Impact of Insulin-Like Growth Factor-I on bovine Follicular dinamics

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I dedicate this thesis to my mother, who passed away in June 2020.

May Allah grant my mother Jannatul-Firdaus

Ameen

I love you, Mom. I want to be with you in Jannah.

PREFACE

The experiments described in this dissertation were carried out at the Laboratory of Reproductive Physiology, Graduate School of Environmental and Life Science (Ph.D. course), Okayama University, Japan, from November 2021 to March 2024, under the supervision of Professor Koji KIMURA.

This dissertation has not been previously submitted in whole or in part to a council, a university, or any other professional institute for a degree, diploma, or other professional qualification.

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CHAPTER 1

GENERAL INTRODUCTION

Studying and understanding reproductive physiology has allowed the development of new biotechnologies that improved animal production and helped in the solution of classic physiological problems that affect the reproduction of domestic animals (1).

THE OVARIES AND FOLLICLES

The term "ovary" is derived from the Latin word "ovum," meaning egg (2). In cattle, ovaries are located at the side and slightly below the uterine horns in the pelvic cavity, whereas in multiparous cows they are situated in the abdominal cavity (3). The ovaries have two parts, cortex and medulla. The surface epithelium, tunica albuginea, numerous ovarian follicles, and corpus luteum (CL) constitute the cortex, while the medulla consists of numerous blood vessels, lymphatics, and nerves (4).

Follicles are blister-like structures and fluid-filled, containing developing oocytes or eggs (5). There are two different pools of follicles on the ovaries of cattle, the non-growing pool that contains primordial follicles, and the growing pool that contains primary, secondary, and tertiary follicles (6). The primordial follicles enter the growth phase by leaving the arrested pool and grow into primary follicles, whereby the oocytes increase in size and the surrounding squamous pre-granulosa cells become cuboidal and proliferate to form a layer of the cells around the growing oocyte (7). A follicular wave is defined as the synchronous growth of a group of small antral follicles, from which a single follicle is selected to become the dominant follicle (8,9). Rajakoski in 1960 proposed for the first time, that follicle development in cattle occurs in a follicular wave pattern (10). The number of follicular waves that occur during the

bovine estrous cycle can vary between two or three waves (8). During folliculogenesis and recruitment for follicle selection, a cohort of small follicles usually 4-6 mm in diameter begin their final growth (11). The recruitment of a cohort of follicles is associated with the initiation of expression of messenger-ribonucleic acid (mRNA) for cholesterol side-chain cleavage enzyme (*P450scc*) and aromatase (*CYP19A1*) in granulosa cells (12). This stage is initiated by the high-level production of follicle-stimulating hormone; FSH (13). Three to six follicles with a diameter of 4 to 5 mm occur after the recruitment of follicles into a follicular wave (14). The beginning of the difference in growth rates between the two largest follicles is termed follicle deviation (15). In cattle, follicle deviation occurs when the largest follicle reaches a mean diameter of 8.5 mm (16). For mono-ovulatory species, such as humans, horses, and cattle, only one single follicle is selected as a dominant within each follicular wave and it is able to ovulate (17). Follicle selection in monovular species is the process wherein only one follicle develops from a wave of growing follicles and continues to grow and ovulate. Several physiological changes have been associated with follicular selection, including deviation in follicular growth rates, decreased circulating FSH, increased circulating estradiol-17β (E2), expression of luteinizing hormone receptors (*LHCGR*) on granulosa and theca cells, and increased free insulin-like growth factor (IGF)-1 (16, 18, 19, 20) in follicular fluid. One of the recruited follicles is subsequently selected from the cohort for continued growth (11), whereas the subordinate follicles become atretic (11). The selected follicle acquires *LHCGR* in the granulosa cells and more free IGF-1 compared with the subordinate follicles (11). The selected follicle becomes larger than other follicles and becomes capable of surviving in a low FSH environment (11). The dominant follicle grows approximately 1 to 2 mm per day and reaches its maximum size of 10 to 20 mm (8).

GONADOTROPHIN HORMONES

The gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus and stimulates the secretion of both FSH and luteinizing hormone (LH) by gonadotropic cells of the adenohypophysis (21). The process of follicle development is tightly regulated by FSH and LH, and intraovarian regulators (22). It has been shown that antral follicles (≥ 4 mm) are responsive to the FSH and emerged by an increase in the circulating FSH concentration in blood (13, 23). At the beginning of selection, all the growing follicles ≥ 5 mm contribute to the decline in FSH concentrations, it is not known whether these growing follicles play a direct role in the initial decline in FSH concentrations (24, 25). The suppression of FSH secretion prevents the emergence of a new group of growing follicles and it enables only future dominant follicle to survive (25). Regardless of whether the dominant follicle continues to use the low concentrations of FSH, the follicle apparently begins to use LH at the beginning of deviation (16, 23). Thus, the deviation process has been proposed as the morphological manifestation of the dominant follicle selection (26, 27). LHCGR starts arising in GCs of the future dominant follicle around the beginning of deviation, which induces the steroidogenic capacity in the dominant follicle (25). In a normal cycle, the elevation in pulsatile secretion of LH influences the final maturation and development of antral follicles (28). The role of LH in the follicular selection process has shown that the selected follicle opportunistically switches from being predominantly FSH-dependent to being supported by LH (29). LH stimulates androgen synthesis within the ovarian theca cells (TCs). Androgens also indirectly maintain follicular health as they are the indispensable substrate for E2 production, which is essential for follicle growth and survival.

ESTRADIOL AND ESTRADIOL RECEPTORS

Estrogen is a primary female sex hormone and is primarily synthesized by the ovarian follicle (30). Theca interna cells produce androstenedione (A4) and testosterone (T) which diffuse into the granulosa cells. The granulosa cells synthesize E2 from androgenic precursors produced by the theca interna cells (31). E2 is the primary signal to the brain that induces the expression of estrus, but only in the absence of progesterone (P4) (32). E2 also plays important roles in growth and development, and the regulation of reproductive cycles. E2 has been reported to play the following roles within the ovary, including 1) stimulating the development and maturation of rat ovarian follicles (33); 2) increasing the expression of FSH receptor (*FSHR*) and *LHCGR* by granulosa cells in rats (34) and 3) modulating steroid production by granulosa and theca cells, as demonstrated in cattle (35). E2 has an effect on follicle development along positive or negative feedback mechanisms to the hypothalamus and pituitary gland. As the ovarian follicles grow and differentiate, increasing amounts of E2 are produced, which up-regulate the synthesis and release of the pituitary gonadotrophins (FSH and LH), thereby promoting ovarian follicular growth (36). E2 stimulates proliferation and induces the expression of *FSHR*, *LHCGR*, *aromatase*, and IGF-1 production in granulosa cells (33). Circulating E2 concentrations begin to increase at the time of deviation as a result of increased E2 production by granulosa cells of the future dominant follicle (15, 37). In cattle, follicular-fluid concentrations of E2 began to increase differentially in large follicle than in subordinated follicle near the expected beginning of deviation (38, 18).

E2 is known to elicit rapid effects on cellular function by binding to a membrane-bound estradiol receptor (*ER*) (39). The *ER* is a member of the superfamily of steroid nuclear receptors (40, 41, 42). *ER* is located within the nucleus (43, 44). Two major forms of *ER* are presently known: *ESR1* and *ESR2* (45). *ESR1* predominates in theca and is upregulated as follicles become more reactive to E2 (46). *ESR2* was observed in granulosa cells of primary, secondary,

and mature follicles but not in theca cells (47). The bovine *ESR2* mRNA expression in the granulosa cells decreases with increasing follicular size (48). By contrast, the mRNA expression of *ESR1* in theca tissue increases continuously during the final growth of the bovine follicles (49). A previous study has shown that the expression of *ESR2* mRNA is high in bovine small follicles (≤4 mm), and there may be a role for *ER* in accelerating the growth of small follicles (50). *ER* plays an important role in maintaining ovarian granulosa cell differentiation, follicle and oocyte growth, development, and ovulation.

IGF SYSTEM

The IGF system in the ovary provides an integrated, cooperating, local network that is regulated by auto/paracrine and endocrine mechanisms to promote growth and differentiation in the ovary (51). The IGF system is composed of different elements which are two ligands (IGF-I and IGF-II) and two types of receptors (*IGF1R* and *IGF2R*). The *IGF1R* mediates most of the somatomedin-like actions of both IGF-I and II (52). The TCs have greater levels of *IGF2* mRNA in dominant follicles. It appears that IGF2 acts through *IGF1R*, even though specific *IGF2R* exists in TCs and GCs (53). The components of the IGF system in mammals include IGF-1 and IGF-2, along with their receptors. This superfamily comprises six high-affinity IGF-binding proteins (IGFBP-1, -2, -3, -4, -5, and -6). Additionally, there are several low-affinity IGFBP-related proteins (IGFBP-rP) (52). Four IGFBPs (BP-2, -3, -4 and -5) have been detected in the follicular fluid of cattle (38).

The indispensable tetralogy for follicle selection and final maturation of antral follicles in bovine follicles involves activating the IGF system (36). For the acquisition of follicle dominance free IGF1 is required, which decreases in the subordinate follicle but remains elevated in the dominant follicle (18) due to the breakdown of IGFBP by the IGFBP protease

(54). The intrafollicular factors that are candidates for activation of deviation include those related to the IGF system (55).

Increasing free IGF-1 in follicular fluid is one of the physiological changes that are associated with other factors in bovine follicle selection (18). The IGFs function as modulators of gonadotropin action on granulosa and theca cells (29). The intrafollicular effects of the gonadotropins are mediated by the IGF-1 system and E2 (56). IGF-1 can stimulate granulosa cell proliferation and synergize with gonadotrophins to promote the differentiation of follicle cells (57) and induce the expression of *FSHR* and aromatase in granulosa cells during the preantral-to-antral transition (58). *In vitro* studies with bovine granulosa cells indicate that IGF-1 stimulates the production of E2 and A4 (59). The elevated levels of E2 and IGF-1 in the follicular fluid of large follicles compared to subordinate follicles indicate their roles in follicular deviation in cattle (29, 18). Taken together, IGF-1 plays a crucial role in follicular growth.

The aims of the present study are to investigate the effects of IGF-1 on various factors involved in follicular growth and deviation such as *ESR2*, *LHCGR*, *FSHR*, *IGF1R*, proliferative cell nuclear antigen (*PCNA*), steroidogenic mRNA expression, steroid hormones production, and cell proliferation in bovine follicles.

CHAPTER 2

Effects of Insulin-like growth factor-1 on the mRNA expression of estradiol receptors, steroidogenic enzymes, and steroid production in bovine follicles

INTRODUCTION

The selection of a single dominant follicle is associated with differential growth rates in a cohort of developing follicles that maintain the ovulatory capabilities of cattle. The growth rate change between the largest and the second largest follicles is defined as follicular deviation (16). During the common growth phase that precedes deviation, the follicle-stimulating hormone (FSH) concentrations decrease (60), and the follicular fluid concentrations of estradiol 17β (E2) begin to increase in the future dominant follicle (18). E2 promotes follicular growth and aromatase activity in granulosa cells (GCs) (35, 61). The action of E2 is mediated by estradiol receptors (*ER*) (39). One of ERs, *ESR2* was detected mainly in GCs, whereas *ERα* in theca cells (TCs) (62). Previous studies have shown that *ESR2* knockout mice exhibited delayed follicular growth (63) and fail to exhibit efficient ovulation even when treated with exogenous gonadotropins (64). These findings suggest that *ESR2* plays a crucial role in follicular development and ovulation.

