Sericin Accelerates the Production of Hyaluronan and Decreases the Incidence of Polyspermy Fertilization in Bovine Oocytes During In Vitro Maturation

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Abstract. Fetal bovine serum (FBS) has been widely used as a supplement in the maturation medium of bovine oocytes in vitro. However, serum contains many undefined factors and is potentially infectious to humans and animals. As a serum replacement, we evaluated the feasibility of using the silk protein, sericin, derived from the cocoons of silkworm. To examine the rates of oocyte maturation and fertilization, cumulus-oocyte complexes were cultured in TCM-199 supplemented with 0.01%, 0.05%, 0.1% or 0.15% sericin or 5% FBS. The sizes of the perivitelline space that might relate to polyspermy, the expressions of Has2 and CD44 mRNA, the amount of hyaluronan (hyaluronic acid: HA) contained in the oocytes and the rates of blastocyst formation following insemination were then compared between the oocytes cultured with 0.05% sericin and 5% FBS, because the polyspermy rates in oocytes cultured with 0.05% sericin were significantly lower than in those cultured with 5% FBS. After in vitro maturation (IVM), the mean size of the perivitelline space was significantly greater in oocytes cultured with sericin than in those cultured with FBS, although the rates of nuclear maturation, fertilization and blastocyst formation of oocytes under both IVM conditions were not significantly different. The expression of HAS2 and CD44 mRNA and the amount of HA in the denuded oocytes cultured with 0.05% sericin were significantly greater than in those cultured with FBS. These results indicate the feasibility of sericin as an alternative protein supplement for IVM in bovine oocytes.

Key words: Hyaluronan production, Perivitelline space, Polyspermy, Sericin

Fetal bovine serum (FBS) is widely used as protein source in embryo culture media. In culture, oocyte maturation and embryo development are supported by the contents of FBS and/or BSA, such as growth factors, chelators of heavy metals and other beneficial components [1, 2]. However, these protein sources contain various unknown components and might be contaminated with pathogens, such as prions and viruses. Moreover, when serum is used in the culture medium, the freeze resistance of blastocysts decreases [3, 4], the kinetics of development and gene expression are altered [5, 6] and a higher incidence of large offspring syndrome after embryo transfer is induced [7]. But then it was reported that nuclear maturation of oocytes, embryo cleavage and development were suppressed when bovine immature oocytes were cultured in medium without hormonal and serum supplements [2]. Therefore, a substitute for serum proteins in bovine in vitro maturation (IVM) has been desired.

Sericin is a family of serine (Ser)-rich silk proteins of silkworms that glues fibroin fibers together to form a robust cocoon [8]. It accounts for 20–30 wt% of the Bombyx mori cocoon fiber and is usually removed by the degumming process during silk processing. Sericin consists of three major protein components, and the molecular weight of the most abundant component is > 250 kDa, which corresponds to the Ser1C protein encoded by the Ser1 gene [9]. Ser1C is rich in hydrophilic amino acids, such as Ser (38.0%), threonine (9.9%), asparagine (8.6%) and aspartic acid (6.0%). Such a unique amino acid composition gives sericin a highly hydrophilic nature. This nature provides sericin with antioxidant action [10]. Sericin is now used as a replacement for FBS in cell culture media [11, 12], and it was reported that sericin stimulated the proliferation of insect [11] and mammalian [12] cells, and improved viability following cryopreservation of many cell lines [13, 14]. In addition, sericin is used not only as a replacement for FBS but also as a supplement for culture medium containing FBS. Recently, improvement of embryo development by supplementation of bovine embryos with sericin was reported [15].

