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ORIGINAL ARTICLE

Ultrastructure changes in buffalo (*Bubalus bubalis*) oocytes before and after maturation *in vitro* with sericin

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ABSTRACT

The aim of this research was to identify the changes in the cytoplasmic ultrastructure of immature and matured oocytes in buffalo (*Bubalus bubalis*). Oocytes were matured *in vitro* in tissue culture medium–199 with and without sericin, and then analyzed by light and transmission electron microscopy. The experiment result showed that the nuclear maturation rate of buffalo oocytes was significantly higher in the presence of sericin (80.6%) than without sericin (68.1%) ($P < 0.05$). The immature oocytes were characterized by cortical granule clusters in the ooplasm and the absence of perivitelline space (PVS). In contrast, the oocytes matured either with or without sericin showed the formation of PVS, erected microvilli, the migration of cortical granules to the cytoplasmic periphery, and the clear appearance of the mitochondria and vesicle in the oolemma. Interestingly, matured oocytes with sericin have smaller cortical granules than do immature oocytes ($P < 0.05$). In conclusion, supplementation of 0.05% sericin in the maturation medium can enhance the maturation rate of buffalo oocytes. Several cytoplasmic ultrastructures were relocated and modulated during the *in vitro* maturation process of buffalo oocytes: PVS development, cortical granules migration to periphery, and mitochondria and vesicles in the cortical region. The ultrastructure was similar between the groups with and without sericin.

Key words: *buffalo oocytes, in vitro maturation, sericin, TEM, ultrastructure.*

INTRODUCTION

In vitro maturation (IVM) of oocytes is an important step in supporting the success of *in vitro* embryo production (IVEP) techniques. During the maturation process, the oocyte cytoplasm will undergo some structural and biochemical changes to support the developmental competence of the embryo (Gordon 2003). In many species, the maturation of the preovulatory oocyte is characterized by progressive changes in the cytoplasmic components of its organization and distribution (Van Blerkom *et al.* 1990). Cytoplasmic maturation is a series of complex events, including the redistribution of cytoplasmic organelles and cytoskeletal filament dynamics (Ferreira *et al.* 2009), whereas molecular maturation includes the transcription, storage and expression of the mRNAs by the chromosomes (Ferreira *et al.* 2009), protein synthesis and RNA transcription (Mao *et al.* 2014). During the transition from the germinal vesicle to the metaphase II stage, the ultrastructure of bovine oocytes, such as mitochondria, ribosomes, endoplasmic reticulum, cortical granules and the Golgi complex, was rearranged (Ferreira *et al.* 2009).

It is well known that the success of the maturation process depends on various factors, such as reactive oxygen species (ROS), that can affect the quality of the oocyte development. ROS can damage DNA, increase cell apoptosis and cause cell death (Dröge

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2002; Alvarez 2003).They can also induce cell death by autophagy, a self-catabolic process involving the sequestration of cytoplasmic components by exhaustion or damage to organelles (Shrivastava *et al.* 2011). At high levels, ROS cause DNA damage because of the disturbance of the mitochondrial membranes (Rocha-Frigoni *et al.* 2016). Since radicals are highly reactive and unstable, ROS can interact with several molecules to bind electrons and make them stable (Deleuze & Goudet 2010). Therefore, these interactions may ultimately lead to cellular damage, including lipid peroxidation, because of major phospholipids in the membrane and the oxidation of amino acids and nucleic acids (Bain *et al.* 2011).

Sericin is a protein from silkworm cocoons (Takasu *et al.* 2007), that was recently reported to act as an antioxidant that potentially decreases levels of ROS (Dash *et al.* 2008). Kato *et al.* (1998) reported the antioxidant activity of sericin could suppress lipid peroxidation and inhibit tyrosinase activity. Additionally, sericin prevents death of insect cells (Takahashi *et al.* 2003) and increases the maturation and fertilization rate of sheep oocytes (Yasmin *et al.* 2015). Until now, there has been no report about the influence of sericin on ultrastructure changes in the maturation process of buffalo oocytes. Therefore, the purpose of the present study was to examine the ultrastructure aspects and nuclear maturation ability of buffalo oocytes matured with sericin.

