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Research article

Cryoprotective effect of sericin on re-freezing of bull spermatozoa and subsequent embryonic development after *in vitro* fertilization

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Abstract

Sericin, a protein produced by silk worm, has been used to improve quality of frozen-thawed spermatozoa in several species. However, the cryoprotective effect of sericin on re-freezing of spermatozoa has not been reported. This study was conducted to investigate the putative cryoprotective effect of sericin on re-freezing of bull spermatozoa followed by an assessment of in vitro fertilizing capacity of sericin-supplemented re-frozen spermatozoa. In experiment 1, the effect of 0.5% sericin supplemented in semen extender on re-freezing of spermatozoa was examined. Highly motile frozen-thawed spermatozoa were prepared with Percoll discontinuous gradient centrifugation prior to re-freezing. Refrozen-thawed spermatozoa were examined for motility, viability, membrane integrity, and acrosome integrity. In experiment 2, the effect of sericin-supplemented re-frozen spermatozoa on fertilizing capacity and subsequent embryonic development were determined using in vitro embryo production approach. Bovine oocytes were fertilized with three groups spermatozoa, re-frozen/control, re-frozen/sericin, and once frozen. The results showed that the total motility of re-frozen thawed spermatozoa in the sericin supplemented group was higher (53.9 % vs. 41.5%, $p<0.05$) than that of the control group whereas viability, membrane integrity, and acrosome integrity were unaffected. The proportion of cleaved oocytes in re-frozen/sericin-supplemented group was comparable to those in the re-frozen/control and once frozen groups. Additionally, the percentages of blastocyst development from cleaved oocytes were similar among the three groups. In conclusion, the results of this study demonstrated that pretreatment of frozen-thawed spermatozoa with 0.5% sericin improved motility after re-frozen thawed without affecting the in vitro fertilizing capacity and subsequent embryonic development.

Keywords: Bull spermatozoa, In vitro fertilization, Re-freezing, Sericin

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INTRODUCTION

During the past few years, re-freezing of frozen-thawed bull spermatozoa has become an interesting topic in semen cryopreservation. Re-freezing of bull spermatozoa has gained attention for two major applications. Firstly, semen of young bulls might be cryopreserved in large volume containers such as 12 mL test tubes or 8 mL cryogenic glass tubes and re-frozen later in straws with only the semen of selected bulls after the completion of genetic value determination (Arav et al., 2002; Saragusty et al., 2009). Secondly, it may be required to produce sexed spermatozoa from frozen-thawed semen of bulls that were no longer in the production or bulls that reside in a long distance from the sorting facility. Being able to re-freeze sexed spermatozoa derived from once frozen-thawed semen would expand the utilization of sperm sexing technology. Recent research has shown that sperm sex-sorting efficiency from frozen-thawed semen was relatively high but the re-cryopreservation method remains to be improved (Hollinshead et al., 2002; de Graaf et al., 2006; Underwood et al., 2009a).

Spermatozoa have been re-frozen successfully in a wide range of mammals albeit drastic reductions in post-thawed sperm quality, especially in sperm motility (human, Polcz et al., 1998; goat, Hollinshead et al., 2004; rabbit, Si et al., 2006; horse, Choi et al., 2006; cattle, Maxwell et al., 2007; mouse, Aoto et al., 2007; brown bear, Alvarez-Rodriguez et al., 2013; giant panda, Santiago-Moreno et al., 2016). However, the fertilization capacity of re-frozen thawed spermatozoa has been assessed in only few studies. The first successful in pregnancies was reported in cattle following artificial insemination (AI) with re-frozen-thawed bull spermatozoa using the multi-thermal gradient semen freezing technique (Arav et al., 2002). In sheep, frozen-thawed ram spermatozoa were sex-sorted, re-frozen, and successfully used with in vitro fertilization and embryo transfer to produce offspring of pre-determined sex (Hollinshed et al., 2004; Morton et al., 2006). In horse, re-frozen stallion spermatozoa were used with intracytoplasmic sperm injection (ICSI) and resulted in comparable blastocyst development to that of once frozen spermatozoa (Choi et al., 2006). In rabbit, AI with re-frozen semen was resulted in reduced pregnancy and kindling rates compared to once-frozen semen (Si et al., 2006). In cattle, sex-sorted bull spermatozoa derived from frozen semen could be re-frozen and used for in vitro embryo production as efficient as once frozen-thawed spermatozoa (Underwood et al., 2010).

