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Hydrogen peroxide concentration and DNA fragmentation of buffalo oocytes matured in sericin-supplemented maturation medium

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Abstract

This study was conducted to investigate the effects of sericin on meiotic maturation, hydrogen peroxide (H_2O_2) concentration and deoxyribonucleic acid (DNA) fragmentation in buffalo oocytes. Oocytes were matured *in vitro* in tissue culture medium (TCM-199) with *in vitro* maturation (IVM) groups under several conditions, namely without bovine serum albumin (-BSA), (+BSA), and 0.025%, 0.05%, 0.1% and 0.25% (w/v) sericin. The results showed that supplementation of the maturation medium with 0.05% sericin significantly increased the rate of oocytes that reach metaphase II compared with other groups, except for the 0.025% sericin-treated group. Intracellular H_2O_2 concentrations in oocytes of all groups were measured using 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA). The concentration of H_2O_2 in matured oocytes treated with 0.05% sericin was lower than in other groups. DNA fragmentation and the nuclear status of oocytes were examined using the terminal deoxyribonucleotidyl transferase-mediated dUTP digoxigenin nick end-labelling (TUNEL) method. The total proportion of TUNEL-positive oocytes at the MII stage was lower in the 0.05% sericin group. The results indicate that addition of 0.05% sericin to the maturation medium may improve nuclear maturation and may inhibit DNA fragmentation in oocytes by decreasing H_2O_2 concentrations.

Keywords: antioxidant, apoptosis, *in vitro* maturation, reactive oxygen species

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Introduction

In buffalo, the efficiency of *in vitro* embryo production (IVEP) is still low (Neglia et al., 2003; Drost, 2007). Approximately only 20% of buffalo oocytes that are fertilized *in vitro* form blastocysts (Gasparrini, 2002; Nandi *et al*., 2002; Manjunatha *et al*., 2008). This inefficiency in buffalo IVEP could be associated with the quality of the oocytes at the beginning of the maturation process, although oocytes were selected based on the compaction of cumulus cells and the homogeneity of ooplasm (Chauhan *et al*., 1998).

One of the critical factors that could affect the quality of oocytes matured *in vitro* is the oxygen tension under which oocytes are cultured. *In vitro* culture environments differ from *in vivo* conditions. The oxygen tension in *in vitro* culture systems (≈ 20%) is often higher than in the female reproductive tract (3% to 9%) (Rocha-Frigoni *et al*., 2016). This condition is believed to possibly induce the generation of reactive oxygen species (ROS), such as superoxide anions, hydroxyl radicals and hydrogen peroxide (H_2O_2) (Morado *et al*., 2009). Reactive oxygen species can modify cell function and endanger the survival of cells that cause fragmentation of DNA, protein oxidation and lipid peroxidation (Yang *et al*., 1998). High oxygen tension during *in vitro* maturation and culture might contribute to decreased oocyte quality (Hashimoto *et al*., 2000) and alter the development of mammalian embryos (Karja *et al*., 2004). Several enzymatic and non-enzymatic

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antioxidant mechanisms have been developed that protect oocytes and embryos against the harmful effects of ROS (Johnson & Nasr-Esfahani, 1994). Antioxidants have the roles that they can i) prevent the formation of ROS, ii) inactivate oxidants and iii) possibly limit the deleterious effects of oxidants or repair oxidative damage (Agarwal *et al*., 2004).

