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The potency of sericin as an alternative protein in collection and maturation media to support *in vitro* bovine oocyte maturation

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ABSTRACT

The purpose of this study was to evaluate the potency of sericin as a substitute for BSA (bovine serum albumin) to support the *in vitro* maturation of bovine oocytes. Cumulus-oocytes complexes were collected with BSA or 0,1% sericin, and then matured either in media with BSA or sericin. The maturation rate was evaluated based on the meiotic status of oocytes. The incidence of DNA fragmentation in oocytes was assessed by TUNEL. The percentage of oocytes reaching the MII stage in the media either with BSA or sericin was significantly higher than those of oocytes collected and matured in BSA only. Although the maturation rate of oocytes collected with BSA and then matured with sericin was comparable to oocytes in BSA-BSA groups ($P>0.05$) the rate was similar to oocytes collected with sericin and then even matured with BSA or sericin ($P>0.05$). There was no significant difference in the incidence of DNA fragmentation among the treatment groups ($P>0.05$), the indices of DNA-fragmented oocytes were found around 17-20 %. In conclusion, Sericin has the potency to replace BSA as a source of protein either in collection media and/or in maturation media as well as potentially preventing the incidence of DNA fragmentation in oocytes.

Keywords: Bovine, DNA fragmentation, In vitro maturation, Meiotic competence, Sericin

INTRODUCTION

The success of in vitro embryo production (IVEP) is well known to be affected by the quality of oocytes subjected to in vitro maturation before being fertilized. A suboptimal in vitro environment will impair oocyte competence and subsequent embryo development. The embryo production from slaughterhouse ovaries begins with the collection of oocytes from the follicles in the ovary. During collection and maturation procedures, oocytes are exposed to media immediately after released from the follicle, therefore suitable media is required to maintain the quality and the viability of the oocytes. Media for the collection and maturation of oocytes in vitro have been widely developed. To date, it is usual to add serum or serum-derived protein such as bovine serum albumin (BSA) in media to improve and ensure the success of culture in vitro

(Arias *et al.* 2022). Serum and its contents are reported to induce cell proliferation and stimulate growth of the cell (Karsmarski *et al.* 2022). It is also reported that serum or serum albumin has the ability as an antioxidant to scavenge reactive oxygen species and chelate heavy metals (Lin and Wang 2021). However, the use of animal origin sera in media has become a consideration regarding the possibility of contamination by pathogenic agents such as bovine spongiform encephalopathy (Banafshi *et al.* 2021). Thus, alternative protein supplements instead of serum and BSA in culture media have an important meaning for the development of serum-free media. There are limited reports of alternative proteins as a substitute for serum or serum albumin in supporting the success of the culture systems in vitro since reducing serum in the media may damage cell growth and even lead to cell death.

Research in recent years found that sericin as a substitute for serum can support the growth and attachment of mammalian cells in vitro (Liu *et al.* 2016; Cao and Zhang 2015) Sericin is a protein extracted from silkworms, *bombyx mori*, often utilized for cosmetics and pharmacological (Qin *et al.* 2020) In IVEP, there is various results regarding of the addition of sericin for supporting embryo development in vitro. It was reported that the addition of 0.5% sericin in maturation media in combination with 10% fetal ovine serum increased the ovine oocyte maturation rate and the number of embryos developed into the blastocyst stage after in vitro fertilization (Aghaz *et al.* 2015). However, Hajarian *et al.* (2017) found that there was no significant effect of sericin on the maturation of Sanjabi sheep oocytes when sericin is added to the maturation medium in combination with 10% of fetal ovine serum. In porcine, Do *et al.* (2014) reported that 1% sericin did not have an effect on the development of the blastocyst stage but had an effect on the maturation and fertilization rate as well as the quality of the embryos. In our previous study, we found that sericin supplemented in collection and maturation media combined with 0.3% BSA improved the maturation rate of bovine oocytes (Satrio *et al.* 2022). From the results of our study, it is not certain sericin alone can replace BSA since the addition of sericin is still in combination with BSA, especially in oocyte collection media. Therefore, the objective of this study was to develop a serum-free media during collection and maturation procedure for bovine oocytes. For this aim, we examine the potency of sericin to substitute BSA in collection and maturation media on the promotion of in vitro nuclear maturation of bovine oocytes. Since sericin also offers protection against oxidative stress (Do *et al.* 2014; Sangwong *et al.* 2016), the incidence of nuclear DNA fragmentation in matured oocytes was also examined.