Insulin-like growth factor-1 (IGF-1) plays a central role in bovine follicular growth. It has been proposed that IGF-1 is required for follicular dominance in cattle (16) and may be involved in the mechanism of deviation by increasing the E2 concentration in follicles in cattle (18) or responsiveness of GCs to their specific gonadotropin receptors (65). IGF-1 increases the FSH responsiveness of GCs by increasing the FSH receptor (*FSHR*) expression in mice (66). In an *in vitro* study, IGF-1 increased the expression of luteinizing hormone receptors (*LHCGR*)

in GCs of small follicles in cattle (60). However, no study has been conducted to determine the effects of IGF-1 on mRNA expression of *ESR2* in bovine GCs either in *in vitro* or *in vivo*.

Androgens are substrates for E2 synthesis, which is crucial for follicular growth and selection. During steroidogenesis, androstenedione (A4) is synthesized by the actions of steroidogenic acute regulatory (*STAR*) (67), cytochrome P450 side chain cleavage (*CYP11A1*), cytochrome P450 17α-hydroxylase (*CYP17A1*) (68), 17*β*-hydroxysteroid dehydrogenase (*HSD17B*) and 3β-hydroxysteroid dehydrogenase (*HSD3B*) in TCs under the LH stimulation and is used as the substrate for E2 synthesis by cytochrome P450 aromatase (*CYP19A1*) under the FSH stimulus interacting with specific receptors in GCs (68, 69). The biosynthesis of P4 is regulated by *HSD3B* (70), and P4 is converted to testosterone (T) by the action of *HSD17B* in TCs (71). In previous studies, IGF-1 was found to enhance the mRNA of *CYP11A1, HSD3B,* and *CYP19A1* in bovine GCs *in vitro* (72, 73). IGF-1 also significantly promoted the mRNA of *STAR, CYP19A1*, and *HSD3B* in mice cultured TCs (74). However, the effects of IGF-1 on steroidogenic enzymes in bovine TCs are not well known. Several studies have shown that A4 and T stimulate the growth and development of follicles in cows (75). The *in vitro* culture of mouse preantral follicles with T and A4 enhances follicular growth and development (76, 77). IGF-1 in cattle enhances androgen synthesis from TCs (78). The *in vivo* effects of IGF-1 on follicular fluid factors were studied in heifers where the second largest follicle was injected with IGF-1 at the expected beginning of deviation and increased the follicular fluid concentrations of A4 after 3 h (79). However, little is currently known about the effects of IGF-1 on steroid hormone production in bovine TCs.

Taking together the findings described above, we hypothesized that IGF-1 increases the expression of *ESR2* mRNA in GCs and A4 production in the theca cells of small follicles, which are the predominant factors for the development and selection of follicles. Therefore, in the present study, we aimed to determine the effects of IGF-1 on *ESR2* expression in bovine GCs

of small and mid-sized follicles. In addition, the effects of IGF-1 on mRNA expression of steroidogenic proteins and steroid hormones (A4, P4, and T) production were examined in bovine GCs and TCs derived from small and medium follicles.

MATERIALS AND METHODS

Collection of bovine ovaries

The ovaries of healthy cows were collected from a local abattoir after the exsanguination and transported to the laboratory on ice to obtain TCs and GCs from small (<6 mm) and medium (7-8 mm) diameter follicles, as previously described (80). The ovaries were washed with sterile saline and 70% ethanol and kept in physiological saline at 4°C until the GCs aspiration and follicles dissection for the collection of TCs. For unculture, GCs were collected from each pair of ovaries between days 1-4 post-ovulation, at which point the first follicular wave is normally initiated (81); therefore, it is easy to select the healthy growing follicles in the ovaries.

Theca and granulosa cells isolation and culture

Bovine TCs were enzymatically harvested from dissected small and medium follicles as described previously (82, 83). Briefly, follicles were dissected from ovaries, and the tissues surrounding the follicles were removed using forceps. The dissected follicles were cut into hemispheres and gently scraped with forceps to remove GCs and a part of the theca cell layer. The thin theca layer thus obtained was rinsed with DMEM (Sigma-Aldrich, St. Louis, MO, USA) containing 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.1% bovine serum albumin (BSA, Nacalai Tesque, Kyoto, Japan) then the theca layer was minced in DMEM and treated with 6600 units/ml collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 8000 units/ml DNase (DNP2, BBI Solutions, Newport, UK), 1 mg/ml dispase (Godo Shusei Co., Ltd., Chiba, Japan) and 1mg/ml hyaluronidase (Nacalai Tesque). Cell dissociation was allowed to continue for 60 min at 37°C for continuous stirring at 800 rpm. After incubation, the cell suspension was separated from undigested tissue by filtration through metal meshes (100

µm x 2, 80 µm x 2), and the dispersed theca cells were centrifuged (800 x g, 5 min at 4°C) and resuspended in 50 mM Tris-NH_4CL for hemolysis after discarding the supernatant. Dispersed cells were washed two times with DMEM (Sigma-Aldrich) containing 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.1% BSA and cell viability was estimated at 90–95% by trypan blue exclusion test.

The GCs and follicular fluid were collected aseptically from small and medium follicles using a 2.5 ml disposable syringe with a 24-gauge needle and transferred to a plastic Petri dish filled with phenol red-free DMEM/Ham's F-12, 1:1 (v/v); (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific), 20 µg/ml amphotericin B (Sigma Aldrich, Inc.), and 50 IU heparin sodium salt (Nacalai Tesque). GCs with follicular fluid were centrifuged (800 x g, 5 min at 4°C) and resuspended in Tris-NH_4CL for hemolysis after discarding the supernatant. The cell suspensions were centrifuged again and resuspended in DMEM (Sigma-Aldrich) containing 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.1% BSA and then were filtered through metal meshes (100 µm x 2, 80 µm x 2) to remove cumulus-oocyte complexes (COCs). After centrifugation, the supernatant was discarded, and the washing step was repeated twice. The cell suspensions were then resuspended in an appropriate volume of phenol red-free DMEM/F-12 containing 10% FBS and 20 µg/ml gentamicin (Sigma-Aldrich). The viability of GCs was determined at 75-80% by the exclusion of trypan blue. The dispersed GCs and TCs separately were cultured at 1.0 x 10^5 viable cells per 1 ml in phenol red-free DMEM/F-12 culture medium containing 10% FBS in 75-cm^2 culture flasks (20 ml/flask; Greiner Bio-One, Frickenhausen, Germany) and cultured under 95% air and 5% CO_2 at 38.5°C for 2–3 days. The medium was changed at 24 h intervals. When the cultured cells reached 80–90% confluence, they were passaged with 0.1% bovine trypsin (Sigma-Aldrich) and sterile phosphate-buffered saline (PBS). Dispersed cells in four replicate wells were preincubated with

and without IGF-1 by culturing for 24 h in a serum-free medium in the absence (control) and in the presence of various concentrations of IGF-1.

Total RNA extraction and quantitative RT-PCR

Total RNA was extracted from cultured and non-cultured GCs and TCs using RNAiso plus (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. The extracted RNA from each sample was quantified and 2 µg of each total RNA was reverse transcribed using ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo, Osaka, Japan).

The mRNA expressions of bovine *ESR2, FSHR, CYP19A1, LHCGR, STAR, CYP11A1, CYP17A1*, *HSD3B*, *HSD17B*, *β-actin* (*ACTB*), and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were determined according to a previously described method (84). The expression of each mRNA was quantified using Brilliant III Ultra-Fast SYBER Green qPCR Master Mix with Low ROX (Agilent Technologies, Santa Clara, CA, USA), starting from 2 ng of reverse-transcribed total RNA. To standardize the relative level of *ESR2, FSHR*, and *CYP19A1* mRNA expression in GCs, *ACTB* was used as an internal control, and to standardize the relative levels of *LHCGR, STAR, CYP11A1, CYP17A1*, *HSD3B* and *HSD17B* in TCs, *GAPDH* was used as an internal control. Five housekeeping genes, *MRPL4*, *H2A*, *GAPDH*, *ACTB*, and *18S*, were tested to determine the optimal reference gene for quantitative RT-PCR data from isolated and cultured bovine GCs and TCs separately. *ACTB* for GCs and *GAPDH* for TCs were selected as reference genes using NormFinder (85) because the expression of *ACTB* and *GAPDH* was the most stable among the samples compared to other genes. For the quantification of mRNA expression levels, PCR was performed using the AriaMx Real-Time PCR System (Agilent Technologies). The amplification was conducted with an initial hot start at 95ºC for 30 sec, followed by 45 cycles of denaturation at 95ºC for 10 sec, annealing at the temperature indicated

in Table 1 for 10 sec, and extension at 72ºC for 15 sec, followed by dissociation program (95°C for 1 min, temperature indicated in Table 1 to 95ºC at 0.5°C intervals, 5 sec per interval). Serial dilutions (20–20,000,000 copies) of each PCR product extracted from the agarose gel were used as standards to analyze the respective mRNA expression levels.

Enzyme immunoassay (EIA)

The concentrations of A4, P4, and T in the culture medium were determined using enzyme immunoassays, as described previously (86). The A4 standard curve ranged from 3.9062 to 4000 ng/ml. The intra- and inter-assay coefficients of variation of the TCs of small and medium follicles were, on average 5.81% and 11.52%, 5.14% and 15.01% respectively. The P4 standard curve ranged from 0.0078125 to 2 ng/ml. The intra- and inter-assay coefficients of variation of the small and medium follicles TCs averaged were 4.22% and 12.57%, 3.53%, and 10.64% respectively. The T standard curve ranged from 0.625 to 12.8 ng/ml. The intra- and inter-assay coefficients of variation of TCs of small and medium follicles averaged were 4.16% and 16.34%, 7.8%, and 14.32% respectively. To fit the range of standard concentrations, the culture media was diluted 10–40 times in assay buffer.

DNA assay

The DNA content of the cultured TCs was measured as previously described (87). Briefly, after disrupting the cells with an ultrasonic homogenizer, cell lysates were incubated with Hoechst 33258 (Sigma-Aldrich). After 10 min of incubation, the fluorescence of each sample and standard was measured using a microplate fluorometer (Fluoroskan Ascent, Labsystems, Waltham, USA). The standard curve ranged from 0.625 to 30 μg/ml.

Experiment.1: The *ESR2* mRNA expression in uncultured granulosa cells

The three largest healthy follicles (i.e. well vascularization and having a transparent follicular wall with fluid) were dissected from paired ovaries in a dish placed on ice and classified into three size categories according to their diameter (small, <6 mm; medium 7–8 mm and large, ≥9 mm) on days 1-4 post ovulation. GCs were obtained by hemisecting follicles at room temperature in a dish placed on ice. GCs were collected from each follicle after centrifugation, immediately mixed with 400 µl of RNAiso plus (Takara Bio Inc), and stored at -80°C until the extraction of total RNA for quantitative RT-PCR.

