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Effect of Sericin Supplement in Tris-egg Yolk Citrate Extender on Quality of Cryopreserved Semen from Thai Native Bulls

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Abstract

We investigated the effect of different concentrations of sericin supplement in Tris-egg yolk extender on the post-thawed Thai native bull semen quality. Semen collected by electro-ejaculator once a week for four consecutive weeks from four Thai native bulls were divided into four aliquot and diluted with Tris-egg yolk extender containing 0% (control), 0.1%, 0.5%, and 1.0% sericin and frozen. Thawed semen samples were evaluated for sperm motions characteristics, sperm viability, plasma membrane and acrosomal integrity, and lipid peroxidation. Significant differences ($p < .05$) were observed in total motility, progressive motility, VAP, and VCL with 0.1% and 0.5% sericin showing the highest values for these motion parameters. Similarly, there was significant difference in the percentage of HOS reactive spermatozoa among the treatments ($p < .05$) with the highest HOS reactive sperms in semen cryopreserved in extender containing 1.0% followed by 0.5% sericin, and the lowest in 0% sericin. Further, 0.1% sericin showed significantly higher percentage of viable spermatozoa than the rest of the treatments. Additionally, the lipid peroxidation tended to decrease with increasing concentration of sercin in the extender. Sericin supplement in Tris-egg yolk extender has beneficial effect on the post-thaw sperm motility and viability. Supplement of sericin at 0.1% and 0.5% in extenders improved semen cryopreservation and quality.

Key words: Antioxidant, Triple Fluorescent staining, Sericin, Thai Native Bull

Introduction

Artificial Insemination (AI) has become one of the most cost effective and commonly used assisted reproductive technologies in cattle breeding for genetic improvement through dissemination of superior genes. In developed countries, it has become a routine procedure on the farm while it is being increasingly used in cattle in developing countries. For AI, production of high quality frozen semen is of primary importance. However, cryopreservation damages the spermatozoa as they are exposed to a series of drastic changes in their physical and chemical environment. This damage could be as high as 50% (Watson, 2000). The detrimental effects, in terms of sperm structure, biochemical and functional damage result in reduction of sperm motility, plasma membrane integrity and fertilising ability. The primary site of cryo-injury to spermatozoa is in the plasma membrane (Hammerstedt *et al*., 1990; Krogenæs *et al.*, 1994). One of the causes of sperm damage due to cropreservation is oxidative stress (Mazur *et al.*, 2000). Phospholipids, essential for the structure, function, and integrity of the plasma membrane, are vulnerable to attack by reactive oxygen species (ROS), initiating a lipid peroxidation cascade, which seriously compromise the functional integrity of the membranes (Alvarez and Storey, 1992). Lipid peroxidation level is negatively correlated with motility, plasmalemma integrity, and fertility of bull semen (Kasimanickam *et al.*, 2007).

Semen is naturally endowed with antioxidants, both enzymatic (catalase, glutathione peroxidase, superoxide dismutase) and non-enzymatic (vitamin C, vitamin E, glutathione, cysteine), which protect the sperm from reactive oxygen species mediated injuries Bilodeau *et al.*, 2000; Andrabi, 2009; Garg *et al*., 2009). However, cryopreservation and thawing reduces

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the level of indigenous antioxidants in bull semen (Alvarez and Storey, 1992; Stradaioli *et al.*, 2007) by dilution of semen with extender and excessive generation of ROS molecules (Andrabi, 2009; Kumar *et al.*, 2011). This reduction in the antioxidant level is insufficient (Baumber *et al.*, 2005; Nichi *et al.*, 2006) to protect the sperm from lipid peroxidation and to maintain its integrity during the processes of cryopreservation and thawing. Addition of antioxidant improves progressive motility, and plasma membrane integrity and DNA integrity (Bucak *et al.*, 2010). Therefore, fortification of extenders with antioxidants is recommended to reduce the cryodamage to spermatozoa. Several antioxidants such as vitamin C, vitamin E, catalase, superoxide dismutase, glutathione peroxidase, cysteine, Thiols, butylated hydroxyanisole, *n*-propyl gallate, deferoxamine mesylate, glutamine, hyaluronan, hypotaurine, butylated hydroxytoluene, methionine, inositol, carnitine, taurine, and cysteamine have been added to semen extenders for improving the semen quality in a variety of animal species.

