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Improved Maturation Rate of Bovine Oocytes Following Sericin Supplementation in Collection and Maturation Media

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ABSTRACT

Sericin is a water-soluble protein produced by silk cocoons and known to have antioxidant activity. This study is aimed to analyze the nuclear maturation and the quality of bovine oocytes in the collection and *in vitro* maturation (IVM) medium supplemented with sericin. Bovine oocytes were collected using a collection medium supplemented with sericin in 0 (control) concentrations, 0.1%, 0.5%, and 1%. Selected oocytes were then matured for 24 h at 38.5 °C in 5% CO_2 and evaluated for nuclear maturation. In the subsequent experiment, oocytes were collected and matured with or without 0.1% sericin at 38.5 °C in 5% CO_2. Matured oocytes were counterstained with terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) and Hoechst 33342. Matured oocytes were characterized by oocytes that reached the MII stage. The results showed that supplementation of 0.1% sericin in the collection medium increased the number of oocytes reaching the metaphase II (MII) stage compared to the control group ($p<0.05$). In the next experiment, sericin 0.1% in the collection and *in vitro* maturation media increased ($p<0.05$) the percentage of oocytes reaching the MII stage compared to control without sericin supplementation. Furthermore, the number of fragmented DNA in the oocytes showed no differences in all groups. It can be concluded that supplementation of 0.1% sericin in the collection and *in vitro* maturation media improved the nuclear status without affecting DNA fragmentation.

Keywords: bovine oocyte; collection medium; in vitro; maturation; sericin

INTRODUCTION

Oocyte quality is one of the essential factors in *in vitro* embryo production (IVEP). The oocytes' qualities can be evaluated based on their morphologies (Ajduk & Zernacka-Goetz, 2013; Hoshino, 2018). Oocytes with excellent morphological qualities produce embryos with good developmental potentials leading to a high pregnancy rate (Ajduk & Zernacka-Goetz, 2013). The qualities of oocytes matured *in vitro* were lower than the of oocytes matured *in vivo* (Lee *et al*., 2018). The low quality of oocytes matured *in vitro* is likely caused by the stress conditions produced from the beginning of animal slaughtering until IVEP processes. The occlusion of blood flow reduces energy and oxygen supplies after the animal is slaughtered. These conditions will make the ovaries are under ischemic conditions (Wang *et al*., 2011). In the ischemic conditions, reactive oxygen species (ROS) are generated due to reoxygenation (Febretrisiana *et al*., 2015; Granger & Kvietys, 2015) and induce oxidative stress (Lobo *et al*., 2010; Wang *et al*., 2017). Moreover, oxidative stress also occurs during IVEP. It is known that oxygen concentration in an *in vitro* environment is higher than that in the *in vivo* environment in the body (Cetica *et al*., 2001), which are 21% and 2%-9%, respectively (Jagannathan *et al*., 2016). Oxidative stress causes damage to the macromolecules, namely chromosomes (Cetica *et al*., 2001), DNA (Lobo *et al*., 2010), proteins (Lobo *et al*., 2010; Wang *et al*. 2017; Wang *et al*., 2018), lipids, and carbohydrates (Lobo *et al*., 2010; Wang *et al*., 2017). Furthermore, oxidative stress also affects vital physiological processes, including cell cycle (Patterson *et al*., 2019), nuclear transport (Kodiha & Stochaj, 2012), and programmed cell death (apoptosis) (Cetica *et al*., 2001; Khazaei & Aghaz, 2017).

Oxidative stress can be minimized by antioxidant supplementation into the medium, and sericin is one of such antioxidants. Sericin is produced by silk cocoons (*Bombyx mori*), and this molecule is a water-soluble protein (Kato *et al*., 1998; Terada *et al*., 2002; Chlapanidas *et al*., 2013). Serine is the highest amino acid in the sericin molecule and known to have antioxidant activity (Kato *et al*., 1998; Terada *et al*., 2002; Chlapanidas *et al*., 2013). Chlapanidas *et al*. (2013) report that sericin is often supplemented in the culture medium. Sericin as an antioxidant prevents

