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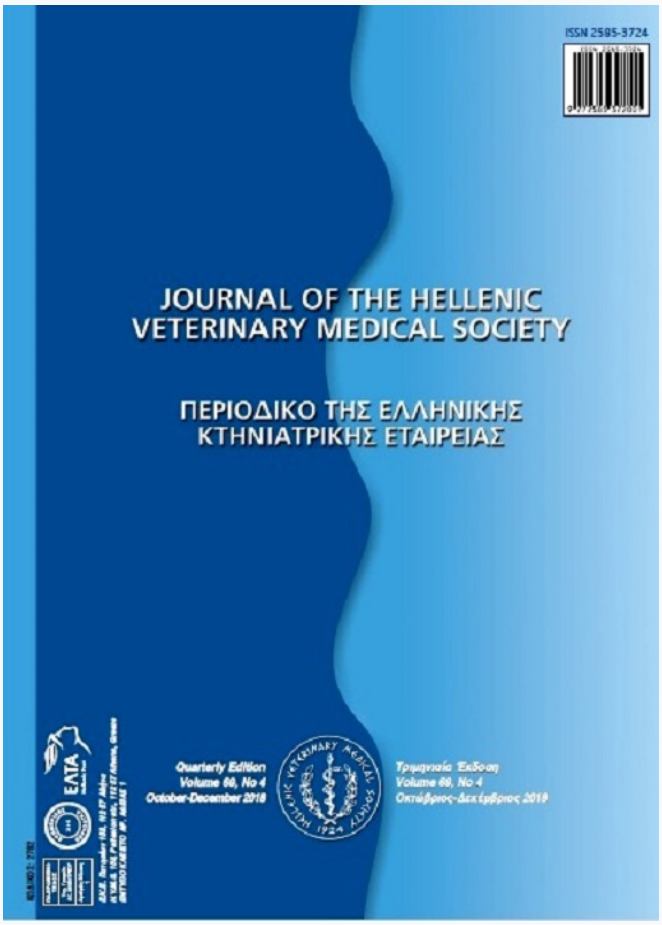

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Comparative effects of addition of superoxide dismutase and reduced glutathione on cryopreservation of Sahiwal bull semen

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ABSTRACT. The present study aimed to investigate effects of superoxide dismutase (SOD) and reduced glutathione (GSH) on the quality of frozen-thawed semen of Sahiwal bulls. Semen was collected twice a week for 8 weeks by artificial vagina from six Sahiwal bulls, kept at the Semen Production Unit Qadirabad, Sahiwal-Pakistan. After gross and microscopic evaluation, qualifying semen ejaculates were divided into 10 equal aliquots and diluted in extenders enriched with no antioxidants (control); or supplemented with either SOD (50, 100 and 200 IU/mL), or GSH (0.5, 1 and 2 mM) or their combinations (50 IU/mL SOD and 0.5 mM GSH, 100 IU/mL SOD and 1 mM GSH and 200 IU/mL SOD and 2 mM GSH). Samples were then frozen and stored in liquid nitrogen at -196°C for 24 h. The following parameters were evaluated for semen quality: post-thawed sperm motility, viability, acrosome and membrane integrity. According to the results, sperm motility, viability, acrosome and membrane integrity were significantly ($P<0.05$) higher in samples treated either with 100 IU/mL of SOD; 1 mM and 2 mM of GSH or 50 IU/mL of SOD plus 0.5 mM of GSH. In conclusion, semen quality might be improved by supplementing semen extenders with 100 IU/mL of SOD; 0.5 and 1 mM of GSH and combination of 50 IU/mL and 0.5 mM of SOD and GSH, respectively