It is known that glycosaminoglycans, including hyaluronan (hyaluronic acid: HA), are synthesized in cumulus cells in the response to stimulation with FSH and LH [16, 17] and that HA is
the predominant molecule of the extracellular matrix in cumulus oocyte complexes (COCs) [18–21]. In the bovine, it has been reported that the transcriptional upregulation of the hyaluronan synthase 2 (HAS2) and hyaluronan receptor (CD44) genes and expression of these proteins in COCs are important prerequisites for final oocyte maturation facilitating cumulus expansion [22], sperm penetration and fertilization [23], and both genes in cumulus cells are biomarkers for prediction of the competence of an oocyte to complete maturation, to undergo successful fertilization and to reach the blastocyst stage [24]. In pigs, the hyaluronan-CD44 interaction is required for meiotic resumption in the oocyte maturation process [25]. HA is present not only in the extracellular matrix in cumulus cells but also in the perivitelline space [26–29], and one of its properties is to retain large volumes of water [29]. Therefore, when HA accumulates in the perivitelline space, it causes it to enlarge. Recently, it has been clarified that the incidence of polyspermy of oocytes with a large perivitelline space is significantly lower than that of oocytes with a small perivitelline space in sows and mice [30–34]. It has also been suggested that HA in the perivitelline space is synthesized and secreted by oocytes themselves and not by cumulus cells [34].

In the present study, to determine the ability of sericin to replace FBS for bovine oocyte maturation in vitro, we evaluated its effect on maturation, fertilization (polyspermy) and development of oocytes to blastocysts. In addition, the size of the perivitelline space was measured in oocytes cultured with sericin and compared with that of oocytes cultured with FBS. The expression of HAS2 and CD44 mRNA in oocytes and cumulus cells and the amount of HA in a medium containing denuded oocytes (DOs) were examined using quantitative real-time RT-PCR and ELISA.

Materials and Methods

Preparation of sericin
Sericin was derived from the silkworm cocoons of the Sericin Hope race, which were maintained at the National Institute of Agrobiological Science. Sericin Hope was developed by crossbreeding and Nd mutant (naked pupa) and the high cocoonyielding strain KC853 [35]. These silkworms spin cocoons made almost exclusively of sericin (98.5%), so sericin is obtained very effectively. The sericin proteins from the Sericin Hope cocoons exhibit three major distinct bands in SDS-polyacrylamide gel electrophoresis [36, 37], and these components are hydrolyzed by autoclaving. Cocoon shells were stored at 2–10°C until preparation. An aqueous solution of intact sericin was prepared by a procedure described previously with a slight modification [38]. Briefly, cocoon shells were solubilized with 8 M lithium bromide at 5% (wt/vol) by stirring at 95°C for 30 min. A one-fourth volume of 0.5 M glycine-NaOH buffer (pH 9.0) was added to render the solution slightly basic. After centrifugation, the supernatant was dialyzed against deionized water with a membrane (Spectra/Por MWCO 12,000–14,000; Spectrum Laboratories, Rancho Dominguez, CA, USA), and then against 0.1 mM sodium carbonate buffer (pH 8.0–9.0). This buffer prevents irreversible aggregation of sericin by maintaining the pH of the solution at about 7.5 during dialysis. The dialyzed solution was centrifuged to remove precipitates. The final concentration of sericin solution was approximately 1% (wt/vol), and the solution was autoclaved at 120°C for 15 min.

In vitro maturation of oocytes
Bovine ovaries were obtained from a local abattoir. COCs were collected by aspiration from follicles 2–8 mm in diameter. Collected COCs were washed three times in PBS supplemented with 3 mg/ml BSA (crystallized BSA, Sigma-Aldrich, St. Louis, MO, USA), 100 IU/ml penicilline G potassium (Meiji Seika, Tokyo, Japan) and 100 μg/ml streptomycin (Meiji Seika). The COCs were cultured in groups of 50 in four-well dishes (Nunc, Roskilde, Denmark) with 500 μl TCM-199 (Gibco-BRL, Grand Island, NY, USA) supplemented with 1, 5, 10 and 15% sericin solution (substantially 0.01, 0.05, 0.1 and 0.15% sericin/medium) or 5% FBS (Tissue Culture Biologicals, Tulare, CA, USA), 1 μg/ml estradiol (17b-estradiol, Sigma) and 0.002 AU/ml FSH (Antrin R, Kawasakimitaka, Tokyo, Japan) for 20–22 h at 38.5°C in an atmosphere of 5% CO2 in air with maximum humidity. After IVM, the nuclei of oocytes were stained with 1% orcein, and the metaphase II (MII) rates were examined.