a stress oxidative in the oocytes by decreasing the production of hydrogen peroxide (H_2O_2) (Isobe *et al*., 2014; Gustina *et al*., 2019) and inhibiting lipid peroxidation (Kato *et al*., 1998). The hydroxyl groups are responsible for the antioxidant action by chelating trace elements such as copper and iron (Kato *et al*., 1998). In IVEP, supplementation of sericin in the maturation medium increases the nuclear maturation of oocytes of buffalo (Gustina *et al*., 2017; Gustina *et al*., 2019) and sheep (Yasmin *et al*., 2015). The process of oocyte collection consists of the COCs aspiration of all ovaries, precipitation of aspirated fluid, and grading of the oocytes under a microscope. This process is conducted before the process of IVEP, and oxidative damage of the oocytes may occur. However, to the best of our knowledge, there are few reports regarding the sericin supplementation into the collection medium and its effects on the quality of bovine oocytes. So far, sericin was typically supplemented in the *in vitro* maturation medium only. This is an essential gap in knowledge and reproductive technology because there is an urgent need to improve the quality of oocytes produced *in vitro*. Therefore, this research was conducted to determine the effects of sericin supplementation in the collection and *in vitro* maturation media on the meiotic competence and frequency of DNA fragmentation of bovine oocytes.

MATERIALS AND METHODS

Experimental Set Up

This study was divided into two experiments. In the first experiment, we examined the impacts of sericin (pure sericin, 163-22683, FUJIFILM Wako Pure Chemical Industries Corporation, Japan; PT Nabelin Indonesia-Supplier) supplementation in the collection medium on nuclear maturation of bovine oocytes. For this purpose, COCs were collected using collection medium supplemented with different concentrations of sericin (0 (control), 0.1%, 0.5%, and 1%) prior to IVM. The nuclear status of the oocytes was evaluated after *in vitro* maturation for 24-26 h.

In the second experiment, we evaluated the effects of sericin supplementations in the collection and maturation media on the nuclear status and DNA fragmentation of bovine oocytes. Oocytes were collected using a collection medium supplemented with sericin 0% (control) and/or sericin 0.1% (the best concentration from the first experiment). The oocytes were then matured in three groups, which were group of collection and maturation without sericin (-Ser 0.1%; -Ser 0.1%); group of collection with 0.1% sericin and maturation without sericin (Ser 0.1%; -Ser 0.1%); group of collection with 0.1% sericin and maturation with sericin 0.1% (Ser 0.1%; +Ser 0.1%). The nuclear maturation and the number of DNA fragmentation of bovine oocytes were examined after maturation culture for 24-26 h.

Oocytes Collection

Bovine ovaries were collected from an abattoir and were transported to the laboratory with a transportation medium at 35-37 °C in less than five hours. Transportation medium contains 0.9% sodium chloride supplemented with 0.001 g/mL streptomycin sulfate (MEIJI, Indonesia) and 100 IU/mL penicillin-G (MEIJI, Indonesia). The process of oocyte collection consists of the COCs aspiration of all ovaries, precipitation of aspirated fluid, and grading of the oocytes. The COCs were aspirated from follicles with diameters ranging from 3-10 mm. The aspirated fluid was accumulated into a sterile centrifuge tube, and the oocytes were precipitated for 10-15 minutes. After that, the supernatant/follicular fluids were removed from the tube and replaced by a collection medium. The collection medium contained phosphate-buffered saline (PBS), 0.001 mg/mL streptomycin sulfate (Sigma-Aldrich, S9137), 100 IU/mL penicillin G (Sigma-Aldrich, P4687), and 0.3% BSA (bovine serum albumin) (Sigma-Aldrich, A7030) with/without sericin. The oocytes selection was conducted under the stereomicroscope. Only oocytes with homogeneous ooplasm and compact cumulus cells were used.

Oocytes Maturation *In Vitro (IVM)*

The selected COCs were washed three times and matured *in vitro* in a maturation medium covered by mineral oil (Sigma-Aldrich, M5310). The medium of maturation consisted of TCM-199 (tissue culture medium-199) (Sigma-Aldrich, M4530), 0.3% BSA (Sigma-Aldrich, A7030), 10 IU/mL human chorionic gonadotrophin (hCG) (ChorulonTM, MSD Animal Health), 1 μg/mL estradiol (Sigma-Aldrich, E1024), 0.1 IU/mL FSH (follicle-stimulating hormone) (Vetoquinol N-A inc, Canada), and 50 μg/mL gentamycin (Sigma-Aldrich, G1264). The maturation medium was pre-equilibrated in a cell culture incubator with humidity and CO_2 for two h. The oocytes were maturated in 100 μL droplets (10-15 oocytes) of maturation medium in petri dishes and incubated for 24 h at 38.5 °C in 5% CO_2.

