Journal of Reproduction and Development, Vol. 60, No 4,

—Original Article—

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

Misa HOSOE[1)], Nao YOSHIDA[2)], Yutaka HASHIYADA[3)], Hidetoshi TERAMOTO[1)], Toru TAKAHASHI[1)#] and Sueo NIIMURA[4)]

[1)]National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan
[2)]Graduate School of Science and Technology, Niigata University, Niigata 950-2181, Japan
[3)]National Livestock Breeding Center, Nishigo, Fukushima 961-8511, Japan
[4)]Faculty of Agriculture, Niigata University, Niigata 950-2181, Japan
[#]Present: Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, Iwate 020-8550, Japan

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

(**J. Reprod. Dev. 60:** 268–273, 2014)

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

Received: October 18, 2013
Accepted: March 20, 2014
Published online in J-STAGE: April 21, 2014
©2014 by the Society for Reproduction and Development
Correspondence: M Hosoe (e-mail: hosoe@affrc.go.jp)
This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <http://creativecommons.org/licenses/by-nc-nd/3.0/>.

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 cocoon-yielding strain KCS83 [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 cocoon exhibits 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% CO_2 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 25×10^6 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% CO_2 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 in100 µl drops of CR1aa medium [41] with 5% FBS at 38.5 C in an atmosphere of 5% CO_2 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 (C_T) 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′-GACAGGCATCTAACGAACCGA-3′ and 5′-TTCAGTAAGGCACTTGGATCGA=3′, for *CD44*: 5′- TCGGATACC AGAGACTACGGCT-3′ and 5′- GTCCAGCTGATTCAGATGCGT-3′, for *GAPDH*: 5′-ACCCAGAAGACTGTGGATGG-3′ and 5′-CAACAGACACGTTGGGAGTG-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 pellucida - 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 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 was 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 solution was removed, 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, 1:5,000) 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

Table 1. Rate of polyspermy in bovine oocytes after IVM with sericin and FBS and following IVF

Medium with	No. of oocytes inseminated	No. of oocytes fertilized (%)	
		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)^{a}
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.

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

Medium with	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.

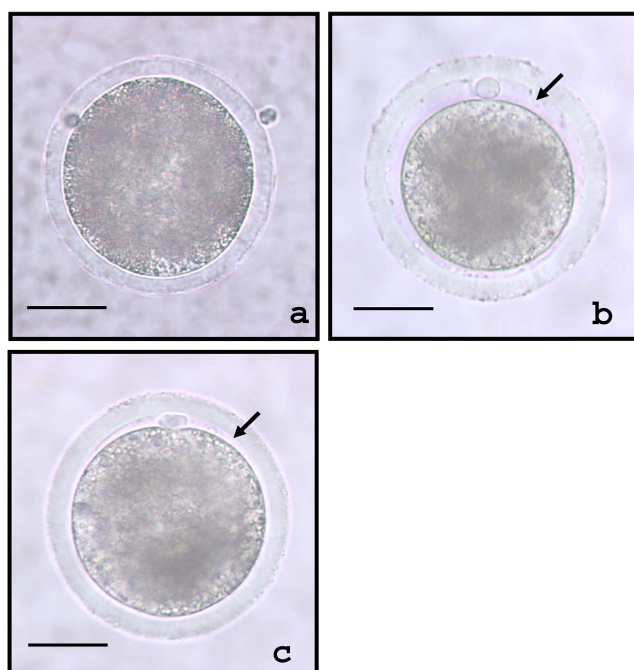

Fig. 1. Bovine oocytes cultured for 0 (a) or 22 h in TCM-199 medium with 0.05% sericin (b) or 5% FBS (c). The perivitelline space is not present in the oocyte immediately after collection from the antral follicle (a). The perivitelline space (arrow) is larger in the oocytes cultured with sericin (b)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

