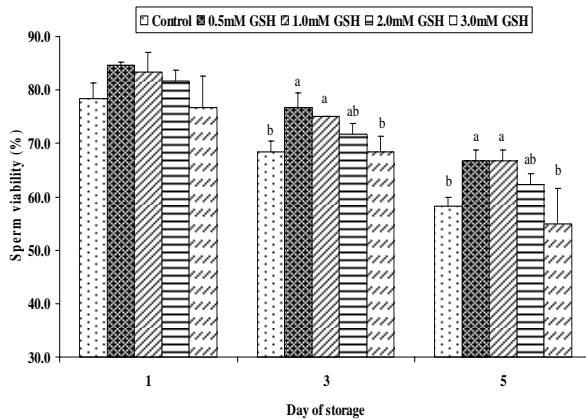


Fig. 2. Effect of glutathione (GSH) in tris-citric egg yolk extender on the viability of buffalo bull spermatozoa at 1st, 3rd, and 5th day of storage at 5°C. Bars with different letters differ ($P < 0.05$) at a given day.

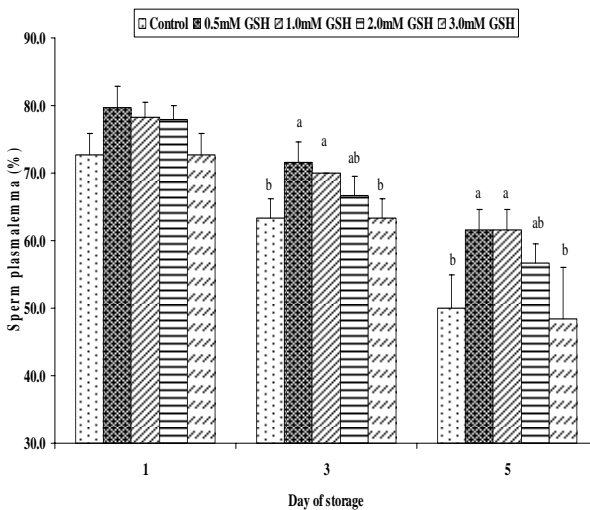


Fig. 3. Effect of glutathione (GSH) in tris-citric egg yolk extender on the plasma membrane integrity of buffalo bull spermatozoa at 1st, 3rd, and 5th day of storage at 5°C. Bars with different letters differ ($P < 0.05$) at a given day.

Effect of glutathione on acrosomal integrity (%) of buffalo bull spermatozoa

The data on the acrosomal integrity of buffalo bull spermatozoa is presented in Figure 4. Percentage of buffalo bull spermatozoa with intact acrosomes did not differ in all the experimental extenders at 1st day of storage. Sperm with intact

acrosomes were higher ($P < 0.05$) at 3rd and 5th day of storage in extender containing glutathione 0.5 mM (91.3 ± 0.6 , 86.3 ± 0.6) as compared to extender containing glutathione 3.0mM (88.3 ± 1.5 , 84.0 ± 1.0) and Control (89.0 ± 1.0 , 84.0 ± 1.0).

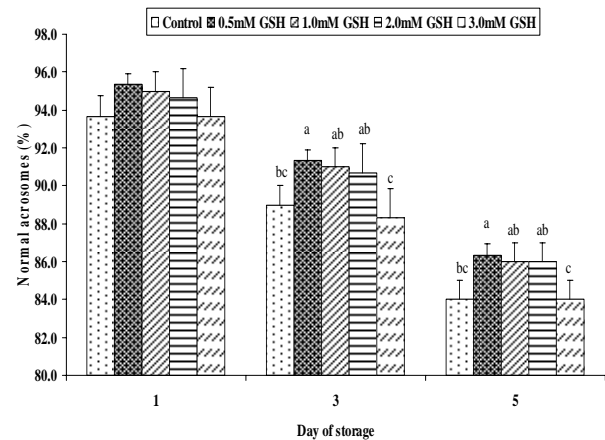


Fig. 4. Effect of glutathione (GSH) in tris-citric egg yolk extender on the normal apical ridge of buffalo bull spermatozoa at 1st, 3rd, and 5th day of storage at 5°C. Bars with different letters differ ($P < 0.05$) at a given day.