MATERIALS AND METHODS

Oocytes Collection and In Vitro Maturation

The oocytes used in this study were collected from ovaries obtained from slaughterhouse. These ovaries were a waste product of slaughtered bovine therefore the permission of the ethical committee was not required. The ovaries were brought to the laboratory in physiological NaCl at 35-37 °C supplemented with 100 IU/mL Penicillin-G (Sigma-Aldrich, USA) and 10 mg/mL Streptomycin sulfate (Sigma-Aldrich, USA). Using an 18-gauge needle, follicles with a diameter of 3-6 mm were aspirated to release the oocytes along with the follicular fluid. Collected oocytes and follicular fluid were pooled in a 15 ml tube and placed in a water bath at 37°C. Follicular fluid was then discarded and replaced with collection media collection before oocyte selection. The sample was then placed on a petri dish for oocyte selection based on the number of cumulus cells and the homogeneity of the oocyte cytoplasm. Collection media consist of phosphate-buffered saline (PBS) supplemented with 0.3% bovine serum albumin (BSA, Sigma-Aldrich, USA) or 0,1% pure sericin (cat no163-22683-Wako). Selected cumulus oocytes complexes were then matured in tissue culture media -199 (TCM-199) (Sigma-Aldrich, USA) supplemented with 0.3% BSA or 0,1% pure sericin in 5% CO_2 at 38.5 °C for 24 h. Maturation media was also added with 10 IU/ml follicle-stimulating hormone (Vetoquinol N.-A inc, Canada), 10 IU/mL human chorionic gonadotrophin (Chorulon^{TM}, MSD Animal Health), 1 µg/mL estradiol (Sigma-Aldrich, USA) and 50 µg/mL gentamycin (Sigma-Aldrich, USA). After being matured, oocytes were evaluated for nuclear maturation based on the meiotic status of oocytes.

Examination of the DNA fragmentation rate was carried out by TUNEL assay.

Maturation Status of Oocyte

After maturation, CoCs in each treatment group were pipetted in PBS solution containing 0.25% hyaluronidase (Sigma-Aldrich, USA) to release cumulus cells. The denuded oocytes were then fixed in acetic acid: methanol (1: 3 V/V) for 48-72 hours and stained using 1% orcein in a 45% acetic acid solution. The resultant oocytes were then examined under a phase-contrast microscope (Olympus IX 70, Japan) to determine their meiotic maturation status. An oocyte with unclear chromosome configuration or fragmented was classified as a degenerated oocyte.

TUNEL Assay

At the end period of in vitro maturation, samples of oocytes from each group were subjected to analysis of DNA fragmentation as described by Satrio *et al.* (2022) with a minor modification, briefly, the denuded oocytes were washed in PBS containing 3 mg/ml polyvinyl alcohol (Sigma-Aldrich, USA), fixed in paraformaldehyde (Sigma-Aldrich, USA) in PBS for overnight at 4 ºC in 3.7% (w/v). The resultant oocytes were then permeabilized in PBS with 0.1% (v/v) Triton-X100 (Sigma-Aldrich, USA). After being permeabilized for 40 minutes, oocytes were blocked in a solution of 100 mg BSA in 10 mL PBS overnight at 4 ºC. Before the examination, oocytes were washed in PBS/PVA and incubated in fluorescein-conjugated 2'-deoxyuridine-5'-triphosphate (dUTP) and terminal deoxynucleotidyl transferase (TdT) (TUNEL reagent; Roche Diagnostics Corp., Tokyo, Japan) for 1 h at 38.5 ºC in 5% CO_2. Stained oocytes were counterstained with 25 µg/mL Hoechst 33342 (Sigma-Aldrich, USA, H33342) for 30 minutes. Oocytes were examined under a Laser Scanning Confocal Microscopy Zeiss LSM 710 equipped with two standard filter sets, a filter with an excitation wavelength of 450–490 nm and a barrier filter of 520 nm to detect fluorescent isothiocyanate (FITC). A filter with an excitation wavelength of 330–380 nm and a barrier filter of 420 nm to detect the Hoechst 33342. The occurrence of DNA fragmentation was determined by TUNEL labelled nuclei.

Statistical Analysis

The percentages of oocytes reaching each stage of meiosis and the proportions of oocytes with DNA-fragmented were subjected to arc sin transformation before analysis, and then were tested using *IBM SPSS statistics* program version 24.0 by *One-Way Analysis of Variance* (ANOVA) and continued by using *Duncan's Multiple Range Test* (DMRT) when expected values were ≤5. Differences at a probability value (P) of 0.05 or less were significant.