Keywords: Semen, Superoxide Dismutase, Reduced Glutathione, Sahiwal Bull

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INTRODUCTION

It is over debated that repeated cooling and freeze-thawing process induce oxidative stress in semen samples, leading to poor viability and fertility of spermatozoa (Stradaioli *et al*., 2007). Lead factor of oxidative stress is the excessive reactive oxygen species (ROS) generation (Budai *et al*., 2014) that profoundly dissolves lipids, proteins and DNA. Since sperm cells are surrounded by protective polyunsaturated fatty acids membrane (Choudhary *et al*., 2010) and thus, provide an opportunity for the ROS to break them. Another key factor that spread the oxidative stress (OS) is the presences of lipids double bonds sperm membranes (Uysal and Bucak, 2007). Antioxidant system of spermatozoa is also compromised during semen manipulation and due to excessive production of oxygen radicals (Stradaioli *et al*., 2007). Sperm dispose of most of their cytoplasm during terminal stages of differentiation, so they lack endogenous enzymatic defense mechanisms, resulting in overall stress situation (Beheshti *et al*., 2011).

In order to counteract the deleterious effect of OS, semen is naturally provided with SOD that can neutralize OS and defend spermatozoa. It is demonstrated in some previous studies that SOD supplementation can improve sperm parameters in different species (Cocchia *et al*., 2011; Asadpour *et al*., 2012; Perumal, 2014). Morever, GSH, a non-enzymatic antioxidant, has exhibited similar physiological function as does SOD. As reported earlier by many researchers (De Oliveira *et al*., 2013; Ismail and Darwish, 2011; Kaeoket *et al*., 2008), GSH can neutralize the negative impacts of OS on semen quality. However, there is a huge gap in literature regarding the optimum dose effect of SOD and GSH on cryopreservation of Sahiwal bull semen. Therefore, the present study was conducted to monitor the effects of SOD and GSH alone and in combinations on frozen-thawed Sahiwal bull semen.

MATERIALS AND METHODS

The stock extender contained tris-hydroxymethyl-aminomethane (2.42%; w/v), citric acid (1.34%; w/v), fructose (0.1%; w/v), glycerol (7%; v/v), egg yolk (20%; v/v), streptomycin sulphate (1mg/mL), procaine penicillin (400 IU/mL), and benzyl penicillin (500 IU/mL). Ten experimental extenders were prepared as shown in Table 1. The groups S1, S2 and S3 were supplemented with 50, 100 and 200 IU/mL of SOD; the R1, R2 and R3 groups were added with 0.5, 1 and 2 mM of GSH; while SR1, SR2 and SR3 groups included 50 IU/mL SOD and 0.5 mM GSH, 100 IU/mL SOD and 1 mM GSH and 200 IU/mL SOD and 2 mM GSH, respectively. CSR group was kept as a control (no antioxidant added). The antioxidants used in this study were purchased from Sigma–Aldrich Chemicals, St. Louis, MO, USA.

Semen ejaculates were collected in graduated plastic tubes using artificial vagina (42˚C) twice a week for a period of eight weeks. Semen ejaculates having acceptable color (creamy white/yellow), volume >2.0 mL, mass activity >3+, sperm motility percentage >60% and sperm concentration >500 × 10^6/mL were selected. Qualifying semen ejaculates were split into10 aliquots and diluted in 10 different experimental extenders so that each of diluted semen contained a concentration of 50 × 10^6 motile spermatozoa per mL. Diluted semen samples were cooled to 4˚C within 2 h and equilibrated

Table 1: Different doses of superoxide dismutase and reduced glutathione added in Tris- based extender

Serial No	Group	Antioxidant	Dose
1	S1	Superoxide dismutase	50 IU/mL
2	S2	Superoxide dismutase	100 IU/mL
3	S3	Superoxide dismutase	200 IU/mL
4	R1	Reduced glutathione	0.5 mM
5	R2	Reduced glutathione	1.0 mM
6	R3	Reduced glutathione	2.0 mM
7	SR1	Superoxide dismutase + Reduced glutathione	50 IU/mL + 0.5 mM
8	SR2	Superoxide dismutase + Reduced glutathione	100 IU/mL + 1.0 mM
9	SR3	Superoxide dismutase + Reduced glutathione	200 IU/mL + 2.0 mM
10	CSR	Superoxide dismutase + Reduced glutathione	No antioxidant

at 4°C for 4 h. Then semen samples were filled in 0.5 mL French straws (IMV, France) with suction pump in a cold cabinet. Semen straws were kept in liquid nitrogen vapors for 10 min and then plunged and stored in liquid nitrogen (-196°C). After 24 h of freezing, semen straws were thawed in a water bath (37°C for 30 sec) and assessed for sperm motility, viability, acrosomal integrity and plasma membrane integrity.