Sericin is a family of proteins that covers fibroin, and as the glue in silkworm cocoons (Takahashi *et al*., 2003). Sericin is known to have a skin moisturizing and antiwrinkle action owing to its high serine content (Engel *et al*., 1987). Currently, sericin is used for various purposes, including functional biomaterials (Altman *et al*., 2003; Cao & Zhang, 2016), pharmacological purposes (Prommuak *et al*., 2008), medical uses (Prasong, 2011; Kunz *et al*., 2016), and food supplements (Zhang, 2002). Sericin is a high molecular weight protein with adhesive properties (Aramwit *et al*., 2010) and is derived from silkworm cocoons (Dash *et al*., 2008). Sericin has ROS-scavenging, anti-tyrosinase, anti-elastase (Chlapanidas *et al*., 2013) and antibacterial properties (Sarovart *et al*., 2003). It can prevent cell death and supports the growth of cells (Agarwal *et al*., 2004), increasing the growth of fibroblast cells (Aramwit *et al*., 2009), and stimulates mammalian cell proliferation (Terada *et al*., 2002). Sato *et al*. (2011) explained that sericin promotes growth in a variety of cells and affects mitosis in mammalian cells through various signalling pathways. Furthermore, Aramwit *et al*. (2009) reported that with its high methionine content, sericin can promote the fastest growth rate of fibroblast cells. Methionine increases the accumulation of extracellular matrix components such as collagen type I and activates matrix metalloproteinases-9 as an active part in extracellular matrix modelling. Kato *et al*. (1998) demonstrated the antioxidant action of sericin by showing it suppresses *in vitro* lipid peroxidation and inhibits tyrosinase activity in rat brains. Previous studies by Chlapanidas et al. (2013) on in vitro stimulated peripheral blood mononuclear cells showed that sericin can serve as an ROS-scavenger. Following its addition to the culture medium for embryo production in vitro, sericin increased the percentage of maturation and in vitro fertilization of sheep oocytes in a medium without BSA (Yasmin *et al*., 2015), and increased the quality and pre-implantation development of bovine embryos that were cultured individually (Isobe et al., 2012). However, the role of sericin in DNA fragmentation or the concentration of H_2O_2 in oocytes matured *in vitro* has not been studied. Therefore, the objective of this study was to evaluate DNA fragmentation and the concentration of H_2O_2 of buffalo oocytes matured in maturation medium supplemented with sericin.

Materials and Methods

Buffalo ovaries were brought from the abattoir to the laboratory in 0.9% NaCl supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin sulphate. Each ovary was sliced with a scalpel blade to release the cumulus-oocyte complexes in phosphate buffered saline (PBS) supplemented with 0.3% bovine serum albumin (BSA) (Sigma-Aldrich) and antibiotics. Oocytes that were surrounded by compact cumulus layers and uniform ooplasm (cumulus-oocyte complexes) were used for this study. The basic maturation medium comprised tissue culture medium-199 (TCM-199) (Sigma, USA) supplemented with 10 IU/mL pregnant mare serum gonadotrophin (Intergonan) (Intervet Deutschland GmbH), 10 IU/mL human chorionic gonadotrophin (Chorulo) (International Intervet BV Boxmeer-Holland), and 50 ug/mL gentamycin (Sigma, USA). Selected oocytes were washed three times and divided into groups, namely those matured in maturation medium without BSA (-BSA group), those matured with BSA only (+BSA group), and without BSA but with sericin (Wako Pure Chem. Industries, Ltd. Osaka, Japan) in varying concentrations [0.025% (w/v) (0.025 Ser group), 0.05% (0.05 Ser group), 0.1% (0.1 Ser) or 0.25% (0.25 Ser)]. Oocytes were matured at a temperature of 38.5 °C with 5% CO_2 for 24 hours.

After maturation, oocytes were denuded from the cumulus cells by repeated pipetting in PBS, which contained 0.25% hyaluronidase enzyme (Sigma, USA). After denudation, oocytes were fixed in methanol (1:3 v/v), for 48 to 72 hours and then stained with aceto-orcein (2% orcein and 45% acetic acid). Nuclear maturation was observed under a phase-contrast microscope and classified based on the stage of maturation as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), a-telophase I (ATI), metaphase II (MII) and degenerated.

