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Sericin supplementation improves semen freezability of buffalo bulls by minimizing oxidative stress during cryopreservation

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ABSTRACT

The variety of mammalian cells has been successfully cryopreserved by use of the silk protein sericin due to its strong free-radical-scavenging and potent antioxidant activity. The present study was conducted to examine the protective role of sericin on buffalo spermatozoa during cryopreservation. Semen of four breeding bulls was collected twice a week using artificial vagina technique. The ejaculates of four bulls were pooled, divided into five equal fractions, diluted with the extender supplemented with different concentrations of sericin (0, 0.25, 0.5, 1.5 and 2%) and then cryopreserved. Post-thawed motility was objectively assessed by computer assisted sperm analyzer. Sperm plasma membrane integrity was assessed by hypo-osmotic swelling test (HOST). Malondialdehyde (MDA) concentration, glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities were determined in frozen–thawed extended seminal plasma by spectrophotometry. The extender supplemented with 0.25, 0.5 and 1% sericin resulted in the higher sperm motility and GPx acivity. Furthermore, plasma membrane integrity and SOD activity were found to be higher ($P<0.05$) in group supplemented with 0.25 and 0.5% sericin ($P<0.05$). The MDA concentration was found to be significantly lower ($P<0.05$) in 0.25 and 0.5% sericin treated groups than control and other treated groups. In conclusion, the supplementation of 0.25–0.5% sericin in semen extender improves frozen–thawed semen quality through protecting sperm from oxidative stress.

1. Introduction

Semen cryopreservation is an important technique for long-term storage of sperm, required for wide application of artificial insemination for genetic improvement of buffalo but its application has been reported on a limited scale in buffalo, because of poor freezability of buffalo spermatozoa when compared to cattle (Andrabi et al., 2008; Andrabi, 2009). In addition, conception rate in buffaloes inseminated with frozen–thawed semen under field condition is very low (30%) (Chohan et al., 1992; Anzar et al., 2003) as compared to cattle frozen–thawed spermatozoa. Primary target of damage in sperm cells is plasma membrane (Morris and Clarke, 1981; Kumar et al., 2014) and it mainly occurs when cell membrane undergoes the phase transition during the cryopreservation (Darin-Bennett and White, 1977). On the other hand, mammalian sperm cells are highly susceptible to lipid peroxidation (LPO) by free radicals such as O^{2-} and H_2O_2 which leads to the structural damage of sperm membranes during

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cryopreservation (Alvarez and Storey, 1989; Sinha et al., 1996). A wide variety of antioxidants have been tested to minimize the cryo-damage by elimination of free radicals during semen cryopreservation in different species (Ball et al., 2001; Paulenzen et al., 2002; Ghosh and Datta, 2003).

The differences in the sensitivity of their sperm to freezing among species are largely attributed to compositional variation in the sperm plasma membrane (Bailey et al., 2000). One of the possible causes of lower freezability of buffalo bull semen compared to cattle is due to the differences in the lipid ratio of the spermatozoa (Jain and Anand, 1976; Andrabi, 2009). Therefore, there is a need to identify suitable cryoprotectant for minimizing the loss due to the cryodamage and make improvement in freezability of buffalo spermatozoa.

Sericin a water-soluble globular protein (a protein hydrolysate) derived from silkworm *Bombyx mori*, and represents a family of proteins whose molecular mass ranges from 10 to 310 kDa (Wei et al., 2005). It consists of 18 kinds of amino acids most of which have strong polar side groups such as hydroxyl, carboxyl, and amino groups (Wei et al., 2005). Sericin is rich in aspartic acid as well as serine (Kwang et al., 2003), which has a high content of the hydroxyl group. Studies have shown that sericin suppress lipid peroxidation (Kato et al., 1998), preventing cell death (Masakazu et al., 2003), protect from freezing (Tsujimoto et al., 2001) and various types of stress (Sasaki et al., 2005). In addition, sericin suppressed carcinogenesis and tumour promotion by reducing oxidative stress in murine skin (Zhaorigetu et al., 2003). There are evidences of successful use of sericin in cryopreservation of many mammalian cells like human hepatocytes (Miyamoto et al., 2010), islet cells (Ohnishi et al., 2012), adipose tissue derived stem cells (Miyamoto et al., 2012) and bovine embryo (Isobe et al., 2013). However, there is no report available related to use of sericin in the freezing media to minimize the damages to the spermatozoa during cryopreservation. Therefore, the present study was undertaken to optimize the concentration of silk protein sericin suitable in semen freezing media for improving freezability of buffalo spermatozoa.

2. Materials and methods

2.1. Sericin

Sericin, a protein derived from the silkworm cocoon, was purchased from Sigma–Aldrich Chemicals Pvt Limited (Cat No.: S5201).