Sperm with progressive motility were assessed using a phase contrast microscope at 200X by placing semen sample on a pre-warmed (37°C) glass slide and covered with a cover slip. The sperm viability was determined by eosin-nigrosin stain as per method of Salisbury and Van-Demark, 1978. It was assessed by counting 200 spermatozoa under phase contrast microscope (400X). Sperm plasma membrane integrity was evaluated by hypo-osmotic swelling test (HOST) as described by Andrabi *et al*. (2008). The sperm acrosomal integrity was judged by mixing 500 µl of semen with 50 µl of 1% formaldehyde citrate in a test tube and observing a drop of sample under phase contrast microscope at 1000X as described by Asr *et al*. (2011).

The obtained data was analyzed using one-way analysis of variance. The differences in groups were compared by Duncan's Multiple Range Test using SPSS (version 20.0, IBM Corp. Armonk, NY). Value having $P<0.05$ was considered statistically significant.

RESULTS

The results of present study showed that SOD, GSH and their combination efficiently improved sperm motility, viability, acrosome integrity and membrane integrity. The analysis of data revealed that S2, R1, R2 and SR1 groups had significantly ($P<0.05$) higher frozen-thawed motility as compared to control as shown in Table 2. The highest motility was achieved in R2 as compared to all other groups ($P<0.05$). However, non-significant difference was present between S2 and SR1 groups. The viability percentages were significantly ($P<0.05$) higher in S2, R1, R2 and SR1 groups as compared other groups, while R1, R2 and SR1 groups had non-significant difference among each other.

The acrosomal integrity of S2, R1, R2 and SR1 groups were significantly ($P<0.05$) higher than other groups. The highest acrosome integrity was seen in R2 group ($P<0.05$). However, the difference of acrosome intergrity was non-significant between R1 and SR1 ($P>0.05$). The functional membrane integrity was significantly ($P<0.05$) high in S2, R1, R2 and SR1 groups as compared to all other groups. Moreover, the membrane integrity of spermatozoa was not different in R1, R2 and SR1 groups ($P>0.05$).

DISCUSSION

In the current study, we have evaluated effects of different concentrations of SOD and GSH along with their combinations on post thaw semen quality parameters (motility, viability, acrosomal integrity and membrane functional integrity) of Sahiwal Bulls. These parameters are known as important indices for the evaluation of

Table 2: Mean (±SE) values for motility, viability, membrane integrity and acrosomal integrity of post-thawed Sahiwal bull semen

Treatment	Motility %	Live Sperm %	HOST %	Acrosomal Integrity %
S1	44.00 ± 0.408 ^{e}	54.69 ± 0.514 ^{d}	46.69 ± 0.514 ^{cd}	65.00 ± 0.612 ^{d}
S2	50.31 ± 0.313 ^{c}	65.31 ± 0.313 ^{b}	50.31 ± 0.514 ^{b}	68.31 ± 0.514 ^{c}
S3	45.00 ± 0.408 ^{e}	50.00 ± 0.408 ^{e}	44.69 ± 0.313 ^{d}	59.69 ± 0.717 ^{f}
R1	52.69 ± 0.514 ^{b}	69.00 ± 0.612 ^{a}	51.00 ± 0.408 ^{ab}	72.00 ± 0.408 ^{b}
R2	55.00 ± 0.408 ^{a}	70.31 ± 0.514 ^{a}	52.69 ± 0.514 ^{a}	75.00 ± 0.408 ^{a}
R3	44.38 ± 0.625 ^{e}	54.69 ± 0.514 ^{d}	45.06 ± 0.544 ^{d}	63.00 ± 0.612 ^{de}
SR1	51.69 ± 0.514 ^{c}	69.06 ± 0.739 ^{a}	51.00 ± 0.612 ^{ab}	70.00 ± 0.612 ^{bc}
SR2	47.38 ± 0.625 ^{d}	57.31 ± 0.514 ^{c}	45.69 ± 0.514 ^{d}	62.00 ± 0.612 ^{ef}
SR3	40.00 ± 0.408 ^{f}	49.06 ± 0.739 ^{e}	39.69 ± 0.120 ^{e}	57.06 ± 0.544 ^{g}
CSR	47.69 ± 0.514 ^{d}	59.00 ± 0.612 ^{c}	48.00 ± 0.612 ^{c}	64.69 ± 0.514 ^{d}