The concentrations of H_2O_2 of matured oocytes were measured using DCHFDA (Sigma) as described by Karja *et al*. (2006) with minor modifications. Oocytes in each group were incubated for 15 min in maturation medium containing 10 µM DCHFDA, and then washed in fresh medium before being placed on glass slides and covered with cover slips. Fluorescence emissions were recorded with a digital camera (Zeiss AxioCam HRc, Germany) attached to a fluorescence microscope (Zeiss Axio Imager A2) after excitation at 480 nm and 510 nm emission. The fluorescent image was converted to TIFF files using Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA), then analysed with ImageJ software 1.47 (Sun Microsystems, Inc., California, USA). The fluorescent image was measured by counting the number of pixels after colour inversion. The fluorescence intensity represents the intracellular H_2O_2 concentration.

Fragmentation of DNA and nuclear status of matured oocytes in each group were analysed using the combined technique for nuclear staining and TUNEL (*in situ* cell death detection system) (Roche Diagnostics Corporation, Indianapolis, IN, USA) (procedure modified after those described by Wongsrikeao *et al*. (2004)). Oocytes were fixed overnight at 4 °C in 3.7% (wt/vol) paraformaldehyde diluted in PBS. After overnight fixation, the oocytes were washed four times in PBS, which contained 3% (w/v) polyvinyl alcohol (PVA) and permeabilized in 0.5% (v/v) Triton-X100 for 1 hour, and then incubated in a blocking solution (PBS + 10 mg/ml BSA) overnight at 4 °C. After being washed in PBS-PVA, positive controls and all treated oocytes were incubated in fluorescein conjugated dUTP and TdT (TUNEL reagent) at 38.5 °C for 1 hour in the dark. As positive controls, one to two oocytes per TUNEL analysis were incubated in 1000 IU/mL deoxyribonuclease I (DNase I) (Sigma) for 20 min. Negative controls were incubated in fluorescein dUTP without TdT. After TUNEL, oocytes were washed three times in PBS-PVA, and later stained with 50 ug/mL propidium iodide (PI) for 20 min to label all nuclei. Oocytes were washed extensively in blocking solution and placed on a glass slide and covered with a glass cover. The oocytes were examined under a fluorescence microscope (Zeiss Axio Imager A2) using excitation at a wavelength of 488 nm and 568 nm to detect the reaction of TUNEL and PI. The images were captured with a digital camera (Zeiss AxioCam HRc, Germany) (Figure 1). To assess the relationship between nuclear status and DNA fragmentation, chromatin staining of oocytes was used, classify them based on configuration from GV to MII. Those oocytes with diffusely stained cytoplasm characteristics of non-viable cells and those in which chromatin was not visible were excluded from DNA fragmentation analyses.

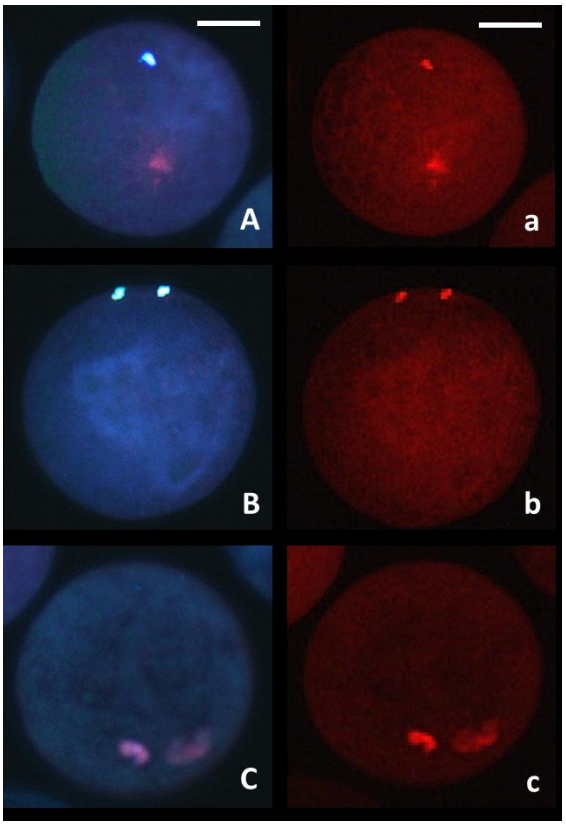

Figure 1 DNA fragmentation in buffalo oocytes stained by terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end-labelling (TUNEL) (left) and propidium iodide (PI) (right). MII-oocyte TUNEL-negative (red nucleus) with the polar body TUNEL-positive (A,a); MII-oocyte TUNEL-positive (bright nucleus) (B,b); and MII-oocyte TUNEL-negative (C,c)
Scale bars represent 40 µm