Sericin, a protein constituent of silk secreted by silkworm, *Bombyx mori* Linnaeus, is a highly hydrophilic macromolecular protein comprising of 18 amino acids, predominantly serine (40%), glycine (16%), glutamic acid, aspartic acid, threonine, and tyrosine. Molecular weight of Sericin protein ranges from 10 to 400 kDa (Kato *et al.*, 1998; Takasu *et al.*, 2002) depending on the extraction methods, temperature, pH, and processing time (Zhang, 2002; Freddi *et al.*, 2003). In addition to its multiple properties and potential uses, Sericin has anti-oxidant property, which suppresses lipid peroxidation and inhibits tryosinase activity (Kato *et al.*, 1998). Due to this property, sericin has been used to replace animal serum in cell cultures and cell cryopreservation; and several favourable results have been reported. Supplementation with 1% sericin in medium for embryo culture improves the maturation and quality of embryos (Do *et al.*, 2014). Isobe *et al.* (2012) reported that damage to zona pellucida, survival, and development of frozen-thawed in-vitro derived embryos in freezing medium supplemented with 0.1%, 0.5%, and 1.0% sericin were similar to those embryos frozen in freezing medium supplemented with 0.4% bovine serum albumin (BSA) and 20% fetal bovine serum (FBS). They also showed that the transfer of in-vivo derived embryos frozen in medium containing 0.5% sericin improves pregnancy rate. Sasaki *et al.* (2005) also found cell culture medium containing sericin to be as effective as conventional medium containing fetal bovine serum in the cryopreservation of cell. Further, 0.05% sericin in oocyte culture reduces the rate of polyspermy and increases prevetilline space (Hosoe *et al.*, 2014).

While sericin is successfully used in embryo culture and cryopreservation, use of sericin in semen cryopreservation is not studied except for a study by Kumar *et al.* (2015) as semen extender for cryopreservation of buffalo semen. Their report shows that supplementation of semen extenders for freezing with 0.25%, 0.5%, and 1% sericin improves total motility, progressive motility and plasma membrane integrity, and decreases the rate of lipid peroxidation. However, higher concentration above 1% is reported to have detrimental effect on sperm motility, membrane integrity, antioxidant activity, and lipid peroxidation. Given its antioxidant property, sericin could protect spermatozoa from lipid peroxidation and improve sperm survival and semen quality. Thus, the objective of this study was to investigate the effect of supplementing different concentrations of sericin in Tris egg yolk extenders on the post-thawed semen quality of Thai native bull.

Materials and Method

The experiment was conducted on the Beef farm of agricultural faculty, Khon Kean University during the month of October, 2014. Four Thai Native bulls were stall-fed with concentrates containing 14% (w/w) protein, *ad libitum* of grass with free access to mineral salt blocks and water. Using Randomized Completely Block Design (RCBD), semen was collected once a week from each bull with an electro-ejaculator for four weeks. The ejaculates from each bull with an acceptable progressive motility (> 70%) and concentration (> 800 x 10^6 spermatozoa/ml) were selected, split into four equal aliquots and extended with Tris-egg yolk citrate (control) and Tris-egg yolk citrate fortified with 0.1%, 0.5%, and 1% sericin.