In vitro fertilization and culture of inseminated oocytes
In vitro fertilization (IVF) of oocytes was carried out as reported previously [39]. Briefly, COCs after IVM were transferred into 50 μl of fertilization medium as defined by Brackett and Oliphant (BO medium) [40] containing 2% crystallized BSA (Sigma) and 1% heparin (1,000 units/ml Novo-Heparin, Novo, Denmark). Frozen bull semen were thawed and poured into BO medium containing 10 mM caffeine-sodium benzoate (Sigma). The sperm suspension was washed twice by centrifuging at 500 g for 5 min. The sperm number was measured and adjusted to 25×10⁶ sperm/ml. Then 50 μl of sperm suspension was added to fertilization medium containing 20–25 COCs. Co-incubation of oocytes and sperm was carried out for 6 h at 38.5°C in an atmosphere of 5% CO2 in air with maximum humidity. At 18–20 h after IVF, the rates of monospermic and polyspermic fertilization were examined by staining with 1% orcein. Oocytes were considered to have undergone monospermic fertilization if they contained two pronuclei and to have undergone polyspermic fertilization if they contained three or more pronuclei. Data from four independent cultures were pooled and analyzed.

To examine the rates of cleavage and development to the blastocyst stage, COCs were cultured in TCM199 with 5% FBS and in TCM199 with 0.05% sericin, which resulted in a lower polyspermic fertilization rate than that with 5% FBS. After IVF, the putative zygotes were cultured in groups of 20 in 100 μl drops of CR1aa medium [41] with 5% FBS at 38.5°C in an atmosphere of 5% CO2 in air with maximum humidity for 8 days. The experiment was replicated seven times.

RT-PCR
After IVM, oocytes and cumulus cells in 50 COCs were separated by vortexing and/or repeated aspiration with a narrow-bore Pasteur pipette. Sample collections was performed three times. After collection, all cells were treated with ISOGEN (Nippon Gene, Toyama, Japan) and stored at −80°C until RNA extraction. Total RNA extraction and reverse transcription were performed as previously reported [42]. To examine the quantity of HAS2 and CD44 expression, COCs were cultured in TCM199 with 0.05% sericin and 5% FBS.

Quantitative real-time RT-PCR analyses for HAS2 and CD44 mRNA in oocytes and cumulus cells were carried out by the SYBR Green assay as previously reported [43, 44]. The thermal cycling
conditions included initial sample incubation at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec and at 60 °C for 1 min. The cycle threshold values (CT) indicated the quantity of the target gene in each sample, and the sequence of the target gene was determined in real time using an Mx3000P QPCR system (Stratagene, La Jolla, CA, USA). Standard curves were generated for each gene by serial dilution of pGEM-cloning vectors containing HAS2, CD44 and GAPDH cDNAs to quantify the amplified products. Real-time RT-PCR was performed using primers (for HAS2: 5′-GACAGGCCATCTAAGCAAGCG-3′ and 5′-TTTGATGGAGATCGA-3′, for CD44: 5′-TCGGATACCGAGACTACGGCT-3′ and 5′-GTCCAGTCGATTAGATGGCT-3′, for GAPDH: 5′-ACCAGAGAACGTGGATGGG-3′ and 5′-CAACAGACAGTTGGAGTGG-3′) that generated 167-, 78-, and 177-bp fragments, respectively. The GenBank accession numbers for bovine HAS2, CD44 and GAPDH are NM_174079, NM_174013 and U85042, respectively.

**Measurement of the perivitelline space**

To examine the size of the perivitelline space, COCs were cultured in TCM199 with 0.05% sericin and 5% FBS. The size of the perivitelline space (inner diameter of zona pel-lucida - cytoplasm diameter)*1/2 was calculated according to a previously described method [34] immediately after collection and after IVM. To measure the size of the perivitelline space and the amount of HA, cumulus cells surrounding oocytes were removed by repeated aspiration with a narrow-bore Pasteur pipette in 0.1% hyaluronidase (Sigma). Following IVM, only oocytes with a first polar body in the perivitelline space were selected to measure the size of the perivitelline space. All sample collections were performed in three independent cultures.

