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Short communication

# Enhanced *in vitro* developmental competence of sheep embryos following sericin supplementation of the *in vitro* maturation and *in vitro* culture media

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ABSTRACT

The objective was to determine the effects of various sericin concentrations (0, 0.1, 0.5, 1, and 2.5%) during *in vitro* oocyte maturation (IVM) and *in vitro* embryo culture (IVC) on ovine embryo development. Pubertal Sanjabi ewes' oocytes were aspirated from slaughterhouse derived ovaries and matured *in vitro*. Semen was collected from a healthy fertile ram, capacitated and the *in vitro* matured oocytes were fertilized using capacitated spermatozoa. The IVM and IVC media were supplemented with 0, 0.1, 0.5, 1 and 2.5% of sericin. Addition of 0.5% of sericin during IVM and IVC significantly increased cleavage and blastocyst rates ($85.48 \pm 1.57$ and $50.07 \pm 3.04$, respectively) compared with 0 and 0.1% of sericin. However, a too high concentration (2.5%) of sericin during IVM and IVC decreased cleavage and blastocyst rates compared with low concentrations ($P < 0.05$). In conclusion, appropriate concentrations (0.1 and 0.5%) of sericin promoted ovine blastocyst formation *in vitro*, although a high concentration of sericin suppressed embryo development.



## 1. Introduction

Using *in vitro* embryo production (IVEP) allows the production of a large number of offsprings from live or slaughtered animal (Guler et al., 2000) for research, genetic improvement or commercial purposes (Accardo et al., 2004). Although substantial progress has been made to improve efficiency of *in vitro* production protocol in livestock, the rate of normally developing embryos is still lower than that of *in vivo*-derived embryos (Arias-Alvarez et al., 2011).

Sericin a water-soluble globular protein (a protein hydrolysate) derived from silkworm *Bombyx mori*, and represents a family of proteins whose molecular mass ranges from 10 to 310 kDa (Tao et al., 2005). The sericin of *B. mori* were found to enhanced the attachment and growth of mouse fibroblasts when used as a substratum (Minoura et al., 1995). Similar preparations accelerated the proliferation of mammalian cells (Terada et al., 2002), improved their performance in the serum-free cultures (Terada et al., 2005), and enhanced attachment of the cultured human skin fibroblasts (Tsubouchi et al., 2005). Inaba et al. (2010) results indicated the feasibility of sericin as an alternative protein supplement for bovine embryo culture. Sericin is rich in aspartic acid as well as serine (Cho et al., 2003), which has a high content of the hydroxyl group. This nature provides sericin with antioxidant action (Takahashi et al., 2005).

One of the major differences between the *in vivo* and the *in vitro* environment for the embryo is the oxygen tension. The atmospheric conditions of the IVEP are 5% $CO_2$ in air. It means 3–4 times more $O_2$ than in the oviduct (Mastroianni and Jones, 1965) resulting in increased reactive oxygen species (ROS) production (Agarwal et al., 2006). It has been reported that ROS induce mitochondrial dysfunction, DNA, RNA, and protein damage (Finkel and Holbrook, 2000), and inhibit sperm-oocyte fusion (Aitken et al., 1993). *In vivo*, the damaging effects of ROS are usually prevented or limited by endogenous antioxidants. These include enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), and lipid- and water-soluble antioxidants such as vitamins C, E, and uric acid (Zusterzeel et al., 1999). However, the level of antioxidants was lower than *in vivo* during *in vitro* culture of oocyte and embryo, because oocytes or embryos do not benefit from maternal antioxidant protection. It seems likely that embryos cultured *in vitro* might be exposed to oxidative stress for which their defense mechanisms are insufficient to protect their delicate cellular structures. To protect oocytes and embryos from oxidative

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stress during IVM and IVC, antioxidants can be added to culture medium.

However, to our knowledge, effects of sericin on the cleavage and blastocyst rates of ovine embryos have not yet been confirmed. Therefore, the objective of the present study was to determine if sericin treatments during IVM and IVC media would improve blastocyst rate in sheep.

## 2. Material and methods

All chemicals and reagents were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

### 2.1. Collection of sheep oocytes and in vitro maturation (IVM)

Ovine ovaries were obtained from an abattoir and transported to the laboratory within 1–2 h in a 0.9% saline solution, at 35–37 °C temperature. Follicles with diameters between 2–6 mm were aspirated with a 21-G needle attached to a 5-ml syringe. Only cumulus-oocyte complexes with compact cells and homogeneous cytoplasm were selected for IVM. Groups of 6–7 cumulus-oocyte complexes were cultured in 50 μl droplets of IVM medium consisting of [(TCM-199 with Earle's Salts, L-glutamine and 25 mM *N*-2-hydroxyethylpiperazine-*N*-2'-ethanesulfonic acid (HEPES), Gibco, UK) with 0.5 μg/ml FSH, 5 μg/ml LH, 1 μg/ml estradiol 17-ß, 50 μg/ml of gentamycin sulfate and 10% (v/v) fetal ovine serum (FOS)], under mineral oil. Oocytes were kept for 22–24 h in a humidified incubator maintained at 38.5 °C and 5% $CO_2$ in air.