The results of the meiotic maturation rate, concentration of H_2O_2, and total proportion of TUNEL-positive oocytes are presented as the mean ± SEM. Data were analysed by one-way analysis of variance (ANOVA). Statistical analysis was performed using SPSS for Windows version 21. $P \leq 0.05$ was the criterion for statistical significance.

Results

The highest proportion of oocytes reaching MII was found when oocytes were cultured in 0.05% sericin (89.2%) ($P \leq 0.05$) (Table 1). Although the MII rate of oocytes that were cultured in 0.025% sericin was significantly higher than that of oocytes cultured in (-BSA) ($P \leq 0.05$), the rate was not significantly different from oocytes in the (+BSA) group. The proportion of oocytes reaching MII decreased when the concentration of sericin was increased ($P < 0.05$) (79.3%, 77.0%, respectively, for 0.1 and 0.25% groups).

Table 1 Meiotic maturation of buffalo oocytes with supplementation of sericin in maturation medium

Groups of IVM	No. oocytes examined	GV (No)	GVBD (No)	MI (No)	AI/TI (No)	MII (No)	Degenerated (No)
(-BSA)	89	2.4 ± 1.8 (2)	12.8^{a} ± 5.0 (10)	10.2 ± 3.1 (10)	0.0 ± 0.0 (0)	71.9^{a} ± 3.2 (65)	2.6 ± 1.8 (2)
(+BSA)	85	1.1 ± 1.1 (1)	3.1^{b} ± 2.2 (3)	17.6 ± 4.5 (13)	0.0 ± 0.0 (0)	73.8^{ab} ± 3.0 (64	1.9 ± 1.3 (2)
0.025 Ser	84	0.8 ± 0.8 (1)	3.3^{b} ± 1.6 (3)	8.3 ± 2.9 (6)	2.8 ± 1.4 (3)	83.0^{bc} ± 2.4 (69)	1.9 ± 1.3 (2)
0.05 Ser	95	0.0 ± 0.0 (0)	2.5^{b} ± 1.3 (3)	7.7 ± 2.2 (8)	0.0 ± 0.0 (0)	89.2^{c} ± 2.6 (83)	0.6 ± 0.6 (1)
0.1 Ser	97	0.6 ± 0.6 (1)	4.4^{ab} ± 2.5 (5)	10.8 ± 4.3 (11)	1.0 ± 1.0 (1)	79.3^{ab} ± 3.6 (76)	3.9 ± 2.1 (3)
0.25 Ser	87	1.0 ± 1.0 (1)	7.7^{ab} ± 4.4 (7)	12.2 ± 4.1 (10)	0.0 ± 0.0 (0)	77.0^{ab} ± 4.3 (67)	2.1 ± 1.5 (2)

Results show the mean of percentage ± SE
Means with different superscripts ($^{a, b, c}$) in the same column differ significantly ($P \leq 0.05$)
Ser: sericin; BSA: bovine serum albumin; AI/TI: anaphase I and telophase I; GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; MII: metaphase II; No: number of oocytes

Fluorescent photomicrographs of buffalo oocytes stained with DCHFDA are shown in Figure 2. The concentration of intracellular H_2O_2 in oocytes is shown in Figure 3. The concentration of H_2O_2 in oocytes treated with 0.05% sericin (33.6 FIU/oocyte) was significantly lower ($P \leq 0.05$) than in the (-BSA) group (39.9 FIU/oocyte), but not significantly different ($P > 0.05$) from other groups of (+BSA), namely 0.025%, 0.1% and 0.25% (34.4%, 34.7%, 35.1% and 37.9%, respectively).

