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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). Moreover, 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<sup>6</sup>/mL were selected. Qualifying semen ejaculates were split into 10 aliquots and diluted in 10 different experimental extenders so that each of diluted semen contained a concentration of 50 × 10<sup>6</sup> 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 integrity 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 ( $\pm$ SE) values for motility, viability, membrane integrity and acrosomal integrity of post-thawed Sahiwal bull semen

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

<sup>abcdefg</sup> 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 spermatogenesis (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 (Khelifaoui *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.

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## LIST OF ABBREVIATIONS

Superoxide dismutase – SOD  
 Reduced glutathione – GSH  
 Reactive oxygen species – ROS  
 Oxidative stress – OS