Experiment 2: Effects of IGF-1 on the mRNA expression of *ESR2*, *FSHR* and *CYP19A1* in cultured granulosa cells

Cultured GCs of small and medium follicles were seeded at a density of 1.0 X 10^5 viable cells in 1 ml phenol red-free DMEM/F-12 to each well of 24-well plates (Greiner Bio-One). The cells were incubated at 38.5°C, and 2 days after seeding, they were incubated with medium (v/v) containing 0.1% BSA, 5 µg/ml holotransferrin (Sigma-Aldrich), and 2 µg/ml insulin (Sigma- Aldrich) and treated with various concentrations (10, 50, and 100 ng/ml) of IGF-1 (Bio Vision, Waltham, MA, USA) for 24 h. In our study, the concentrations of IGF-1 were selected based on previous studies (60, 72). In each group, the cells were mixed with 400 µl of RNAiso plus in a 1.5 ml tube and stored at -80°C for subsequent RNA extraction.

Experiment 3: Effects of IGF-1 on the mRNA expression of *LHCGR*, *STAR*, *CYP11A1*, *HSD17B1*, *CYP17A1* and *HSD3B* in cultured theca cells

The culture condition for experiment 3 was the same as for experiment 2, but in this experiment, the cultured TCs were treated with various concentrations (10, 50, and 100 ng/ml) of IGF-1 for 24 h. In each group, the cells were mixed with 400 µl of RNAiso plus in a 1.5 ml tube and stored at -80°C for subsequent RNA extraction.

Experiment 4: Effects of IGF-1 on steroid hormones (A4, P4 and T) production by cultured bovine theca cells

To determine the effects of IGF-1 on A4, P4, and T production, cultured TCs from small and medium follicles were seeded at a density of 0.5×10^5 viable cells in 250 µl phenol red-free DMEM/F-12 in 48 well plates (250 µl/well; Greiner Bio-One). The cells were incubated at 38.5°C, and 2 days after seeding, they were incubated with medium (v/v) containing 0.1% BSA, 5 µg/ml holotransferrin (Sigma-Aldrich), and 2 µg/ml insulin (Sigma- Aldrich) and treated with different concentrations (10, 50, and 100 ng/ml) of IGF-1 for 24 h. After incubation, the medium was collected in a 1.5 ml tube, the concentrations of A4, P4, and T were measured by enzyme immunoassay, and the DNA content was measured to standardize the androgen and P4 concentrations.

Purity of granulosa and theca cells

Isolated GCs and TCs were separately cultured at 1.0 x 10^5 viable cells per 1 ml in phenol red-free DMEM/F-12 culture medium containing 10% FBS in 6 well plates and cultured under 95% air and 5% CO_2 at 38.5°C for 2 days. The GCs and TCs were checked under the microscope (FSX100, Olympus, Tokyo, Japan) to determine the purity of isolated cells. TCs were thin, long, and spindle-shaped while GCs were thick, flat-shaped, and smaller than TCs. The purity of isolated GCs and TCs were 95% and 90%, respectively (Fig. 1).

STATISTICAL ANALYSIS

All experimental data are shown as the mean ± SEM and normality and homoscedasticity were confirmed using the Shapiro-Wilk test and Brown-Forsythe test using R (88) respectively and analyzed by ANOVA followed by Tukey's multiple comparison tests using R. The statistical level of significance was considered at $P < 0.05$.

RESULTS

Experiment 1. *ESR2* mRNA expression in uncultured granulosa cells

The three largest healthy follicles were dissected from each pair of ovaries (Days 1–4 post-ovulation). The *ESR2* mRNA expressions in GCs from small (<6 mm), medium (7–8 mm), and large (≥9 mm) follicles were shown in Fig. 2. The *ESR2* mRNA expression in the GCs was low in large follicles (≥9 mm), moderately higher in medium follicles (7–8 mm) and highest in small follicles (<6 mm) ($p<0.05$). The *ESR2* mRNA expression*s* were higher in small follicles than in medium and large-sized follicles.

Experiment 2. Effects of IGF-1 on the expression of mRNA of *ESR2*, *FSHR*, and *CYP19A1* in cultured granulosa cells

GCs were treated with different doses of IGF-1 for 24 h. Results showed that the mRNA expression of *ESR2* (Fig. 3), *FSHR* (Fig. 4), and *CYP19A1*(Fig. 5) significantly increased ($p<0.05$) at a dose of 100 ng/ml of IGF-1 in cultured GCs of small follicles. However, the other doses did not have any effect on the mRNA expression of *ESR2, FSHR,* and *CYP19A1* in GCs of small follicles. In medium follicles, IGF-1 (10–100 ng/ml) increased the *ESR2* mRNA expression, but only IGF-1 (100 ng/ml) significantly ($p<0.05$) increased the expression of *CYP19A1* mRNA. The *FSHR* mRNA expression in cultured GCs of medium follicles was not affected by IGF-1 treatment for 24 h.

Experiment 3: Effects of IGF-1 on the mRNA expression of ***LHCGR***, ***STAR***, ***CYP11A1***, ***CYP17A1***, ***HSD17B1*** and ***HSD3B*** in cultured theca cells

The cultured TCs were treated with selected doses of IGF-1 for 24 h. IGF-1 (100 and 50-100 ng/ml) increased significantly ($p<0.05$) the mRNA expression of *LHCGR* and *HSD17B* in small and medium follicles, respectively. IGF-1 (100 ng/ml) increased the expression of *STAR* mRNA in TCs of small follicles while IGF-1 (10–100 ng/ml) enhanced *STAR* mRNA expression in TCs of medium follicles. IGF-1 (50 and 100 ng/ml) significantly raised ($p<0.05$) the *CYP11A1* mRNA expression in the TCs of small and medium follicles, respectively. IGF-1 (10-100 ng/ml) increased the *CYP17A1* mRNA in TCs of small follicles, while IGF-1 (50-100 ng/ml) significantly increased the *CYP17A1* mRNA in TCs of medium follicles. IGF-1 (100, 50-100 ng/ml) significantly enhanced the *HSD3B* and *HSD17B1* mRNA in TCs of small and medium follicles, respectively (Fig. 6).

Experiment 4. Effects of IGF-1 on androstenedione, progesterone, and testosterone production in cultured bovine theca cells.

The cultured TCs were treated with different doses of IGF-1 (10, 50, and 100 ng/ml) for 24 h. IGF-1 (50 ng/ml) increased A4 production in TCs of small follicles, while IGF-1 (10-50 ng/ml) significantly enhanced ($p<0.05$) the production of A4 in TCs of medium follicles (Fig. 7A). Furthermore, IGF-1 significantly enhanced P4 production in TCs (50-100 ng/ml in small follicles, 100 ng/ml in medium follicles, respectively) (Fig. 7B). However, there was no effect of IGF-1 on the production of T in TCs of either small or medium follicles (Fig. 7C).

Table 1. Sequences of primers used for quantitative RT-PCR

Gene	Forward and reverse primers	Accession no.	Product size (bp)	Temperature (°C)
ESR2	5'-GCCAGCCGTCAGTTCTGTAT-3' 5'-CAACTGCTCCCACTAGCCTT-3'	NM_174051	121	58.5
FSHR	5'-TGCAGTCGAACTGAGGTTTG-3' 5’-ATGTAGTTTGGGCAGGTTGG-3'	NM_174061.1	154	60
CYP19A1	5’-CAACAGCAGAGAAGCTGGAAGACA-3’ 5’-CACCAACAACAGTCTGGATTTCCCT-3'	NM_174305.1	225	60
ACTB	5'GAGAAGAGCTACGAGCTTCCTGACG-3' 5'-AGGATTCCATGCCCAGGAAGGAAGG-3'	NM_173979.3	106	60
LHCGR	5'-GATAGAAGCTAATGCCTTTGACAAC-3’ 5'-CCAGAATGAAATTAAATTCAGAGGAG-3'	NM_174381.1	197	60
STAR	5’-CAACAGCAGAGAAGCTGGAAGACA-3’ 5’-CACCAACAACAGTCTGGATTTCCCT-3’	NM_174189.3	133	60
HSD17B1	5'-TAAACCTGGTCACCGACTGC-3’ 5'-CTGCCAGGGATGTAGGCAAA-3'	NM_001035395.2	126	57
CYP11A1	5’-TAAGCAAGATGCTGCAAATG-3’ 5’-CTTGGCAGGAATCAGGTAAT-3'	NM_176644.2	137	59
GAPDH	5'-CACCCTCAAGATTGTCAGCA-3’ 5'-GGTCATAAGTCCCTCCACGA-3'	NM_001034034.2	103	60

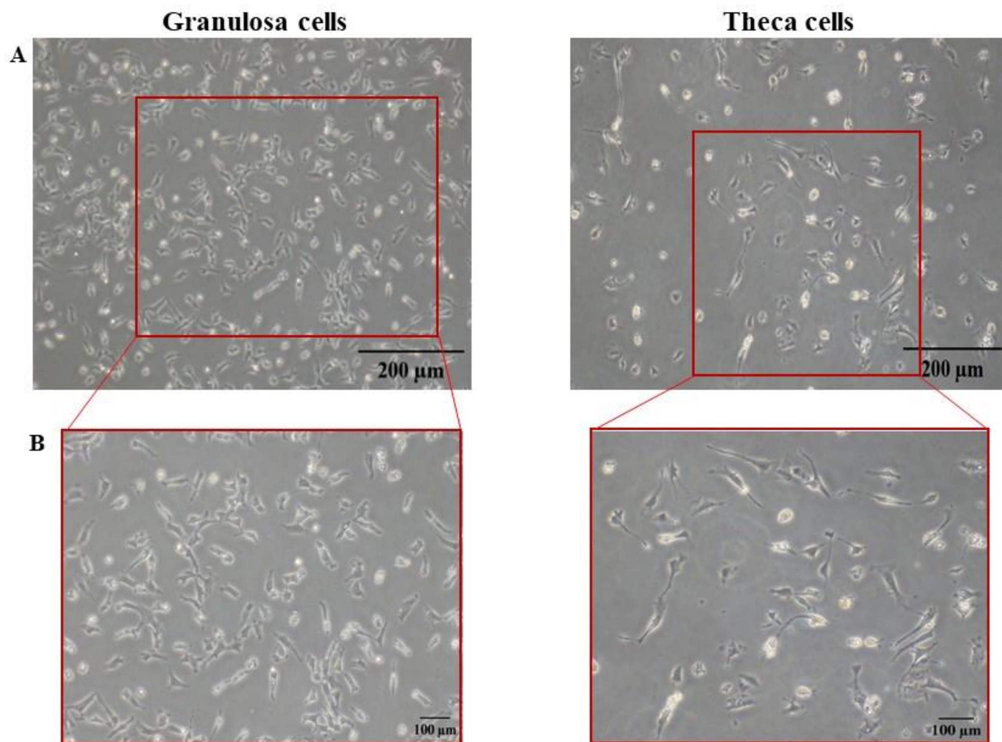


Fig. 1. Isolated bovine granulosa (GCs, A) and theca cells (TCs, B) were separately cultured at 1.0 x 10^5 viable cells per 1 ml in phenol red-free DMEM/F-12 culture medium containing 10% FBS for 2 days and checked under the microscope. TCs were thin, long, and spindle-shaped while GCs were thick, flat-shaped, and smaller than TCs.