### 2.2. Oocyte maturation assessment: nuclear stage

Oocyte nuclear stage in meiosis was determined after aceto-orcein staining. Briefly, the oocytes were denuded by gentle pipetting and then fixed for at least 24 h in ethanol fixative solution (1:3). Afterward, they were placed on a clean glass slide and overlaid with a square cover slip that was held up by four droplets of a vaseline-paraffin mixture (40:1). Thereafter, the oocytes were stained for 2 min with 1% aceto-orcein before washing with a mixture of distilled water, glycerol, and acetic acid (3:1:1). Finally, the nuclear maturation was recorded under a phase contrast microscope (Olympus B201; Olympus). (Ghaffarilaleh et al., 2014).

### 2.3. Sperm preparation and fertilization

In this experiment fresh semen was collected from a fertile healthy ram into a sterilized 15 ml plastic centrifuge tube, using the artificial vagina. Raw semen (50 μl), was placed in 2 ml sperm-TALP medium (Parrish et al., 1988), in two test tubes, and the sperm allowed to swim-up during incubation for 1–2 min at 38.5 °C, under 5% $CO_2$ in air. After maturation, matured oocytes were taken from the maturation medium drops 22–24 h of maturation. The expanded cumulus cells of the oocytes were removed by repeated gentle pipetting in fertilization-TALP medium, using a sterilized Pasteur pipette. Then a maximum of 5–7 oocytes were transferred to 48 μl drops of IVF medium (IVF-TALP medium) supplemented with 6 mg/ml BSA and 2 μg/ml heparin. Insemination was carried out by adding 1–2 × $10^6$ spermatozoa/ml, and PHE (penicillamine, 20 μmol/l; hypotaurine, 10 μmol/l; epinephrine, 1 μmol/l). Oocytes were co-incubated with spermatozoa for 6–8 h at 38.5 °C and 5% $CO_2$ in humidified air atmosphere.

### 2.4. In vitro culture (IVC)

The putative zygotes were taken from the fertilization drops after 6–8 h of co-incubation. After washing, the putative zygotes were then placed in 60 μl of simplex optimization medium supplemented by amino acides (KSOM-aa) in a 60 mm Petri dish, covered with mineral oil and incubated until day 8 after fertilization at 38.5 °C in a humidified atmosphere of 5% $CO_2$. Cleavage was assessed after 48 h of culture, and the numbers of embryos developing to the morula and blastocyst stages were assessed on days 4 and 6, respectively (day 0 = day of IVF). In the present study, a two step-culture system was used. The first KSOM-aa1 medium contained 0.8% crystallized BSA (w/v) was used for the first 48 h. Then, the medium was replaced by the second step (KSOM-aa2) containing 0.8% FOS (v/v) for the remaining 6 days of culture.

### 2.5. Experimental design

In this experiment, the effects of sericin supplementation during IVM and IVC were investigated. The same concentrations of sericin were added to both, maturation and culture medium (designated 0/0,0.1/0.1, 0.5/0.5, 1/1 and 2.5/2.5 (w/v) sericin groups, respectively).

### 2.6. Statistical analysis

This experiment was replicated at least 5 times. Differences in nuclear maturation (MII %), and embryo development (cleavage and blastocyst rates) between the experimental groups were analyzed by one-way ANOVA, using the GLM procedure and LSMEANS statement of SAS (SAS for Windows, version 9.1). The Duncan's multiple range tests were used to test the differences between the treatments. A P value less than 0.05 denoted a statistically significant difference.

## 3. Results

As shown in Table 1, the addition of 0.5% of sericin during IVM and IVC increased ($P < 0.05$) cleavage and blastocysts rates compared with the control (table.1). However, supplementation of a high concentration (2.5/2.5%) of sericin during IVM and IVC decreased cleavage and blastocyst rates, compared with 0/0, 0.1/0.1, and 0.5/0.5% sericin groups ($P < 0.05$). No significant differences were recorded in terms of morula rate, when sericin was supplemented at a concentration of 0/0, 0.1/0.1 and 1/1%, while the 0.5/0.5% of sericin supplementation significantly ($P < 0.05$) increased the percentage production of morula (Table 1).

## 4. Discussion

Improvement of culture the culture system is of great importance for gamete and embryo manipulation and production *in vitro*. Two of the major problems encountered in the culture system have been oxidative stress (Michel et al., 2013) and suboptimal culture medium (Mirshamsi and Shabankareh, 2012) which have adverse effects on oocytes and embryos (Gardner, 2007). To improve this situation, special attention has been accorded to the addition of antioxidants and supplementations to the culture system (Tao et al., 2010), and sericin is one of the supplements (Terada et al., 2002) and antioxidants being tested (Isobe et al., 2012). Evaluating it based on its chemical structure, sericin is a glue protein of silk, has recently been investigated for its activities in the biotechnological field.

In the present experiment, a significant increase in the cleavage and blastocyst rates was observed after supplementation of 0.5% of sericin in the IVM and IVC medium. The sericins of *Bombyx mori (B. mori)* were found to function as an antioxidant (Isobe et al., 2012; Do et al., 2014), a useful culture medium supplement for stimulating the proliferation of mammalian cells (Terada et al., 2002). The antioxidant effects of sericin could be one of the reasons responsi-