MATERIALS AND METHODS

Oocyte collection and IVM

Buffalo ovaries were obtained from a local abattoir and transported to the laboratory in a 0.9% physiological saline solution supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin sulphate. Cumulus-oocyte complexes (COCs) were collected by slicing the ovaries with a surgical blade in phosphate–buffered saline (PBS) supplemented with 0.3% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA), and 100 IU/mL penicillin and 100 µg/mL streptomycin sulphate. COCs surrounded by more than three cumulus layers and homogeneous ooplasm were selected and used for this study. The maturation medium used for oocyte IVM consisted of tissue culture medium–199 (TCM-199; Sigma, St. Louis, MO, USA) supplemented with 10 IU/mL human chorionic gonadotrophin (hCG, Chorulon, Intervet International B. V., Boxmeer, The Netherlands), 10 IU/mL pregnant mare serum gonadotrophin (PMSG, Intergonan, Intervet GmbH, Tönisvorst, Germany) and 50 µg/mL gentamycin (Sigma). The selected COCs were washed three times in the maturation medium and then transferred into 100 µL droplets of the maturation medium supplemented with 0.05% sericin (Wako Pure Chem. Industries, Ltd., Osaka, Japan) or without sericin. Concentration of sericin was taken from our previous result that sericin in concentration of 0.05% gave the best effect on nuclear maturation of buffalo oocytes *in vitro* (unpublished data). In the group without sericin, BSA 0.3% was used as a protein source. The oocyte maturation was carried out at 38.5°C in a humidified atmosphere of 5% CO_2 for 24 h.

Matured oocytes were denuded from the cumulus cells by repeated pipetting in PBS containing 0.25% hyaluronidase (Sigma). After denudation, the oocytes were washed, placed on a slide and then covered with a cover slip that was propped up by a mixture of vaseline stripes : paraffin (9:1). The oocytes were fixed in a solution of acetic acid : methanol (1:3 v/v) for 48-72 h and then stained with aceto-orcein (2% orcein and 45% acetic acid). The stained oocytes were observed under a phase-contrast microscope and classified based on the meiosis stage from germinal vesicle (GV) to metaphase II (MII).

Transmission electron microscopy (TEM) ultrastructure changes in immature and IVM oocytes with sericin

Immature COCs were selected and fixed shortly after slicing. The cumulus cells of COCs were removed with 0.25% of hyaluronidase (Sigma) along with repeated pipetting. Both immature and matured oocytes were fixed in 2.5% glutaraldehyde in 0.1 mol phosphate buffer (pH 7.2–7.4) at room temperature (15 min) and at 4°C (15 min). The oocytes were post-fixed with OsO4 (1%) and then dehydrated in ascending concentrations of ethanol embedded in Spurr's resin (Sigma-Aldrich). Semi-thin sections (0.5–1 µm) were serially cut on an ultramicrotome (Reichert Ultracut S). Semi-thin sections were stained with toluidine blue and examined under a light microscope to determine the target cells. Thin sections (60–90 nm) were collected on copper grids, stained with uranyl acetate for 1 h and triple lead for 20 min and then examined by TEM (JEOL 1010; Tokyo, Japan). For description by TEM, the organelles, cytoplasmic membrane and zona pellucida (ZP) were observed, and then the organelle size was measured using a scale bar (AxioVision microscope software; Carl Zeiss MicroImaging GmbH, Jena, Germany).

Statistical analysis

Data of the oocytes' nuclear maturation were presented as percentage ± SEM and then analyzed by analysis of variance (ANOVA). Data of the TEM images of immature and matured oocytes are presented by descriptive comparison. Meanwhile, data of the ultrastructure size of cortical granules, mitochondria and vesicles in immature oocytes, oocytes matured with sericin, and oocytes matured without sericin, were analyzed by ANOVA. The means were compared using the Duncan and least significant differnece tests. All statistical analyses were performed using SPSS for Windows (IBM Corp., Armonk, NY, USA), version 21. The values were considered statistically significant when $P < 0.05$.

RESULTS AND DISCUSSION

Sericin is a fibrous protein produced by silkworms that has been widely investigated in the biotechnological field (Aramwit *et al.* 2009). In our study, we found that supplementation with 0.05% sericin in the maturation medium increased the percentage of oocytes that reached the MII phase ($P < 0.05$) (Table 1). These data supported a previous study reported by Yasmin *et al.* (2015), who found that sericin in the maturation medium could support the nuclear maturation competence of sheep oocytes. It might be the methionine and cysteine in sericin that play an important role in the attachment and growth of mammalian cells (Hynes 1986; Aramwit *et al.* 2009). According to Aramwit *et al.* (2009) sericin from silkworm cocoons with white shells contains 3.39 mol% methionine and 0.44 mol% cysteine, higher than other silk strains.