2.2. Semen collection

Four healthy Murrah buffalo breeding bulls (age 3–5 years) of progeny testing program of the institute were maintained with standard feeding and management practices. Semen of these bulls was collected twice a week using artificial vagina technique and freezing was performed for ten times for the study. Pre-freezing sperm motility was assessed subjectively under phase contrast microscope equipped with a warm stage (37 °C) at 400× magnification and only ejaculates having ≥70% sperm motility were cryopreserved. In order to eliminate individual differences in bulls, the ejaculates were mixed in a pool for balancing the sperm contribution of each bull.

2.3. Semen processing

The pooled semen was divided into five equal fractions and diluted to final concentration 80 million sperms/mL using AndroMed® (Minitube, Germany) semen extender supplemented with different concentrations of sericin (0, 0.25, 0.5, 1.5 and 2%). Thereafter, the extended semen was slowly cooled to 4 °C and equilibrated for a period of 4 h in a cold cabinet (IMV, L'Aigle, France). Equilibrated semen was then loaded into 0.25 mL plastic straws (IMV, L'Aigle, France) and frozen with a programmable biological freezer (Mini Digi-cool, IMV Technologies, L'Aigle, France) as described earlier in this laboratory (Kumar et al., 2014).

2.4. Objective assessment of sperm motility

Sperm percent motility was assessed using computer assisted sperm analyzer (CASA) system (IVOS 12.1, Hamilton-Thorne Biosciences, Beverly, MA, USA) as described earlier (Kumar et al., 2014). Three straws for each treatment were thawed separately in a water bath at 38 °C for 45 s and five optical fields around the central reticulum of the chamber were used to count spermatozoa.

2.5. Assessment of plasma membrane integrity

Plasma membrane integrity was evaluated using hypo-osmotic swelling test (HOST) as described by Jeyendran et al. (1984). The assay was performed by mixing 100 μL of frozen–thawed semen with 1 mL hypo-osmotic solution (0.735 g sodium citrate $2H_2O$ and 1.351 g fructose in 100 mL distilled water). After incubation for 60 min at 37 °C, sperm tail bending/coiling was assessed by placing 15 μL of well-mixed sample on a warm slide (37 °C) under light microscopy at 400× magnifications. At least 200 spermatozoa were observed per slide/per straw. Sperm had coiled tail after HOST was considered intact plasma membrane.

2.6. Determination of antioxidant enzymes activity and lipid peroxidation

Semen from three straws per treatment was pooled and used for biochemical analyses. After thawing, the spermatozoa were separated from extended seminal plasma by centrifugation (1000 × *g* for 10 minimum, at room temperature). The supernatant was used for the estimation of glutathione peroxidase (GPx), superoxide dismutase (SOD) and malondialdehyde (MDA) activity as per instruction of kit manufacturer (Cayman Chemicals Company).

2.6.1. Determination of glutahione peroxidase acivity

The glutahione peroxidase (GPx) activity was assessed using Cayman GPx assay kit. Briefly, 100 μL of assay buffer, 50 μL of co-substrate mixture and 20 μL standards/samples were added in each designated wells on the

plate. The reaction was initiated by adding 20 μL of cumene hydroperoxide to all the wells being used. The absorbances were taken in every min at 340 nm using plate reader to obtain at least five points. The standard curve was plotted using the GPx standards, and the activity of GPx for each sample was calculated from the standard curve and expressed as nmol/min/mL.

2.6.2. Determination of superoxide dismutase activity

The SOD activity was determined using Cayman SOD assay kit. Briefly, 200 μL of the diluted radical detector and 10 μL of standards/samples were added in each designated wells on the plate. The reaction was initiated by adding 20 μL of diluted xanthine oxidase to all the wells and incubated on a shaker for 20 min at room temperature. The standard curve was plotted using the SOD standards, and the activity of SOD for each sample is calculated from standard curve and expressed as U/mL.

2.6.3. Determination of malondialdehyde concentration

The level of MDA in the extended seminal plasma was determined using TABARS assay kit (Cayman Chemical Company). Briefly, to each tube 100 μL of samples/standards, 100 μL of SDS solution and 4 mL color reagent was added. The mixture was boiled in a boiling water bath for 1 h. After 1 h, the samples and standards were removed immediately and placed in ice bath for 10 min to stop the reaction. After cooling, the suspension was centrifuged for 10 min at 1600 × *g* at 4 °C. The 150 μL suspensions were loaded into the colorimetric plate and absorbance was measured at 535 nm. The standard curve was prepared using the MDA standards, and the value of MDA for each sample is calculated from standard curve and expressed as μM/mL.

2.7. Statistical analysis

The data obtained in this study was subjected to statistical analysis as per standard methods described by Snedecor and Cochran (1989). The mean values of the percentages of total sperm motility, progressive sperm motility, plasma membrane integrity and enzyme activity were compared using Duncan's multiple range test by one-way analysis of variance (ANOVA) procedure; and differences were considered significant at $P < 0.05$.

