Theriogenology 97 (2017) 113–123

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Relationship between *in vitro* growth of bovine oocytes and steroidogenesis of granulosa cells cultured in medium supplemented with bone morphogenetic protein-4 and follicle stimulating hormone

Kenichiro Sakaguchi [a], Weiping Huang [a, b], Yinghua Yang [a], Yojiro Yanagawa [a], Masashi Nagano [a, *]

[a] *Laboratory of Theriogenology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, 060-0818, Japan*

[b] *Anhui Provincial Laboratory of Animal Genetic Resources Protection and Breeding, College of Animal Sciences and Technology, Anhui Agricultural University, Hefei, Anhui, China*

ARTICLE INFO

Article history:
Received 26 September 2016
Received in revised form
21 April 2017
Accepted 21 April 2017
Available online 23 April 2017

Keywords:
BMP-4
Early antral follicle
FSH
In vitro growth
Maturational competence
Steroidogenesis

ABSTRACT

Bone morphogenetic protein-4 (BMP-4) and FSH play important regulatory roles in follicular growth and steroidogenesis *in vivo*. The purpose of this study was to investigate the effects of BMP-4 and FSH on *in vitro* growth (IVG) and steroidogenesis of bovine oocyte-cumulus-granulosa complexes (OCGCs). We cultured OCGCs collected from early antral follicles (0.5–1 mm) in medium without BMP-4 and FSH for 4 days and investigated the appearance of OCGCs and their steroidogenesis. During the first 4 days of IVG, morphologically normal OCGCs produced more estradiol-17β (E_2), but less progesterone (P_4). Morphologically normal OCGCs were subjected to an additional culture in medium supplemented with BMP-4 (0, 10, and 50 ng/mL) and FSH (0 and 0.5 ng/mL) until day 12. We examined the viability and steroidogenesis of OCGCs after 8 and 12 days of culture. Oocyte growth, characteristics of granulosa cells, and the maturational competence of oocytes were also investigated. On day 8, the viability of OCGCs cultured without FSH was higher in the 10 ng/mL BMP-4 group than in the 50 ng/mL BMP-4 group ($P < 0.05$). No significant difference was observed in the viability of groups cultured with FSH, regardless of the addition of BMP-4, and FSH improved the viability of 50 ng/mL BMP-4 group similar to 10 ng/mL BMP-4 group. The total number of granulosa cells was larger in 10 ng/mL BMP-4 group cultured with FSH than in 50 ng/mL BMP-4 group cultured with FSH on day 8 ($P < 0.05$). E_2 production decreased from days 8–12, and P_4 production increased throughout IVG culture, regardless of the addition of BMP-4 and FSH ($P < 0.05$). No significant differences in E_2 production were observed between groups from days 4–8, regardless of whether BMP-4 was added without FSH; however, E_2 production in the group cultured with 50 ng/mL BMP-4 was suppressed by FSH. BMP-4 suppressed E_2 production from days 8–12, regardless of whether FSH was added. The group cultured with 10 ng/mL BMP-4 without FSH showed the lowest P_4 production among all groups for all culture periods. OCGCs that produced mature oocytes tended to secrete more E_2 and less P_4 than OCGCs that produced immature oocytes. In conclusion, until day 8 of the IVG culture, P_4 production by OCGCs was suppressed by the addition of 10 ng/mL BMP-4 in the absence of FSH, without inhibiting E_2 production. These conditions appear to mimic growing follicles until day 8 and mimic degenerating follicles from days 8–12 of culture.

1. Introduction

A large number of primordial follicles exist in mammalian ovaries, and granulosa cells multiply and oocytes become developmentally competent as they grow. However, most follicles degenerate during follicular growth, and only a small proportion of follicles develop sufficiently to undergo ovulation [1,2]. If it is

* Corresponding author.
E-mail addresses: ksaka@vetmed.hokudai.ac.jp (K. Sakaguchi), huangwp@ahau.edu.cn (W. Huang), young@vetmed.hokudai.ac.jp (Y. Yang), yoji-y@vetmed.hokudai.ac.jp (Y. Yanagawa), mnaga@vetmed.hokudai.ac.jp (M. Nagano).

http://dx.doi.org/10.1016/j.theriogenology.2017.04.030
0093-691X/

possible to develop a culture system that enables early stage oocytes or follicles to grow to the ovulatory stage, more embryos may be produced and an experimental model may also be established to investigate the mechanisms underlying follicular recruitment, selection, and ovulation. In mice, tissue culture of neonatal ovaries combined with *in vitro* growth (IVG) culture of oocyte-cumulus-granulosa complexes (OCGCs) allows pups to be produced from primordial follicles *in vitro* [3,4]. However, in cattle, no studies have currently reported the production of calves from follicles at stages earlier than preantral follicles. Previous studies described bovine calves derived from IVG oocytes that originated from OCGCs in early antral follicles [5–7]; however, their developmental competence to transferable embryos was lower than the competence of *in vivo* matured oocytes [5–13].