Sericin is a glycoprotein produced by silk worm. The silk fibers contain approximately 20%-30% of sericin. Sericin has an unusually high content of serine (30-33%) with the typical secondary structure of a random coil (Kato et al., 1998; Huang et al., 2003). The commercially available sericin is produced by solvent extraction from silk cocoons. Studies showed that supplementation of sericin in semen extender improved quality of frozen-thawed spermatozoa in several species. In buffalo, 0.25-0.5% sericin treatments resulted in higher post-thawed sperm motility and membrane integrity compared to those in control group (Kumar et al., 2015). In goat, inclusion of 0.25% sericin improved the post-thawed sperm quality by enhancing antioxidative capacity and reducing the intracellular enzymes leakage (Reddy et al., 2018). Rabbit spermatozoa pretreated with 0.5 % sericin had higher sperm motility, viability, and acrosome integrity after thawed (Raza et al., 2019). Addition of 0.5 -0.75% sericin improved sperm motility in pig and decreased lipid peroxidation (Ratchmak et al., 2020). Recently, the post-thawed

bull sperm quality was improved by supplementation of 0.25-0.5% sericin in semen extender (Yangngam et al., 2021). Nonetheless, there is no report available related to the cryoprotective effect of sericin on re-freezing of spermatozoa. The objective of this study was to determine whether supplementation with sericin in extender could improve the post-thawed quality and in vitro embryo production efficiency of re-frozen thawed bull spermatozoa.

MATERIALS AND METHODS

Ethical approval

No animal ethics approval is required in this research. Frozen semen for re-freezing and in *vitro* fertilization was requested from a governmental source. Cow ovaries were obtained from a local slaughterhouse.

Study period and location

This research was conducted from October 2019 to November 2021 at the Faculty of Veterinary Medicine, Khon-Kaen University, Khon-Kaen, Thailand. Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA)

Experimental design

Experiment 1 Bull sperm quality after re-frozen with sericin supplemented extender

The experiment was conducted with the selected concentration of sericin at 0.5% that has been shown to improve post-thawed sperm quality consistently after supplemented in semen extender (Kumar et al., 2015; Yangngam et al., 2021). For this experiment, frozen semen from four bulls obtained from the Department of Livestock Development, Thailand were pooled together. Only semen with the initial post-thawed motility above 40 % was used in this study. Motile spermatozoa were selected prior to re-freezing using Percoll centrifugation method. The experiments were repeated for five replications

Experiment 2 Bovine embryonic development after in vitro fertilization with re-frozen spermatozoa

In vitro fertilization (IVF) followed by embryo culture were employed to determine the functional integrity of re-frozen thawed spermatozoa. Frozen semen from single bull was used to produce re-frozen semen. The experimental groups were the following

Group 1, IVF with once frozen-thawed semen

Group 2, IVF with re-frozen semen without sericin (re-frozen/control)

Group 3, IVF with re-frozen semen with 0.5% sericin (re-frozen/sericin)

Experiments were conducted for four replications.

Re-freezing of frozen-thawed bull spermatozoa

Motile sperm separation

Semen from four bulls was pooled together. Motile spermatozoa were separated from immotile sperm for further re-frozen study using discontinuous

Percoll centrifugation technique. The iso-osmotic 90% Percoll solution was prepared by combining nine parts of Percoll PLUS (GE Healthcare Bio-Science AB, Uppsala, Sweden) with one part of 10X tris-citric acid-glucose solution (2.5 M tris-hydroxymethyl aminomethane, 820 mM citric acid, and 690 mM glucose). The 45% Percoll solution was prepared with 1 mL of 90% Percoll and 1 mL of 1X tris-citric acid-glucose solution. Frozen-thawed semen was gently layered on the top of 45% Percoll solution and centrifuged at 700g for 10 min. Sperm pellet was collected in the volume of 100 µL and added to 400 µL of semen extender A (without glycerol).