DISCUSSION

In present study, glutathione addition (0.5-1.0mM) in extender improved the quality (motility, viability, plasma membrane and acrosomal integrity) of cooled buffalo (*Bubalus bubalis*) bull semen; however, the higher concentrations (>1.0mM) were not beneficial to improve the semen quality.

It is reported that chilling of buffalo semen resulted in decreased motility associated with reduced antioxidant activity and higher ROS production (El-Sissy *et al.*, 2007). In present study there was no difference in motility of buffalo bull spermatozoa in all experimental extenders at 1st day of storage. However, on 3rd and 5th day of storage sperm motility was 8-10% higher in extender containing glutathione 0.5 mM and 1.0 mM as compared to control. As definite positive relationship has been reported between level of glutathione and sperm motility (Gadea *et al.*, 2004; Stradaioli *et al.*, 2007), it is suggested that higher motility of the buffalo bull spermatozoa in extender containing glutathione is the result of protection to

the sperm during liquid storage against oxidative stress and ROS deleterious effects. Other related studies on bovine (Munsi *et al.*, 2007; Foote *et al.*, 2002), swine (Funahashi and Sano, 2005) and ovine semen (Bucak and Tekin, 2007) also reported improvement in sperm motility after the addition of exogenous glutathione in extender at ambient temperature.

The number of viable sperm per dose significantly affected the fertility rates in the field (Andrabi *et al.*, 2006). Lower viability of the buffalo bull spermatozoa has been reported due to poor antioxidant activity and increased ROS production during liquid storage (El-Sissy *et al.*, (2007). In present study, no difference was observed in the viability of buffalo bull spermatozoa in all experimental extenders containing different concentrations of glutathione at 1st day of storage at 5°C. However, on 3rd and 5th day of storage sperm viability was better (8-10%) in extender containing glutathione 0.5 mM and 1.0 mM as compared to control. In two, similar studies on ram (Bucak and Tekin, 2007) and boar semen (Funahashi and Sano, 2005), glutathione supplementation of the semen extender resulted in higher percentage of viable spermatozoa stored at ambient temperature.

Plasma membrane integrity is essential for the exchange of metabolites with the environment (Silva and Gadella, 2006) and a biochemically active plasmalemma is necessary to complete the processes of capacitation, acrosome reaction and the oocyte penetration. During liquid storage of buffalo semen (El-Sissy *et al.*, 2007), oxidative stress accelerated the over production of the ROS, resulting in lipid peroxidation of the cell membrane. The resulting oxidative metabolites injured the spermatozoa chemically and physically using different pathways (Cotran *et al.*, 1989). Glutathione plays a vital role in scavenging reactive oxygen intermediates and other radicals with the help of glutathione reductase in cell (Meister and Anderson, 1983). In present study, plasma membrane integrity of buffalo bull spermatozoa did not differ due to experimental extenders at 1st day of storage. However, on 3rd and 5th day of storage, plasma membrane integrity was higher (10%) in extender containing glutathione 0.5 and 1.0 mM as compared to control. The results of our study are similar to the findings of previous

study on the preservation of ovine semen at 5°C in which glutathione addition resulted in a higher percentage of spermatozoa with an intact plasma membrane (Bucak and Tekin, 2007). The higher number of buffalo bull spermatozoa with intact plasma membrane in extender containing glutathione may be due to protection of sperm from membrane damage through glutathione by inhibiting the lipid peroxidation process (Sinha *et al.*, 1996; Gadea *et al.*, 2007).

