Effect of Sericin Supplementation in Collection Medium on Bovine Oocyte Nuclear Maturation

F A Satrio1, N W K Karja1, M A Setiadi1, E M Kain3, M Gunawan2, E Memili3, and B Purwantara1

1 Faculty of Veterinary Medicine, IPB University, Jl. Raya Darmaga, Bogor, 16680, Indonesia
2 Indonesian Institute of Science, Jl. Raya Bogor Km. 46, Bogor, 16911, Indonesia
3 Departement of Animal and Dairy Sciences, Mississippi State University, 4025 Wise Center Box 9815, Mississippi State, Mississippi 39762, USA

Purwantara1959@gmail.com

Abstract. Sericin is a water-soluble globular protein derived from silkworm Bombyx mori and has the competency as an antioxidant. This study was conducted to examine the effect of sericin supplementation in the collection medium on bovine oocyte nuclear maturation. Sericin with different concentration (0 (control), 0.1 %, 0.5 %, and 1%) was added to collection medium and matured for 24 hour at 38.5 °C in 5% of CO2 air. Matured oocytes were stained with acetocarmine and determined the oocyte nuclear stage under a stereomicroscope. After in vitro maturation, 74-87% of oocytes were reached nuclear maturation (metaphase II). The maturation rates of oocytes were significantly higher at 0.1% group (87.7%) (P<0.05) compared to other groups. There was no significant differences were observed between control group (74.6%), 0.5% group (79.4%), and 1% group (78.3%) (P>0.05). These findings showed that supplementation of 0.1% sericin in the collection medium improved the nuclear maturation of bovine oocytes.

1. Introduction

In vitro embryo production (IVEP) consists of several stages, which are oocyte maturation, oocyte fertilization by spermatozoa, and embryo culture [1]. In IVEP, oocytes are sourced from ovaries slaughterhouses or live animals (collected by ovum pick up / OPU). Neglia et al. [2] reported that oocytes from slaughterhouse have maturation rates lower than oocytes collected by ovum pick-up (OPU). Furthermore, Manjunatha et al. [3] reported that buffalo’s embryo from oocyte collected by OPU has in vitro embryo development and blastocyst rate after freezing better than oocyte from the slaughterhouse. The oocytes collected by OPU has blastocyst rate higher than collected by aspiration technique, 36.5% and 20.8%, respectively [4]. It may be induced by oocyte’s stress during IVEP, one of them is oocyte collection processes.

Bovine oocyte collection from slaughterhouse was conducted after an animal’s slaughter. In these conditions, the occlusion of blood flow reduces oxygen and places ovaries under ischemic conditions [5]. The ischemic condition leads the cells to become an anaerobic condition and reoxygenation is known to generate toxic radical oxygen or reactive oxygen species (ROS) [6; 7]. Reactive oxygen species that can be formed are superoxide (O2·−), toxic hydroxyl radicals (·OH), dan hydrogen peroxide (H2O2) [5]. Reactive oxygen species leads to damage to the structure of cells such as DNA [8], carbohydrates, lipids [8; 9] and proteins [8; 9;10]. Reactive oxygen species promote to induce oxidative stress [9] and stimulate a programmed cell death (apoptosis) [11]. In the in vivo condition, oocytes damaged by ROS can be protected by endogenous antioxidants, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) [12]. Otherwise, endogenous antioxidants in the
Oocyte In Vitro Maturation

Selected Cumulus oocyte complex (COCs) were then washed three times in the washing medium covered by mineral oil (Sigma-Aldrich, USA, M5310). The composition of the washing medium is the same as the maturation medium. The oocytes were then matured in 100 ml drops maturation medium for 10-15 oocytes covered by maturation medium (Sigma-Aldrich, USA, M5310) [23]. Maturation medium contains tissue culture medium-199 (TCM-199) (Sigma-Aldrich, USA, M4530) supplemented with 0.3% BSA (Sigma-Aldrich, USA, A7030), 10 IU/ml follicle-stimulating hormone (FSH) (Vetoquinol N.-A inc, Canada), 10 IU/ml human chorionic gonadotrophin (hCG) (ChorulonTM, MSD

oxidative stress condition cannot reduce ROS production, so the exogenous antioxidants are needed. One of the exogenous antioxidants that can be supplemented in the medium is sericin.