Cryopreservation of frozen-thawed bull spermatozoa

Two-step dilution (extender A and B) was employed for re-freezing of bull spermatozoa. The semen extender was the egg yolk-tris-citric extender consisting of 200 mM tris-hydroxymethyl aminomethane, 65 mM citric acid, 55 mM glucose, and 20% (v/v) fresh chicken egg yolk. Motile spermatozoa re-suspended in semen extender A (without glycerol) were cooled to 4ºC in a foam box using a 2-step approach, 30ºC to 12ºC at 0.3ºC/min and held for 30 min prior to cooled down to 4ºC at the same rate. At 4ºC, the total volume of 500 µL of semen extender B containing 10% (v/v) glycerol was slowly added in each volume of 100 µL at 10 min intervals to achieve the final concentration of 5% glycerol. Semen was held at 4ºC for 60 min before loaded in to 0.25 mL straws. Semen was frozen with a controlled-rate freezing equipment (CL-8800, CryoLogic, Victoria, Australia). The freezing protocol was cooling from 5ºC to -8ºC at -1ºC/min and hold for 2 min, -8ºC to -43ºC at -8ºC/min, free fall to -120ºC, and finally straws were plunged into liquid nitrogen.

Sperm quality evaluation

Assessment of sperm motility and kinematic by computer-assisted sperm analysis (CASA)

Motion analysis of spermatozoa was carried out with the CEROS II unit (Hamilton-Thorne, Beverly, MA, USA) at the manufacturer's recommended settings. At least five different fields were selected and scanned for total motility, progressive motility, average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), straightness (VSL/VAP ratio; STR), and linearity (VCL/VAP ratio; LIN).

Assessment of sperm viability using live-dead staining technique

One part of semen was mixed with one part of warm eosin-nigrosin staining solution (0.67% eosin-Y and 10% nigrosin in phosphate-buffered saline, WHO laboratory manual for the examination and processing of human semen) and wait for 30 sec. A small drop of semen and dye mixture was smeared on a warm slide. Two hundred sperm were examined under light microscope at 400X magnification.

Assessment of sperm membrane integrity using hypo-osmotic swelling test (HOS test)

The hypo-osmotic solution with an osmotic strength of 100 mOsmol/kg was used. The solution consists of 9 g/L fructose and 4.9 g/L tri-sodium citrate (Correa and Zavos, 1994). One part of semen was diluted in 20 parts of

hypo-osmotic solution and incubated for 60 min at 37°C. After incubation, two hundred sperm were examined in each sample under light microscope at 400X magnification. Spermatozoa with bending or coiling of tails were classified as membrane intact spermatozoa.

Assessment of acrosome integrity using PNA-FITC staining method (peanut agglutinin from Arachis hypogaea conjugated with fluoresceine-isothiocyanate)

A thin smear of the semen was prepared and air-dried for approximately 10 min. Slides were soaked in cold absolute methanol (4°C) for 30 sec and covered with PNA-FITC (50 µg/mL final conc.) and propidium iodide (10 µg/mL final conc.) for 30 min in a humidified chamber at 4°C. Slides were observed under an epi-fluorescent microscope at 1000X magnification. Spermatozoa stained evenly bright green of FITC all over the acrosomal area were classified as intact acrosome.

In vitro production of bovine embryos

In vitro oocyte maturation (IVM)

Bovine ovaries were collected from a local slaughter house in Khon-Kaen province. Cumulus-oocyte complexes (COCs) with at least three layers of cumulus cells surrounding and homogeneous cytoplasm were selected and washed in TCM-199. Up to 20 selected COCs were cultured in 100 µL of maturation medium comprised of TCM-199 supplemented with 50 µg/mL sodium pyruvate, 25 mM $NaHCO_3$, 0.1 mM cystine, 0.1 mM cysteamine, 10 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES), 5% fetal bovine serum (Gibco, USA.), 10 ng/mL epidermal growth factor (EGF), and 0.025 armor unit/mL of follicle stimulating hormone (FSH) for 23 hours at 38.5°C under 5%CO_2.

In vitro fertilization (IVF)

Motile spermatozoa were selected with Percoll centrifugation technique similar to the above described except that 90% Percoll was prepared with 10X TALP (Tyrode's albumin-lactate-pyruvate; Parrish et al., 1995). The 45% Percoll was prepared with 1 mL of 90% Percoll and 1 mL of TALP-HEPES (Farrell and Bavister, 1984). The sperm pellet in the 90% Percoll layer was washed once by centrifugation with TALP-HEPES. The sperm pellet was re-suspended and counted for sperm concentration. Mature oocytes were inseminated with 1×10^6 spermatozoa/mL for 18-20 h in 100 µL drop of Fert-TALP (Parrish et al., 1988) containing 20 µg/mL heparin and incubated at 38.5°C under 5%CO_2