Tris-egg yolk extender was prepared before dilution and added with 0.1%, 0.5%, and 1% sericin, mixed thoroughly, and 20% chicken egg yolk added. The solution was then centrifuged and divided into two parts; Solution A and Solution B. Solution A was warmed to 37 ^{0}C. In Solution B, 6% Glycerol was added, thoroughly mixed by heating and stirring, and cooled to 5 ^{0}C. The semen aliquots were diluted to obtain concentration of 30 x 10^6 sperm/straw. Solution A was added to the semen at 35 ^{0}C, after which they were cooled to 5 ^{0}C in 2 hour at which equal volume of Solution B containing glycerol was added. After equilibration at 5 ^{0}C for 3 hour, semen was loaded in 0.25 ml straws, exposed to liquid nitrogen vapour for 10 min and thereafter plunged into liquid nitrogen. After

storing for two to three days, semen samples were thawed and examined for post thaw quality.

Colour, consistency, and ejaculate volume were visually assessed and recorded. Mass movement and progressive motility of fresh and equilibrated semen was evaluated under a phase contrast microscope (Olympus, Japan). Sperm concentration was measured with a hemocytometer (Neubauer, Boeco, Germany) after a 1:400 dilution with sodium-citrate solution, while sperm were stained with Eosin-Nigrosin stain to assess viability.

Thawed semen samples were assessed for motility, plasma membrane integrity, and acrosomal integrity. Post-thaw semen motion characteristics were evaluated by computer-assisted semen analysis (CASA, Hamilton-Thorne Biosciences IVOS, Version 12.3, Bevery, MA, USA). The CASA procedure/setting was performed according to the manufacturer's recommendation. The frame rate was set up at 60 Hz (Sundararaman and Edwin, 2005; Bucak *et al*., 2010). Briefly, 10 µl of the thawed semen sample was placed in a 2X-CEL chamber slide (Hamilton-Thorne, Inc, MA, USA) and warmed at 37 ^{0}C prior to analysis. Three different fields were selected and scanned for motility (MOT percentage), progressive motility (PMOT percentage), average pathvelocity (VAP, µm/s), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/sec), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hertz), straightness (a ratio of VSL/VAP; STR, percentage), and linearity (a ratio VCL/VAP; LIN percentage). The mean values from three fields were taken for statistical analyses.

The plasma membrane integrity was evaluated with hyposmotic swelling test (HOST). In this, 0.1 ml of fresh and thawed semen was mixed with 1.0 mL hyposmotic solution. A hypo-osmotic solution (150 mOsm/ml) consisting of sodium citrate (1.47 g per 100 ml) and fructose (2.7 g per 100 ml) and incubated at 37 ^{0}C for 30 minute. After incubation, 10 µl drop was put on a slide, cover slipped and examined under the microscope (40x). The swelling response of sperm tail by tail coiling, type b to g as described by Jeyendran *et al.* (1984) was determined.

Further, triple staining using Propidium Iodide (PI), Fluorescein isothyocyanate (FITC)-conjugated with *Arachis hypogaea* L. agglutinin from peanut (PNA), and 5, 5', 6, 6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) was used to evaluate the viability, plasma membrane integrity, acrosome intactness, and mitochrondrial status. In this, 150 µl of thawed semen was transferred to a tube and diluted with 1 ml Tyrode's albumin, lactate, and pyruvate (TALP) at 37 ^{0}C to obtain a concentration of 4.5 x 10^6 spermatozoa/ml. To this, 2 µl of PI (3 mM, Sigma, 28,707-5, in Dulbecco's phosphate buffer (PBS) (0.5 mg/ml; live/dead sperm viability kit L7011 Invitrogen, USA), 5 µl FITC-PNA (100 µg/mL, Sigma, L-0770, in DPBS), and 2 µl JC1 (500 mM, Molecular Probes, M-7514, in DMSO) was added and incubated for 10 minutes in the dark. After incubation, 10 µl drop of the mixture was placed on the slide, cover slipped, and examined using a phase contrast epifluorescence microscopy (Micromanipulator Olympus IX71) with a triple filter showing a set:UV-2E/C (excitation 340-380 nm and emission 435-485 nm), B-25/C (excitation 465-495 nm and emission 515-555 nm) and G-2E/C (excitation 540-525 nm and emission 605-655 nm) at 400X magnification. At least 200 spermatozoa were counted to determine the percentage of the eight types of spermatozoa as described by Celeghini *et al.* (2007). The PI stains the head of the dead spermatozoa red, while the spermatozoa with intact plasma membrane do not take up the stain and remain colourless, the FITC-PNA conjugate gives the damaged acrosome a green fluorescence, while the JC-1 stains the mid piece of spermatozoa with high mitochondrial potential orange or yellow.