^{abcdefg} values within same column sharing similar superscripts are statistically not different ($P>0.05$).

semen fertility and suggested as primary markers for epididymal maturation and spermatogesis (Morakinyo *et al.,* 2010).

The spermatozoa are susceptible to OS due to cold shock, which reduces the motility of spermatozoa due to decrease in ATP production (Dandekar *et al*., 2002). The present study demonstrated that 100 IU/mL of SOD had significantly (P<0.05) improved motility of post-thawed semen as compared to control. The results of study were almost similar to those reported by El-Sisy *et al*., (2008) and Shoae and Zamiri, (2008).

In present study, the addition of 0.5 and 1.0 mM of GSH in Tris-citric acid extender improved the motility of post-thawed semen. These findings were in accordance with the reports of Ansari *et al*. (2011) and Munsi *et al*. (2007) in bull semen. The post-thaw motility was also significantly higher in 50 IU/mL of SOD plus 1.0 mM of GSH supplemented group as compared to control. The probable reason for increased motility might be due to counteraction of ROS by the antioxidants added in the extender (Bilodeau *et al*., 2001).

The present study revealed that % viability was significantly (P<0.05) higher in 100 IU/mL of SOD supplemented group, which is similar to the results previously reported in buffalo bull semen (El-Sisy *et al*., 2008). Furthermore, sperm viability was significantly higher in 1.0 mM of GSH treated group as compared to control (P<0.05). This is in agreement with findings of previous reports in buffalo bull (Ansari *et al*., 2012) and stallion semen (Khlifaoui *et al*., 2005). The viability was significantly enhanced in combination group containing 50 IU/mL of SOD and 1.0 mM of GSH. Sperm plasma membrane contains high contents of unsaturated fatty acids which are at risk of lipid peroxidation by the oxygen radicals (Nair *et al*., 2006). This lipid peroxidation may damage sperm plasma membrane and may lead to sperm death (Ansari *et al*., 2011). The prevention of freezing damage to the spermatozoa by fortification of antioxidants in extender might be due to limiting the process of lipid peroxidation by the antioxidants.

The plasma membrane prevents spermatozoa from harmful effects of OS and intact plasma membrane is regarded as an index of fertilizing potential of spermatozoa (Jeyendran *et al*., 1984). The results of present study demonstrated higher membrane integrity of spermatozoa in groups containing 100 IU/mL of SOD, 0.5 mM and 1.0 mM of reduced glutathione and 50 IU/mL SOD plus 0.5 mM of GSH, as compared to other groups. The results of this study are in harmony with some previous reports (Perumal *et al*., 2011; Perumal, 2014; Farouzanfar *et al*., 2013 and Ansari *et al*., 2012).

It is well established that oxygen radicals, produced during freezing process, have high affinity to unsaturated fatty acids of sperm plasma membrane. Reaction of oxygen radicals with sperm plasma membrane can cause lipid peroxidation and sperm death (Uysal and Bucak, 2007). However, the antioxidants added in semen extenders can counteract with oxygen radicals and can prevent injury to sperm plasma membrane.

Acrosome integrity is an indication of functional membrane status of spermatozoa (Silva and Gadella, 2006). Acrosome is a secretory organelle derived from golgi/endoplasmic reticulum which contain hydrolytic enzymes. The presence of intact acrosome is needed to facilitate the acrosome reaction of spermatozoa and is essential for the process of fertilization. Along with total antioxidant potential, freeze-thawing process decreases the intact acrosome of spermatozoa (Anzar *et al*., 2010). In the present study, the percentages of spermatozoa with normal acrosomes were significantly (P<0.05) higher in extenders containing 100 IU/mL of SOD than control. Our results were in line with the previous findings on the acrosome integrity of frozen-thawed ram semen (Farouzanfar *et al*., 2013 and Silva *et al*., 2012).