In vitro embryo culture (IVC)

At 18-20 hours post insemination (hpi), presumptive zygotes were transferred to synthetic oviductal fluid (SOF) supplemented with 0.5 mM citrate (Keskintepe and Brackett, 1996), 0.4 mM treonine, and 2 mg/mL bovine serum albumin (BSA). Prezumptive zygotes were cultured in 50 µL drops at 38.5ºC under 5%CO_2. At 72 hpi, only embryos with at least 4 blastomeres were collected for further culture. At 144 hpi, embryos were cultured in the TCM-199 medium with 2% fetal bovine serum and without FSH or EGF.

The cleavage was determined on day 3 and blastocyst development was determined on day 8 after fertilization. To assess the proper speed of development, embryos reaching blastocyst were classified into three stages of blastocyst; early blastocyst, full blastocyst, and expanded blastocyst.

Data analysis

The experimental design was the complete randomized design. The data obtained from sperm quality were analyzed by Student's T-test using IBM SPSS Statistics V.28 and IBM AMOS. The data obtained from embryonic development were analyzed by Chi-square test.

RESULTS

Experiment 1 Effects of sericin on re-freezing of bull spermatozoa

The mean percentages of motile spermatozoa, determined by visual observation, after Percoll was 70.9%. At 4°C prior to re-freezing, the percentages of motile spermatozoa were similar between the control and sericin-added groups (59.6% vs. 64.9%, respectively). The post-thawed quality of re-frozen spermatozoa was shown in Table 1. The CASA analyses revealed that the total motility of re-frozen thawed sperm in sericin-added group was significantly higher than that of control group (53.9% vs. 41.5%, respectively) while the progressive motility was comparable to the control group. No significantly different in sperm kinematic was found, the mean VAP, VSL, VCL, STR, and LIN were similar between two groups. The percentages of viable sperm and membrane intact sperm were comparable between two groups. However, the percentage of acrosome intact sperm in the sericin-added group tended to be higher than that of the control group (58.0% vs. 50.8%, respectively, p= 0.05).

Table 1 Post-thawed quality of bull spermatozoa re-frozen with or without addition of sericin in semen extender. Data are showed in mean±SD.

Parameters	Experimental groups		
	Control	Treatment	P value
CASA analyses			
Total motility (%)	41.5±7.9[a]	53.9±8.6[b]	0.02
Progressive motility (%)	23.2±7.1	33.5±6.8	0.07
VAP (µm/sec)	62.8±8.1	66.0±7.1	0.42
VSL (µm/sec)	55.9±5.7	58.5± 5.1	0.53
VCL (µm/sec)	102.3±16.6	106.7±15.5	0.31
STR (%)	88.6±2.5	89.3±1.7	0.46
LIN (%)	57.3±3.4	58.1±3.5	0.50
Viability (%)	60.6±3.5	58.7±3.9	0.96
Membrane integrity (%)	29.1±6.3	32.1±7.4	0.92
Acrosome integrity (%)	50.8±3.6	58.0±3.6	0.05

Data were pooled from five replications. VAP, average path velocity; VSL, straight linear velocity; VCL, curve linear velocity; STR, straightness (a ratio of VSL/VAP); LIN, linearity (a ratio of VCL/VAP). Values in the same row with different superscripts differed significantly (p< 0.05).

Experiment 2 Bovine embryonic development after *in vitro* fertilization with re-frozen spermatozoa

The cleavage and blastocyst development were shown in Table 2. The results showed that re-frozen control spermatozoa had *in vitro* fertilization capacity comparable to that of once frozen-thawed sperm of the same bull. Similarly, the proportions of cleaved oocytes reaching the blastocyst stage were comparable between two groups. No beneficial effect of sericin was found on fertilization and embryonic development. The percentages of cleavage and blastocyst development in re-frozen sericin group were comparable to those of re-frozen control and once frozen groups.

Table 2 *In vitro* production of bovine embryos using re-frozen thawed bull spermatozoa. Data were pooled from four replications.