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

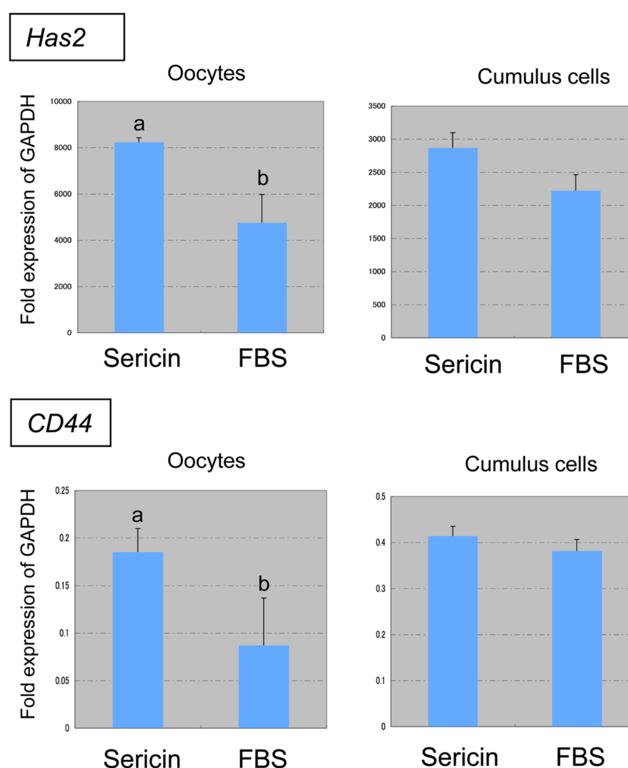

Fig. 2. Quantitative real-time RT-PCR analysis of bovine *HAS2* and *CD44* mRNAs in the oocytes and cumulus cells cultured for 22 h in TCM199 with 0.05% sericin or 5% FBS. The expression of these mRNAs was normalized to the expression of *GAPDH* measured in the same RNA preparation. Results of three independent experiments are summarized and expressed as the mean ± SEM. Different letters above the bars indicate significant differences ($P<0.05$).

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 3. Size of perivitelline space cultured with sericin and FBS

Medium with	No. of oocytes matured	Diameter of cytoplasm (μm)*	Inner diameter of zona pellucida (μm)	Size of the perivitelline space (μm)
0.05% Sericin	95	108.1 ± 0.7	123.0 ± 0.7	14.9 ± 0.7[a]
5% FBS	97	110.1 ± 0.9	122.7 ± 0.7	12.6 ± 0.8[b]

*Means ± SE. Values with different superscripts in the same column are significantly different ($P<0.05$).

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 larger 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 likely 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 decreaseing 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 ooplasmic maturation of bovine oocytes.

Acknowledgment

We appreciate the review of this manuscript prior to submission by SciRevision, and we are grateful to Dr. Y. Hirao for critical reading and editing of the manuscript.

References

1. **Abe H, Hoshi H.** Evaluation of bovine embryos produced in high performance serum-free media. *J Reprod Dev* 2003; **49**: 193–202. [Medline] [CrossRef]
2. **Korhonen K, Kananen K, Ketoja E, Matomäki J, Halmekytö M, Peippo J.** Effects of serum-free in vitro maturation of bovine oocytes on subsequent embryo development and cell allocation in two developmental stages of day 7 blastocysts. *Reprod Domest Anim* 2010; **45**: 42–49. [Medline] [CrossRef]
3. **Abe H, Yamashita S, Satoh T, Hoshi H.** Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. *Mol Reprod Dev* 2002; **61**: 57–66. [Medline] [CrossRef]
4. **Rizos D, Gutiérrez-Adán A, Pérez-Garnelo S, De La Fuente J, Boland MP, Lonergan P.** Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression. *Biol Reprod* 2003; **68**: 236–243. [Medline] [CrossRef]
5. **Abe H, Yamashita S, Itoh T, Satoh T, Hoshi H.** Ultrastructure of bovine embryos developed from in vitro-matured and -fertilized oocytes: comparative morphological evaluation of embryos cultured either in serum-free medium or in serum-supplemented medium. *Mol Reprod Dev* 1999; **53**: 325–335. [Medline] [CrossRef]
6. **Rizos D, Fair T, Papadopoulos S, Boland MP, Lonergan P.** Developmental, qualitative, and ultrastructural differences between ovine and bovine embryos produced in vivo or in vitro. *Mol Reprod Dev* 2002; **62**: 320–327. [Medline] [CrossRef]
7. **Sinclair KD, Young LE, Wilmut I, McEvoy TG.** In-utero overgrowth in ruminants following embryo culture: lessons from mice and a warning to men. *Hum Reprod* 2000; **15**(Suppl 5): 68–86. [Medline] [CrossRef]

