RESEARCH ARTICLE

Influence of Antioxidant Sericin in Tris Extender on Oxidative Markers during Cryopreservation (–196ºC) of Bovine Semen

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Abstract

This investigation was carried out during winter season on the semen of three mature, healthy breeding bulls each of Gir cattle and Murrah buffalo breeds. The aim was to assess the effect of different concentration of antioxidant Sericin (0.0, 0.1, 0.25, 0.50 and 1.0%, w/v) in standard tris fructose egg yolk glycerol (TFYG) extender on cryopreservability of bovine semen based on sperm motility and oxidative markers in seminal plasma of freshly diluted and cryopreserved semen. The mean sperm motility observed in freshly diluted and frozen-thawed Gir bull semen, irrespective of Sericin levels, were 76.93 ± 0.39 and 43.47 ± 0.58 % and in Murrah bulls 78.20 ± 0.38 and 44.10 ± 0.48 %, respectively. The values of malondialdehyde (MDA, µmol/ml) in seminal plasma of freshly diluted and frozen-thawed semen of Gir bulls, irrespective of Sericin levels, were 21.68 ± 0.38 and 24.99 ± 0.56, and in Murrah bulls 21.49 ± 0.57 and 25.60 ± 0.94, respectively. The corresponding values of superoxide dismutase (SOD, U/ml) were 1.77 ± 0.06 and 1.37 ± 0.05 in Gir and 1.18 ± 0.06 and 0.85 ± 0.04 in Murrah bulls, and those of glutathione peroxidase (GPx, nmol/min/ml) 417.10 ± 12.00 and 349.76 ± 11.92 in Gir and 385.71 ± 9.21 and 320.02 ± 9.49 in Murrah bull semen. Sperm motility and activities of all three enzymes differed highly significantly ($p < 0.01$) between stages. SOD was significantly ($p < 0.05$) lower in buffalo than cattle semen. Inclusion of 0.5% and/or 0.25% Sericin in TFYG extender gave better protection to spermatozoa over other levels against ROS mediated injuries as the MDA production was significantly reduced with increased sperm motility and higher levels of SOD and GPx enzymes in the seminal plasma.

Keywords: Antioxidant, Bovine semen, Cryopreservation, Oxidative markers, Sericin, Sperm motility.

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Introduction

Antioxidant potential of silk protein sericin was reported earlier by Kato *et al.* (1998), who explained that sericin could suppress lipid peroxidation and inhibit tyrosinase activity *in vitro* by its scavenging function which may be provided by the chelating effect of hydroxyl groups of hydroxyamino acids (serine and threonine) that are abundantly contained in sericin (Kwang *et al.,* 2003). Recently, Michael and Subramanyam (2014) reported that exposure of isolated cells of midgut and hemocytes to sericin before their exposure to hydrogen peroxide increased the activity of antioxidant enzymes and inhibited oxidative derivatives (protein carbonyl, MDA). Various types of antioxidants both enzymatic and non-enzymatic have been fortified into the semen extenders with varying degree of success by many workers (Tuncer *et al.,* 2010; Varghese *et al.,* 2016; Chikhaliya *et al.,* 2018), thus indicating crucial role of these antioxidants supplementation to the bull and buffalo semen for improving its preservability and fertility. Sericin also possesses the biological activity of preventing cell death during culture and cryopreservation (Masakazu *et al.,* 2003), it is being used in oocyte maturation media and culture media replacing bovine serum albumin and fetal bovine serum (Yasmin *et al.,* 2015; Aghaz *et al.,* 2016). Recently it has also been proved beneficial in sperm cryopreservation (Dorji *et al.,* 2015; Kumar *et al.,* 2015; Demra *et al.*, 2017). Therefore the present study was planned to evaluate the antioxidant and cryoprotective property of sericin through assay of sperm motility and oxidative markers before and after freezing of bovine sperm.