In this study, we observed the changes of oocyte ultrastructure both in oocytes matured with or without sericin, such as mitochondria, cortical granules and vesicles. During the final maturation, instead of cytoplasmic maturation the oocyte acquires a complex cytoplasmic organization consisting of the modification and redistribution of organelles in specific ooplasmic areas (Motta *et al.* 2002). In general, the ultrastructure of buffalo oocytes was similar to that of oocytes in other mammalian species such as cattle (Fair *et al.* 1997; Hyttel *et al.* 1997), sheep (Máximo *et al.* 2012) and camels (Kafi *et al.* 2005). Buffalo oocytes also have lipid droplets, which the oocytes use as a source of energy in the process of maturation (Mondadori *et al.* 2007; Máximo *et al.* 2012). However, some differences were observed that indicate species-specific characteristics. In buffalo, we observed mostly that the mitochondria are in a round shape. Mondadori *et al.* (2007) reported that the mitochondria of buffalo oocytes have transversal cristae but do not have granules. Kacinskis *et al.* (2005) noted that the proliferation of mitochondria is needed to increase the capacity of energy production in cells that are growing. The immature oocytes had no perivitelline space (PVS), with a tight cytoplasmic membrane and clusters of cortical granules in the ooplasm (Fig. 1a). In the matured oocytes, the perivitelline space was formed, and the cortical granules had moved from their clusters to the peripheral area of the ooplasm along the cytoplasmic membrane in oocytes matured both with and without sericin (Fig. 1b and c). Furthermore, erect and bent microvilli in the well-developed PVS, round mitochondria located in the cortical region, and vesicles with different sizes and shapes were found in both groups of matured oocytes. These data indicated that during the cytoplasmic maturation process, some important changes occurred in the organelle structures, such as cortical granules, mitochondria and vesicles. Our data supported previous studies by Mondadori *et al.* (2010a,b) who reported that the PVS developed during the IVM process and became increasingly enlarged over 24 h. This development was associated with the liberation of the microvilli from the ZP, resulting in bent microvilli lying parallel to the ZP. Microvilli play an important role in the absorption and secretion functions of cells (Kafi *et al.* 2005). The peripheral relocation of the organelles is evidence of preparation for the resumption of oocyte meiosis, which occurs due to the increase of metabolic activity (Mondadori *et al.* 2010b).

To evaluate the effect of sericin on the ultrastructure of organelles, we measured the size of the cortical granules, mitochondria and vesicles (Table 2) as specific characters of buffalo oocytes. No significant differences ($P > 0.05$) were found in mitochondria and vesicle size among the groups of oocytes, but we found a decrease in the cortical granule size in oocytes matured with sericin ($P < 0.05$). However, it is unclear how the sericin affected the alteration of the cortical granule size. In mammals, the formation

Table 1 Nuclear maturation rate of buffalo oocytes matured with and without sericin

Maturation	No. oocytes examined	Percentage (*n*) of oocytes at each stage					
		GV	GVBD	MI	AI/TI	MII	Degenerated
Ser(−)	58	0.0 ± 0.0(0)	0.0 ± 0.0(0)	20.3 ± 5.2(13)	2.8 ± 3.2(2)	68.1 ± 3.9(40)^{a}	4.8 ± 4.1(3)
Ser(+)	66	0.0 ± 0.0(0)	1.6 ± 1.6(1)	16.6 ± 3.6(11)	0.0 ± 0.0(0)	80.6 ± 1.3(53)^{b}	1.2 ± 1.2(1)

^{a,b}Values with different superscripts in the same column differ significantly ($P < 0.05$). AI/TI, anaphase I and telophase I; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II. Ser(−); Oocyte matured without sericin in maturation medium. Ser(+); Oocyte matured with sericin in maturation medium.