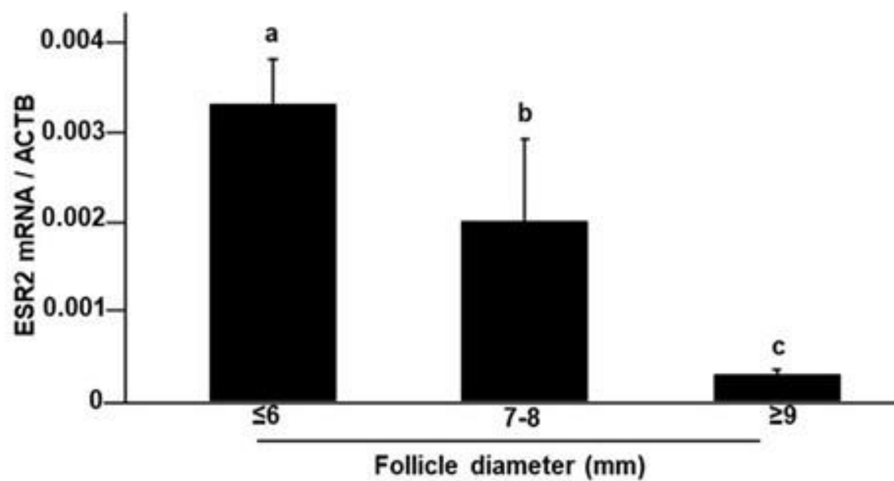


Fig. 2 Gene expression of E2 receptor β (*ESR2*) mRNA relative to the amount of *β-actin* (*ACTB*) in isolated GCs from the three largest follicles in each pair ovary between days 1 and 4 post-ovulation (follicles/group). The expression values are presented as means ± SEM. The follicles were grouped by range as follows: small (<6mm), medium-sized (7-8 mm), and large (≥9 mm) in diameter. Different superscript letters (a-c) indicate significant differences ($p < 0.05$; ANOVA) among groups followed by Tukey's multiple comparison test.

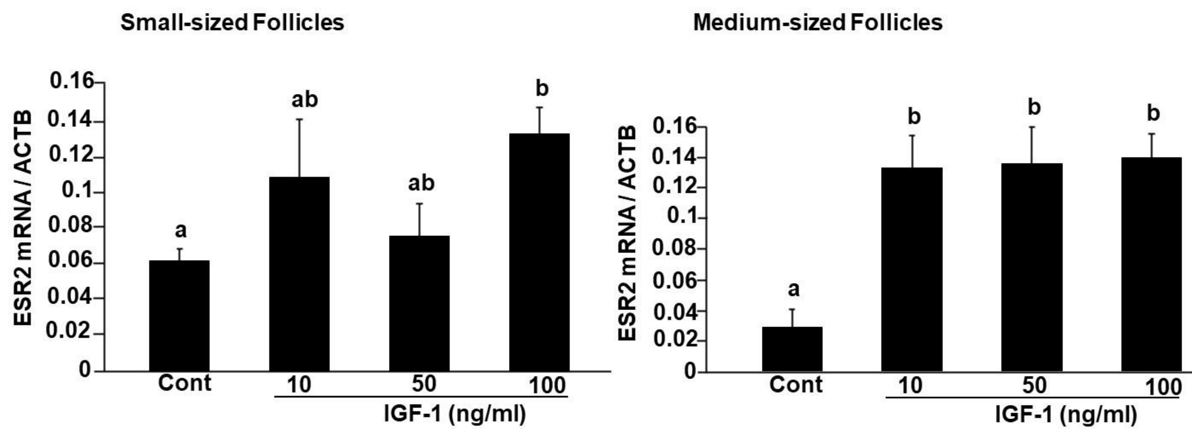


Fig. 3. Effects of IGF-1 on mRNA expression of *ESR2* in GCs. The GCs from small- and medium-sized follicles were exposed to various concentrations of IGF-1 (10, 50, and 100 ng/ml) for 24 h. The genes were quantified relative to *β-actin* (*ACTB*) in cultured GCs from small- and medium-sized follicles. The expression values are presented as means ± SEM of five replicates. Different superscript letters indicate statistically significant differences ($p < 0.05$; ANOVA) as determined by Tukey's multiple comparison test.

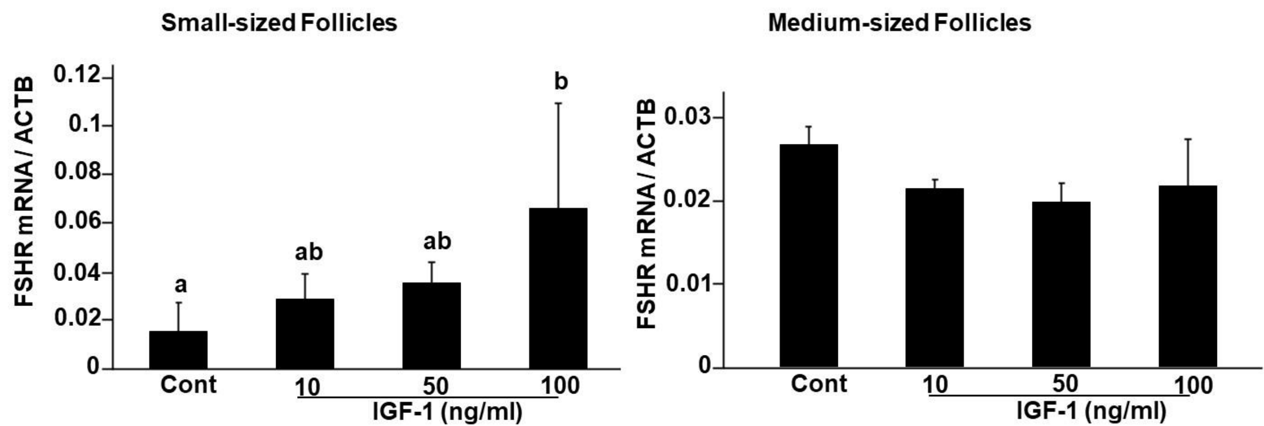


Fig. 4. Effects of IGF-1 on mRNA expression of *FSHR* in GCs. The GCs from small- and medium-sized follicles were exposed to various concentrations of IGF-1 (10, 50, and 100 ng/ml) for 24 h. The genes were quantified relative to *β-actin* (*ACTB*) in cultured GCs from small- and medium-sized follicles. The expression values are presented as means ± SEM of five replicates. Different superscript letters indicate statistically significant differences ($p < 0.05$; ANOVA) as determined by Tukey's multiple comparison test.

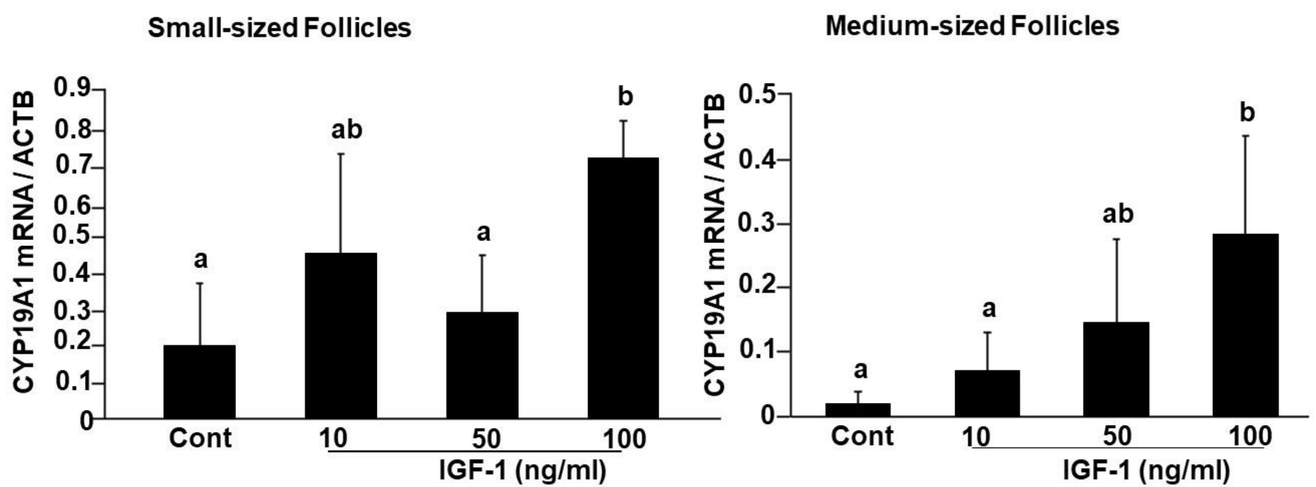


Fig. 5. Effects of IGF-1 on mRNA expression of *CYP19A1* in GCs. The GCs from small- and medium-sized follicles were exposed to various concentrations of IGF-1 (10, 50, and 100 ng/ml) for 24 h. The genes were quantified relative to *β-actin* (*ACTB*) in cultured GCs from small- and medium-sized follicles. The expression values are presented as means ± SEM of five replicates. Different superscript letters indicate statistically significant differences ($p < 0.05$; ANOVA) as determined by Tukey's multiple comparison test.