8. **Craig CL, Riekel C.** Comparative architecture of silks, fibrous proteins and their encoding genes in insects and spiders. *Comp Biochem Physiol B Biochem Mol Biol* 2002; **133**: 493–507. [Medline] [CrossRef]
9. **Garel A, Deleage G, Prudhomme J-C.** Structure and organization of the Bombyx mori sericin 1 gene and of the sericins 1 deduced from the sequence of the Ser 1B cDNA. *Insect Biochem Mol Biol* 1997; **27**: 469–477. [Medline] [CrossRef]
10. **Takahashi M, Tsujimoto K, Kato Y, Yamada H, Takagi H, Nakamori S.** A sericin-derived peptide protects sf9 insect cells from death caused by acute serum deprivation. *Biotechnol Lett* 2005; **27**: 893–897. [Medline] [CrossRef]
11. **Kato N, Sato S, Yamanaka A, Yamada H, Fuwa N, Nomura M.** Silk protein, sericin, inhibits lipid peroxidation and tyrosinase activity. *Biosci Biotechnol Biochem* 1998; **62**: 145–147. [Medline] [CrossRef]
12. **Terada S, Sasaki M, Yanagihara K, Yamada H.** Preparation of silk protein sericin as mitogenic factor for better mammalian cell culture. *J Biosci Bioeng* 2005; **100**: 667–671. [Medline] [CrossRef]
13. **Terada S, Kumagai T, Ogawa A, Ishimura J, Miki M, Ozaki K, Sasaki M, Yamada H.** Transfecting anti-apoptosis genes into a hepatoma cell line in order to improve cryopreservation. *Low temp Med* 2003; 29: 1–5.
14. **Sasaki M, Kato Y, Yamada H, Terada S.** Development of a novel serum-free freezing medium for mammalian cells using the silk protein sericin. *Biotechnol Appl Biochem* 2005; **42**: 183–188. [Medline] [CrossRef]
15. **Isobe T, Ikebata Y, Onitsuka T, Wittayarat M, Sato Y, Taniguchi M, Otoi T.** Effect of sericin on preimplantation development of bovine embryos cultured individually. *Theriogenology* 2012; **78**: 747–752. [Medline] [CrossRef]
16. **Eppig JJ.** The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation, and cumulus expansion. *Dev Biol* 1982; **89**: 268–272. [Medline] [CrossRef]
17. **Ball GD, Wieben ED, Byers AP.** DNA, RNA, and protein synthesis by porcine oocyte-cumulus complexes during expansion. *Biol Reprod* 1985; **33**: 739–744. [Medline] [CrossRef]
18. **Eppig JJ.** FSH stimulates hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles. *Nature* 1979; **281**: 483–484. [Medline] [CrossRef]
19. **Eppig JJ.** Regulation of cumulus oophorus expansion by gonadotropins *in vivo* and *in vitro*. *Biol Reprod* 1980; **23**: 545–552. [Medline] [CrossRef]
20. **Ball GD, Bellin ME, Ax RL, First NL.** Glycosaminoglycans in bovine cumulus-oocyte complexes: morphology and chemistry. *Mol Cell Endocrinol* 1982; **28**: 113–122. [Medline] [CrossRef]
21. **Salustri A, Yanagishita M, Underhill CB, Laurent TC, Hascall VC.** Localization and synthesis of hyaluronic acid in the cumulus cells and mural granulosa cells of the preovulatory follicle. *Dev Biol* 1992; **151**: 541–551. [Medline] [CrossRef]
22. **Schoenfelder M, Einspanier R.** Expression of hyaluronan synthases and corresponding hyaluronan receptors is differentially regulated during oocyte maturation in cattle. *Biol Reprod* 2003; **69**: 269–277. [Medline] [CrossRef]
23. **Gutnisky C, Dalvit GC, Pintos LN, Thompson JG, Beconi MT, Cetica PD.** Influence of hyaluronic acid synthesis and cumulus mucification on bovine oocyte in vitro maturation, fertilisation and embryo development. *Reprod Fertil Dev* 2007; **19**: 488–497. [Medline] [CrossRef]
24. **Assidi M, Dufort I, Ali A, Hamel M, Algriany O, Dielemann S, Sirard MA.** Identification of potential markers of oocyte competence expressed in bovine cumulus cells matured with follicle-stimulating hormone and/or phorbol myristate acetate *in vitro*. *Biol Reprod* 2008; **79**: 209–222. [Medline] [CrossRef]