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Materials and Methods

The study was conducted during the winter season from November to February (2018-19) on the semen of three mature, healthy Gir cattle and three Murrah buffalo bulls, aged 5-7 years, maintained at Sperm Station of the College. All these bulls were in good health and under optimal veterinary

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care. They were maintained in nearly identical nutritional and managerial conditions throughout the period of study and were under regular weekly twice semen collection schedules using AV. For the present study, the ejaculates (6/bull, 18 per breed) were evaluated once in a week for routine physico-morphological attributes. The ejaculates with >75% initial motility were divided into five equal aliquots. One aliquot was extended with standard tris-citrate-fructose-yolk-glycerol (TFYG) extender as Control and the rests four were extended with TFYG added with antioxidant additive Sericin (Sigma-Aldrich, USA) @ 0.10, 0.25, 0.50 and 1.00% W/V to study their comparative efficacy on sperm motility and oxidative markers just after dilution and after cryopreservation (-196°C) of bovine semen.

The French mini straws (at least 15) filled and sealed from each diluted aliquot using automatic filling and sealing machine (IS4 System, IMV Technologies, France) were racked and cooled to 4–5°C within 60-90 minutes and further equilibrated at the same temperature for 4 hrs in cold handling cabinet (IMV, France). Just before freezing, the samples were evaluated for pre-freeze sperm motility. Freezing of the straws was carried out using a programmable bio-freezer (Digitcool 5300 CE ZH 350, IMV, France) using a previously tested freezing curve for bovine semen. After 18 hrs of frozen storage, the straws of each split-sample were thawed in a water bath at 37°C for 30 seconds. Post-thaw motility was assessed under phase-contrast microscope (40x) fitted with a Biotherm.

The freshly diluted and frozen-thawed semen samples (2.0 ml each) of different aliquots were centrifuged at 1000 g for 10 minutes. The supernatant (plasma) separated was stored at -20°C in a deep freeze. The determination of activities of antioxidant enzymes, *viz.,* glutathione peroxidase (GPx), superoxide dismutase (SOD) and malondialdehyde (MDA) in pre- and post-thaw plasma was carried out using commercial kits (Cayman Assay Kits, USA, Cat No. 706002, 705003 & 703102) according to the instructions of the manufacturer. The data was analyzed statistically using ANOVA, DMRT, and t-test (Snedecor and Cochran, 1994).

Results and discussion

Oxidative stress is known to deteriorate the quality of frozen semen (El-Sisy *et al.,* 2007) that results in the production of ROS (Baumber *et al.,* 2005), which subsequently increases lipid peroxidation levels in the cryopreserved spermatozoa (El-Sisy *et al.,* 2007; Kadirvel *et al.,* 2009) affecting its plasma membrane and DNA integrity and thereby fertility potential. The mean sperm motility and concentrations of oxidative markers, *viz.,* malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) observed in seminal plasma of freshly diluted and frozen-thawed semen of Gir cattle and Murrah buffalo bull processed in conventional standard tris fructose egg-yolk glycerol extender (TFYG) as control, and TFYG added with antioxidant Sericin at the concentration of 0.10, 0.25, 0.50 and 1.00% (w/v) are presented in Table 1. Statistical analysis revealed that there were highly significant differences ($p < 0.01$) in all these traits between different levels of Sericin and between stages of cryopreservation, but not between breeds/species.

Sperm Motility and Lipid Peroxidation

The mean sperm progressive motility observed in freshly diluted and frozen-thawed semen of Gir bulls in TFYG extender, irrespective of Sericin levels, were 76.93 ± 0.39 and 43.47 ± 0.58 % and in Murrah bulls 78.20 ± 0.38 and 44.10 ± 0.48 %, respectively. The corresponding mean values of malondialdehyde (MDA) production recorded in seminal plasma of freshly diluted and frozen-thawed semen of Gir bulls were 21.68 ± 0.38 and 24.99 ± 0.56 µmol/mL, and in Murrah bulls 21.49 ± 0.57 and 25.60 ± 0.94 µmol/