Types of spermatozoa	No. of oocytes	% Cleavage	% Blastocyst from cleavages	% Blastocyst from oocytes	% Expanded blastocysts from total blastocysts
Once frozen	130	85.8 %	36.9 %	31.7 %	61.5 %
Re-frozen/control	121	80.2 %	35.1 %	28.1 %	71.0 %
Re-frozen/treatment	125	81.6 %	35.7 %	29.1 %	66.7 %

DISCUSSION

Although cryopreservation of bull spermatozoa is generally considered a routine procedure, reports on re-freezing of bull spermatozoa are limited. In human, it is a common practice to perform several repeated freeze-thaw cycles to maximize the use of donor spermatozoa for various reasons (Polcz et al., 1998). However, under most circumstances, those spermatozoa were destined to be used for intracytoplasmic sperm injection (ICSI) under which the sperm motility or even viability was not required (Wakayama and Yanagimachi, 1998; Ahmadi and Ng, 1999). In cattle, on the contrary, AI and IVF are still the main approaches in utilizing frozen semen. Hence, preserving the sperm motility remains a challenge in re-freezing of bull spermatozoa. The results of this study demonstrated an improvement in bull sperm motility following re-freezing and thawing using sericin treatment and also showed that re-frozen thawed bull spermatozoa had the *in vitro* fertilizing ability and subsequent embryonic development similar to that of the once frozen-thawed spermatozoa. Successful re-freezing of frozen-thawed bull spermatozoa was first reported by Arav et al. (2002) and resulted in pregnancies following AI but the motility of re-frozen thawed sperm was not reported. Later, a discontinuous density gradient centrifugation (PureSperm®) was used to select motile spermatozoa prior to re-freezing (Maxwell et al., 2007). In this study, the use of discontinuous density gradient centrifugation was carried out to increase the quality of spermatozoa derived from the first freeze-thaw. We obtained an average sperm motility at 70.9 % after Percoll discontinuous density gradient centrifugation during this study. Unlike other studies, we prepared isotonic 90% Percoll with 10X Tris-citric-glucose buffer. This allowed the sperm pellet to be re-suspended directly in Tris-egg yolk extender without washing by centrifugation preventing the further sperm lost from another centrifugation process. Under our conditions, approximately 40% of motile spermatozoa were recovered (data not shown)

which were slightly higher than previous studies (Samardzija et al.,2006; Maxwell et al., 2007, Machado et al., 2009). The percentage of Percoll carried over was calculated to be at 9% which proved to be harmless to re-freezing of bull spermatozoa.

In experiment 1, we examined the cryoprotective effect of sericin on post-thawed qualities of re-frozen bull spermatozoa. In general, acceptable the post-thawed sperm qualities were obtained in both control and sericin supplemented groups. Sericin has been shown to exert the cryoprotective effect to spermatozoa in various species (buffalo Kumar et al., 2015; goat Reddy et al., 2018; human Aghaz et a., 2020; rabbit Raza et al., 2019; horse Nasirabadi et al., 2019; mouse Ghasemi et al., 2019; pig Ratchmak et al., 2020; cattle Yangngam et al., 2021). Therefore, it is interesting to determine whether sericin also exert such effect on re-freezing of bull spermatozoa. Our results showed that supplementation with 0.5% sericin improved total sperm motility and tended to improve the progressive motility of re-frozen thawed spermatozoa. Since the percentage of motile spermatozoa assessed at 4°C before re-freezing in sericin-treated group was only slightly higher than that of the control group, it suggested that sericin had minimal effect during cooling step but exerted the beneficial effect mainly on freezing step or possibly after thawed by prolonging sperm motility.

The exact mechanism on how sericin can protect spermatozoa against freeze-thaw injuries remains unknown. Initially, sericin was chosen to replace serum for cryopreservation of mammalian cells owing to its antioxidative property from previous reports (Kato et al., 1998; Sasaki et al., 2005). Pregnancies were obtained after transfer of cryopreserved bovine embryos using 0.5% sericin as a serum replacement confirming the cryoprotective effect of sericin (Isobe et al., 2013). Studies conducted with cryopreserved buffalo and goat semen revealed that sericin possessed cryoprotective effect by enhancing the antioxidant status, according to the level of superoxide dismutase and glutathione peroxidase detected, in sperm-free semen extender containing seminal plasma (Kumar et al., 2015; Reddy et al., 2018). However, whether the increasing in antioxidant status took place before freezing or after thawing was unclear. A study in rabbit spermatozoa showed that sericin could protect cryopreserved spermatozoa by enhancing sperm osmotic tolerance to hypo-osmotic conditions that spermatozoa must encounter during thawing from rapid influx of water (Raza et al., 2019). Additionally, sericin treatment has been shown to improve mitochondrial function in cryopreserved boar spermatozoa (Ratchamak et al., 2019).