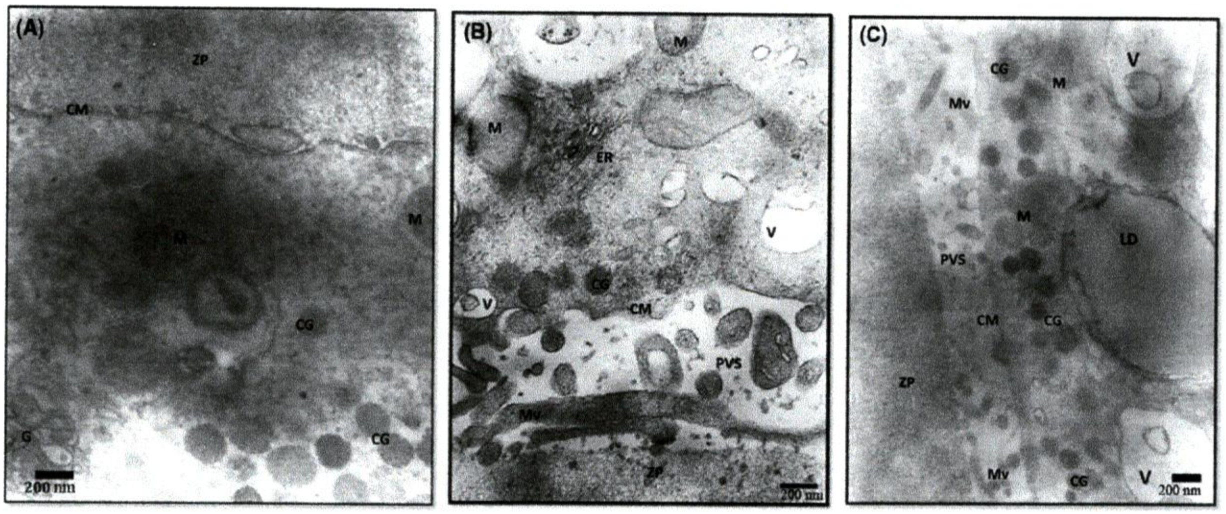

Figure 1 Transmission electron micrograph (TEM) of buffalo oocytes: (a) immature, (b) matured (-sericin), and (c) matured (+sericin). The Golgi complex (G); zona pellucida (ZP); mitochondria (M); cortical granules (CG); microvilli (Mv); vesicle (V); perivitelline space (PVS); cytoplasmic membrane (CM), endoplasmic reticulum (ER), and lipid droplet (LD) (TEM, ×20 000).

Table 2 Organelle ultrastructure size of immature and matured oocytes (with and without sericin)

Treatments	Organelle ultrastructure size (nm)		
	Cortical granule	Mitochondria	Vesicle
Immature	230.2 ± 18.1^{a}	438.9 ± 63.1	492.1 ± 17.2
Matured (− Ser)†	$183.0 \pm 2.6^{a,b}$	411.1 ± 23.0	354.1 ± 110.3
Matured (+ Ser)‡	154.5 ± 4.7^{b}	362.6 ± 66.0	633.6 ± 110.3

^{a,b}Values with different superscripts in the same column differ significantly ($P < 0.05$). Ser(−); †Oocyte matured without sericin in maturation medium. Ser(+); ‡Oocyte matured with sericin in maturation medium.

of cortical granules derived from the Golgi complex undergoes hypertrophy and proliferation (Abbott & Ducibella 2001). The cortical granules are believed to be necessary for the processes of zona hardening and polyspermy blocks during gamete fusion (Conner *et al.* 1997; Hyttel *et al.* 1997). In this study, the organelle size was similar between the groups of oocytes matured with and without sericin. This finding suggests that sericin has no effect on the ultrastructure of the organelles of buffalo oocytes.

In conclusion, a supplementation of 0.05% sericin in the maturation medium can improve the nuclear maturation of buffalo oocytes. During the maturation process, morphological changes occurred in the ultrastructure of organelles. In morphometry, sericin affected only the cortical granules, but the ultrastructure was similar between the groups of oocytes matured with and without sericin.

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REFERENCES

Abbott AL, Ducibella T. 2001. Calcium and the control of mammalian cortical granule exocytosis. *Frontiers in bioscience: a journal and virtual library* **6**, D792–D806.

Alvarez JG. 2003. DNA fragmentation in human spermatozoa: significance in the diagnosis and treatment of infertility. *Minerva Ginecologica* **55**, 233–239.

Aramwit P, Kanokpanont S, De-Eknamkul W, Kamei K, Srichana T. 2009. The effect of sericin with variable amino-acid content from different silk strains on the production of collagen and nitric oxide. *Journal of Biomaterials Science* **20**, 1295–1306.

Bain NT, Madan P, Betts DH. 2011. The early embryo response to intracellular reactive oxygen species is developmentally regulated. *Reproduction, Fertility and Development* **23**, 561–575.

Conner S, Leaf D, Wessel G. 1997. Members of the SNARE hypothesis are associated with cortical granule exocytosis in the sea urchin egg. *Molecular Reproduction and Development* **48**, 106–118.

Dash R, Acharya C, Bindu PC, Kundu SC. 2008. Antioxidant potential of silk protein sericin against hydrogen peroxide-induced oxidative stress in skin fibroblasts. *BMB reports* **41**, 236–241.

Deleuze S, Goudet G. 2010. Cysteamine supplementation of in vitro maturation media: a review. *Reproduction in Domestic Animals* **45**, e476–e482.

Dröge W. 2002. Free radicals in the physiological control of cell function. *Physiological Reviews* **82**, 47–95.