3. Results

Findings of the study revealed that sericin in semen freezing media influenced the sperm motility and membrane integrity following the freezing-thawing process (Fig. 1). The percentages of total motility (TM) and progressive sperm motility (PM) was higher ($P < 0.05$) in the freezing medium having three different concentrations 0.25, 0.5 and 1% sericin while it decreased ($P < 0.05$) in the samples with 1.5 and 2% level of sericin. Furthermore, sericin 0.25 and 0.5% treatment resulted in greater plasma membrane integrity than control and other treated group ($P < 0.05$). The effect of sericin supplementation in the freezing extender on antioxidant activities in extended seminal plasma is shown in Table 1. The extender supplemented with 0.25 and 0.5% sericin, significantly ($P < 0.05$) enhanced SOD activity compared to the control. However, increased dose of sericin (1 and 1.5%) decreased SOD activity, compared to the extender supplemented with 0.25 and 0.5% sericin ($P < 0.05$). Addition of 2% sericin in the freezing media showed the lowest level of SOD activity, compared to all the groups ($P < 0.05$). The GPx acivity was found higher in the extender supplemented with sericin at the concentration of 0.25, 0.5 and 1% compared to the control ($P < 0.05$),

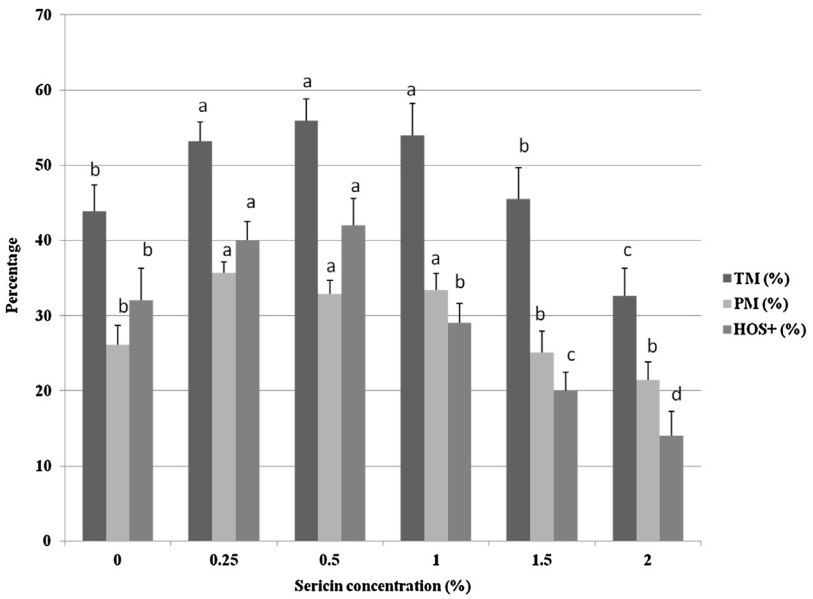

Fig. 1. Effect of sericin concentration on post-thaw sperm motility and membrane integrity ($n = 30$); [abcd] Different superscripts under different concentration for each parameter indicate significant difference ($P < 0.05$); TM: total motility; PM: progressive motility; HOS^+: hypo-osmotic swelling test.

Table 1
Effect of sericin concentration on antioxidant enzymes (superoxide dismutase, SOD, glutathione peroxidase (GPx) activity and malondialdehyde (MDA) concentration in seminal plasma of frozen–thawed buffalo semen ($n = 10$).

Parameters	Sericin concentration (%)					
	0	0.25	0. 5	1	1.5	2
SOD (U/mL)	29.65 ±2.35[b]	40.60 ±1.65[a]	39.42 ±2.67[a]	32.75 ±2.34[b]	28.77 ±1.54[b]	23.16 ±3.25[c]
GPx (nmol/min/mL)	13.5 ± 0. 35[b]	28.90 ±0.23[a]	25.50 ±0. 8[a]	23.61 ±0.12[a]	15.72 ±0. 73[b]	16.41 ±0.60[b]
MDA (μM/mL)	1.47 ±0.21[b]	0.66 ±0.43[a]	0.50 ±0.45[a]	1.93 ±0.56[b]	2.25 ±0.29[b]	11.03 ±0.67[c]

[abcd] Different superscripts in rows indicate significant difference ($P < 0.05$).

while 1.5 and 2% sericin groups had no significant difference to control. In the sericin treated groups (0.25 and 0.5%.) a significant ($P < 0.05$) decrease in MDA concentration in extended seminal plasma was obtained compared to control. The highest level of MDA concentration was found to be in 2% sericin treated group in comparison to other treatment and the control groups ($P < 0.05$).