After incubation, bovine oocytes were denuded by repeated pipetting in PBS containing 0.25% hyaluronidase (Sigma-Aldrich, H3506). Furthermore, denuded oocytes were washed three times in a collection medium and placed on a glass slide overlaid with a coverslip supported by paraffin mixture: vaseline stripes (1:9). The slide was fixed using the fixative solution (methanol: acetic acid (3:1 v/v)) for 48 h. The slide was then stained with 2% aceto-orcein and evaluated under a phase-contrast microscope (Olympus IX 70, Japan). The oocytes were classified based on their nuclear maturation stages as a germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), anaphase and telophase I (A/T), and metaphase II (MII). Matured oocytes were characterized by oocytes that reached the MII stage.

Analysis of DNA Fragmentation of Oocytes

DNA fragmentation of oocytes was determined based on the modified procedures reported by Karja *et al*. (2006) using a combined technique of simultaneous nuclear staining and deoxynucleotidyl transferase (TdT)

dUTP nick-end labeling (TUNEL). Denuded oocytes were washed four times using PBS supplemented with 3 mg/mL polyvinyl alcohol (Sigma-Aldrich, P8136) (PBS/PVA). They were fixed in a fixative solution (3.7% paraformaldehyde (Sigma-Aldrich, P6148) in PBS) at 4 °C overnight. Furthermore, the oocytes were washed four times in PBS/PVA and permeabilized in PBST (0.1% (v/v) *Triton*-X100 (Sigma-Aldrich, T9284) in PBS) for 40 minutes. The oocytes were then incubated in blocking solution (100 mg BSA/10 mL PBST) at 4 °C overnight. The oocytes were washed four times in PBS/PVA and were incubated with fluorescein-conjugated 2'-deoxyuridine-5'-triphosphate (dUTP) and terminal deoxynucleotidyl transferase (TdT) (TUNEL reagent; Roche Diagnostics Corp., Tokyo, Japan) at 38.5 °C in 5% CO_2 for one h. Following TUNEL, the oocytes were dipped three times in PBS and incubated with 25 µg/mL Hoechst 33342 (Sigma-Aldrich, H33342) for 30 minutes. Moreover, the oocytes were rinsed four times in blocking solution and three times in PBS/PVA solution. The stained oocytes were placed into 5–8 µL antifade reagent on a glass slide overlaid with a coverslip supported by vaseline stripes: paraffin mixture (9:1). The stained oocytes were examined under a Laser Scanning Confocal Microscopy Zeiss LSM 710. This research used two standard filters: an excitation wavelength of 450–490 nm and barrier filter 520 nm to detect fluorescent isothiocyanate (FITC) and an excitation wavelength of 330–380 nm and barrier filter 420 nm to determine the nuclear status of the oocytes stained with Hoechst 33342. The nuclei labeled TUNEL were evaluated to assess DNA fragmentation of the oocytes.

Statistical Analysis

The data of nuclear status and DNA fragmentation were shown as the mean ± SEM. Each experiment was repeated five times. In each experiment group, the oocytes were randomly distributed. The data were analyzed using One-Way Analysis of Variance (ANOVA), and Duncan's Multiple Range Test (DMRT) was performed if there is any significant difference among the treatments at $p<0.05$. Statistical analysis was conducted using IBM® SPSS® Statistics version 24.0 (IBM Corp., Armonk, NY, US).

RESULTS

Experiment 1. Sericin in the Collection Medium and its Effect on Nuclear Maturation of Bovine Oocytes

The stage of oocyte maturation was started with the stage of germinal vesicle (GV), which was characterized by a condensation of the oocytes or slightly diffused chromatin. After that, the oocytes that possessed clumped or strongly condensed chromatin that formed an irregular network of individual bivalents or a metaphase plate but no polar body were classified as a metaphase I (MI) stage. The matured oocytes were characterized by those that reached the MII stage (oocytes with either a polar body or two chromatin masses) (Figure 1). Table 1 showed that all experiments showed lower percentages of oocytes reaching the MI stage than the MII stage. Furthermore, the matured oocytes (MII stage) were ranged from 75.3% to 87%. The percentages of oocytes reaching the MII stage in the group with sericin 0.1% (87.0 ± 3.1) were significantly higher ($p<0.05$) com-