RESULTS AND DISCUSSION

Meiotic Status of Oocytes

The exposure of the oocytes directly after release from the follicle into media with sericin without the presence of BSA improved the maturation rate of bovine oocytes *in vitro* in this study. As shown in Table 1, when the oocytes exposure to collection media with 0.1% sericin directly after being released from the follicle before maturing in the media containing BSA or 0,1 % sericin (Ser-BSA group or Ser-Ser group, respectively), the percentage of oocytes reached MII phase was significantly higher compared to those of oocytes collected and matured in media with BSA only (BSA-BSA group) (P<0.05). The maturation rate of oocytes in the BSA-Ser group

Table 1. Nuclear status of bovine oocytes collected and matured in media supplemented either with BSA and/or Sericin*

Groups	No. of oocytes matured	Percentage ± SEM (No.) of oocytes at each stage***				Percentage of degenerated oocytes (n)
		GV	MI	AT	MII	
BSA-BSA	106	0.0 ± 0.0 (0)	25.1 ± 3.7 (27) [a]	0.0 ± 0.0 (0)	74.9 ± 3.7 (79) [a]	0.0 ± 0.0 (0)
BSA-Ser	102	0.0 ± 0.0 (0)	16.6 ± 3.9 (16) [ab]	0.9 ± 0.9 (1)	81.9 ± 3.0 (84) [ab]	0.9 ± 0.9 (1)
Ser-BSA	127	0.0 ± 0.0 (0)	13.7 ± 2.6 (17) [b]	0.6 ± 0.6 (1)	84.4 ± 1.9 (107) [b]	0.6 ± 0.6 (2)
Ser-Ser	136	0.0 ± 0.0 (0)	7.6 ± 2.9 (11) [b]	0.9 ± 0.9 (4)	88.3 ± 2.5 (119) [b]	1.5 ± 0.9 (2)

*Seven replicate trials were carried out. ** BSA-BSA: Oocytes were collected and matured in media supplemented with BSA, BSA-Ser: Oocytes were collected in media supplemented with BSA and then matured in media supplemented with sericin, Ser-BSA: Oocytes were collected in media supplemented with sericin and then matured in media supplemented with BSA, Ser-Ser: Oocytes were collected and matured in media supplemented with sericin. ***GV: germinal vesicle, MI: metaphase I, AT: anaphase I and telophase I, MII: metaphase II. [a-b] values with different superscript letters are significantly different (p<0.05).

was similar to oocytes in BSA-BSA groups (P>0.05) and to those of oocytes in Ser-BSA and Ser-Ser groups (P>0.05). These data indicated that sericin has the potential to replace BSA as a source of protein either in collection media and/or in maturation media. In addition, Gustina *et al.* (2017) reported that ultrastructure changes of buffalo oocytes when oocytes matured with sericin are similar to BSA, indicating the possibility of replacing-BSA with sericin.

These data supported our previous study that supplementation of 0.1% sericin into in vitro maturation media improved the meiotic competence of oocytes (Satrio *et al.*, 2022). Similarly, Aghaz *et al.* 2015) also reported that 0.5% sericin in IVM media supported nuclear maturation of Sanjabi ewes oocytes in vitro. It is reported that sericin may play an important role in the attachment and growth of cells (Liu *et al.* 2016). Hosoe *et al.* (2014) found that sericin accelerated the expression of CD44 mRNA and the amount of hyaluronan production in oocytes more than FBS. Hyaluronan (hyaluronic acid: HA) is a linear polysaccharide belongs to the family of glycosaminoglycans (GAGs) (Nagyova 2018), and is known to be produced by actively dividing cells during mitosis (Hascall 2019). The function of HA via CD44 (principal cell surface receptor HA) is responsible for the inhibition of apoptosis (Worku *et al.* 2017), meiotic resumption of oocytes and cytoplasmic maturation of oocytes (Richani *et al.* 2021). Hajarian *et al.* (2017) results suggested that sericin supplementation during oocyte culture supports embryonic development by increasing HA production. Therefore, the beneficial effects of sericin in the collection and/or in maturation media in the present study are expected through the increasing the HA production, which could increase the meiotic competence of bovine oocytes matured in vitro.

DNA Fragmentation of Oocytes

The TUNEL-positive oocytes at the MII stage are shown in Figure 1. There was no significant difference in the incidence of DNA fragmentation was found among the treatment groups (P>0.05) (Table 2). Of the oocytes that reached the MII stage, the indices of DNA-fragmented oocytes were found around 17-20 %.