4. Discussion

Buffalo bull sperm is known to be rich in polyunsaturated fatty acids in its plasma membrane (Jain and Anand, 1976; Tatham, 2000) making them sperm highly susceptible to lipid peroxidation (LPO). LPO of the sperm membrane ultimately leads to the impairment of sperm function due to ROS, altering sperm motility, membrane integrity and fertility, through oxidative stress (Alvarez and Storey, 1989; Aitken et al., 1993). Sericin has been reported as a novel cryopreservation agent of mammalian and insect cell lines (Sasaki et al., 2005). We extended this property of sericin to semen cryopreservation. This is the first report to the best of our knowledge in which supplementation of 0.25 and 0.5% sericin in the freezing extender improved antioxidant status of freezing media and semen quality. Further, MDA concentration was reduced significantly by adding 0.25 and 0.5% sericin as compared to control and other treated groups. The finding of the present study is consistent with finding of Bucak et al. (2007) where it was deduced that antioxidant additives exhibited cryoprotective activity on sperm in moderate doses, but increasing doses of antioxidant additives would result in a hypertonic property of extender which impairs sperm functions. In the present study also, the extender supplemented with 0.25 and 0.5% sericin, resulted in the higher sperm motility, higher plasma membrane integrity and lower lipid peroxidation. Our finding was consistent with the observation with Terada et al. (2002) who reported that 1% sericin had harmful effects on the various mammalian cell lines. Thus, freezing media supplemented with sericin reduces the harmful effect of lipid peroxidation thereby resulting in significantly greater post-thaw semen quality. The antioxidative systems control the balance between production and neutralization of ROS and protect spermatozoa against peroxidative damage (Griveau and Le Lannou, 1997). SOD is an important component of the enzymatic antioxidant system and there is a positive effect of SOD activity on the sperm membrane integrity following the cryopreservation process (Lasso et al., 1994). In this study, 0.25 and 0.5% sericin treated groups showed higher activity of SOD in extended seminal plasma. Similarly, glutathione peroxidase plays an important role in the elimination of hydrogen peroxide (Meister and Anderson, 1983). The elevation of SOD and GPx activity was indicative of improved antioxidant capacity, which was similar to the finding of other workers who used various antioxidants (Aisen et al., 2005; Bucak et al., 2007, Hu et al., 2010). The exact antioxidative as well as scavengering mechanisms of sericin protein remain to be elucidated. However, Kato et al. (1998) hypothesized that the scavenging function may be provided by the chelating effect of hydroxyl groups of hydroxyamino acids (serine and threonine) that are abundantly contained in sericin. Many workers have also identified the strong free-radical-scavenging activity and potent antioxidant activity of silk sericin (Fan et al., 2009; Takechi et al., 2014). Dash et al. (2008) reported that sericin decreased lactate dehydrogenase, catalase, and thiobarbituric acid reactive substance (all parameters of oxidative stress) in H_2O_2-treated cells. Likewise, this study also revealed that sericin act as potent antioxidant and free radical scavenger that reduces lipid peroxidation and protects sperm from free radicals and oxidative damage.

Sericin also possesses the biological activity of preventing cell death and promoting cellular growth (Masakazu et al., 2003). It also served as a protectant against various stresses such as cryoprotectants, ethanol, surfactants, heating and cooling stresses (Sasaki et al., 2005). On the basis of above finding, it can be hypothesized that sericin also protect the sperm cells during dilution, cooling and cryoprotectant toxicity through unknown mechanism. These factors can damage the plasma membrane structure and functions which affect normal sperm functions (Hammerstedt et al., 1990) and can reduce mobility and fertilizing ability (Maxwell and Watson, 1996).

The beneficial effects of sericin in cryopreservation of various mammalian cells have been reported in many studies. For example, Ohnishi et al. (2012) successfully cryopreserved islet cells to increase the practicality of clinical islet transplantation by use of the silk protein sericin. Miyamoto et al. (2012) cryopreserved human adipose tissue-derived stem/progenitor cells using sericin that

have applications in both regenerative medicine and cell transplantation. Further, Isobe et al. (2013) successfully cryopreserved in vitro fertilized embryo by using freezing medium supplemented with sericin and showed no differences in the percentages of recipients with pregnancy, abortion, stillbirth, and normal calving between sericin-based freezing media and serum-supplemented media.

In conclusion, sericin supplementation improves post-thaw motility, membrane integrity and antioxidant status of buffalo cryopreserved semen. Thus, the supplementation of 0.25–0.5% sericin in semen extender improved frozen–thawed semen quality by preventing oxidative stress.

Author contributions

PK and DK performed evaluation of semen quality after supplementation of sericin. PK and PS evaluated antioxidant status in the samples and performed statistical analysis. P Singh, PK and PS designed the work and prepared the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality in this experiment.

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