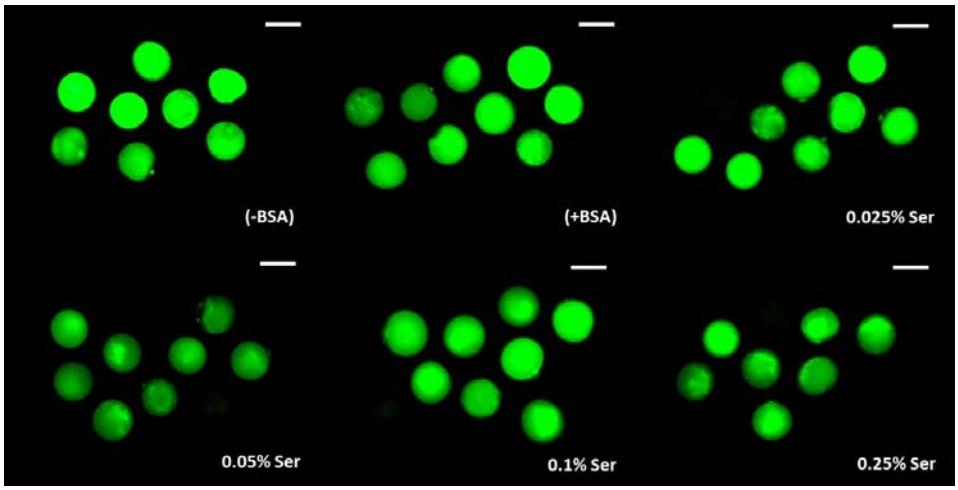

Figure 2 Fluorescent photomicrographs of buffalo oocytes stained with 2',7'-dichlorodihydrofluorescein diacetate sericin
Scale bars represent 140 µm

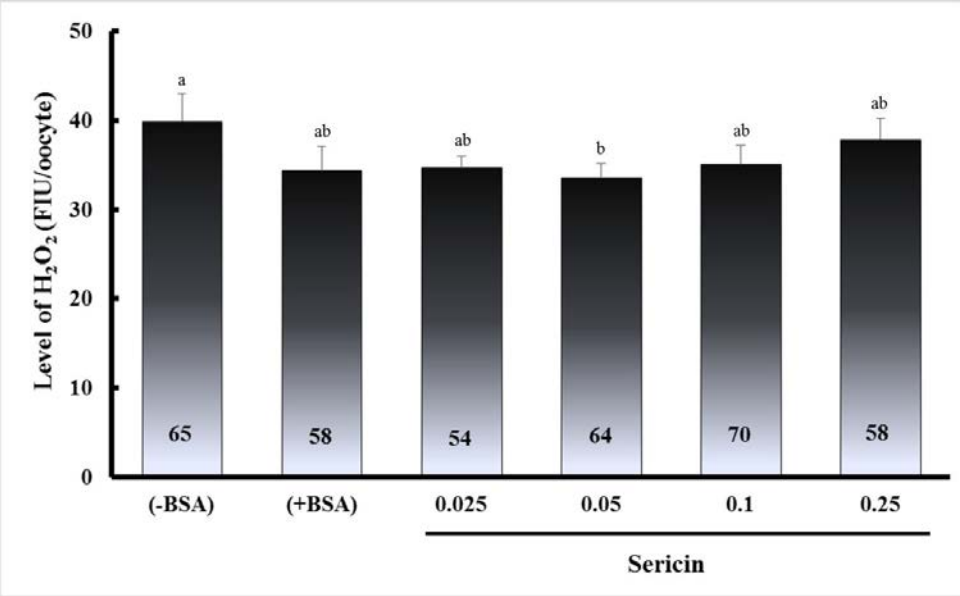

Figure 3 Hydrogen peroxide concentration in buffalo oocytes matured with and without sericin
FIU: values represent fluorescence intensity units
Within each end point, bars with different letters (a, b) are significantly different ($P \leq 0.05$)
The numbers on the bars represent the number of oocytes used for the assay