Fair T, Hulshof SCJ, Hyttel P, Greve T, Boland M. 1997. Oocyte ultrastructure in bovine primordial to early tertiary follicles. *Anatomy and Embryology* **195**, 327–336.

Ferreira EM, Vireque AA, Adona PR, Meirelles FV, Ferriani RA, Navarro PAAS. 2009. Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. *Theriogenology* **71**, 836–848.

Gordon I. 2003. *Laboratory Production of Cattle Embryos*, 2nd edn. CABI, London, GB.

Hynes RO. 1986. Fibronectins. *Scientific American* **254**, 42–51.

Hyttel P, Fair T, Callesen H, Greve T. 1997. Oocyte growth, capacitation and final maturation in cattle. *Theriogenology* **47**, 23–32.

Kacinskis MA, Lucci CM, Luque MCA, Báo SN. 2005. Morphometric and ultrastructural characterization of Bos indicus preantral follicles. *Animal Reproduction Science* **87**, 45–57.

Kafi M, Mesbah F, Nili H, Khalili A. 2005. Chronological and ultrastructural changes in camel (*Camelus dromedarius*) oocytes during in vitro maturation. *Theriogenology* **63**, 2458–2470.

Kato N, Sato S, Yamanaka A, Yamada H, Fuwa N, Nomura M. 1998. Silk protein, sericin, inhibits lipid peroxidation and tyrosinase activity. *Bioscience, Biotechnology, and Biochemistry* **62**, 145–147.

Mao L, Lou H, Lou Y, Wang N, Jin F. 2014. Behaviour of cytoplasmic organelles and cytoskeleton during oocyte maturation. *Reproductive BioMedicine Online* **28**, 284–299.

Máximo DM, Martins da Silva IG, Mondadori RG, Neves JP, Lucci CM. 2012. Ultrastructural characteristics of sheep oocytes during in vitro maturation (IVM). *Small Ruminant Research* **105**, 210–215.

Mondadori RG, Luque MCA, Santin TR, Báo SN. 2007. Ultrastructural and morphometric characterization of buffalo (*Bubalus bubalis*) ovarian preantral follicles. *Animal Reproduction Science* **97**, 323–333.

Mondadori RG, Santin TR, Fidelis AAG, Name KPO, da Silva JS, Rumpf R, *et al*. 2010a. Ultrastructure of *in vitro* oocyte maturation in buffalo (*Bubalus bubalis*). *Zygote* **18**, 309–314.

Mondadori RG, Santin TR, Fidelis AAG, Porfirio EP, Báo SN. 2010b. Buffalo (*Bubalus bubalis*) pre-antral follicle population and ultrastructural characterization of antral follicle oocyte. *Reproduction in Domestic Animals* **45**, 33–37.

Motta PM, Nottola SA, Familiari G, Makabe S, Stallone T, Macchiarelli G. 2002. Morphodynamics of the follicular-luteal complex during early ovarian development and reproductive life. *International Review of Cytology* **223**, 177–288.

Rocha-Frigoni NAS, Leão BCS, Dall'Acqua PC, Mingoti GZ. 2016. Improving the cytoplasmic maturation of bovine oocytes matured *in vitro* with intracellular and/or extracellular antioxidants is not associated with increased rates of embryo development. *Theriogenology* **86**, 1897–1905.

Shrivastava A, Kuzontkoski PM, Groopman JE, Prasad A. 2011. Cannabidiol induces programmed cell death in breast cancer cells by coordinating the cross-talk between apoptosis and autophagy. *Molecular Cancer Therapeutics* **10**, 1161–1172.

Takahashi M, Tsujimoto K, Yamada H, Takagi H, Nakamori S. 2003. The silk protein, sericin, protects against cell death caused by acute serum deprivation in insect cell culture. *Biotechnology Letters* **25**, 1805–1809.

Takasu Y, Yamada H, Tamura T, Sezutsu H, Mita K, Tsubouchi K. 2007. Identification and characterization of a novel sericin gene expressed in the anterior middle silk gland of the silkworm *Bombyx mori*. *Insect Biochemistry and Molecular Biology* **37**, 1234–1240.

Van Blerkom J, Bell H, Weipz D. 1990. Cellular and developmental biological aspects of bovine meiotic maturation, fertilization, and preimplantation embryogenesis in vitro. *Journal of Electron Microscopy Technique* **16**, 298–323.

Yasmin C, Otoi T, Setiadi MA, Karja NWK. 2015. Maturation and fertilisation of sheep oocytes cultured in serum-free medium containing silk protein sericin. *Acta Veterinaria Hungarica* **63**, 110–117.