In addition, in this study it was found that the proportion of oocytes that reached the MII stage was lowest in the oocyte group that was collected and matured in media with BSA only. When the oocytes were collected in media with BSA and matured with sericin, the maturation rate was similar to those oocytes matured with sericin. When oocytes were collected and matured in vitro with sericin, it was able to support the meiotic competence of oocytes. These data indicated the feasibility of sericin as an alterna-

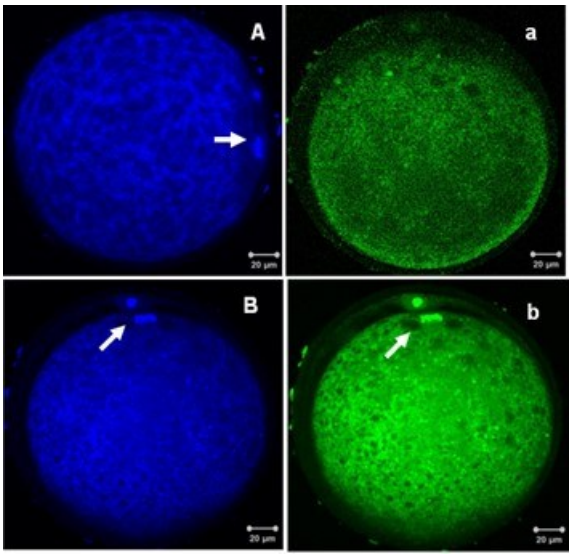

Figure 1. Negative (A) and positive (B) TUNEL of MII stage bovine oocytes. The blue color was oocytes stained by Hoechst 33342 and the green color was stained by deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end-labelling (TUNEL). Scale bars represent 20 µm.

Table 2. DNA Fragmentation of bovine oocytes collected and matured in media supplemented either with BSA or Sericin*

Groups**	No. of oocytes matured	Percentage ± SEM (No.) of oocytes at stage***		Percentage ± SEM (No.) of TUNEL-positive oocytes at stage	
		MI	MII	MI	MII
BSA-BSA	78	27.6 ± 2.9 (22) [a]	72.4 ± 2.9 (56) [a]	44.4 ± 15.1 (8)	17.8 ± 5.4 (11)
BSA-Ser	77	20.4 ± 3.8 (15) [ab]	79.6 ± 3.8 (62) [ab]	33.4 ± 16.1 (6)	20.4 ± 9.9 (13)
Ser-BSA	70	16.7 ± 1.8 (11) [b]	84.0 ± 1.8 (59) [b]	24.8 ± 8.3 (3)	19.5 ± 5.7 (11)
Ser-Ser	65	19.7 ± 4.4 (9) [b]	87.3 ± 4.4 (56) [b]	39.5 ± 15.7 (5)	17.3 ± 6.1 (9)

*Four to five replicate trials were carried out. ** BSA-BSA: Oocytes were collected and matured in media supplemented with BSA, BSA-Ser: Oocytes were collected in media supplemented with BSA and then matured in media supplemented with sericin, Ser-BSA: Oocytes were collected in media supplemented with sericin and then matured in media supplemented with BSA, Ser-Ser: Oocytes were collected and matured in media supplemented with sericin. ***MI: metaphase, MII: metaphase II. [a-b] values with different superscript letters are significantly different ($p<0.05$)

tive protein supplement for collecting bovine oocytes and in vitro maturation as reported by Banafshi *et al.* (2021).

Reactive oxygen species (ROS) are formed during normal cellular metabolism, but when present in high concentrations, they become toxic. Increased levels of ROS can damage various cellular processes. The hydroxyl radical generated from hydrogen peroxide reacting with different transition metals is particularly damaging to DNA, leading to mutagenesis and carcinogenesis (Martemucci *et al.* 2022). It is well established that, ROS generation has been implicated as a major cause of the low percentage of in vitro embryo production, across several species (Sovernigo *et al.* 2017). Therefore, the presence of antioxidants in culture media is expected to overcome ROS (Khazaei and Aghaz 2017). Recently Sericin was nominated as a natural antioxidant (Miguel and Álvarez-López 2020). Sericin is rich in aspartic acid as well as serine (Jena *et al.* 2018), which has a high content of the hydroxyl group (Tian *et al.* 2021). The antioxidative activity of sericin is shown in the peroxidation of linoleic acid and ferrous-ion-chelating ability (Biganeh *et al.* 2022). BSA is also reported to have a role in chelating toxic metal ions or in promoting the uptake of ions (León-Espinosa *et al.* 2021). However, 17-20% of oocytes in the MII stage showed nuclear fragmentation. The incidence of DNA fragmentation in MII stage oocytes did not differ among the treatment group in this study ($P>0.05$). These data indicated that the presence of sericin only as a source of protein in collection and/or in maturation media also has antioxidative activity similar to BSA.

CONCLUSION

In conclusion, the exposure of the oocytes directly after being released from the follicle into media with sericin without the presence of BSA improved the maturation rate of bovine oocytes in vitro. Sericin has the potential to replace BSA as a source of protein either in collection media and/or in maturation media as well as potentially prevent the incidence of DNA fragmentation in oocytes.

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