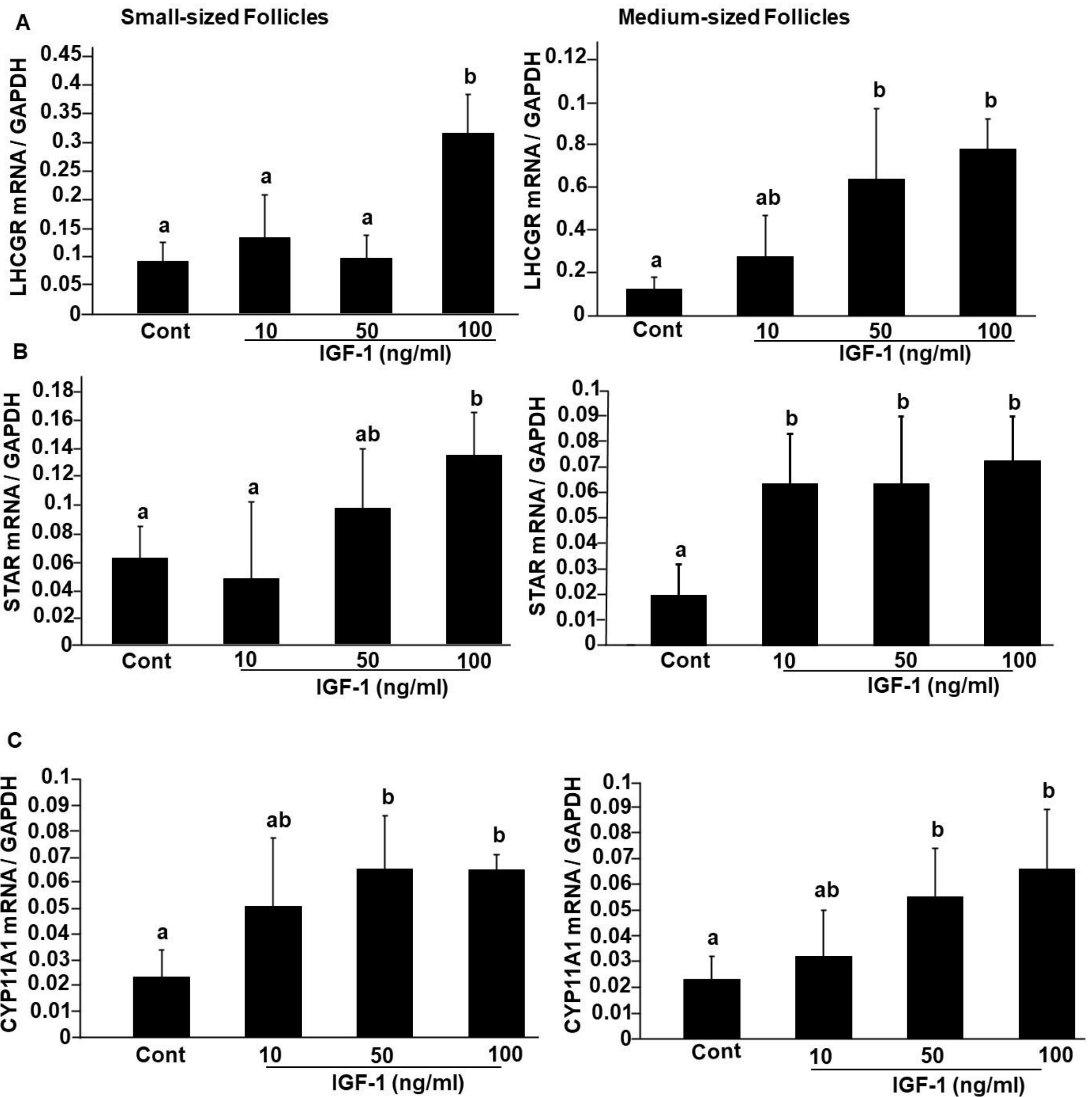

A
Small-sized Follicles
Medium-sized Follicles
LHCGR mRNA / GAPDH
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
a
a
b
Cont
10
50
100
IGF-1 (ng/ml)
0.12
0.1
0.8
0.6
0.4
0.2
0
a
ab
b
b
B
STAR mRNA / GAPDH
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
a
a
ab
b
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
C
CYP11A1 mRNA / GAPDH

Fig. 6. Effects of IGF-1 on mRNA expression of *LHCGR* (A), *STAR* (B) and *CYP11A1* (C) in TCs. The TCs from small- and medium-sized follicles were exposed to various concentrations of IGF-1 (10, 50, and 100 ng/ml) for 24 h. The genes were quantified relative to *GAPDH* in cultured TCs from small- and medium-sized follicles. The expression values are presented as the means ± SEM of five replicates. Different superscript letters indicate statistically significant differences ($p < 0.05$; ANOVA) as determined by Tukey's multiple comparison test.

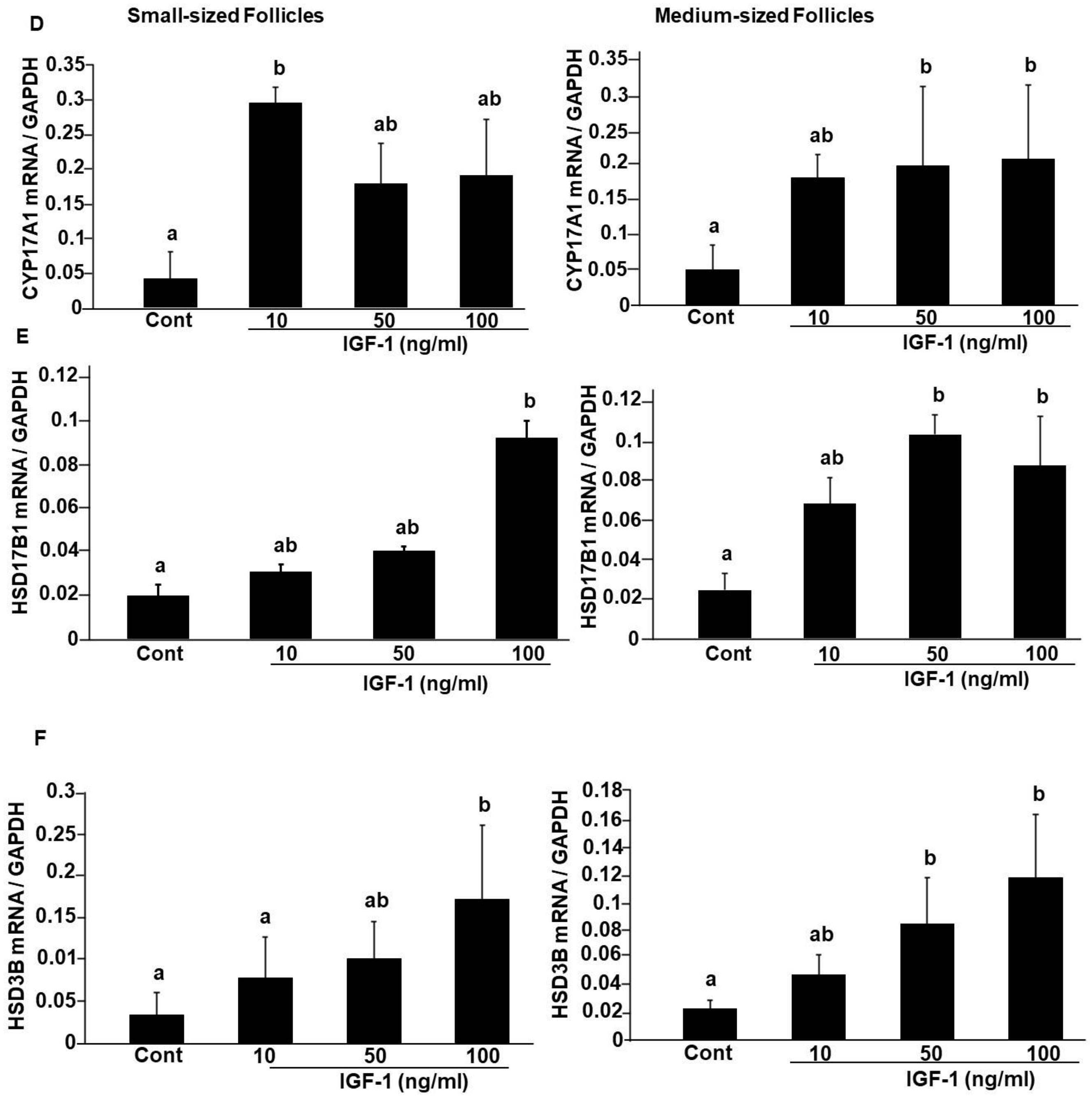
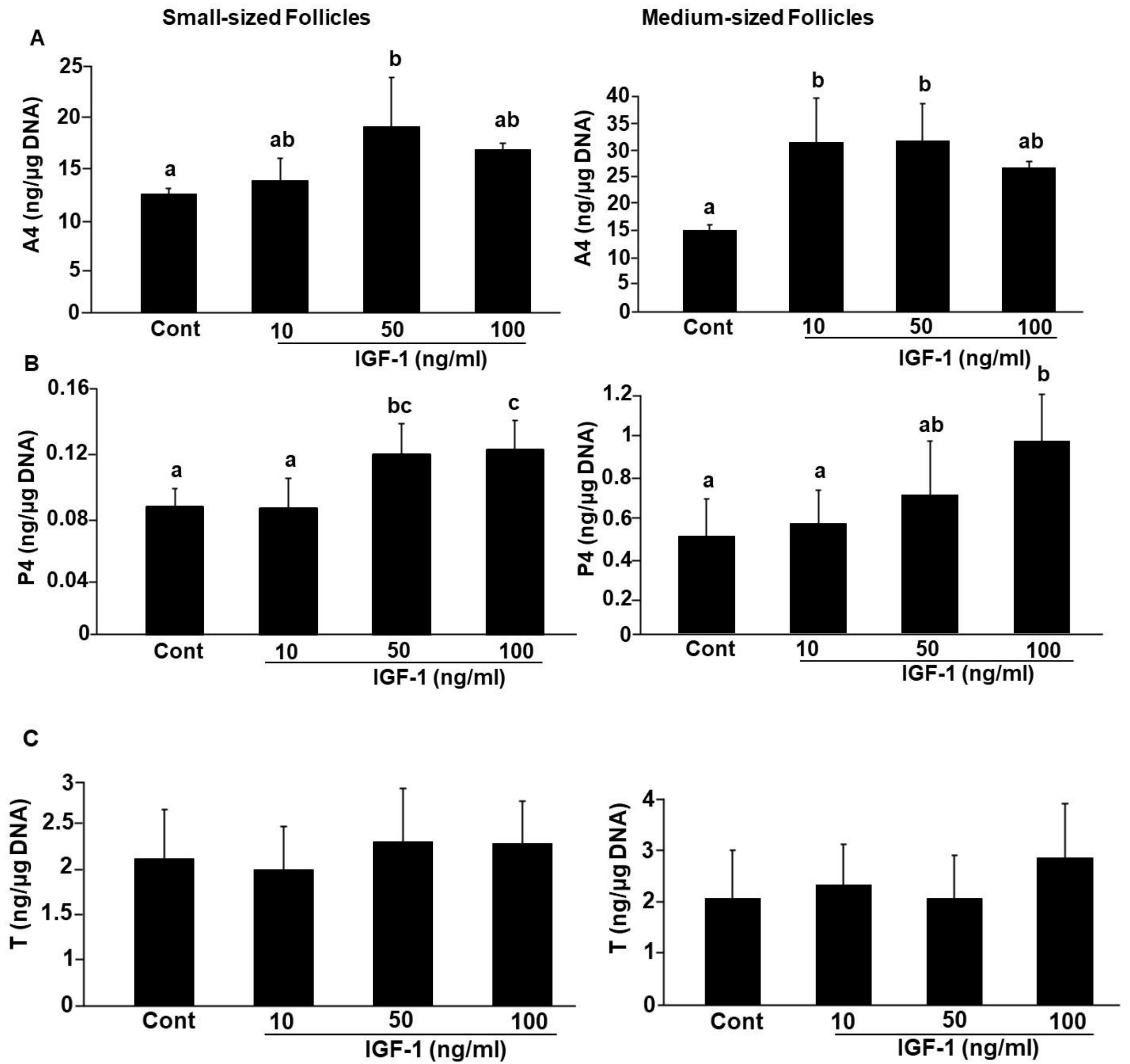

D
Small-sized Follicles
Medium-sized Follicles
CYP17A1 mRNA / GAPDH
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
b
ab
ab
Cont
10
50
100
IGF-1 (ng/ml)
a
ab
b
b
E
HSD17B1 mRNA / GAPDH
0.12
0.1
0.08
0.06
0.04
0.02
a
ab
ab
b
F
HSD3B mRNA / GAPDH
0.3
0.25
0.2
0.15
0.01
0.05
0.18
0.16
0.14
0.12

Fig. 6. Effects of IGF-1 on mRNA expression of *CYP17A1* (D), *HSD17B1* (E), and *HSD3B* (F) in TCs. The TCs from small- and medium-sized follicles were exposed to various concentrations of IGF-1 (10, 50, and 100 ng/ml) for 24 h. The genes were quantified relative to *GAPDH* in cultured TCs from small- and medium-sized follicles. The expression values are presented as the means ± SEM of five replicates. Different superscript letters indicate statistically significant differences ($p < 0.05$; ANOVA) as determined by Tukey's multiple comparison test.