Sperm acrosomal integrity is vital for the acrosomal reaction leading to fertilization (Bailey *et al.*, 2000) and its evaluation effectively predict the fertilization potential of the buffalo bull spermatozoa. It is pertinent to mention that chilling of buffalo semen resulted in lower intact acrosomes associated with decreased antioxidant activity and ROS production (El-Sissy *et al.*, 2007). Stradaioli *et al.*, (2007) suggested that reduction in non-capacitated viable bovine sperm after preservation in frozen state is due to decrease in glutathione contents. In our study acrosomal integrity of buffalo bull spermatozoa remained similar in all extenders at 1st day of storage. However, on 3rd and 5th day of storage, percentage of sperm with intact acrosomes was higher in extender containing glutathione 0.5mM as compared to control. In a similar study on bovine semen stored at refrigeration temperature for five days, higher number of spermatozoa with intact acrosome has been reported with the addition of glutathione in extender (Munsi *et al.*, 2007). A higher percentage of non-capacitated sperm with the supplementation of glutathione has been reported in swine (Funahashi and Sano, 2005) and bovine semen (Gadea *et al.*, 2007). It seems that, the disruption of the sperm plasma membrane was decreased by higher glutathione content in extender through reducing the lipid peroxidation/modulating the overproduction ROS.

It was observed that glutathione level beyond 0.5 mM was non-beneficial for protecting the quality of buffalo bull spermatozoa. The reason might be that the higher concentrations of glutathione are above the physiological requirements to maintain the quality of sperm. In conclusion, addition of glutathione (0.5-1.0mM) in tris-citric egg yolk extender improved the quality of cooled buffalo semen for five days of storage.

However, higher concentrations of glutathione > 1.0mM were non-beneficial for improvement of semen quality parameters. On the basis of these results further studies are required to identify the beneficial role of glutathione addition in semen extender on fertility rate in buffaloes.

REFERENCES

- 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.
- 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. Domest. Anim.*, **43**: 272-278.
- ANDRABI, S.M.H., SIDDIQUE, M., ULLAH, N. AND KHAN, L.A., 2006. Effect of reducing sperm number per insemination dose on fertility of cryopreserved buffalo bull semen. *Pakistan Vet. J.*, **26**: 17-19.
- 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.
- 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.
- BAUMBER, J., BALL, B.A., GRAVANCE, C.G., MEDINA, V. AND DAVIES-MOREL, M.C.G., 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential and membrane lipid peroxidation. *J. Androl.*, **21**: 895-902.
- BRITO, L.F.B., BARTH, A.D., BILODEAU-GOESEEL, S., PANICH, P.L. AND KASTELIC, J.P., 2003. Comparison of methods to evaluate the plasmalemma of bovine sperm and their relationship with in vitro fertilization rate. *Theriogenology*, **60**: 1539-1551.
- BUCAK, M.N. AND TEKIN, N., 2007. Protective effect of taurine, glutathione and trehalose on the liquid storage of ram semen. *Small Rum. Res.*, **73**: 103-108.
- COTRAN, R.S., KUMAR, V. AND ROBINS, S.L., 1989. *Pathologic basis of diseases*, 4th edn, W. B. Saunders Co. Philadelphia, 9-16.
- EL-SISSY, G.A., EL-NATTAT, W.S. AND EL-SHESHTAWY, R.I., 2007. Buffalo semen quality, antioxidants and peroxidation during chilling and cryopreservation. *J. Vet. Res.*, **11**: 55-61.
- FOOTE, R.H., BROCKETT, C.C. AND KAPROTH, M.T., 2002. Motility and fertility of bull sperm: a brief review. *Reprod. Fertil. Dev.*, **5**: 665-673.
- FUNAHASHI, H. AND SANO, T., 2005. Select antioxidants improve the function of extended boar semen stored at 10°C. *Theriogenology*, **63**: 1605-1616.
- GADEA, J., GUMBAO, D., NOVAS, S.C., ZQUEZ, F.A.Z., GRULLO, L.A. AND GARDO, J.C., 2007. Supplementation of the dilution medium after thawing with reduced glutathione improves function and the in vitro fertilizing ability of frozen-thawed bull spermatozoa. *Intl. J. Androl.*, **31**: 1-10.
- GADEA, J., SELLES, E., MARCO, M.A., COPY, P., MATAS, C., ROMAR, R. AND RUIZ, S., 2004. Decrease in glutathione content in boar sperm cryopreservation. Effect of the addition of reduced glutathione to the freezing and thawing extenders. *Theriogenology*, **62**: 690-701.
- GARG, A., KUMARESAN, A. AND ANSARI, M.R., 2008. Effects of hydrogen peroxide (H₂O₂) on fresh and cryopreserved buffalo sperm functions during incubation at 37°C in vitro. *Reprod. Domestic Anim.*, (in press)
- 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. Fertil.*, **70**: 219-228.
- KADIRVEL, G., SATISH, K. AND KUMARESAN, A., 2008. Lipid peroxidation, mitochondrial membrane potential and DNA integrity of spermatozoa in relation to intracellular reactive oxygen species in liquid and frozen-thawed buffalo semen. *Anim. Reprod. Sci.*, (in press)
- MAXWELL, W.M. AND SALAMON, S., 1993. Liquid storage of ram semen: a review. *Reprod. Fertil. Dev.*, **5**: 613-638.
- MAXWELL, W.M.C. AND WATSON, P.F., 1996. Recent progress in the preservation of ram semen. *Anim. Reprod. Sci.*, **42**: 55-65.
- MEISTER, A. AND ANDERSON, M.E., 1983. Glutathione. *Ann. Rev. Biochem.*, **52**: 711-760.
- MUNSI, M.N., BHUIYAN, M.M.U., MAJUMDER, S. AND ALAM, M.G.S., 2007. Effects of exogenous glutathione on the quality of chilled bull semen. *Reprod. Domest. Anim.*, **42**: 358-362.
- NAIR, S.J., BRAR, A.S., AHUJA, C.S., SANGHA, S.P.S. AND CHAUDHRY, K.C., 2006. A 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.
- SANSONE, G., NASTRI, M.J.F. AND FABBROCINI, A., 2000. Storage of buffalo (*Bubalus bubalis*) semen. *Anim. Reprod. Sci.*, **62**: 55-76.
- SHANNON, P. AND VISHWANATH, R., 1995. The effect of