Table 1: Effect of sericin supplementation in Tris extender on Sperm motility (%), Lipid peroxidation (MDA, µmol/ml), Superoxide Dismutase (SOD, U/ml) and Glutathione peroxidase (GPx, nmol/min/ml) activity in seminal plasma of Gir and Murrah bulls after dilution and cryopreservation of semen

Freezing stage	Sericin Levels in Extender	Sperm Motility (%)		MDA (µmol/ml)		SOD (U/m)		GPx (nmol/min/ml)	
		Gir bulls	Murrah bulls	Gir bulls	Murrah bulls	Gir bulls	Murrah bulls	Gir bulls	Murrah bulls
On dilution	0.00%	74.50 ± 0.55^{a}	76.00 ± 0.44^{a}	24.21 ± 0.89^{c}	23.93 ± 1.33^{b}	1.37 ± 0.06^{a}	0.91 ± 0.10^{a}	347.66 ± 28.00^{a}	358.82 ± 18.34^{a}
	0.10%	76.17 ± 0.78^{ab}	77.00 ± 0.82^{a}	21.51 ± 0.75^{ab}	21.81 ± 1.24^{ab}	1.66 ± 0.12^{ab}	1.11 ± 0.14^{a}	396.88 ± 24.96^{ab}	376.19 ± 15.00^{ab}
	0.25%	77.83 ± 0.75^{b}	77.83 ± 0.92^{ab}	22.32 ± 0.89^{bc}	20.86 ± 1.63^{ab}	1.82 ± 0.10^{b}	1.10 ± 0.10^{a}	429.93 ± 25.74^{bc}	386.52 ± 20.78^{ab}
	0.50%	80.50 ± 0.77^{c}	80.83 ± 0.76^{c}	19.67 ± 0.70^{a}	18.73 ± 0.63^{a}	2.20 ± 0.15^{c}	1.65 ± 0.17^{b}	482.45 ± 25.50^{c}	430.00 ± 22.82^{b}
	1.00%	75.67 ± 1.04^{ab}	79.33 ± 0.92^{bc}	20.69 ± 0.70^{ab}	22.11 ± 1.12^{ab}	1.81 ± 0.12^{b}	1.10 ± 0.11^{a}	428.58 ± 21.73^{bc}	377.02 ± 23.17^{ab}
	Average	76.93 ± 0.39^{x}	78.20 ± 0.38^{x}	21.68 ± 0.38^{x}	21.49 ± 0.57^{x}	1.77 ± 0.06^{x}	1.18 ± 0.06^{x}	417.10 ± 12.00^{x}	385.71 ± 9.21^{x}
Post-thaw	0.00%	42.17 ± 1.12^{ab}	42.33 ± 1.01^{a}	26.98 ± 1.19	29.37 ± 2.56^{b}	1.02 ± 0.05^{a}	0.64 ± 0.06^{a}	270.86 ± 23.72^{a}	288.31 ± 19.72^{a}
	0.10%	42.67 ± 1.29^{ab}	43.67 ± 0.93^{a}	24.54 ± 0.94	26.01 ± 2.19^{ab}	1.25 ± 0.09^{ab}	0.82 ± 0.09^{a}	330.12 ± 27.32^{ab}	313.17 ± 16.81^{ab}
	0.25%	45.50 ± 1.03^{bc}	44.83 ± 0.81^{ab}	24.79 ± 1.09	24.53 ± 2.32^{ab}	1.36 ± 0.09^{b}	0.81 ± 0.05^{a}	361.63 ± 26.19^{bc}	326.82 ± 21.34^{ab}
	0.50%	46.33 ± 1.24^{c}	47.33 ± 1.01^{b}	23.89 ± 1.90	24.13 ± 0.89^{a}	1.79 ± 0.12^{c}	1.21 ± 0.09^{b}	418.62 ± 23.57^{c}	358.03 ± 22.00^{b}
	1.00%	40.67 ± 1.51^{a}	42.33 ± 1.37^{a}	24.76 ± 0.87	26.66 ± 1.89^{ab}	1.42 ± 0.10^{b}	0.79 ± 0.06^{a}	367.55 ± 21.84^{bc}	313.77 ± 24.43^{ab}
	Average	43.47 ± 0.58^{y}	44.10 ± 0.48^{y}	24.99 ± 0.56^{y}	25.60 ± 0.94^{y}	1.37 ± 0.05^{y}	0.85 ± 0.04^{y}	349.76 ± 11.92^{y}	320.02 ± 9.49^{y}