Nowadays, sericin has been widely used for the pharmaceutical industry, biomedicines, cosmetics and food industry [13]. Moreover, Chlapanidas et al. [13] also used the sericin for supplemented in the culture medium because sericin can induce cell proliferation [14]. Sericin is a water-soluble globular protein derived from silkworm Bombyx mori [13]. Sericin has 10-310 kDa in the mass molecule [15]. Sericin contains 18 types of amino acids with the highest component being serine [13; 14; 16]. Serine has a strong polar side to the hydroxyl and carboxyl amino acid groups [17]. These groups are known to have antioxidant activity [13; 14; 16]. Sericin as an antioxidant has the potential to inhibit lipid peroxidation [16] and to decrease H₂O₂ levels in the culture medium [17; 19]. In IVEP, sericin has been used for supplementing the maturation medium of sheep [20] and buffalo [21] and has been proven to be able to improve the nuclear maturation. There has not been reported the effect of sericin supplementation in the collection medium. Therefore, the aim of this research is to examine the effect of sericin supplementation in the collection medium on bovine oocyte nuclear maturation

2. Materials and methods

2.1. Chemicals

The main chemical used in this research was Pure sericin™, 163-22683, which was purchased from FUJIFILM Wako Pure Chemical Industries Corporation, Japan. Sigma-Aldrich Inc produced other chemicals unless otherwise stated.

2.2. Ovaries Collection

In this research, ovaries were obtained from a slaughtered house in Bogor. The ovaries were then transported to the laboratory less than 5 hours after the slaughter in the transportation medium at 37 °C. Transportation medium consists of NaCl 0.9%, 100 IU/ml Penicillin-G (MEIJI, Indonesia) and 1 mg/ml Streptomycin (MEIJI, Indonesia).

2.3. Oocytes Collection

Bovine ovaries were washed three times with the transportation medium at 37 °C. Oocytes were collected using aspiration technique [1; 22]. The technique was carried out by aspirating all visible antral follicles with a diameter of 3 mm-10 mm using an 18-gauge needle connected with a 10 mL disposable syringe. The aspirated fluid was collected into centrifuge sterile tube at 10-15 minutes. After that, the supernatant was removed and replaced with the collection medium for 5 minutes. The fluid was placed in a sterile petri dish for collecting the oocytes. Collection media contains phosphate-buffered saline (PBS) supplemented with 0.3% Bovine Serum Albumin (BSA) (Sigma-Aldrich, USA, A7030), 100 IU/ml penicillin G (Sigma-Aldrich, USA, P4687), 0.001 mg/ml streptomycin sulfate (Sigma-Aldrich, USA, S9137) and pure sericin™ (Wako, Japan) with different concentrations (0% (control), 0.1%, 0.5% and 1 %). Oocytes were collected under a stereomicroscope (Olympus IX 70, Japan) and only oocytes surrounded by more than three layers of unexpanded cumulus cells and uniform cytoplasm were selected for in vitro maturation.

2.4. Oocyte In Vitro Maturation

Selected Cumulus oocyte complex (COCs) were then washed three times in the washing medium covered by mineral oil (Sigma-Aldrich, USA, M5310). The composition of the washing medium is the same as the maturation medium. The oocytes were then matured in 100 ml drops maturation medium for 10-15 oocytes covered by maturation medium (Sigma-Aldrich, USA, M5310) [23]. Maturation medium contains tissue culture medium-199 (TCM-199) (Sigma-Aldrich, USA, M4530) supplemented with 0.3% BSA (Sigma-Aldrich, USA, A7030), 10 IU/ml follicle-stimulating hormone (FSH) (Vetoquinol N.-A inc, Canada), 10 IU/ml human chorionic gonadotrophin (hCG) (ChorulonTM, MSD.
Animal Health), 1 μg/ml estradiol (Sigma-Aldrich, USA, E1024) and 50 μg/ml gentamycin (Sigma-Aldrich, USA, G1264). The oocytes were incubated under 5% CO2 at 38.5 °C for 24-26 h.