Malondialdhyde (MDA) concentration, as indices of the lipid peroxidation in the semen samples, was measured using the thiobarbituric acid reaction in accordance with the method described by Partyka *et al*. (2007). Briefly, thawed semen samples were washed 3 times with 3ml TALP followed by centrifugation each time. Thereafter, semen was resuspended in 1ml TALP to which, 0.25 ml each of Ferrous sulfate and Sodium Ascorbate was added and incubated at 37 ^{o}C for one hour in water bath. Thereafter, 1 ml each of TCA and Thiobarbituric acid (TBA) was added to each samples and immersed in boiling water for 10 min followed by immersing in chilled water at 4 ^{o}C for 2 minute. After cooling to room temperature, the samples were centrifuged at 5000rpm for 10 min and the supernatant was used to determine absorbance at 532 nm. The amount of MDA was determined by the applying the regression equation generated by the standard calibration curve of MDA equivalents produced by the acid-catalysed hydrolysis of 1, 1, 3, 3-tetramethoxypropane I (TEP). The MDA concentrations were expressed as nmol/ml.

Data was analysed using the SAS 9.1 statistical software package. General Linear Model (GLM) procedure was used to test the differ-

ence in the mean post-thaw semen quality between different treatments at a significance level of $p < .05$. Correlation between different parameters was investigated with Pearson's correlation coefficient test.

Results and Discussion

Post-thaw sperm motion parameters

Computer-assisted semen analyser (CASA) is commonly used to provide precise and accurate information on different sperm motion characteristics (Gravance and Davis, 1995; Holt and Palomo, 1996). In this study, the effect of different sericin concentrations in Tris-egg yolk citrate semen extender on post-thaw motion characteristics of semen is present in Table 1. The results indicated that there were significant differences ($p < .05$) in total motility between the semen cryopreserved in extender containing sericin compared to the control. Similarly, significant differences were also found in progressive motility between the sericin supplemented group and the control with 0.5% showing the highest value followed by 1%, 0.1%, and 0% (control).

Table 1. Effect of different sericin concentrations in Tris-egg yolk citrate semen extender on post-thaw motion characteristics of semen

	Sericin concentration (%)					
Semen motion parameters	0	0.1	0.5	1	*SEM*	*p* value
Total motility (%)	37.7^{b}	40.6^{a}	41.7^{a}	39.9^{a}	4.7	0.002
Progressive motility (%)	24.6^{b}	26.1^{a}	26.7^{a}	26.4^{a}	2.8	0.002
VAP	76.4^{b}	79.3^{a}	79.5^{a}	76.3^{b}	7.6	0.001
VSL	60.6	62.5	62.6	60.6	6.1	0.174
VCL	129.2^{b}	163.1^{a}	136.1^{ab}	129.8^{b}	12.3	0.001
ALH	6.1	6.2	6.3	6.3	0.7	0.753
BCF	27.0^{b}	45.1^{a}	25.2^{b}	25.4^{b}	6.7	0.029
STR	79.7	79.3	78.9	79.4	4	.67C6
LIN	50.3	50.2	49	49.8	4.1	0.47

Values with different letter superscripts in the same row indicate significant difference at $p < .05$