**HA assay in denuded oocytes**

To examine the amount of HA, COCs were cultured in TCM199 with 0.05% sericin and 5% FBS. The amount of HA in culture medium with oocytes was measured immediately after collection and after IVM. The amount of HA, cumulus cells surrounding oocytes were removed by repeated aspiration with a narrow-bore Pasteur pipette in 0.1% hyaluronidase (Sigma). Following IVM, only oocytes with a first polar body in the perivitelline space were selected to measure the size of the perivitelline space. All sample collections were performed in three independent cultures.

**HA assay in denuded oocytes**

To examine the amount of HA, COCs were cultured in TCM199 with 0.05% sericin and 5% FBS. The amount of HA in culture medium with oocytes was measured immediately after collection and after IVM. The HA assay was carried out by ELISA as reported previously [34, 45]. The DOs were stored in groups of 50 in 60 μl of each culture medium at −20 °C until assayed. Sample collections were performed three times. One hundred microliters of poly-L-lysine solution (Sigma-Aldrich) was dispensed into 96-well plates and incubated at 37 °C for 1 h. The plates were washed and the plates were air-dried. One hundred microliters of bovine vitreous humor HA (MP Biomedicals, Inc., Cleveland, OH, USA; 25 μg/ml in PBS) in PBS was added to the precoated plates, incubated overnight at room temperature and washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS-Tween 20). Microplates were blocked with 1% BSA (Sigma-Aldrich) in PBS at 37 °C for 1 h, washed five times with PBS-Tween 20 and then air-dried. The coated plates were wrapped in polyethylene film and stored at 4 °C until use.

The frozen media containing DOs were thawed and centrifuged at 1500 rpm (×176 g) for 10 min. The supernatants were collected and diluted twofold with TCM199 containing 0.05% sericin or 5% FBS. Sixty microliters of maturation medium containing HA at 0, 0.195, 0.391, 0.781, 1.56, 3.12, 6.25 or 12.5 ng/ml as standard solution and 60 μl of diluted samples were put into tubes with 60 μl of biotinylated HA binding protein (Seikagaku Corp., Tokyo, Japan; 0.175 μg/ml in distilled water) and incubated at 37 °C for 90 min. One hundred microliters of this reaction mixture was dispensed onto HA-coated plates and incubated at 37 °C for 1 h. The plates were washed five times with PBS-Tween 20, and 100 μl of alkaline phosphatase-conjugated avidin (Sigma-Aldrich, 1:5000) was added to each well, incubated at 37 °C for 1 h, washed five times with PBS-Tween 20 and then air-dried. The amount of HA was measured by a colorimetric assay with p-nitrophenyl phosphate (Sigma-Aldrich) as previously reported [33, 44]. Briefly, 100 μl of alkaline phosphatase substrate (p-nitrophenyl phosphate) was dispensed and incubated at 37 °C for 15 min and then 80 μl of 0.2 M NaOH was added to stop the reaction. The absorbance at 405 nm was measured using a microplate reader (Bio-Rad, CA, USA). The assay was carried out in triplicate. In this assay, the lower limit of HA detection is 0.195 ng/ml.

**Statistical analysis**

The size of the perivitelline space was statistically analyzed by one-way analysis of variance (ANOVA). The rate of maturation in cultured oocytes, rates of fertilization in oocytes following insemination and rates of development of oocytes to 2 cell embryos and blastocysts following IVM and IVF were transformed using arcsine transformation and then statistically analyzed with Tukey’s test. The amount of HA was statistically analyzed with the Student’s t-test. A value of P<0.05 was considered to be statistically significant.

**Results**

The maturation rates of the oocytes cultured for 20–22 h with 0.01%, 0.05%, 0.1% or 0.15% sericin or 5% FBS were 67.7% (67/99), 76.9% (90/117), 79.0% (79/100), 76.0% (79/104) and 75.0% (75/100), respectively, and were not significantly different among oocytes cultured with sericin or FBS. As shown in Table 1, the fertilization rates were also not significantly different among oocytes cultured with sericin or FBS. The rate of polyspermic fertilization of oocyte cultured with 0.05% sericin was significantly lower than that of oocytes cultured with 0.15% sericin or 5% FBS. The rates of cleavage and development to blastocysts after IVF did not differ between the oocytes matured with 0.05% sericin (70.2 and 31.5%) and those matured with FBS (77.1 and 32.0%) (Table 2).