Small-sized Follicles
Medium-sized Follicles
A
A4 (ng/µg DNA)
a
ab
b
ab
a
b
b
ab
Cont
10
50
100
IGF-1 (ng/ml)
B
P4 (ng/µg DNA)
a
a
bc
c
a
a
ab
b
C
T (ng/µg DNA)

Fig. 7. Androstenedione (A), Progesterone (B), and Testosterone (C) production by theca cells of small- and medium-sized follicles. Theca cells were cultured with various concentrations of IGF-1 (10, 50, and 100 ng/mL) for 24 h. All values are presented as the means ± SEM of five replicates. Different superscript letters indicate significant differences ($p < 0.05$; ANOVA), followed by Tukey's multiple comparison test.

DISCUSSION

The intraovarian actions of *ESR2* are vital to fully differentiated follicles and are necessary for the enzymatic activity to convert androgen precursors to E2 (89), which is crucial for follicular growth and deviation. The *ESR2* mRNA receptor mediates E2 actions in GCs (63,69,70). In the present study, the expression of *ESR2* mRNA in GCs decreased with follicular size. This result agrees with previous studies that the mRNA *ESR2* decreased in bovine GCs of large follicles (50, 90, 48). These results suggest that higher *ESR2* mRNA expression in GCs of small and medium follicles is crucial for their growth and deviation. In a previous study, IGF-1 increased E2 production in cultured GCs of small follicles (60), which may require greater *ESR2* mRNA expression to maintain its bioactivity. The effect of IGF-1 on *ESR2* mRNA expression in GCs has not been thoroughly investigated in cattle previously. Therefore, we examined the effect of IGF-1 on *ESR2* mRNA expression in bovine GCs. Our results showed that IGF-1(100 ng/ml) increased *ESR2* mRNA expression in the GCs of small follicles, whereas low levels of IGF-1 (10-100 ng/ml) increased *ESR2* mRNA expression in GCs of medium follicles. These results indicate that (i) the effect of IGF-1 on *ESR2* mRNA in the GCs is dose-dependent, (ii) the stimulatory effect of IGF-1 on *ESR2* mRNA supports our hypothesis that IGF-1 increases *ESR2* mRNA expression in GCs of small to maintain E2 bioactivity for follicular growth and deviation, and (iii) the effects of IGF-1 may depend on the presence of *IGF1R* in GCs, requiring further investigation into IGF-1 effects on mRNA expression of *IGF1R* in GCs of different stages of follicles.

The steroid hormone synthesis is controlled by *ESR2* (89), *FSHR* (91), and *CYP19A1* (72) in the GCs of cattle. IGF-1 plays a central role in these interactions with respect to steroidogenesis (92, 56). In the present study, we investigated the effects of IGF-1 on gene expression that regulate E2 synthesis in GCs. Our previous study showed that IGF-1 (1-100

ng/ml) significantly increased E2 production in GCs of small follicles *in vitro* (60). In this study, IGF-1 (100 ng/ml) increased *CYP19A1* and *FSHR* mRNA expressions and even a low dose of IGF-1 (10 ng/ml) stimulated *CYP19A1* mRNA expression in GCs of small follicles, which converts A4 to E2 in GCs. IGF-1 (100 ng/ml) increased *CYP19A1* mRNA expression, but IGF-1 did not affect *FSHR* mRNA expression in GCs of medium follicles. In previous studies, IGF-1(10 ng/ml) increased the mRNA *FSHR* in mice (74) and IGF-1 (50 ng/ml) enhanced *FSHR* and *CYP19A1* mRNA expressions in bovine GCs (2-8 mm) treated for 48 h (72, 73). The species or culture protocol can influence the expression of mRNA of genes. These results clearly stated that IGF-1 significantly enhanced the mRNA of *CYP19A1* and *FSHR* in GCs of small follicles which regulate E2 synthesis that plays a vital role in follicular development.

Androgen is synthesized via steroidogenesis-related enzymes in TCs which are the precursors for E2 (93-94). The binding of LH to its receptor on TCs stimulates the activities of the steroidogenic enzymes necessary for A4 synthesis (19). In our study, IGF-1 significantly increased the expression of *LHCGR* mRNA in TCs in both sized follicles, consistent with previous reports in cultured follicles of mice (74) and in bovine GCs (60). Our study also demonstrated the effects of IGF-1 on three genes regulating A4 synthesis in TCs, namely *STAR, CYP11A1,* and *HSD17B* (13, 95-97, 71). IGF-1 (100 ng/ml) increased the mRNA of *STAR, CYP11A1,* and *HSD17B* in TCs of small follicles, whereas lower doses of IGF-1 enhanced these enzymes in TCs of medium follicles, comparable with previous studies that IGF-1 enhanced mRNA of *STAR* in cultured follicles of mice (74) and *CYP11A1* mRNA in GCs of porcine 98). IGF-1 had little effect on *CYP11A1* mRNA in bovine-cultured TCs in the presence of LH (72). Interestingly, in the present study, IGF-1 alone increased the expression of mRNA of *CYP11A1* in TCs. The conversion of pregnenolone to dehydroepiandrosterone (DHEA) is regulated by *CYP17A1*, and DHEA is subsequently converted to A4 by *HSD17B* and *HSD3B* (68). Previous studies have reported a significant increase in the expression of *CYP17A1* mRNA in bovine

theca cells (TCs) cultured with IGF-1 supplemented medium for 24 hours (99). Additionally, *CYP17A1* mRNA in bovine small-sized follicle TCs was found to increase in response to IGF-1 and LH in vitro (100). However, IGF-I alone has no effect on *CYP17A1* mRNA in cultured rat TCs (100). IGF-1 has been shown to increase *HSD3B* mRNA in bovine granulosa cells (GCs) (74) and in TCs of rats (91). In the present study, IGF-1 increased *CYP17A1* and *HSD3B* mRNA in cultured TCs of both small and medium-sized follicles. The differences in results may indicate that the species or experimental treatment protocol can influence the expression of *CYP17A1* mRNA in TCs. These results indicate that IGF-1 enhances gene expression involved in A4 biosynthesis.

In fact, IGF-1 (50 ng/ml) and IGF-1 (10-100 ng/ml) increased A4 production in TCs of small and medium follicles respectively. This study provides more information that IGF-1 enhances A4 production in TCs of small follicles, which play a key role in follicular growth, and indicates that enhancing A4 production and related gene expression in TCs of small and medium follicles are IGF-1 dose-dependent. Pregnenolone is converted to P4 via *HSD3B* in TCs (68). *In vivo*, no differential changes in P4 concentration were observed in the two largest follicles before the beginning of deviation in heifers (18, 101). IGF-1 increased P4 production in bovine TCs from large follicles (101, 58). In our study, IGF-1 enhanced the P4 production in TCs of small and medium follicles. However, P4 is not crucial for follicular growth but may serve as a great precursor for A4 synthesis in TCs. IGF-1 did not increase T production in cultured TCs from either small or medium follicles. These results show that IGF-1 has the capability to increase the expression of steroidogenic enzymes and A4 production in TCs of small follicles which is a great source for E2 synthesis in GCs that play a crucial role in follicular growth and deviation (Fig. 8).

In conclusion, IGF-1 usually affects pre-antral or early antral follicles. This study shows that IGF-1 is involved in some gene expressions and hormone production which play vital roles

in various aspects of follicular growth of small and medium antral follicles. This study shows that the effects of IGF-1 in small and medium follicles were in a dose-dependent manner. The high dose of IGF-1 (100 ng/ml) was more effective in enhancing the targeted variables in small follicles whereas the lower doses were in medium follicles. Indeed, these results supported our hypothesis that IGF-1 increased the expression of *ESR2* mRNA in GCs and A4 production in TCs of small follicles which enhance follicular growth and is required for follicular deviation.

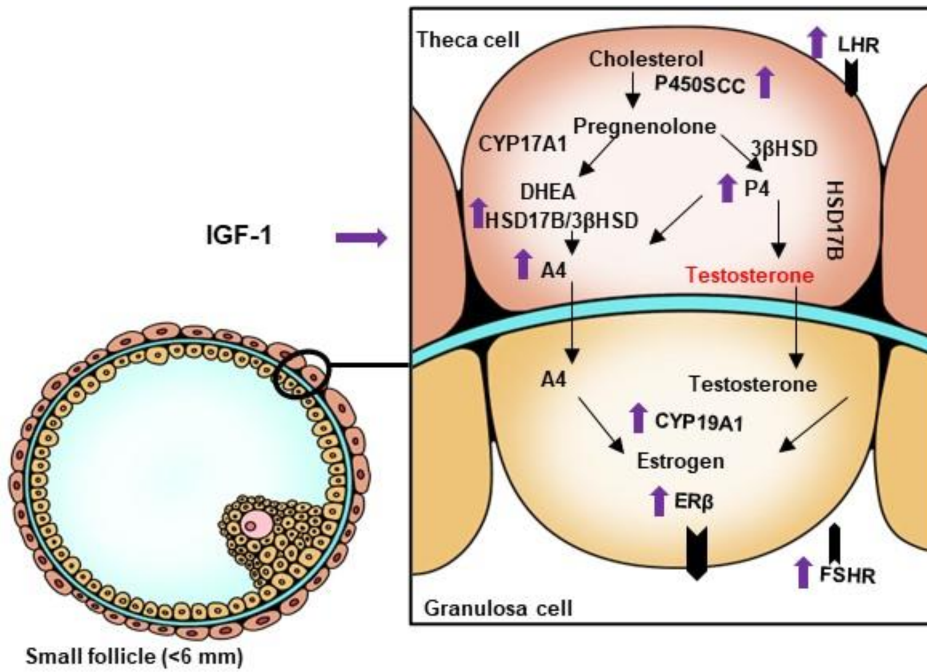


Fig. 8. Illustration describing our conclusion. IGF-1 increased A4 production via enhancing gene expression of steroidogenic enzymes and *LHCGR* in TCs and promoted mRNA of *ESR2* in GCs of small follicles, indicating that IGF-1 is the best choice for enhancing factors involved in bovine follicular deviation and selection.