2.5. Assessment of Oocytes Nuclear Maturation
At the end of the maturation period, the cumulus cells of the oocytes were removed by repeated pipetting and using the hyaluronidase enzyme 0.25% (Sigma-Aldrich, USA, H3506). Denuded oocytes were then placed on an object-glass which was overlaid with a coverslip supported paraffin mixture dan Vaseline stripes (1:9). The slide was fixed using acetic acid: methanol (1:3 v/v) for 48-72 h. The slide was then stained using 2% aceto-orcein and examined under a phase-contrast microscope (Olympus IX 70, Japan) for determining the nuclear status of bovine oocytes. Percentage of oocyte nuclear maturation was determined based on the nuclear status from germinal vesicle (GV) to the metaphase II (MII) stage. Oocytes in which condensed or slightly diffused chromatin could be identified, were classified as being in the germinal vesicle (GV) stage. Oocytes with diffused chromatin in which the nuclear membrane was fragmented before rapidly disappearing to leave only small sacs with double walls, were classified as being in the germinal vesicle breakdown (GVBD) stage. Oocytes that possessed clumped or strongly condensed chromatin that formed an irregular network of individual bivalents (prometaphase) or a metaphase plate but no polar body were classified as being in the metaphase I (MI) stage. Oocytes with either a polar body or two chromatin masses were classified as being in the metaphase II (MII) stage of the maturation process [24].

2.6. Data Analysis
The data of nuclear maturation were presented as the mean ± SEM and were analyzed by One-Way Analysis of Variance (ANOVA). Differences with a probable value of P<0.05 were considered to be statistically significant. If there were any significant differences, further testing was continued by using Duncan’s Multiple Range Test (DMRT). The data were replicated three times. Statistically, the analysis was performed using IBM SPSS statistics program version 24.0.

3. Results and Discussion
Nuclear maturation of bovine oocytes based on the oocyte abilities to develop until the metaphase II stage. Nuclear maturation is the development of the oocytes from diplotene prophase I (germinal vesicle or GV), germinal vesicle breakdown (GVBD), metaphase I, anaphase/telophase until metaphase II (MII) stages of maturation process [25]. A representative overview of bovine oocytes supplemented by sericin in the collection medium was presented in Figure 1. Based on Figure I, we find that the status of nuclear oocytes is metaphase I and metaphase II stages of the maturation process. Moreover, our research also found the degeneration of the bovine oocytes.

Matured oocytes are characterized by nuclear and cytoplasmic maturation [25; 26] Oocytes must have nuclear maturation because only matured oocytes have the ability to be fertilized and to be developed to embryos [22]. The stages of oocyte maturation indicate the intrinsic quality of oocytes. The oocyte’s quality can be evaluated based on the morphology, cellular and molecular. Morphological oocyte quality assessment is an assessment that is often used because the assessment is non-invasive and easy to do. Morphologically, oocytes can be evaluated base on the morphology of cumulus-oocyte complex (COC), cytoplasmic, polar body and meiosis spindle [26]. Morphologically, matured oocytes can be characterized by Oocytes with either a polar body or two chromatin masses [24]. The maturation rate of bovine oocytes supplemented by various sericin concentrations in the collection medium can be shown in Table 1.
Figure 1. Representative overview of bovine nuclear status supplemented by sericin in the collection medium; MI: metaphase I, MII: metaphase II, D: degeneration. The image was taken by using a phase-contrast microscope with 200x magnification.

As depicted in Table 1, the percentage of the bovine oocyte that has reached the metaphase II stage between 74% until 87%. Supplementation 0.1% sericin in the collection medium has a higher percentage of maturation rate of bovine oocytes and has significantly different from the control group (P<0.05). Furthermore, there were no significant differences in nuclear maturation of bovine oocyte were observed between the control group (74.6%), 0.5% group (79.4%), and 1% group (78.3%) (P>0.05).