A high number of sperm with normal acrosome were found in 0.5 mM and 1.0 mM of GSH supplemented groups than control. These results are similar to the findings of Funahashi and Sano (2005) and Gadea *et al*., (2007). Similarly, the acrosome integrity was significantly (P<0.05) higher in combination group containing 50 IU/mL of SOD and 0.5 mM of GSH. The ROS produced during freeze-thaw process interact with the sperm plasma membrane and cause hyper-activation along with pre-mature capacitation of spermatozoa. Hence, it seems that the disruption of sperm acrosomes might be reduced by the addition of exogenous antioxidants in treatment extenders.

In conclusion, supplementation of semen extenders with SOD and GSH in various concentrations can improve the post-thawed semen quality of Sahiwal bulls. However, higher concentrations of these antioxidants have no beneficial effects on semen. Moreover,

the routine inclusion of these antioxidants in semen extenders could be recommended only after performing fertility trials.

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CONFLICT OF INTERESTS

The authors report no conflict of interests.

REFERENCES

Andrabi SMH, Ansari MS, Ullah N, Afzal M (2008) Effect of non-enzymatic antioxidants in extender on post thaw quality of buffalo (*Bubalus bubalis*) bull spermatozoa. Pak Vet J 28(4): 159-162.

Ansari MS, Rakha BA, Ullah N, Andrabi SMH, Akhter S (2011) Glutathione addition in Tris-citric egg yolk extender improves the quality of cooled buffalo (*Bubalus bubalis*) bull semen. *Pak J Zool* 43(1): 49-55.

Ansari MS, Rakha BA, Andrabi SMH, Ullah N, Iqbal R, Holt WV, Akhtar S (2012) Glutathione supplemented tris-citric acid extender improves the post-thaw and in vivo fertility of buffalo (*Bubalus bulalis*) bull spermatozoa. Reprod Biol 12: 271-276.

Anzar M, Rasul Z, Ahmad TA, Ahmad N (2010) Response of buffalo spermatozoa to low temperatures during cryopreservation. Reprod Fertility Develop 22: 871-880.

Asadpour R, Jafari R, Nasarabadi HT (2012) The effect of antioxidant supplementation in semen extenders on semen quality and lipid peroxidation of chilled bull spermatozoa. Iran J Vet Res 13: 246-249.

Asr ST, Beheshti R, Kohram H (2011) The evaluations of tris-citrate acid or Bioxcell extenders on the post-thawed buffalo sperm parameters. Ann Biol Res 2: 360-365.

Beheshti R, Asadi A, Eshratkhah B, Ghalekandi JG, Ghorbani A (2011) The effect of cysteine on post-thawed buffalo bull (*Bubalus bubalis*) sperm parameters. Adv Environ Biol 5(6): 1260-1263.

Bilodeau JF, Blanchette S, Gagnon C, Sirard MA (2001) Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen. Theriogenology 56(2): 275–286.

Budai C, Egerszegi I, Olah J, Javor A, Kovacs A (2014) The protective effect of antioxidants on liquid and frozen stored ram semen – Review. Anim Sci Biotech 47(1): 46-52.

Choudhary R, Chawala VK, Soni ND, Kumar J and Vyas RK (2010) Oxidative stress and role of antioxidants in male infertility. Pak J Physiol 6(2): 54-59.

Cocchia N, Pasolini MP, Mancini R (2011) Effect of SOD (superoxide dismutase) protein supplementation in semen extenders on motility, viability, acrosome status and ERK (extracellular signal-regulated kinase) protein phosphorylation of chilled stallion spermatozoa. Theriogenology 75(7): 1201-1210.

Dandekar SP, Nadkarni GD, Kulkarni VS, Punekar S (2002) Lipid peroxidation and antioxidant enzymes in male infertility. J Postgrad Med 48(3): 186–189.