Table 1. Nuclear status percentage of bovine oocytes supplemented with sericin with different concentrations in the collection medium*

Collection Medium	Number of oocytes examined	Percentage of oocytes at each stage		
		MI	MII	Deg
Ser 0%	154	22.0 ± 1.8 (33)[a]	75.3 ± 0.8 (116)[a]	2.8 ± 1.8 (5)
Ser 0.1%	120	10.3 ± 3.3 (13)[b]	87.0 ± 3.1 (104)[b]	2.8 ± 1.9 (3)
Ser 0.5%	113	19.8 ± 1.9 (23)[a]	80.3 ± 1.9 (90)[ab]	0.0 ± 0.0 (0)
Ser 1%	90	17.8 ± 0.5 (16)[a]	79.3 ± 2.8 (71)[a]	3.3 ± 3.3 (3)
p-value		0.01	0.026	0.681

Note: The data were shown as the mean ± SEM of oocytes percentage (number of oocytes). *= Five replicate trials were carried out. MI= metaphase I; MII= metaphase II; Deg= degeneration; Ser 0%= supplementation of sericin at a dose of 0%; Ser 0.1 %= supplementation of sericin at a dose of 0.1%; Ser 0.5%= supplementation of sericin at a dose of 0.5%; Ser 1%= supplementation of sericin at a dose 1%.
[a,b] Means in the same column with different superscripts differ significantly ($p<0.05$).

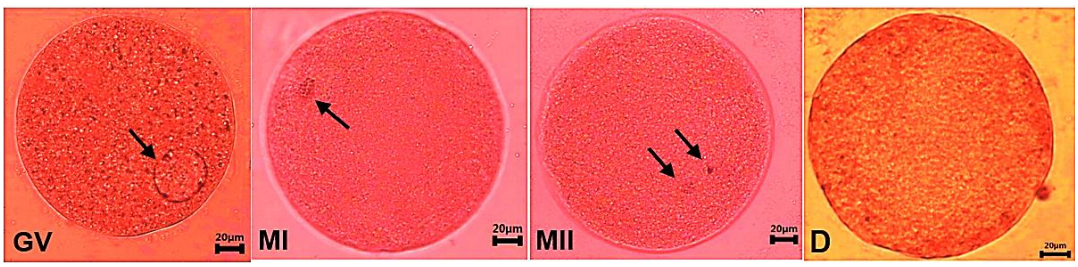

Figure 1. Photomicrograph of nuclear status of bovine oocytes supplemented with sericin in the collection and maturation medium, GV= germinal vesicle; MI= metaphase I; MII= metaphase II; D= degeneration.

pared to control and 1% of sericin, which was similar to 0.5% of sericin.

Experiment 2. Effects of Sericin Supplementation in the Collection and Maturation Media on the Nuclear Maturation and DNA Fragmentation of Bovine Oocytes

As depicted in Table 2, the percentages of oocytes reaching the MII stage in all groups ranged from 74% to 85%. The maturation rates of oocytes collected with sericin and matured with and without sericin were significantly higher ($p<0.05$) than the control.

Data on DNA fragmentation of bovine oocytes in each treatment group or each meiotic stage were combined to investigate any effect of sericin supplementation in the collection and *in vitro* maturation media on DNA fragmentation. Representative images of nuclear status and DNA fragmentation in bovine oocytes are shown in Figure 2. Oversaturation of TUNEL positive occurs due to the specific binding of the terminal deoxynucleotidyl transferase (TdT) enzyme to the 3′-hydroxyl (3′-OH) end of fragmented DNA (Figure 2). The bond will be detected with a certain wavelength from a fluorescent microscope. There were no significant differences in the numbers of DNA fragmentations between the control and the other treatment groups of oocytes collected and maturated with or without sericin (Table 3).

