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Sonic Hedgehog promotes *in vitro* oocyte maturation and term development of embryos in Taiwan native goats

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ABSTRACT

The aim of this study was to investigate the effects of Shh (Sonic Hedgehog) protein on caprine oocyte maturation, early embryo development, and developmental competence after embryo transfer of vitrified-thawed *in vitro*-produced embryos. Cumulus-oocyte complexes (COCs) derived from abattoir were randomly allocated to the *in vitro* maturation (IVM) medium supplemented with 0 (Control), 0.125, 0.25, 0.5, or 1.0 μg mL^{-1} recombinant mouse Shh protein. After IVM, COCs were fertilized with frozen-thawed semen and the presumptive zygotes were cultured on goat oviduct epithelial monolayers in M199 medium for 9 days. Our results showed that supplementation of Shh (0.25 or 0.5 μg mL^{-1}) enhanced oocyte maturation as compared with the control group (92.4% and 95.0% *vs.* 86.2%, $P < 0.05$), yet the effect could be reversed by the simultaneous addition of cyclopamine (an inhibitor of Shh signaling by direct binding to the essential signal transducer Smo). Subsequently, an improved blastocyst rate (66.3 ± 10.9, $P < 0.05$) was observed for the embryos derived from the oocytes matured in the presence of 0.5 μg mL^{-1} Shh compared with the control group (41.4 ± 12.9). Expressions of *Shh*, *SMO* and *Gli1* were observed in the ovaries, granulosa cells, COCs, cumulus cells, oocytes and oviduct epithelia. Notably, *Ptch1* was expressed in nearly all of the aforementioned tissues and cells except cumulus cells. The embryos exhibited a higher survival rates in the Shh-supplemented group (37.5%) compared to those without Shh supplementation (14.8%; $P < 0.05$) after embryo transfer. This study demonstrated the beneficial effects of Shh supplementation on oocyte maturation and subsequent embryo development both *in vitro* and *in vivo*, suggesting a functional existence of Shh signaling during the final stage of folliculogenesis and early embryogenesis in caprine.

1. Introduction

Influenced by macrobiotics and traditional Chinese medicine, goat milk and meat consumption has been popular in Taiwan. Compared to the exotic breeds, Taiwan native goats have much better diseases- and coarse diets-resistant despite of their low productivity. In response to the ever-increasing demand, a large number of Nubian goats were imported to cross-breed with Taiwan native goats during the 1980s [1,2]. This is one of the main reasons for the decreased population of the pure Taiwan native goats, which may soon become endangered. Therefore, reproductive technologies could be an important tool for preserving genetic diversity and rescuing this caprine breeds in Taiwan.

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Generation of high quality goat embryos is an essential step towards further development and application of *in vitro*-produced embryos [3,4]. Previous studies have demonstrated that the growth of preimplantation embryos can be enhanced by supplementation of cytokines and/or growth factors in the culture medium [5]. Hedgehog (Hh) protein, which attracts considerable attention over the past few years, is a paracrine factor that enhances embryonic development [6]. In vertebrates, there are at least three Hh members, namely, Sonic Hh (Shh), Indian Hh (Ihh) and Desert Hh. The signaling of the Hh family peptides is mediated through a membrane bound surface receptor Patched (Ptc) and a membrane associated signal transducer Smoothened (Smo). In the absence of Hh ligand, Ptc suppresses Smo so that no downstream signaling occurs [7,8]; whereas in the presence of Hh, the suppression of Smo is lifted, leading to the activation of intracellular transcription effectors Gli1, Gli2 and Gli3 in vertebrates [9].

In the mouse, Ihh and Dhh are expressed in the granulosa cells of preantral and antral follicles, with the receptor Ptc and the signal transducer Smo expressed in thecal cells. Previous studies also suggested that paracrine signaling of Hh exist between granulosa and theca cells [10,11]. Similarly, Russell et al. [9] reported that Hh ligands, including Ihh, Dhh and Shh, are expressed in both immature and adult mouse ovaries, where the expression of Ptc (Ptc1, Ptc2) and Smo are found in all ovarian tissues. Therefore, the Hh signaling pathway is most likely to be involved in granulosa cell proliferation and oocyte maturation. Spicer et al. [12] demonstrated that the mRNA expression of the Hh-patched signaling molecule *Ihh* in granulosa cells increased, but mRNA expressions of *Smo* and *Ptch1* levels decreased in theca cells of small follicles compared to large follicles in cattle. In cultured bovine theca-interstitial cells, qRT-PCR analyses revealed that the abundance of *Gli1* and *Ptch1* mRNAs increased with Shh treatment. Additional studies have shown that Shh induces proliferation and androstenedione production of cultured bovine theca cells. Moreover, expression and regulation of *Ihh* mRNA in granulosa cells and *Ptch1* mRNA in theca cells may also suggest a potential paracrine role during bovine folliculogenesis. Nguyen et al. [13–15] have also reported that the Shh signaling pathway is active or at least partially active in the porcine ovary, which is likely associated with cytoplasmic and nuclear maturation of oocytes as well as with subsequent embryonic development *in vitro*. Therefore, we propose that this paracrine factor also promotes oocyte maturation and early embryogenesis in goats. To test this hypothesis, the effects of Shh treatment during goat oocyte maturation on both *in vitro* and *in vivo* developmental competence, including oocyte maturation, embryonic development, pregnancy and kidding rates as well as the Shh-related gene expression in reproductive cells/tissues, were all determined.