Immediately after collection, the perivitelline space could not be

| Medium with | No. of oocytes inseminated | No. of oocytes fertilized (%) |
|-------------|----------------------------|-----------------------------|
|             | Total | Polyspermy | Total | Polyspermy |
| 0.01% Sericin (wt/v) | 148 | 108 (73.0) | 21 (14.2)ab |
| 0.05% Sericin | 154 | 107 (69.5) | 4 (2.6)ab |
| 0.1% Sericin | 166 | 120 (72.3) | 18 (10.8)ab |
| 0.15% Sericin | 187 | 143 (76.5) | 38 (20.3) b |
| 5% FBS (v/v) | 192 | 155 (80.7) | 34 (17.7)b |

Values with different superscripts in the same column are significantly different (P<0.05). Experiment was replicated four times.

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270  HOSOE et al.
EFFECT OF SERICIN ON OOCYTE MATURATION

seen in 36.7% (11/30) of oocytes (Fig. 1a) or could rarely be seen in 63.3% (19/30) of oocytes, and the mean size of the perivitelline space was 0.9 ± 0.5 μm. After IVM, the mean size of the perivitelline space increased to 14.9 ± 0.7 μm (n=95) in oocytes cultured with 0.05% sericin (Fig. 1b) and 12.6 ± 0.8 μm (n = 97) in oocytes cultured with 5% FBS (Fig. 1c). The mean size of the perivitelline space was significantly larger in oocytes cultured with sericin than that in oocytes cultured with FBS, although the mean diameter of the cytoplasm and inner diameter of the zona pellucida of oocytes were not significantly different between oocytes cultured with sericin and those cultured with FBS (Table 3).

Expression of HAS2 and CD44 mRNA in DOs matured in the presence of 0.05% sericin was significantly greater (P<0.05) than that of DOs matured with 5% FBS. In contrast, HAS2 and CD44 mRNA expression in cumulus cells was similar after IVM with 0.05% sericin or 5% FBS (Fig. 2).

Immediately after collection from follicles, HA was not detected in DOs. The mean amount of HA per DO after IVM with 0.05% sericin (29.9 ± 3.3 pg) was significantly greater than that of HA per DO after IVM with 5% FBS (19.0 ± 2.1 pg).

Discussion

In the present investigation, we attempted to evaluate sericin derived from silkworm cocoons as a substitute for serum in IVM medium of bovine oocytes. Though the rates of nuclear maturation, fertilization, cleavages and blastocyst formation were not different between the oocytes cultured with sericin and those cultured with FBS, the polyspermy rate after IVF was significantly lower in those cultured with 0.05% sericin than in those cultured with 0.15% sericin or 5% FBS. Furthermore, oocytes cultured with 0.05% sericin had a larger perivitelline space and a larger amount of HA and showed a higher expression of HAS2 and CD44 mRNA than those cultured with 5% FBS. These results indicate, for the first time, the feasibility of sericin derived from an insect as an alternative protein supplement for in vitro maturation in bovine oocytes. Terada et al. reported that 0.01 and 0.1% sericin accelerate mouse hybridoma cell proliferation [12]. Similarly, our results showed that 0.05% sericin was appropriate for bovine oocyte maturation culture.

Table 2. Rate of development in inseminated bovine oocytes matured with sericin and FBS

| Medium       | No. of oocytes cultured | No. of oocytes cleaved (%) | No. of oocytes developed to blastocysts (%) |
|--------------|-------------------------|----------------------------|---------------------------------------------|
| 0.05% sericin| 289                     | 203 (70.2)                 | 91 (31.5)                                  |
| 5% FBS       | 297                     | 229 (77.1)                 | 95 (32.0)                                  |

Experiment was replicated seven times.

Fig. 1. Bovine oocytes cultured for 0 (a) or 22 h in TCM-199 medium with 0.05% sericin (b) or 5% FBS (c). The perivitelline space is not present in the oocyte immediately after collection from the antral follicle (a). The perivitelline space (arrow) is larger in the oocytes cultured with sericin (b) than in those cultured with FBS (c). Scale bar = 50 μm.