SUMMARY

Insulin-like growth factor-1 (IGF-1) plays a crucial role in follicular growth and stimulates steroid hormone production in bovine follicles. Steroid hormones are synthesized by the actions of steroidogenic enzymes, namely *STAR, CYP11A1, HSD3B*, *CYP17A1*, *HSD17B1* and *CYP19A1* in both theca cells (TCs) and granulosa cells (GCs) under the stimulation of gonadotropins. Especially, estradiol 17β (E2) plays a key role in follicular development and selection via estrogen receptors β (*ESR2*) in GCs. We examined the mRNA expression of *ESR2* in GCs of developing follicles and the effect of IGF-1 on the mRNA expression of *ESR2, CYP19A1*, *FSHR* and on *LHCGR*, *STAR*, *CYP11A1*, *HSD17B*, *HSD3B*, *CYP17A1* in cultured GCs and TCs, respectively. We also checked the effect of IGF-1 on androstenedione (A4), progesterone (P4) and testosterone (T) production in TCs. Small follicles (<6 mm) expressed the highest levels of *ESR2* mRNA and medium (7-8 mm) expressed higher than large follicles (≥9 mm) ($p < 0.05$). IGF-1 increased the mRNA expression of *ESR2, CYP19A1*, and *FSHR* in GCs of both sizes of follicles, except for *FSHR* mRNA in medium follicles ($p < 0.05$). IGF-1 significantly increased mRNA expression of *LHCGR, STAR, CYP11A1, CYP17A1, HSD3B* and *HSD17B* in TCs of small and medium follicles ($p < 0.05$). Moreover, IGF-1 increased A4 and P4 production but did not affect the T production in TCs of small and medium follicles. Taken together, IGF-1 up-regulates the steroidogenic enzymes and steroid hormone production suggesting that IGF-1 is crucial for follicle development and selection.

CHAPTER 3

Effects of Insulin-like Growth Factor-1 on Growth Regulatory Factors of Granulosa Cells in Bovine Small Follicles

INTRODUCTION

In follicular development, the endocrine, paracrine, and autocrine factors are involved, which control several processes including the proliferation and differentiation of the follicular cells (102). It has been shown that IGF-1 is important for antral formation, where it is involved in follicle growth, stimulation of somatic cell proliferation, and steroidogenesis (56, 103). In *in vitro* culture of preantral follicles, IGF-1 stimulated the follicular growth in bovines (104). The essential role of IGF-I was confirmed in experiments using IGF-I knockout mice, whose follicles were arrested at the early antral stage and failed to respond to gonadotrophins (66).

In cattle, the mRNA of IGF-1 receptors (*IGF1R*) is expressed in granulosa cells (GCs) throughout follicle development (105, 106) playing roles as a mitosis inducer (107) and apoptosis suppressor (108), and for the formation of the preovulatory follicle (19). However, the regulation of the bioactivity of IGF-1 is still under concern. It has been reported that the bioactivity of IGF-1 is controlled by its association with at least six IGF-binding proteins (110). In contrast, another study revealed that IGF-1 mediates their biological actions via *IGF1R* (105). The unbound or the free fraction of IGF-1 may be the dominant biologically active form (110). As follicles undergo growth, there is a reduction in the level of IGF binding protein- 2 (IGFBP-2), and more IGF-1 would be available for binding with the *IGF1R* (111). These findings show that the bioactivities of IGF-1 may be mediated via *IGF1R* mRNA in the GCs of growing follicles. However, the effects of IGF-1 on mRNA expression of *IGF1R* in GCs remain unknown. To assess the effect of IGF-1 on the expression of *IGF1R* mRNA, we hypothesized that IGF-1 promotes the expression of *IGF1R* mRNA in GCs of small follicles.

The initiation of follicular growth depends on sensitive markers such as proliferating cell nuclear antigen (*PCNA*), a protein expressed by actively growing and proliferating cells (112) which has been suggested to play a key role in ovarian follicle development (113). The follicle growth was initiated *in vitro*, and its initiation was further confirmed through immunolocalization of *PCNA* in cultured and freshly isolated pieces of the ovarian cortex (114). IGF-1 is one of the growth factors in bovine that stimulates both follicular growth (115), and proliferation (116). Despite their evident significance, the specific role of IGF-1 on the expression of *PCNA* mRNA in GCs remains unknown. Therefore, we investigated the effects of IGF-1 on *PCNA* mRNA expression and on GC number in small follicles.

The steroidogenic potential of GCs can be regulated with the interaction of locally produced steroidogenic enzymes which are crucial for follicular growth and selection. IGF-1 plays a central role in these interactions with respect to steroidogenesis (92, 56). The previous studies, using diverse culture systems with different types of follicular cells from various follicular diameters of bovine ovaries, have demonstrated that IGF-1 enhances the mRNA expression of steroid-related enzymes (117, 73, 92). In the present study, we also examined the effect of IGF-1 on *STAR*, *CYP11A1*, *CYP17A1*, *HSD3B,* and *HSD17B1* mRNA expression in cultured GCs of small follicles.

MATERIALS AND METHODS

Collection of bovine ovaries

Freshly excised ovaries of healthy cows were collected from a local abattoir to obtain GCs from small (<6 mm) diameter follicles, as previously described (80). For unculture, GCs were collected from each pair of ovaries between days 1-4 post-ovulation (81).

Granulosa cells isolation and culture

The GCs and follicular fluid were collected aseptically from small follicles using a 2.5 ml disposable syringe with a 24-gauge needle and transferred to a plastic Petri dish filled with phenol red-free DMEM/Ham's F-12, 1:1 (v/v); (Invitrogen) containing 10% calf serum, 20 μg/ml amphotericin B (Sigma Aldrich), and 50 IU heparin sodium salt (Nacalai Tesque). The further procedure for isolation GCs was identical to the one explained in Chapter Two's methods and materials section. Dispersed GCs in four replicate wells were preincubated with and without IGF-1 by culturing for 24 h in a serum-free medium in the absence (control) and in the presence of various concentrations of IGF-1.

Total RNA extraction and quantitative RT-PCR

Total RNA was extracted from cultured and fresh GCs using RNAiso plus (Takara Bio Inc) according to the manufacturer's instructions. The extracted RNA from each sample was quantified and 2 μg of each total RNA was reverse transcribed using ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo).

The mRNA expression of *IGF1R*, *PCNA*, *STAR*, *CYP11A1*, *CYP17A1*, *HSD3B,* and *HSD17B1* mRNA, Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and β-actin (*ACTB*) were determined according to a previously described method (83). The expression of each mRNA was quantified using Brilliant III Ultra-Fast SYBER Green qPCR Master Mix with Low ROX (Agilent Technologies), starting from 2 ng of reverse-transcribed total RNA. To standardize the relative level of genes in uncultured GCs, *GAPDH* was used as an internal control, and to standardize the relative levels of genes in cultured GCs, *ACTB* was used as an internal control. The internal control was selected using NormFinder (84). For the quantification of mRNA expression levels, PCR was performed under the following conditions using the AriaMx Real-Time PCR System (Agilent Technologies) The amplification was conducted with an initial hot start at 95ºC for 30 sec, followed by 35 cycles of denaturation at 95ºC for 10 sec, annealing at the temperature indicated in Table 2 for 10 sec, and extension at 72ºC for 15 sec, followed by dissociation program (95°C for 1 min, temperature as indicated in Table 1, to 95ºC at 0.5°C intervals, 5 sec per interval). Serial dilutions (20–20,000,000 copies) of each PCR product extracted from the agarose gel were used as standards to analyze the respective mRNA expression levels. The sequences of primers were listed in Table 2.

Cell proliferation

The DNA content of the GCs was measured as previously described (87). Briefly, after disrupting the cells with an ultrasonic homogenizer, cell lysates were incubated with Hoechst H 33258 (Sigma-Aldrich). After 10 min of incubation, the fluorescence of each sample and standard was measured using a microplate fluorometer (Fluoroskan Ascent). The standard curve ranged from 0.625 to 30 µg/ml.

Experiment.1: The *IGF1R*, *PCNA*, *STAR*, *CYP11A1*, *HSD3B*, *CYP17A1* and *HSD17B1* mRNA expression in uncultured granulosa cells

The three largest healthy follicles were dissected from paired ovaries and classified into three size categories according to their diameter (small, <6 mm; medium-sized, 7–8 mm; and large, ≥9 mm) on days 1-4 post ovulation as described previously (118). GCs were collected from each follicle after centrifugation, immediately mixed with 400 µl of RNAiso plus (Takara Bio Inc) and stored at -80°C until the extraction of total RNA for quantitative RT-PCR.

Experiment 2: Effects of IGF-1 on the mRNA expression of *IGF1R*, *PCNA*, and steroidogenic-related enzymes in cultured granulosa cells

Cultured GCs of small were seeded at a density of 1.0 X 10^5 viable cells in 1 ml phenol red-free DMEM/F-12 to each well of 24-well plates (Greiner Bio-One). The cells were incubated at 38.5°C, and after the cells reached confluence, they were incubated with medium (v/v) containing 0.1% BSA, 5 µg/ml holotransferrin (Sigma-Aldrich), and 2 µg/ml insulin (Sigma- Aldrich) and treated with different concentrations (10, 50, and 100 ng/ml) of IGF-1 (Bio Vision) for 24 h. In each group, the cells were mixed with 400 µl of RNAiso plus in a 1.5 ml tube and stored at -80°C for subsequent RNA extraction.

Experiment 3: Effects of IGF-1 on granulosa cell proliferation

The isolated GCs of small follicles were cultured at 1.0 x 10^5 viable cells per 1 ml in phenol red-free DMEM/F-12 culture medium containing 10% calf serum in 75-cm^2 culture flasks (20 ml/flask) under 95% air and 5% CO_2 at 38.5°C for 24 h. GCs were passaged with

0.1% bovine trypsin (Sigma-Aldrich) and sterile phosphate-buffered saline (PBS). GCs were seeded at a density of 5.0 X 10^3 viable cells in 1 ml phenol red-free DMEM/F-12 to each well of 24-well plates. The cells were incubated at 38.5ºC with medium (v/v) containing 0.1% BSA, 5 µg/ml holotransferrin (Sigma-Aldrich), and 2 µg/ml insulin (Sigma- Aldrich) and treated with (10, 50 and 100 ng/ml) IGF-1 for 0, 12, 24 and 48 h. In each group, the cells were mixed with 100-150 µl of DNA assay buffer in 1.5 ml tube and stored at -30°C for subsequent DNA assay.

STATISTICAL ANALYSIS

All experimental data are shown as the mean ± SEM and normality and homoscedasticity were confirmed using the Shapiro-Wilk test and Brown-Forsythe test using R (88), respectively and analyzed by ANOVA followed by Tukey's multiple comparison tests using R. In Fig. 3, the raw data were subjected to a logarithmic transformation (LOG 10) to ensure parametric properties, the two-way ANOWA test was performed among the two factors at the same time. The statistical level of significance was considered at $P<0.05$.

RESULTS

Experiment 1. The *IGF1R*, *PCNA*, *STAR*, *CYP11A1*, *HSD3B*, *CYP17A1* and *HSD17B1* mRNA expression in uncultured granulosa cells

The three largest healthy follicles (i.e. well vascularization and having a transparent follicular wall with fluid) were dissected from each pair of ovaries (Days 1–4 post-ovulation). The *IGF1R*, *PCNA*, and steroidogenic-related enzymes mRNA expression in GCs from small (<6 mm), medium (7–8 mm), and large (≥9 mm) follicles were investigated. The *IGF1R* and *CYP17A1* mRNA expression in the GCs was low in small follicles, slightly higher in medium follicles, and highest in large follicles (Fig. 1A, B; $p<0.05$). The mRNA expressions of *STAR*, *HSD3B* and *HSD17B1* were significantly highest in medium follicles among these three groups (Fig. 1C, D, E). The *PCNA* and *CYP11A1* mRNA expressions were higher in large follicles compared to the small and medium follicles (Fig. 1F, G). The *IGF1R* mRNA expression was slightly higher in GCs of large follicles than in medium and small follicles (Fig. 1A).