2. Materials and methods

2.1. Chemicals

All chemicals used in the present study were purchased from Sigma-Aldrich (St Louis, MO, USA), unless stated otherwise.

2.2. Oocyte recovery and in vitro maturation (IVM)

During the breeding season, ovaries of adult goats were collected from a local abattoir and transported to the laboratory in saline at 38 °C within 2.5 h. Ovaries were further washed in warm saline, and oocytes were harvested by slicing all visible follicles (1–5 mm in diameter) with a blade and then flushed with TCM 199 (Gibco, 12340–030, Grand Island, USA) supplemented with 100 UI mL^{-1} heparin, 40 mg mL^{-1} gentamycin and 10 mM mL^{-1} HEPES. Only oocytes surrounded by multilayered, unexpanded cumulus cells and finely granulated ooplasm (Grades 1 and 2) were used for IVM [16]. The cumulus-oocyte complexes (COCs) were washed three times in the maturation medium (TCM 199 supplemented with 10% FCS, 10 μg/mL FSH, 10 μg mL^{-1} LH, 0.2 mM sodium pyruvate, 1 μg mL^{-1} estradiol 17β, 10 ng mL^{-1} EGF and 100 μM cysteamine), and then cultured in 4-well dishes (Nunc, Roskilde, Denmark) containing 0.5 mL of maturation medium and 20 to 30 oocytes per well. The COCs were incubated for 24 h at 38.5 °C in a humidified atmosphere containing 5% CO_2 in air [17].

2.3. In vitro fertilization (IVF) and embryo culture

Motile spermatozoa from frozen-thawed semen of Taiwan native goats were separated by washing and centrifugation (10 min at 900 × g) with washing medium (2 mM $CaCl_2 \cdot 2H_2O$, 521 μM $MgCl_2 \cdot 6H_2O$, 112 mM NaCl, 4 mM KCl, 819 μM $NaH_2PO_4 \cdot H_2O$, 3.69 mM $NaHCO_3$, 1.25 mM Sodium pyruvate, 13.9 mM D-glucose, 6 mg mL^{-1} Bovine serum albumin, 50 μg mL^{-1} Gentamycin, and 20% Goat serum). Viable spermatozoa were diluted with an appropriate volume of fertilization medium (the washing medium supplemented with 20 μg mL^{-1} heparin and 0.8 μg mL^{-1} caffeine) to a concentration of 1×10^7 spz mL^{-1} and then capacitated for 15 min at 38.5 °C in an incubator containing 5% CO_2 in humidified air. Cumulus cells were removed by gentle pipetting and oocytes were washed three times with fertilization medium. Groups of 20–30 oocytes were transferred into a 4-well dish containing 450 μL of fertilization medium covered with 350 μL of mineral oil. For fertilization, capacitated sperm (50 μL) were added into the wells at the final concentration of 1×10^6 spz mL^{-1} and then co-incubated for 18 h [17]. After IVF, fertilized embryos were co-cultured with goat oviduct epithelial cell (GOEC) monolayer in a 4-well dish containing 500 μL culture medium (TCM 199 plus 10% FCS) for 9 days and half of the culture medium was renewed every 48 h. The GOEC monolayer was prepared based on a previous study by Mermillod *et al.* with some modifications [18]. Briefly, the mucosa layer was mechanically expelled by squeezing the oviduct, collected from the abattoir, with a sterile microscope slide onto the bottom of a Petri dish. The epithelial fragments were washed three times in TCM199 medium and placed in 4-well dishes containing 500 μL of TCM 199 supplemented with 10% (v/v) FCS and 80 μg mL^{-1} of gentamycin and then cultured at 38.5 °C in a humidified atmosphere of 5% CO_2. The GOEC monolayers were established 2 days prior to being used for embryo co-culture. A half of the embryo culture medium was renewed every 48 h during *in vitro* development.

2.4. Recipients does

Crossbred does at ages of 3–4 years were used as recipients for embryos transfer. The does were housed indoors and fed with 0.3 kg/day of concentrates with free access to quality hays and water. Experiments were carried out during the breeding seasons (spring and autumn) of goats in Taiwan. Recipient animals were synchronized for estrus by inserting a vaginal releasing device containing 366 mg progesterone (Controlled Internal Drug Release, CIDR, EAZI-breed, Rydalmere, Australia) for 11 days. Two days before CIDR removal, the recipients received 500 IU eCG (Sera-Gona, China Chemicals, Taipei, Taiwan) and 125 mg cloprostenol (Estrumate, Schering-Plough, Baulkham, Australia). Behavioral estrus was observed 15–30 h following CIDR removal. Preoperative treatment, anesthesia, surgery for embryo transfer and post-operation care were performed as described previously according to the IACUC guidelines (approval number# LRIIACUC 101003) [19,20].