Table 1 Nuclear maturation of bovine oocytes supplemented by various sericin concentrations in the collection medium

| Collection medium | Number of oocyte (n) | % Mean ± SEM (n) of oocyte at each stage |
|-------------------|---------------------|----------------------------------------|
|                   | GV                  | MI                                     | A/T                                    | MII                                   | Deg                                    |
| Ser 0 %           | 102                 | 0.0 ± 0.0 (0)                         | 23.0 ± 2.3 (23)                        | 0.0 ± 0.0 (0)                         | 74.6 ± 0.7 (76)                        | 2.3 ± 2.3 (3)                          |
| Ser 0.1 %         | 95                  | 0.0 ± 0.0 (0)                         | 11.1 ± 4.4 (11)                       | 0.0 ± 0.0 (0)                         | 87.7 ± 4.0 (83)                       | 1.1 ± 1.1 (1)                          |
| Ser 0.5 %         | 90                  | 0.0 ± 0.0 (0)                         | 20.6 ± 2.4 (19)                       | 0.0 ± 0.0 (0)                         | 79.4 ± 2.4 (71)                       | 0.0 ± 0.0 (0)                          |
| Ser 1 %           | 73                  | 0.0 ± 0.0 (0)                         | 17.6 ± 0.9 (13)                       | 0.0 ± 0.0 (0)                         | 78.3 ± 3.8 (57)                       | 4.2 ± 4.2 (3)                          |

Notes: GVBD: Germinal Vesicle, MI: Metaphase I, A/T: anaphase/telophase, MII: Metaphase II, Deg: Degeneration, Ser: Sericin. a,b Values with different superscript in the same column are significantly different (P<0.05).

The process of oocyte collection is one of the processes in IVEP that is very susceptible to ROS production. Oxidative stress occurred when ROS production exceeded the antioxidant capacity to do scavenging. In sufficient quantities, ROS functions for gene expression, cell signaling, and redox balance [9]. Reactive oxygen species is included in radical species, such as superoxide anion radical (O$_2^.$). Superoxide anion radical is produced under normal condition and stimulate the other ROS formation. The function of Superoxide anion radical is a regulator of oxidative chain reactions. After O$_2^.$ dismutation, hydrogen peroxide (H$_2$O$_2$) passes the cell through its membrane. The presence of O$_2^.$, H$_2$O$_2$, and iron triggers the Haber-Weiss reaction, which generates the toxic hydroxyl radicals [9]. Hydroxyl radical is an oxygen formation that is very reactive, toxic, and leads to oxidative damage [9].

The oxidative damage at the cellular level, especially in mitochondria, can increase to change permeabilization of the mitochondrial outer membrane. These conditions stimulate the release of intermembrane space protein such as cytochrome c [27;28]. Cytochrome c will bind the apoptotic protease activating factor 1, which increases the level of capcase-9 and capcase-3 that cause apoptosis [28]. The balance between generation and elimination of ROS is the main factor needed by mammals
for metabolic function [26]. In general, the purpose of antioxidants as scavengers for ROS is to maintain the balance of oxidants and antioxidants in the cells [8; 29]. Sericin contains serine, amino acid hydroxyl groups, which is known to have antioxidant action by scavenger against ROS through chelation with trace elements [13;16]. The previous studies are in line with our findings, which is supplementation of sericin in the collection medium can improve the maturation rate of bovine oocytes. Sericin may inhibit the Haber-Weiss reaction because it will chelation the iron, so that hydroxyl radical (·OH) will fail to form. This condition leads to protect the oxidative stress and inhibits the apoptosis. Therefore, bovine oocytes can develop until the metaphase II (MII) stage of the maturation process.

In conclusion, our finding indicates that supplementation of 0.1% sericin in the collection medium improves the maturation rate of bovine oocytes. Further study is necessary to evaluate the effectivity of sericin supplementation in the collection medium on the oocyte’s qualities, embryonic development, and embryonic qualities.