25. **Yokoo M, Kimura N, Sato E.** Induction of oocyte maturation by hyaluronan-CD44 interaction in pigs. *J Reprod Dev* 2010; **56**: 15–19. [Medline] [CrossRef]
26. **Talbot P, DiCarlantonio G.** Ultrastructure of opossum oocyte investing coats and their sensitivity to trypsin and hyaluronidase. *Dev Biol* 1984; **103**: 159–167. [Medline] [CrossRef]
27. **Dandekar P, Talbot P.** Perivitelline space of mammalian oocytes: extracellular matrix of unfertilized oocytes and formation of a cortical granule envelope following fertilization. *Mol Reprod Dev* 1992; **31**: 135–143. [Medline] [CrossRef]
28. **Dandekar P, Aggeler J, Talbot P.** Structure, distribution and composition of the extracellular matrix of human oocytes and cumulus masses. *Hum Reprod* 1992; **7**: 391–398. [Medline]
29. **Talbot P, Dandekar P.** Perivitelline space: does it play a role in blocking polyspermy in mammals? *Microsc Res Tech* 2003; **61**: 349–357. [Medline] [CrossRef]
30. **Funahashi H, Cantley TC, Stumpf TT, Terlouw SL, Day BN.** Use of low-salt culture medium for *in vitro* maturation of porcine oocytes is associated with elevated oocyte glutathione levels and enhanced male pronuclear formation after *in vitro* fertilization. *Biol Reprod* 1994; **51**: 633–639. [Medline] [CrossRef]
31. **Wang WH, Abeydeera LR, Prather RS, Day BN.** Morphologic comparison of ovulated and *in vitro*-matured porcine oocytes, with particular reference to polyspermy after *in vitro* fertilization. *Mol Reprod Dev* 1998; **49**: 308–316. [Medline] [CrossRef]
32. **Kitagawa T, Niimura S.** Relationship between the size of perivitelline space and the incidence of polyspermy in porcine oocytes. *Bull Facul Agric Niigata Univ* 2006; **59**: 21–26.
33. **Ueno S, Niimura S.** Size of perivitelline space and incidence of polyspermy in mouse oocytes matured *in vivo* and *in vitro*. *J Mamm Ova Res* 2008; **25**: 44–49. [CrossRef]
34. **Ueno S, Yoshida N, Niimura S.** Amount of hyaluronan produced by mouse oocytes and role of hyaluronan in enlargement of the perivitelline space. *J Reprod Dev* 2009; **55**: 496–501. [Medline] [CrossRef]
35. **Mase K, Iizuka T, Okada E, Miyajima T, Yamamoto T.** A new silkworm race for sericin production, "Sericin Hope" and its product, "Vergin Sericin. *J Insect Biotechnol Sericology* 2006; **75**: 85–88.
36. **Takasu Y, Yamada H, Tsubouchi K.** Isolation of three main sericin components from the cocoon of the silkworm, Bombyx mori. *Biosci Biotechnol Biochem* 2002; **66**: 2715–2718. [Medline] [CrossRef]
37. **Teramoto H, Nakajima K, Takabayashi C.** Preparation of elastic silk sericin hydrogel. *Biosci Biotechnol Biochem* 2005; **69**: 845–847. [Medline] [CrossRef]
38. **Teramoto H, Kameda T, Tamada Y.** Preparation of gel film from Bombyx mori silk sericin and its characterization as a wound dressing. *Biosci Biotechnol Biochem* 2008; **72**: 3189–3196. [Medline] [CrossRef]
39. **Hosoe M, Shioya Y.** Distribution of cortical granules in bovine oocytes classified by cumulus complex. *Zygote* 1997; **5**: 371–376. [Medline] [CrossRef]
40. **Brackett BG, Oliphant G.** Capacitation of rabbit spermatozoa *in vitro*. *Biol Reprod* 1975; **12**: 260–274. [Medline] [CrossRef]
41. **Walker SK, Hill JL, Kleemann DO, Nancarrow CD.** Development of ovine embryos in synthetic oviductal fluid containing amino acids at oviductal fluid concentrations. *Biol Reprod* 1996; **55**: 703–708. [Medline] [CrossRef]
42. **Herath CB, Shiojima S, Ishiwata H, Katsuma S, Kadowaki T, Ushizawa K, Imai K, Takahashi T, Hirasawa A, Tsujimoto G, Hashizume K.** Pregnancy-associated changes in genome-wide gene expression profiles in the liver of cow throughout pregnancy. *Biochem Biophys Res Commun* 2004; **313**: 666–680. [Medline] [CrossRef]