TUNEL results (Table 2) revealed no significant differences in the proportion of DNA fragmentation in oocytes at all of the nuclear stages after maturation among all groups ($P > 0.05$). However, the 0.05% sericin group has the lowest percentage mean of DNA fragmentation (7.1) in oocytes that remained at the MII stage, whereas the (-BSA), (+BSA), 0.025, 0.1 and 0.25 groups had mean DNA fragmentation rates in oocytes that remained at MII of 15.8, 9.4, 9.1, 9.2 and 10.1, respectively ($P > 0.05$).

Table 2 Nuclear DNA fragmentation of buffalo oocytes matured with sericin

Groups of IVM	No. oocytes examined	Percentage of TUNEL-positive oocytes				
		GV	GVBD	MI	ATI	MII
(-BSA)	58	0.0 ± 0.0 (0/0)	1.6 ± 1.6 (1/1)	10.1 ± 3.5 (6/14)	1.6 ± 1.6 (1/1)	15.8 ± 3.1 (9/42)
(+BSA)	64	0.0 ± 0.0 (0/0)	0.0 ± 0.0 (0/0)	7.8 ± 3.1 (4/13)	1.4 ± 14 (1/2)	9.4 ± 3.0 (6/49)
0.025 Ser	65	0.0 ± 0.0 (0/0)	0.0 ± 0.0 (0/0)	6.9 ± 2.9 (5/20)	1.8 ± 1.8 (1/1)	9.1 ± 4.5 (6/44)
0.05 Ser	65	0.0 ± 0.0 (0/0)	1.6 ± 1.6 (1/1)	4.9 ± 2.4 (3/11)	0.0 ± 0.0 (0/0)	7.1 ± 2.9 (5/53)
0.1 Ser	61	0.0 ± 0.0 (0/0)	0.0 ± 0.0 (0/0)	6.1 ± 2.3 (4/18)	1.3 ± 1.2 (1/1)	9.2 ± 3.2 (6/42)
0.25 Ser	63	1.4 ± 1.4 (1/1)	1.8 ± 1.8 (1/1)	3.0 ± 2.0 (2/11)	0.0 ± 0.0 (0/0)	10.1 ± 4.5 (6/50)

Results show the mean of percentage ± SEM, with the number of terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end-labelling (TUNEL)-positive oocytes/total number of examined oocytes
AI/TI: anaphase I and telophase I; GV: germinal vesicle; GVBD: germinal vesicle breakdown
MI: metaphase I; MII: metaphase II

Discussion

During maturation and culturing, various factors can affect the quality and development of oocytes and embryos, especially environmental conditions. Oxygen tension during the culture process is influential on embryo development. Culture conditions under high oxygen tension have a negative effect that can induce the generation of ROS (Rocha-Frigoni *et al*., 2016). ROS can alter most types of cellular molecules, and block the development of pre-implantation embryos (Guerin *et al*., 2001).

In this study, the addition of 0.05% sericin significantly increased the percentage of meiotic maturation of buffalo oocytes compared with the (-BSA) and (+BSA) groups. These data are consistent with the findings of Isobe *et al*. (2012) that the addition of 0.5% sericin can improve the quality and preimplantation development of bovine embryos. In sheep, maturation and fertilization rates can be improved with the addition of 0.1% sericin (Yasmin *et al*., 2015). On the other hand, the authors found that the level of nuclear maturation in the high concentration sericin group (0.25%) showed a significant decrease, possibly owing to a toxic effect of sericin. According to Aramwit *et al*. (2010), sericin can increase collagen production, depending on the concentration, but high concentrations can cause cellular toxicity.

Furthermore, in this study, 0.05% sericin supplementation reduced the concentrations of intracellular H_2O_2 compared with the (-BSA) group. Dash *et al*. (2008) reported that sericin reduced levels of catalase, lactate dehydrogenase and the activity of malondialdehyde (markers of oxidative stress) in fibroblast cells exposed H_2O_2. Isobe *et al*. (2012) reported that sericin may have a unique antioxidant potential. The mechanism of the antioxidant effect of sericin is chelation of trace elements, because of its high content of hydroxyl amino acids (serine and threonine) (Kato *et al*., 1998).