Acknowledgments
This research was supported by USAID through Sustainable Higher Education Research Alliances (SHERA) Program-Center for Collaborative Research Animal Biotechnology and Coral Reef Fisheries (CCR ANBIOCORE) and Ministry of Research, Technology, and Higher Education through Pendidikan Magister Menuju Doktor untuk Sarjana Unggul (PMDSU) scholarship.

References
[1] Gordon I 2003 Laboratory Production of Cattle Embryos 2nd Edition (Willingford: CABI publishing)
[2] Neglia G, Gasparrini B, Caracciola di-Brienza V, Palo R D, Campanile G, Presicce G A and Zicarelli L 2003 Theriogenology 59 1123
[3] Manjunatha B M, Gupta P S P, Ravindra J P, Devaraj M and Nandi S 2008 Anim. Reprod. Sci. 104 419
[4] Merton J S, de Roos A P W, Mullaert E, de Ruigh L, kaal L, Vos P L A M and Dieleman S J 2003 Theriogenology 59 651
[5] Wongsrakeao P, Otoi T, Karja N W K, Agung B, Nii M and Nagai T 2005 J. Reprod. Develop. 51 87
[6] Epstein F H and McCord J M 1985 N. Engl. J. Med. 312 159
[7] Iwata H, Ohota M, Hashimoto S and Nagai Y 2003 Zygote. 11 1
[8] Lobo V, Patil A, Phatak A and Chandra N 2010 Pharmacogn. Rev. 4 119
[9] Wang S, He G, Chen M, Zou T, Xue W and Liu X 2017 Oxid. Med. Cell. Longev. 1
[10] Young I S and Woodside J V 2001 J. Clin. Pathol. 54 176
[11] Webster K A, Discher D J, Kaiser S, Hernandez O, Sato B and Bishoprie N H 1999 J. Clin. Invest. 104 239
[12] Zusterzeel P L, Peters W H, De B, Knapen M F, Merkus H M and Steegers E 1999 Obstetric and Gynecology 94 1033
[13] Chlapanidas T, Faragó S, Lucconi G, Perteghella S, Galuzzi M, Mantelli M, Avanzini M A, Tosca M C, Marazzi M, Vigo D, Torre M L and Faustini M 2013 Int. J. Biol. Macromol. 58 47-56
[14] Terada S, Nishimura T, Sasaki M, Yamada H and Miki M 2002 Cytotechnology 40 3
[15] Tao W, Li M and Xie R 2005 Macromolecular materials and engineering 290 188
[16] Kato N, Sato S, Yamanaka A, Yamada H, Fuwa N and Nomura M 1998 Biosci. Biotechnol. Biochem. 62 145
[17] Chao J A, Prasad G S, White S A, Stout C D and Williamson J R 2003 J. Mol. Biol. 326 999
[18] Dash R, Acharya C, Bindu P C and Kundu S C 2008 BMB Rep. 41 236
[19] Gustina S, Karja N W K, Hasbi H, Setiadi M A and Supriatna I 2017 S. Afr. J. Anim. Sci. 49 227
[20] Yasmin C, Otoi T, Setiadi M A and Karja N W K 2015 Acta Vet. Hung. 63 110
[21] Gustina S, Hasbi H, Karja N W K, Setiadi M A and Supriatna I 2017 ASJ. 1
[22] Setiadi M A and Karja N W K 2013 KKH. 7 150
[23] Hasbi H, Gustina S, Karja N W K, Supriatna I and Setiadi M A 2017 *Indonesian Trop. Anim. Agric.* 40 7
[24] Shirazi A and Sadeghi N 2007 *Small Ruminant. Res.* 69 103
[25] Adhikari D and Liu K 2014 *Mol. Cell. Endocrinol.* 382 410
[26] Wang Q and Sun Q-Y 2007 *Reprod. Fertil. Dev.* 19 1
[27] Murphy M P 2009 *Biochem. J.* 417 1
[28] Tiwari M, Prasad S, Tripathi A, Pandey A N, Ali I, Singh A K, Shrivastav T G and Chaube S K 2015 *Apoptosis* 20 1019
[29] Khazei M and Aghaz F 2017 *Int. J. Fertil. Steril.* 11 63