Fig. 2. Quantitative real-time RT-PCR analysis of bovine HAS2 and CD44 mRNAs in the oocytes and cumulus cells cultured for 22 h in TCM199 with 0.05% sericin or 5% FBS. The expression of these mRNAs was normalized to the expression of GAPDH measured in the same RNA preparation. Results of three independent experiments are summarized and expressed as the mean ± SEM. Different letters above the bars indicate significant differences (P<0.05).
It has been reported that supplementation of a culture medium containing 5% FBS with 0.5% sericin increased blastocyst formation and protected embryos against oxidative stress due to the antioxidant potential derived from the remarkably high content of hydroxyl amino acids in sericin [15]. It has also been reported that supplementation with 1% sericin had no harmful effects on embryonic development [15]. In contrast, Terada et al. reported that the addition of 1% sericin to culture medium (not including another protein source) had harmful effects on the mouse hybridoma cells [12]. FBS might hyde advantageous effects of sericin and rescue embryos from the harmful effects of sericin. Evaluation of the effects of sericin on embryo development as a replacement for FBS will be needed in the future.

Recently, it has been suggested that there is a relationship between the size of the perivitelline space and the incidence of polyspermy in porcine and mouse oocytes [30–34]. The reason for the high incidence of polyspermy in oocytes with small perivitelline spaces is still unclear. Proteins and HA are present in the perivitelline space of oocytes before ovulation [26–29, 46], and secretions from oviducts [47–54] are also present after ovulation. When the perivitelline space enlarges, the substances in the perivitelline space increase in quantity. These have been suggested to physically obstruct the movement and attachment of sperm to the plasma membrane of oocytes [26, 28]. HA has also been reported to have an inhibitory effect on membrane fusion [55–57]. Therefore, it is speculated that large amounts of HA in the enlarged perivitelline space may prevent the fusion of extra sperm and oocytes, resulting in a lower incidence of polyspermy in oocytes with larger perivitelline spaces. In the present investigation, the amount of HA in DOs cultured with sericin was significantly lower than in DOs cultured with FBS, and the polyspermy rate in oocytes cultured with 0.05% sericin was significantly lower than that in oocytes cultured with FBS. Our results showed that there is a relationship among the amount of HA in the perivitelline space, the size of the perivitelline space and the incidence of polyspermy in bovine oocytes, previously reported in pig and mouse oocytes.

In mammals, the HAS family consists of three known isozymes, HAS1, HAS2 and HAS3 [58,59], and it has been reported that HAS2 is mainly responsible for rapid HA synthesis in bovine COCs and granulosa cells [22]. On the other hand, Ueno et al. [34] suggested that HA produced by HAS2 in cumulus cells is less likely to pass through the zona pellucida, and the HA involved in enlargement of the perivitelline space is produced by the oocytes themselves rather than by cumulus cells. This hypothesis is in good agreement with our result, namely that the increase in HA production was associated with enlargement of the perivitelline space in oocytes cultured with sericin.

CD44 is the principal cell-surface receptor for extracellular matrix hyaluronan, and it has been reported that the function of hyaluronan via CD44 is responsible for inhibition of apoptosis [60], meiotic resumption of oocytes [25] and cytoplasmic maturation of oocytes and sperm-egg interaction [22]. It has also been reported that the expression of CD44 mRNA greatly increased during in vitro maturation of COCs [22]. Our result shows that sericin accelerated the expression of CD44 mRNA in oocytes more than FBS, which might imply a beneficial effect of sericin on oocyte maturation. It has been suggested that oocyte-derived local factors play a role in regulating CD44 expression during IVM without endocrine signals [22]. Also, it has been proposed that HAS2 mRNA expression is under the control of growth differentiation factor 9 (GDF9) [61] and is an important prerequisite for initiating HA-mediated effects during final oocyte maturation, as well as sperm-egg interactions [22, 24]. The optimal amount of sericin might accelerate the function of oocyte-derived factors regulating HA during maturation culture in oocytes, consequently decreasing polyspermy after insemination.

In conclusion, it was clarified that the addition of sericin instead of FBS to maturation medium enlarged the perivitelline space, increased HA production and decreased polyspermic fertilization in bovine oocytes. These findings suggest that sericin, as an alternative protein supplement for in vitro maturation, plays a beneficial role in the ooplasmatic maturation of bovine oocytes.

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