There was strong positive correlation between total motility and progressive motility ($R^2 = 0.93$, $p = 0.0001$), total motility and viability ($R^2 = 0.64$, $p = .0001$). Total motility and progressive motility observed in this study are consistent with those reported by Leite *et al.* (2010) and Büyükleblebici *et al.* (2014) in semen from Gyr bulls, Rengarajan (2004) in *Bos indicus* Linnaeus, and Kathiravan *et al.* (2005) in *Bos indicus* x *Bos Taurus,* but much lower than those reported by Anzara *et al.* (2011) and Sundararaman *et al*. (2012) in Jersey bulls. This could be due to genetic variability in semen motion characteristics as reported by several authors (Rengarajan, 2004; Hoflack *et al.*, 2007). The difference could also be due to the semen collection method used. In the present study, semen was collected by the electro-ejaculator method which has been reported to be thinner in consistency, have lower number of spermatozoa, and lower post-thaw recovery (Leon *et al*., 1991). In this study, sericin supplement in extender improved total motility and progressive motility suggesting the beneficial effect of sericin on sperm motility.

There was significant difference ($p < .05$) in the velocity parameters (VAP and VCL) in semen extender containing 0.1% and 0.5% sericin as compared to the control. However, there was no significant difference ($p > .05$) the treatments for other motion parameters, namely, VSL, ALH, STR, LIN where significant bull and ejaculate effect was observed. However, the significant treatment x ejaculate interaction on total motility, progressive motility, VAP, VCL and bull x ejaculate effect on other motion parameters such as VSL, ALH, STR and LIN could be due to the variability among bulls as reported by Farrell *et al.* (1997); Parkinson and Whitfield (1987).

Combined motion parameters assessed by CASA, especially progressive motility, VAP, and VSL, have been reported to be highly correlated to fertility (Amann, 1989; Farrell *et al.*, 1998). It would be interesting to see the effect beyond the level used in this study. The significant difference found among the treatments in other semen motion characteristic such as VAP and VCL suggests the beneficial effect of sericin on sperm motion characteristics and

supports the result of total motility and progressive motility. Kathiravan *et al.* (2008) reported that progressive motility alone is responsible for 62.6% of the variation in fertility rate of bulls, and when combined with VAP and VSL and percent hypoosmotic reacted spermatozoa, the contribution rose to 66.1% indicating the high association of CASA assessed sperm motion characteristics to fertility.

Post-thaw semen viability

The HOS test is a simple, inexpensive and easily applicable method (Quintero-Moreno *et al.*, 2009) for assessment of sperm viability and is reported to correlate highly with progressive motility, hamster oocyte penetration test (Jeyendran *et al.*, 1992), in-vitro fertilisation (IVF) results in human (van der Venn *et al.*, 1986), and with pregnancy rates in pigs (Pérez-Llano *et al.*, 2001). The mean± SD of HOS test reactive spermatozoa are presented in Table 2. The difference in the percent HOS reactive spermatozoa was significantly different between the spermatozoa cryopreserved in extender with sericin as compared to the control. The highest percentage of HOS reactive spermatozoa was observed in semen cryopreserved in extender containing 1%, followed by 0.5%, 0.1%, and 0% sericin.Strong positive correlation was observed between total motility and percentage of HOS reactive spermatozoa (R^2 = 0.75, p = .0001), and between progressive motility and HOS reactive spermatozoa (R^2 = 0.91, p = .0001). The percentage of HOS reactive spermatozoa in this study was in the range reported by Muzafer *et al.* (2012). In this study, correlation between percentage of HOS reactive spermatozoa and total motility and progressive motility was consistent with those reported (r^2 = 0.87) by Kasimanickam *et al.* (2006). Higher percentage of HOS positive spermatozoa in extender containing sericin compared with spermatozoa cryopreserved in extender without sericin is an indication of the beneficial effect of sericin on the plasma membrane integrity. There was strong correlation between progressive motility and viable spermoatozoa (R^2 = 0.82, p = .0001), and between HOS and viability (R^2 = 0.77, p = .0001).