The primary roles of follicular cells are to support the growth and maturation of oocytes, as well as the production of sex steroid hormones by granulosa cells. Therefore, in order to mimic *in vivo* follicular growth via an *in vitro* culture system, the growth of oocytes and production of sex steroid hormones by granulosa cells both need to be investigated. A previous study that combined histological observations with the measurement of sex steroid hormones in follicular fluid revealed that estradiol-17β (E_2) concentrations in growing follicles increased as the follicles grew, with a peak at estrus in cattle, and the degeneration of follicles led to increases in progesterone (P_4) concentrations [14]. Furthermore, E_2 concentrations in dominant follicles increased concomitantly with follicular development and the E_2/P_4 ratio also increased; however, subordinate follicles showed low E_2/P_4 ratios [14,15]. These findings indicate that a culture system of OCGCs that produces more E_2 and less P_4 is needed to mimic *in vivo* dominant follicular development. In conventional IVG, serum has typically been added to culture media to promote cell growth and survival [5–7]. However, granulosa cells cultured in media containing serum luteinize, compromise E_2 production, and begin to produce P_4 [16,17]. Previous studies attempted to culture OCGCs in serum-free media [18,19]; however, the oocytes derived from serum-free cultures had low maturational competence and low fertilizability. Thus, a culture system for OCGCs that produces oocytes with high developmental competence and inhibits the luteinization of granulosa cells under culture conditions containing serum needs to be developed. As shown in our previous study, the addition of bone morphogenetic protein-4 (BMP-4) to the growth medium inhibited the luteinization of granulosa cells [20].

BMP-4 is produced by theca cells in bovine follicles, and its receptor is primarily expressed in oocytes and granulosa cells [21]. An *in situ* hybridization analysis of rat ovaries revealed that the expression level of BMP-4 increases during follicular growth [22]. Based on previous studies of cultured granulosa cells without oocytes, BMP-4 promotes E_2 production by inhibiting apoptosis [23] and promoting aromatase (P450arom) activity in cattle [21]. In addition, P_4 production is inhibited by the suppression of steroidogenic acute regulatory protein (StAR) in cattle [24] and sheep [25] and cholesterol side chain cleaving (P450scc) in sheep [25] at the messenger RNA (mRNA) and protein levels. Moreover, BMP-4 promotes the FSH-mediated activation of E_2 production, which is increased in the presence of oocytes [26]. On the other hand, P_4 production was inhibited in a manner independent of the presence of oocytes in an *in vitro* study of rat granulosa cells [26]. Therefore, the addition of BMP-4, which compensates for the lack of theca cells, and FSH to the medium of IVG for bovine OCGCs may promote oocyte growth by promoting E_2 production and inhibiting P_4 production. According to our recent report, the addition of BMP-4 (10 ng/mL) to an IVG culture suppressed P_4 production and did not affect oocyte growth, nuclear maturation, or fertilization, but impaired subsequent embryonic development and, at a higher concentration (50 ng/mL), even compromised the viability of OCGCs by suppressing the proliferation of granulosa cells [20]. In that study [20], we cultured OCGCs in medium that contained high concentrations of E_2 (1 μg/mL) to increase the E_2/P_4 ratio similar to a dominant follicle; therefore, we were unable to correctly investigate the effects of BMP-4 and FSH on the steroidogenesis of granulosa cells. In the present study, we added BMP-4 and FSH to the growth medium without the addition of E_2 and examined the production of sex steroid hormones from individually cultured OCGCs. We also retrospectively analyzed the correlation between the steroidogenesis of OCGCs during the IVG culture and the nuclear maturation of the corresponding oocytes.

2. Materials and methods

2.1. Chemicals

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

2.2. Collection of OCGCs and the IVG culture

Bovine ovaries obtained from a local abattoir were stored in plastic bags at 20 °C and transported to the laboratory within 6–10 h of their collection. After the ovaries were washed three times with physiological saline, slices of ovarian cortex tissues (<1 mm thickness) were prepared using a surgical blade (no. 11) and stored in tissue culture medium 199 (TCM-199; Thermo Fisher Scientific, Roskilde, Denmark) supplemented with 0.1% polyvinyl alcohol, 25 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 10 mM sodium bicarbonate, and 50 μg/mL gentamicin sulfate (isolation medium, pH 7.4) at 37 °C, as described elsewhere [8]. Under a stereomicroscope, early antral follicles (0.5–1 mm in diameter) were dissected from sliced ovarian tissues using a surgical blade (no. 20) and fine forceps in a 90-mm petri dish that had a 1-mm^2 scale on its bottom (FLAT Co., Ltd., Chiba, Japan). OCGCs were isolated from early antral follicles using a pair of fine forceps and subjected to IVG as previously described [27]. The growth medium was HEPES-buffered TCM-199 supplemented with 0.91 mM sodium pyruvate, 5% (v/v) fetal calf serum (FCS; Invitrogen), 4 mM hypoxanthine, 4% (w/v) polyvinylpyrrolidone (MW 360,000), 50 μg/mL ascorbic acid 2-glucoside (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 55 μg/mL cysteine, 50 μg/mL gentamicin sulfate, and 10 ng/mL androstenedione (A_4) as a precursor for E_2 [11]. OCGCs with oocytes surrounded by a cumulus investment and an attached mural granulosa-cell layer (Fig. 1) were cultured individually in a 96-well culture plate (Primaria 353872, Corning Life Sciences, Tewksbury, MA, USA) with 200 μL of growth medium at 39 °C for 12 days in humidified air with 5% CO_2. At the onset of the IVG culture, OCGCs were photographed under an inverted microscope (CK 40, Olympus, Tokyo, Japan) with an attached CCD camera (Moticam 2000, Shimadzu Rika Corporation, Tokyo, Japan). The diameters of the oocytes were assessed using software (Motic Images Plus 2.2s, Shimadzu). Every 4 days of the IVG culture, half (100 μL) of the growth medium was replaced with the same amount of fresh medium. Spent media collected at 4, 8, and 12 days of culture were stored at −30 °C until steroid hormone assays were conducted.

2.3. Evaluation of OCGC morphology

After 4 days of IVG culture, the morphological appearance of OCGCs was examined (Fig. 1). OCGCs with an evenly granulated ooplasm that were completely enclosed by several layers of healthy cumulus and granulosa cells were defined as normal. When OCGCs