Means within the column bearing different superscripts between sericin levels (a,b,c), and between stages (x,y) differ significantly ($p < 0.05$).

mL, respectively. These values differed highly significantly ($p < 0.01$) between stages, but not between breeds/species. The mean motility and MDA levels observed on dilution of Gir and Murrah bull semen in control TFYG extender and TFYG with Sericin @ 0.10, 0.25, 0.50 and 1.00 % were almost similar between breeds, however MDA activity in post-thawed seminal plasma was higher in buffalo semen than Gir bull semen, probably due to more lipid peroxidation, though did not differ significantly (Table 1). The initial and post-thaw values of MDA recorded in TFYG extender without and with antioxidant sericin followed the inverse trend to that of sperm motility percent during the steps of cryopreservation. MDA is the product of lipid peroxidation; higher the MDA greater is lipid peroxidation. The values of sperm motility were significantly ($p < 0.05$) higher and MDA lower at both the stages for both the breeds/species in TFYG with 0.50 and/or 0.25 % sericin than the higher or lower levels and control tris extender.

The present findings on sperm motility and lipid peroxidation were in harmony with Dorji *et al.* (2015) and Kumar *et al.* (2015), who reported significantly better sperm motility with MDA levels to be non-significantly (1.3 vs 1.5 nmol/mL) and significantly (0.50 ± 0.45 vs. 1.47 ± 0.21 nmole/ml) lower in Thai and Murrah bull semen, respectively, cryopreserved in TFYG extender with Sericin @ 0.50 % compared to control extender and the values for 0.1, 0.25 and 1.0 % Sericin were intermediate. Our findings also concurred well with those of Shaikh *et al.* (2016), who found significantly ($p < 0.05$) higher motility and lower lipid peroxidation (MDA, 20.06 ± 0.13 µmol/ml) in Kankrej bull semen cryopreserved in TFYG extender supplemented with trehalose at 100 mM compared to 50 or 150 mM and control extender. Sariozkan *et al.* (2009) reported significantly higher MDA in TFYG with taurine 2 mM compared to control extender, but no significant effect was observed with cysteine 2 mM. Chhillar *et al.* (2012) found significantly ($p < 0.05$) lower rate of H_2O_2 production, lipid peroxidation in spermatozoa cryopreserved in presence of 50 and 100 mM trehalose compared to control TFYG extender. Chikhaliya *et al.* (2018) reported significantly ($p < 0.05$) lower mean MDA levels at pre-freeze and post-thaw stage (30.74 ± 0.55 and 15.95 ± 0.74 µmol/ml) in Gir bull semen frozen in Andromed extender supplemented with taurine at 50 mM as compared to 25 or 75 mM. Kurmi *et al.* (2018) also evidenced beneficial effect of Vitamin E, 1 mM and 2 mM, in tris extender in terms of inhibited lipid peroxidation as indicated by lower MDA production and higher sperm quality including DNA integrity of ram semen.