Triple staining of spermatozoa with PI/FITC -PNA and JC-1 can simultaneously evaluate the plasma membrane integrity, acrosome intactness and mitochondrial status at the same time (Graham *et al.*, 1990; Celeghini *et al.*, 2007). An intact plasma membrane, intact acrosome, and high mitochondrial potential represent the viable spermotozoa and are considered important for fertilisation. The results showed significantly higher percentage of spermatozoa with intact plasma membrane, intact acrosome, and high mitochondrial potential (IPIAHM), in 0.1% sercin than the rest of the treatments. However, spermatozoa with intact plasma, intact acrosome and high mitochondrial potential (IPIAHMP) found in this study is lower than those reported by Celeghini *et al*. (2007). The difference could be due to the extender used in this study as the percentage categories of spermatozoa in fluorescent staining could be underestimated due to the interference from egg yolk particles (Nagy *et al.*, 2003). Nevertheless, the significantly higher IPIAHM spermatozoa, which represents the viable spermatozoa found in 0.1% sericin, is suggestive of the protective effect of sericin on sperm viability.

Lipid peroxidation

The MDA, as a measure of lipid peroxidation, was assessed by spectrometer based on the thiobarbituric acid reaction absorbance. The mean and SEM values for MDA for different levels of sericin in the extender are presented in Table 2. No significant difference was found among semen cryopreserved in extender containing 0%, 0.1%, 0.5%, and 1% sericin. However, there is a decreasing trend in the MDA levels with increasing concentration of sericin as shown in Table 2. MDA is an indicator of lipid perxidation in semen. MDA values obtained in this study by using different antioxidants such as superoxide dismutase and glutathione, reduced glutathione, glutathione peroxidase and catalase were similar to the range reported by Sariözkan *et al.* (2009), Asadpour *et al.* (2012), and Muzafer *et al*. (2012), but lower than those reported by Bansal and Bilaspuri (2009) and Büyükleblebici *et al.* (2014). However, lower MDA concentration in the semen cryopreserved in extender containing sericin as compared to the control indicates the antioxidant property of sericin, which is supported by Kumar *et al.* (2015). The mechanism of action of sericin as an antioxidant is not clearly understood but has been suggested to be due to high content of hydroxy amino acids and chelating of trace elements such as Fe^+ by the hydroxyl groups (Kato *et al*., 1998). Further, the low MDA levels in the control in this study suggest that Thai Native bulls may have inherently low level of seminal peroxidation.

Table 2. HOS reactive and viable spermatozoa, and MDA concentration in frozen-thawed semen at different levels of sericin supplementation in Tris egg yolk extender

	Level of sericin (%)					
	0	0.1	0.5	1	*SEM*	*p* value
HOS reactive (%)	34.4^{c}	36.1^{b}	37.1^{ab}	38.0^{a}	2.9	0.001
Viable spermatozoa (%)	30.9^{b}	33.4^{a}	31.5^{ab}	31.9^{ab}	7.5	0.001
MDA(nmol/ml)	1.5	1.3	1.3	1.2	0.6	0.183

Values with different letter superscripts in the same row indicate significant difference at $p < .05$

Conclusions

Significant difference among the treatments was observed in semen motion characteristics such as total motility, progressive motility, VAP, VCL and HOS reactive spermatozoa in semen from Thai Native bulls. Additionally, decreasing trend in lipid peroxidation was observed with increasing concentration of sericin in the extender. This research suggested that sericin supplement in Tris-egg yolk extender has beneficial effect on the post thaw motility and viability of spermatozoa. Supplementation of 0.1% and 0.5% sericin in extenders could improve quality of cryopreserved semen. This is the first study on the effect of sericin as an antioxidant on bull semen cryopreservation. However, further studies are needed to validate the result. Evaluation of sericin with different extenders with or without egg yolk, the level of sericin used, evaluation of sericin in semen collected by different methods in addition to use of larger number of experimental animals and replicates are suggested to validate the effect of sericin and establishing it as a cryoprotectant for semen cryopreservation.

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