43. **Ushizawa K, Takahashi T, Hosoe M, Ishiwata H, Kaneyama K, Kizaki K, Hashizume K.** Global gene expression analysis and regulation of the principal genes expressed in bovine placenta in relation to the transcription factor AP-2 family. *Reprod Biol Endocrinol* 2007; **5**: 17. [Medline] [CrossRef]
44. **Hayashi K-G, Ushizawa K, Hosoe M, Takahashi T.** Differential genome-wide gene expression profiling of bovine largest and second-largest follicles: identification of genes associated with growth of dominant follicles. *Reprod Biol Endocrinol* 2010; **8**: 11. [Medline] [CrossRef]
45. **Kongtawelert P, Ghosh P.** A method for the quantitation of hyaluronan (hyaluronic acid) in biological fluids using a labeled avidin-biotin technique. *Anal Biochem* 1990; **185**: 313–318. [Medline] [CrossRef]
46. **Talbot P, Dicarlantonio G.** The oocyte-cumulus complex: ultrastructure of the extracellular components in hamsters and mice. *Gamete Res* 1984; **10**: 127–142. [CrossRef]
47. **Okada A, Inomata K, Nagae T.** Spontaneous cortical granule release and alteration of zona pellucida properties during and after meiotic maturation of mouse oocytes. *Anat Rec* 1993; **237**: 518–526. [Medline] [CrossRef]
48. **Chung SO.** Volume changes during the preimplantation stages of mouse egg development. *Yonsei Med J* 1973; **14**: 63–90. [Medline]
49. **Buhi WC, Alvarez IM, Kouba AJ.** Secreted proteins of the oviduct. *Cells Tissues Organs* 2000; **166**: 165–179. [Medline] [CrossRef]
50. **Buhi WC.** Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein. *Reproduction* 2002; **123**: 355–362. [Medline] [CrossRef]
51. **Kapur RP, Johnson LV.** An oviductal fluid glycoprotein associated with ovulated mouse ova and early embryos. *Dev Biol* 1985; **112**: 89–93. [Medline] [CrossRef]
52. **Kapur RP, Johnson LV.** Selective sequestration of an oviductal fluid glycoprotein in the perivitelline space of mouse oocytes and embryos. *J Exp Zool* 1986; **238**: 249–260. [Medline] [CrossRef]
53. **Buhi WC, O'Brien B, Alvarez IM, Erdos G, Dubois D.** Immunogold localization of porcine oviductal secretory proteins within the zona pellucida, perivitelline space, and plasma membrane of oviductal and uterine oocytes and early embryos. *Biol Reprod* 1993; **48**: 1274–1283. [Medline] [CrossRef]
54. **Buhi WC, Alvarez IM, Kouba AJ.** Oviductal regulation of fertilization and early embryonic development. *J Reprod Fertil Suppl* 1997; **52**: 285–300. [Medline]
55. **Vollet JJ, Roth LE.** Cell fusion by nascent membrane induction and divalent cation treatment. *Cryobiol* 1974; **9**: 249–262.
56. **Kujawa MJ, Tepperman K.** Culturing chick muscle cells on glycosaminoglycan substrates: attachment and differentiation. *Dev Biol* 1983; **99**: 277–286. [Medline] [CrossRef]
57. **Orkin RW, Knudson W, Toole BP.** Loss of hyaluronate-dependent coat during myoblast fusion. *Dev Biol* 1985; **107**: 527–530. [Medline] [CrossRef]
58. **Watanabe K, Yamaguchi Y.** Molecular identification of a putative human hyaluronan synthase. *J Biol Chem* 1996; **271**: 22945–22948. [Medline] [CrossRef]
59. **Spicer AP, Olson JS, McDonald JA.** Molecular cloning and characterization of a cDNA encoding the third putative mammalian hyaluronan synthase. *J Biol Chem* 1997; **272**: 8957–8961. [Medline] [CrossRef]
60. **Kaneko T, Saito H, Toya M, Satio T, Nakahara K, Hiroi M.** Hyaluronic acid inhibits apoptosis in granulosa cells via CD44. *J Assist Reprod Genet* 2000; **17**: 162–167. [Medline] [CrossRef]
61. **Gui LM, Joyce IM.** RNA interference evidence that growth differentiation factor-9 mediates oocyte regulation of cumulus expansion in mice. *Biol Reprod* 2005; **72**: 195–199. [Medline] [CrossRef]