Hydrogen peroxide penetrates biological membranes (Silva & Coutinho, 2010). The mechanism of damage by H_2O_2 in cultured oocytes involves ROS derivatization. Reactive oxygen species can spread and pass through cell membranes and alter cellular molecules, such as proteins, lipids and nucleic acids, resulting in alterations in the mitochondria, embryo cell blockage, depletion of adenosine triphosphate (ATP) and DNA fragmentation (Guerin *et al*., 2001). DNA fragmentation indicates apoptosis, in which the generation of ROS can result in DNA strand breaks that arrest the cell cycle or even cause cell death (Fahrudin *et al*., 2002). Therefore, the authors also observed the index of DNA fragmentation in oocyte nuclei matured with sericin. Apoptosis is a normal process during preimplantation embryo development, which may be due to environmental stress and chromosome abnormalities (Matwee *et al*., 2000). Apoptosis is characterized by loss of phospholipid balance in the plasma membrane, chromatin condensation, DNA fragmentation and blebbing of the plasma membrane. In fragmentation, the cell becomes condensed and divides into several fragments, causing cytoplasmic condensation, condensed nuclei (Wyllie *et al*., 1980), and DNA breakdown, resulting in DNA fragments of 180 - 200 base pairs (Elmore, 2007).

This study demonstrated that although sericin can enhance the number of oocytes that reach MII, there were no significant differences in H_2O_2 concentration or the total of oocytes undergoing DNA fragmentation in all treatment groups. Although the reasons for this finding are currently unclear, the authors presume that it may be due to the effect of glutathione (GSH), which is a natural intracellular antioxidant. Levels of intracellular GSH increase during the oocyte maturation process in the ovary, and peak when oocytes reach MII (Deleuze & Goudet, 2010). Similar to other species, buffalo oocytes can synthesize GSH *de novo* during IVM (Gasparrini *et al*., 2003). Nevertheless, the results of this study show that the addition of sericin to maturation media may serve as a replacement for BSA to increase the maturation rate, and reduce the concentration of H_2O_2 and the proportion of DNA fragmentation. Bovine serum albumin is the main soluble protein in the circulatory system and has many physiological functions. BSA serves as a depot protein and as a transport protein for a variety of compounds (Papadopoulou *et al*., 2005). It is generally used as a supplement in culture medium. Although BSA can protect cells and has many advantages, there are sanitary risks such as disease transmission, and it may serve as a contamination vector (Thibier, 2006). BSA is not only expensive in the long term, but it leads to enhanced risk of many zoonotic diseases (Cao & Zhang, 2016). This finding is in agreement with other studies, which were reported by Yasmin *et al*. (2015) in sheep oocytes, and Terada *et al*. (2002) in several mammalian cell lines in which sericin was used to accelerate cell proliferation. Sericin may serve as an alternative supplement in serum-free media for cell culture (Tunma *et al*., 2013). Yanagihara *et al*. (2006) reported that sericin is a powerful alternative to the production of the adenoviral vector in human cells.

Conclusion

In conclusion, the current research findings suggest that supplementation of 0.05% sericin in the maturation medium enhances meiotic maturation of buffalo oocytes. Additionally, sericin can replace BSA as a supplement in serum-free medium, which at 0.05% may decrease the concentration of intracellular H_2O_2 in buffalo oocytes. Further research is warranted to evaluate the effects of sericin supplementation in the quality and development of buffalo embryos.

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Author's Contributions

SG contributed to the conception and design of the study, data collection, statistical analysis, interpretation of data and drafting the manuscript. HH contributed data collection, statistical analysis and interpretation of data. NWKK, MAS, and IS were involved in conception and design of study, and critical revision of manuscript.

Conflict of Interest Declaration

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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