- optimal and suboptimal concentrations of sperm on the fertility of fresh and frozen bovine semen and a theoretical model to explain the fertility differences. *Anim. Reprod. Sci.*, **39**: 1–10.
- SHARMA, N.C. AND SAHNI, K.L., 1988. *Fertility of frozen and liquid semen in buffaloes*. 2nd World Buffalo Congress New Delhi, India, 100.
- SIKKA, S.C., 2004. Role of oxidative stress and antioxidants in andrology and assisted reproductive technology. *J. Androl.*, **24**: 5-18.
- SILVA, P.F.N. AND GADELLA, B.M., 2006. Detection of damage in mammalian sperm cells. *Theriogenology*, **65**: 958-978.
- SINHA, M.P., SINHA, A. K., SINGH, B. K. AND PRASAD, P.L., 1996. The effect of glutathione on the motility, enzyme leakage and fertility of frozen goat semen. *Theriogenology*, **41**: 237–243.
- 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.
- VISHWANATH, R., PITT, C.P. AND SHANNON, P., 1996. Sperm numbers, semen age and fertility in fresh and frozen bovine semen. *Proc. N.Z. Soc. Anim. Prod.*, **56**: 31–34.
- VISHWANATH, R. AND SHANNON, P., 1997. Do sperm cells age? A review of the physiological changes in sperm during storage at ambient temperature. *Reprod. Fertil. Dev.*, **9**: 321-332.
- VISHWANATH, R. AND SHANON, P., 2000. Storage of bovine semen in liquid and frozen state. *Anim. Reprod. Sci.*, **62**: 23-53.
- WEITZE, K.F., 1991. Long term storage of extended boar semen in: Johnson LA, 2nd Intl. conf. on Boar semen preservation, USA. *Reprod. Domest Anim.*, 231-253.

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