In this study, the percentage of acrosome intact spermatozoa tended to be improved in the sericin treated group compared to control. This is in agreement with previous studies showing that the detrimental effect of freeze-thaw on acrosomes was reduced by sericin treatment (Reddy et al., 2018; Aghaz et al., 2020; Raza et al., 2019). The velocity, linearity, and straightness kinematics of re-frozen thawed spermatozoa were also determined in this present study. Our results showed that sericin treatment was unable improve the motility characteristics of re-frozen thawed spermatozoa. In fact, effect of sericin treatment on sperm kinematics has never been determined before in previous reports. Nonetheless, the kinematic values obtained in this study were comparable to those of re-frozen thawed bull spermatozoa in previous reports (Maxwell et al., 2007; Underwood et al., 2009b).

In experiment 2, IVF was employed to determine the fertilizing capacity and subsequent embryonic development of re-frozen spermatozoa of the same bull. Although our IVF data were obtained from semen of a single bull, the results demonstrated that bull spermatozoa retained their full functional integrity after two repeated freeze-thaw cycles. Since the percentages of cleavage and blastocyst development as well as expanded blastocyst stage embryos were comparable between once-frozen thawed and both of re-frozen thawed spermatozoa groups, it strongly indicated that there was no severe damage occurring to the re-frozen thawed spermatozoa. Nevertheless, the percentages of cleavage and blastocyst formation of this study concur with previous studies in using re-frozen thawed spermatozoa for IVF (Hollished et al., 2004; Underwood et al., 2010).

To achieve acceptable IVF results, it is common to separate motile spermatozoa from immotile sperm using various techniques, mainly swim-up and a discontinuous gradient centrifugation. Percoll discontinuous gradients centrifugation has been shown to be more efficiency in motile sperm recovery compared to swim-up method (Parrish et al., 1995; Mehmood et al., 2009; Arias et al., 2017). In this experiment, all groups of spermatozoa were prepared with Percoll discontinuous gradients centrifugation prior to IVF. Therefore, it was understandable that fertilization and blastocyst development in both of re-frozen thawed spermatozoa groups were comparable to that of once frozen-thawed spermatozoa, despite a drastic reduction in initial post-thawed motility (65% for once frozen vs. 45-50% for re-frozen, determined by visual observation). In fact, the percentages of sperm motility after Percoll centrifugation prior to IVF were comparable among the sperm groups (80% for once frozen vs. 75-80% for re-frozen, determined by visual observation). Percoll discontinuous gradients centrifugation has been shown to effectively improve post-thawed quality of spermatozoa in previous reports (Oliveira et al., 2012; Noguchi et al., 2015).

The lack of improvement in fertilization capability of sericin-treated re-frozen spermatozoa, compared to re-frozen/control sperm, was not unexpected owing to the fact that the spermatozoa used for IVF in all groups were selected with the Percoll centrifugation technique, as mentioned earlier. Although a number of studies have reported the benefit of sericin in cryopreservation of various mammalian spermatozoa, the reports on fertilization capability of sericin-treated cryopreserved spermatozoa are limited and inconclusive. A study conducted in rabbit showed that supplementation of extender with 0.5% sericin improved post-thawed quality of rabbit spermatozoa but abolish the fertility after using for artificial insemination (Raza et al., 2019). On the other hand, extender supplemented with 0.5% sericin was showed to improve the fertilization capability of the frozen-thawed epididymal mouse spermatozoa (Ghasemi et al., 2019). Clearly, the harmful effect of sericin on fertilization capability of spermatozoa was not found in this study. This contradicting result might be partially explained by different between sperm preparation and sperm utilization approaches used among the studies.

CONCLUSION

This study demonstrated that sericin supplementation in extender improved motility of re-frozen thawed bull spermatozoa but unable to further enhance fertilization capacity after IVF. However, re-frozen thawed bull spermatozoa, both control and treatment, had a comparable efficiency of the in vitro embryo production to that of once frozen thawed under the condition described.

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AUTHOR CONTRIBUTIONS

Boualy Saphungthong: re-freezing of spermatozoa and post-thawed sperm evaluation
Sujira Thammawung: in vitro production of bovine embryos
Saksiri Sirisathien: in vitro production of bovine embryos and finalizing the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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