De Oliveira, RA, Wolf CA, Viu MADO, Gambarini ML (2013) Addition of glutathione to an extender for frozen equine semen. J Equine Vet Sci 33: 1148-1152.

El-Sisy, GA, El-Nattat,WS, El-Sheshtawy RI (2008) Effects of superoxide dismutase and catalase on viability of cryopreserved buffalo spermatozoa. Global Vet 2(2): 56–61.

Farouzanfar M, Ershad SF, Husseini SM, Hajian M, Hosseini SO, Abid A, Tavalaee M, Shahverdi A, Dizaji AV, Esfahani MHN (2013) Can permeable super oxide dismutase mimetic agents improve the quality of frozen-thawed ram semen. Cryobiology 66: 126-130.

Funahashi H, 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 SC, Zquez FAZ, Grullo LA, Gardo JC (2007) Supplementation of 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.

Ismail LK, Darwish SA (2011) Effect of glutathione (GSH) on microscopic parameters and DNA integrity in Egyptian buffalo semen during liquid and frozen storage. J Reprod Infert 2(3): 32-40.

Jeyendran RS, Van Der Ven HH, Perez-Pelaez M, Craboand BG, Zaneveld LJD (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.

Kaeoket K, Tantiparinyakul K, Kladkaew W, Chanapiwat P, Techakumphu M (2008) Effect of different antioxidants on quality of cryopreserved boar semen in different breeds. Thai J Agri Sci 41: 1-9.

Khlifaoui M, Battut I, Bruyas JF, Ghatagnon G, Trimeche A, Tainturier D (2005) The effects of glutamine on post-thaw motility of stallion spermatozoa: an approach of the mechanism of action at spermatozoa level. Theriogenology 63: 138-149.

Morakinyo, AO, Achema, PU, & Adegoke, O A (2010) Effect of Zingiber officinale (Ginger) on sodium arsenite-induced reproductive toxicity in male rats. African Journal of Biomedical Research 13(1), 39-45.

Munsi MN, Bhuiyan MMU, Majumder S, Alam MGS (2007) Effects of exogenous glutathione on the quality of chilled bull semen. Reprod Domest Anim 42: 358-362.

Nair SJ, Brar AS, Ahuja CS, Sangha SPS, Chaudhry KC (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.

Perumal P (2014) Effect of superoxide dismutase on semen parameters and antioxidant enzyme activities of liquid stored (5˚C) Mithun (*Bos frontalis*) semen. J Anim 14: 1-9.

Perumal P, Selvaraju S, Selvakumar S, Barik A, Mohanty D, Mishra P (2011) Effect of pre-freeze addition of cysteine hydrochloride and reduced glutathione in semen of crossbreed jersey bulls on sperm parameters and conception rates. Reprod Domes Anim 46: 636-641.

Salisbury GW, Van-Demark NL (1978) Physiology of Reproduction and Artificial Insemination of Cattle.1st Ed, WH Freeman and Company, Sanfrancisco, USA.

Shoae A, Zamiri MJ (2008) Effect of butylated hydroxyl toluene on bull spermatozoa frozen in egg yolk-citrate extender. Anim Reprod Sci 104: 414-418.

Silva EC, Cajueiro JF, Silva SV, Soares PC, Guerra MM (2012) Effect of an antioxidants resveratrol and quercetin on in vitro evaluation of frozen ram semen. Theriogenology 77: 1722-1726.

Silva, PFN, Gadella BM (2006) Detection of damage in mammalian sperm cells. Theriogenology 65: 958-978.

Stradaioli G, Noro T, Sylla L, Monaci M (2007) Decrease in glutathione (GSH) content in bovine sperm after cryopreservation: comparison between two extenders. Theriogenology 67: 1249-1255.

Uysal O, Bucak MN (2007) Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen. Acta Vet Brno 76: 383-390.

LIST OF ABBREVIATIONS

Superoxide dismutase – SOD

Reduced glutathione – GSH

Reactive oxygen species – ROS

Oxidative stress – OS