DISCUSSION

Supplementation of 0.1% sericin in the collection media improved the nuclear maturation of the bovine oocyte. Oxidative damage may occur during the process of oocyte collection (the COCs aspiration of all ovaries, precipitation of aspirated fluid, and grading of the oocytes under a microscope). The collection of oocytes in the *in vitro* environment has many factors that promote the formation of ROS. The increasing level of ROS might lead to oxidative stress and induce apoptosis (Fan *et al.*, 2017). The initial step in the formation of ROS production is O_2^{-}. H_2O_2 passes the cell through its membrane, following O_2^{-} dismutation. The presence of H_2O_2, iron, and O_2^{-} will trigger the Haber-Weiss reaction and will generate toxic hydroxyl radicals (Wang *et al*., 2017). The increased ROS level induces the expression of the apoptotic gene ratio (*Bcl-2* and *Bax*) in the mitochondria (Tiwari *et al*., 2015). Sericin had a high hydroxyl amino acid (serine), which had the potential to be an antioxidant (Kato *et al.*, 1998; Isobe *et al.*, 2012). Sericin decreases the level of ROS in cultures by preventing

Table 2. Nuclear status percentage of bovine oocytes supplemented with sericin in the collection and/or maturation medium*

Medium		Number of oocytes examined	Percentage of oocytes at each stage			
Collection	IVM		GV	MI	MII	Deg
– Ser 0.1%	– Ser 0.1%	155	0.0 ± 0.0 (0)	24.2 ± 2.0 (38)[a]	74.8 ± 2.0 (115)[a]	1.0 ± 0.6 (2)
Ser 0.1%	– Ser 0.1%	160	0.5 ± 0.5 (1)	13.8 ± 1.7 (22)[b]	85.1 ± 2.2 (136)[b]	0.5 ± 0.5 (1)
Ser 0.1%	Ser 0.1%	147	0.0 ± 0.0 (0)	12.2 ± 2.5 (20)[b]	85.2 ± 1.8 (124)[b]	2.7 ± 2.0 (3)
		P-value	0.361	0.001	0.001	0.468

Note: The data were shown as the mean ± SEM of oocytes percentage (number of oocytes). * Nine replicate trials were carried out. GV= germinal vesicle; MI= metaphase I; MII= metaphase II; Deg= degeneration; IVM= in vitro maturation; Ser 0.1 %= supplementation of sericin at a dose of 0.1%; -Ser 0.1%= without supplementation of sericin. [a,b] Means in the same column with different superscripts differ significantly ($p<0.05$).

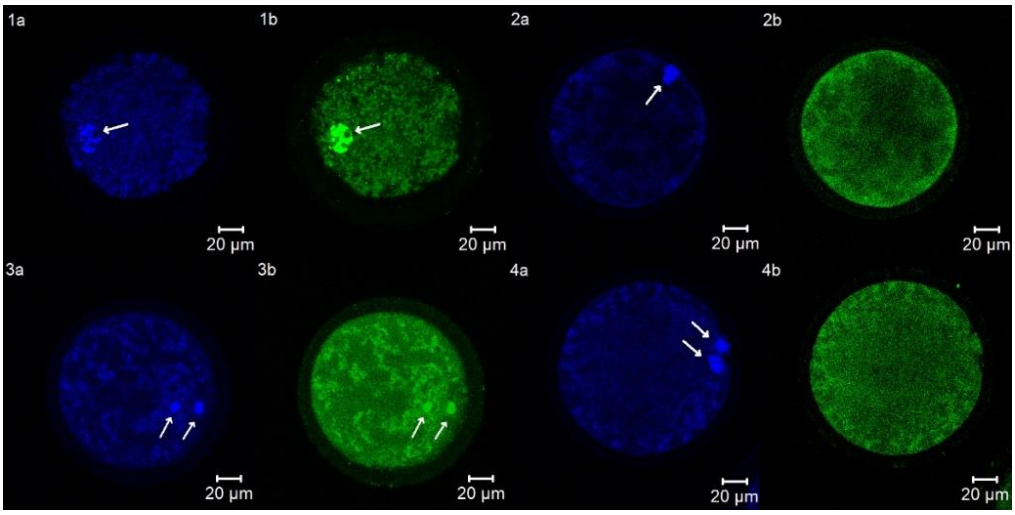

Figure 2. Nuclear status and DNA fragmentation of bovine oocytes stained by Hoechst 33342 (blue channel) (a) and terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end-labeling (TUNEL; green channel) (b); 1a and 2a= metaphase I; 1b= condensed chromatin (arrow) or TUNEL positive of metaphase I; 2b= TUNEL negative of metaphase I; 3a and 4a= Metaphase II; 3b= condensed chromatin (arrow) or TUNEL positive of metaphase II; 4b= TUNEL negative of metaphase II.