Superoxide Dismutase and Glutathione Peroxidase Activity

The mean values of superoxide dismutase (SOD) activity observed in seminal plasma of freshly diluted and frozen-thawed Gir bull semen cryopreserved in TFYG extender, irrespective of Sericin levels, were 1.77 ± 0.06 and 1.37 ± 0.05 U/mL, and in Murrah bulls 1.18 ± 0.06 and 0.85 ± 0.04 U/mL, respectively. The corresponding mean values of glutathione peroxidase (GPx) activity of freshly diluted and frozen-thawed Gir bull semen were 417.10 ± 12.00 and 349.76 ± 11.92 nmol/min/mL and in Murrah bulls 385.71 ± 9.21 and 320.02 ± 9.49 nmol/min/ml, respectively. The activity of both the enzymes differed highly significantly ($p < 0.01$) between stages, and even between breeds/ species, being lower in buffalo than cattle semen (Table 1). The values of both SOD and GPx enzymes were significantly higher at both initial and post-thaw stages for both the breeds/species in TFYG with 0.50 and/or 0.25 % Sericin as compared to 0.1 or 1.0 % level, and in control tris extender.

These findings on SOD and GPx activity were in harmony with the previous report of Sariozkan *et al.* (2009), with cysteine 2 mM as compared to control for cryopreserved bull semen. Kumar *et al.* (2015) reported significantly ($p < 0.05$) higher post-thaw SOD (39.42 ± 2.67 U/ml) and GPx (25.50 ± 0.8 nmol/min/mL) activity in buffalo semen cryopreserved using TFYG extender with Sericin @ 0.50 % as compared to the higher or lower levels and control suggesting protective role of Sericin against ROS mediated sperm cell cryoinjury. However, the sericin levels at 1.5 and 2.0 % were reported to be toxic/detrimental to buffalo sperm by Kumar *et al.* (2015) as revealed by the highest MDA production and lowest SOD and GPx activity compared to lower levels of sericin. The similar beneficial effect of trehalose 100 mM on oxidative stress was also observed by Shaikh *et al.* (2016) in Kankrej bull semen cryopreserved in TFYG extender.

The role of Sericin as cell-protective, cryoprotective, antioxidant with the ability to eliminate free radicals has been reported during the cryopreservation of various cells and tissues (Isobe *et al.,* 2013). During the present study, an increase in percentage of sperm motility with reduced oxidative stress as indicated by less production of MDA and higher activity of SOD and GPx in Sericin supplemented semen showed its antioxidant and cryoprotective effect. Our study is also in agreement with that of Kumar *et al.* (2015) , who observed a cryoprotective and antioxidant action of sericin on frozen-thawed bovine semen. Various types of antioxidants both enzymatic and non-enzymatic, have been fortified into the semen extenders with varying degree of success by many workers (Tuncer *et al.,* 2010; Chikhaliya *et al.,* 2018). Antioxidant potential of silk protein sericin by its scavenging function was reported earlier by Kwang *et al.* (2003) and Michael and Subramanyam (2014). These workers support the crucial role of these antioxidants supplementation to the bull and buffalo semen for improving its preservability and fertility. Sericin also possesses the biological activity of preventing cell death during cryopreservation and has been proved beneficial in sperm cryopreservation by a few researchers (Dorji *et al.,* 2015; Kumar *et al.,* 2015; Demra *et al.*, 2017). Though supplementation of Sericin at 0.5 % or 0.25 % in

TFYG extender improved the freshly extended as well as post-thawed motility of bovine sperms with reduced oxidative stress in the present study, the final recommendation of its routine use in semen extender could, however, only be made after performing actual controlled fertility trials.

Conclusion

From the results of the present study, Sericin @ 0.50 and/or 0.25% appeared an optimal effective dose to reduce cryodamage and improve post-thaw sperm motility in both cattle and buffalo semen. Sericin supplementation to the extender reduced the oxidative stress and enhanced inherent antioxidant enzyme activity (SOD, GPx) protecting lipid peroxidation and sperm membrane integrity. Further, reduction in oxidative stress due to sericin supplementation resulted in enhanced sperm motility in both freshly extended and cryopreserved bovine semen irrespective of breed or species specificity in its action or tolerance. However, *in vivo* fertility trials are warranted to prove its ultimate goal of using as cryoprotectant in semen preservation.

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