Table 3. DNA fragmentation percentage of bovine oocytes supplemented with sericin in the collection and/or maturation medium*

Treatment medium		Number of oocytes examined	Percentage of TUNEL-positive oocytes at each stage	
Collection	IVM		MI	MII
– Ser 0.1%	– Ser 0.1%	64	25.0 ± 15.8 (4/14)	33.2 ± 2.9 (16/50)
Ser 0.1%	– Ser 0.1%	60	20.8 ± 12.5 (2/7)	23.8 ± 4.1 (11/53)
Ser 0.1%	Ser 0.1%	62	62.5 ± 23.9 (5/8)	30.5 ± 3.4 (17/54)
		P-value	0.317	0.113

Note: MI= Metaphase I; MII= Metaphase II; IVM: in vitro maturation; Ser 0.1 %= supplementation of sericin at a dose of 0.1%; – Ser 0.1%= without supplementation of sericin; [a,b] Means in the same column with different superscripts differ significantly ($p<0.05$); * Five replicate trials were carried out.

H_2O_2-induced oxidative stress (Dash *et al.*, 2008). The mechanism of this antioxidant action is the chelation of trace elements derived from the high content of amino acid hydroxyl (Kato *et al.*, 1998). Therefore, the collection of oocytes using media with 0.1% sericin supplementation will have more oocytes competent to develop and mature.

On the other hand, sericin also increases hyaluronan levels and accelerates CD44 mRNA expression (Hosoe *et al.*, 2014). Nagyova (2018) reports that hyaluronan (hyaluronic acid) has glycosaminoglycans, which are synthesized in the extracellular matrix in COCs as a response to stimulation with FSH and LH (Salustri *et al.*, 2017). The CD44 is the principal cell surface receptor for extracellular matrix hyaluronan (Yokoo *et al.*, 2010; Hosoe *et al.*, 2014). The hyaluronan function via CD44 is responsible for cytoplasmic (Hosoe *et al.*, 2014) and nuclear (Yokoo *et al.*, 2010) maturation of oocytes. The interaction between Hyaluronan and CD44 induces tyrosine phosphorylation of Cx43, which inhibits cAMP transport from cumulus cells into the oocytes and leads to MPF activation and the resumption of the meiotic oocyte (Yokoo *et al.*, 2010). Therefore, supplementation of 0.1% sericin in the collection and maturation media can improve the nuclear maturation rate of bovine oocytes.

This study demonstrated that sericin could improve the nuclear maturation of oocytes, and there were no significant differences in the amounts of DNA fragmentation in the oocytes in all treatment groups. Although this process was not mechanistically well-understood, the authors speculate that it could be due to the effects of glutathione from the oocytes. The glutathione levels may still be sufficient to carry out its function as an antioxidant. Glutathione, a tripeptide thiol (γ-glutamyl cysteinyl glycine), is an endogenous non-enzymatic antioxidant naturally present in the oocytes (Lobo *et al.*, 2010; Khazaei & Aghaz, 2017; Wang *et al.*, 2017). Glutathione has an essential role in protecting the cells from oxidative damage through protective action against ROS (Jiao *et al.*, 2013; Adeoye *et al.*, 2018). This action is facilitated by the interactions with its associated enzymes, such as glutathione peroxidase and glutathione reductase. Glutathione peroxidase will catalyze the reduction of hydrogen peroxide and lipid peroxides in the presence of GSH (reduced form), which is converted to GSSG (oxidized form). Moreover, GSSG is minimized by glutathione reductase in the presence of NADPH, which is generated mainly in the pentose phosphate pathway. NADPH then regenerates GSH through glutathione reductase activity (Jiao *et al.*, 2013; Wang *et al.*, 2017). These findings are significant because the new knowledge generated can improve oocyte maturation and enhance the assisted reproductive technologies (ART) for livestock, humans, and endangered species.

CONCLUSION

Supplementation of 0.1% sericin in the collection and *in vitro* maturation media increases the meiotic competence without affecting bovine oocytes' DNA fragmentation. Further study is necessary to evaluate the effect of sericin supplementations in the collection and *in vitro* maturation media on embryonic development after IVF and embryo quality.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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