Experiment 2: Effects of IGF-1 on the mRNA expression of *IGF1R*, *PCNA*, and steroidogenic-related enzymes in cultured granulosa cells

GCs were treated for 24 h with various doses of IGF-1. IGF-1 (10-100 ng/mL) significantly increased ($p<0.05$) the mRNA expression of *IGF1R*, whereas the mRNA expression of *PCNA* significantly increased at the dose of 100 ng/ml of IGF-1 (Fig. 2A, B). However, the other doses of IGF-1 did not have any effect on the mRNA expression of *PCNA* in GCs. IGF-1 (50-100 ng/ml) enhanced the mRNA expression of *STAR* and *CYP11A1* while IGF-1 (100 and 10-100 ng/ml) significantly increased the mRNA expression of *HSD3B* and

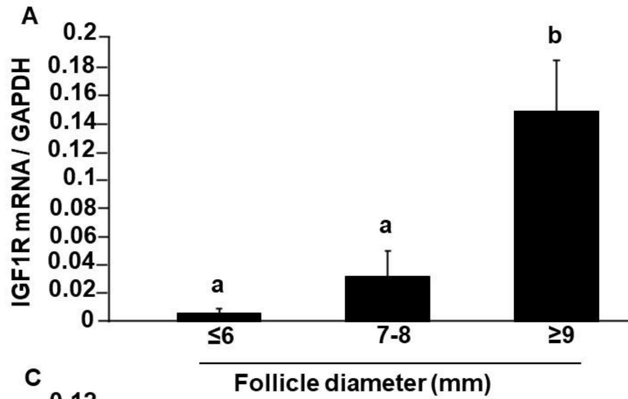
CYP17A1, respectively (Fig. 2C, D, E, F; $p<0.05$). IGF-1 did not affect the mRNA expression of *HSD17B1* in cultured GCs of small follicles for 24 h (Fig. 2G).

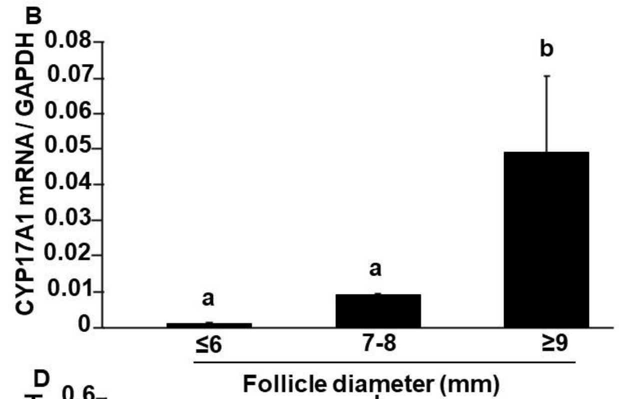
Experiment 3: Effects of IGF-1 on granulosa cell proliferation

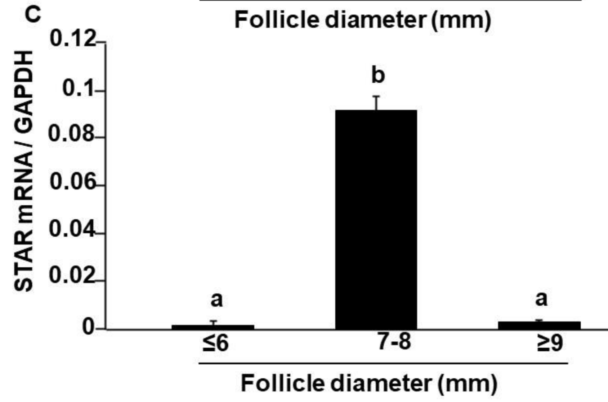
The 24 h cultured GCs were treated with IGF-1(10, 50 and 100 ng/ml) for 0, 12, 24 and 48 h. IGF-1 (50-100 ng/ml) increased significantly ($p<0.05$) the proliferation of GCs at 48h compared to 0 and 12 h of the incubation time base. In contrast, IGF-1 (100 ng/ml) significantly increased the GC proliferation in comparison to the control group with a dose base. The other doses of IGF-1 did not affect the proliferation of GCs in small follicles (Fig. 3).

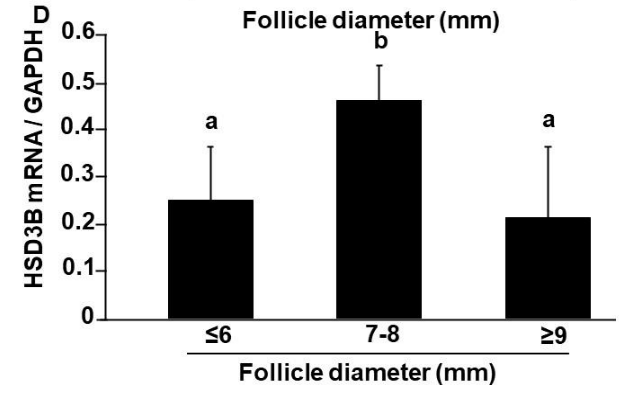
Table 2. Sequences of primers used for quantitative RT-PCR

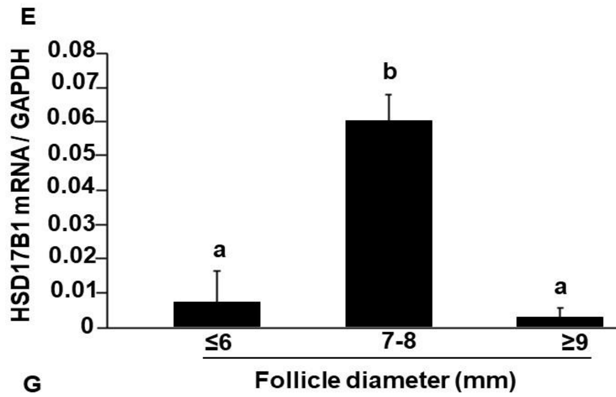
Gene	Forward and reverse primers	Accession no.	Product size (bp)	Temperature (^{o}C)
IGF1R	5'-GATCCCGTGTTCTTCTACGTTC-3'	X54980	101	59
	5'-AAGCCTCCCACTATCAACAGAA-3'			
PCNA	5'AGGGCTTCGACACTTACCG-3'	NM_176644.2	137	59
	5'-CTTGGCAGGAATCAGGTAAT-3'			
ACTB	5'GAGAAGAGCTACGAGCTTCCTGACG-3'	NM_173979.3	106	60
	5'-AGGATTCCATGCCCAGGAAGGAAGG-3'			
HSD3B	5'-TCCCGGATGAGCCTTCCTAT-3'	NM_174343.3	116	58
	5'-ACTAGGTGGCGGTTGAAGCA-3'			
CYP17A1	5'-GACGTTGTGGTCAACCTGTG-3'	NM_174304	142	59
	5'-CTGCTCCAAAGGGCAAGTAG-3'			
STAR	5'-CAACAGCAGAGAAGCTGGAAGACA-3'	NM_174189.3	133	60
	5'-CACCAACAACAGTCTGGATTTCCCT-3'			
HSD17B1	5'-TAAACCTGGTCACCGACTGC-3'	NM_001035395.2	126	57
	5'-CTGCCAGGGATGTAGGCAAA-3'			
CYP11A1	5'-TAAGCAAGATGCTGCAAATG-3'	NM_176644.2	137	59
	5'-CTTGGCAGGAATCAGGTAAT-3'			
GAPDH	5'-CACCCTCAAGATTGTCAGCA-3'	NM_001034034.2	103	60
	5'-GGTCATAAGTCCCTCCACGA-3'			

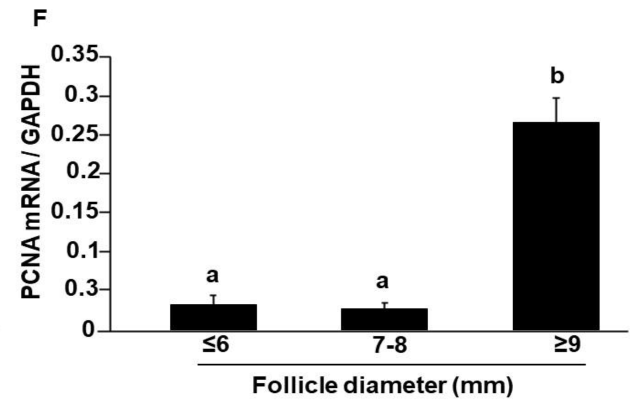

A
IGF1R mRNA / GAPDH
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
a
a
b
≤6
7-8
≥9
Follicle diameter (mm)


B
CYP17A1 mRNA / GAPDH
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
a
a
b
≤6
7-8
≥9
Follicle diameter (mm)


C
STAR mRNA / GAPDH
0.12
0.1
0.08
0.06
0.04
0.02
0
a
b
a
≤6
7-8
≥9
Follicle diameter (mm)


D
HSD3B mRNA / GAPDH
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
a
≤6
7-8
≥9
Follicle diameter (mm)


E
HSD17B1 mRNA / GAPDH
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
a
b
a
≤6
7-8
≥9
Follicle diameter (mm)


F
PCNA mRNA / GAPDH
0.35
0.3
0.25
0.2
0.15
0.1
0.3
0
a
a
b
≤6
7-8
≥9
Follicle diameter (mm)

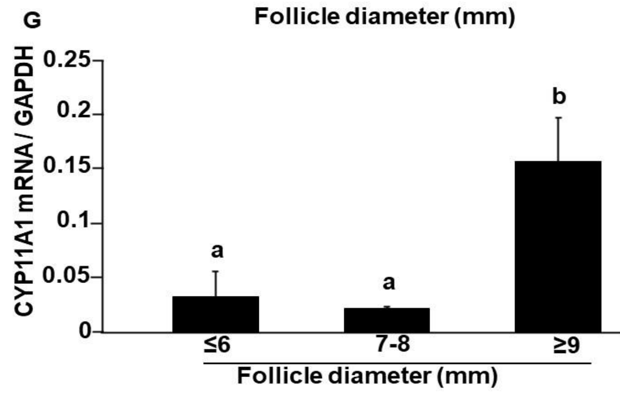

G
CYP11A1 mRNA / GAPDH
0.25
0.2
0.15
0.1
0.05
0
a
a
b
≤6
7-8
≥9
Follicle diameter (mm)

Fig. 1. Gene expression of *IGF1R* (A), *CYP17A1* (B), *STAR* (C), *HSD3B* (D), *HSD17B1* (E), *PCNA* (F), *CYP11A1* (G) mRNA relative to the amount of *GAPDH* in isolated granulosa cells from the three largest follicles in each pair ovary between Day 1 and Day 4 post-ovulation. Expression values are presented as means ± SEM, n=3-5. Follicles were grouped by range as follows: <6mm, 7-8 mm, and ≥9 mm in diameter. Different superscripts (a-b) indicate significant differences ($p < 0.05$; ANOVA) followed by Tukey’s multiple comparison test.

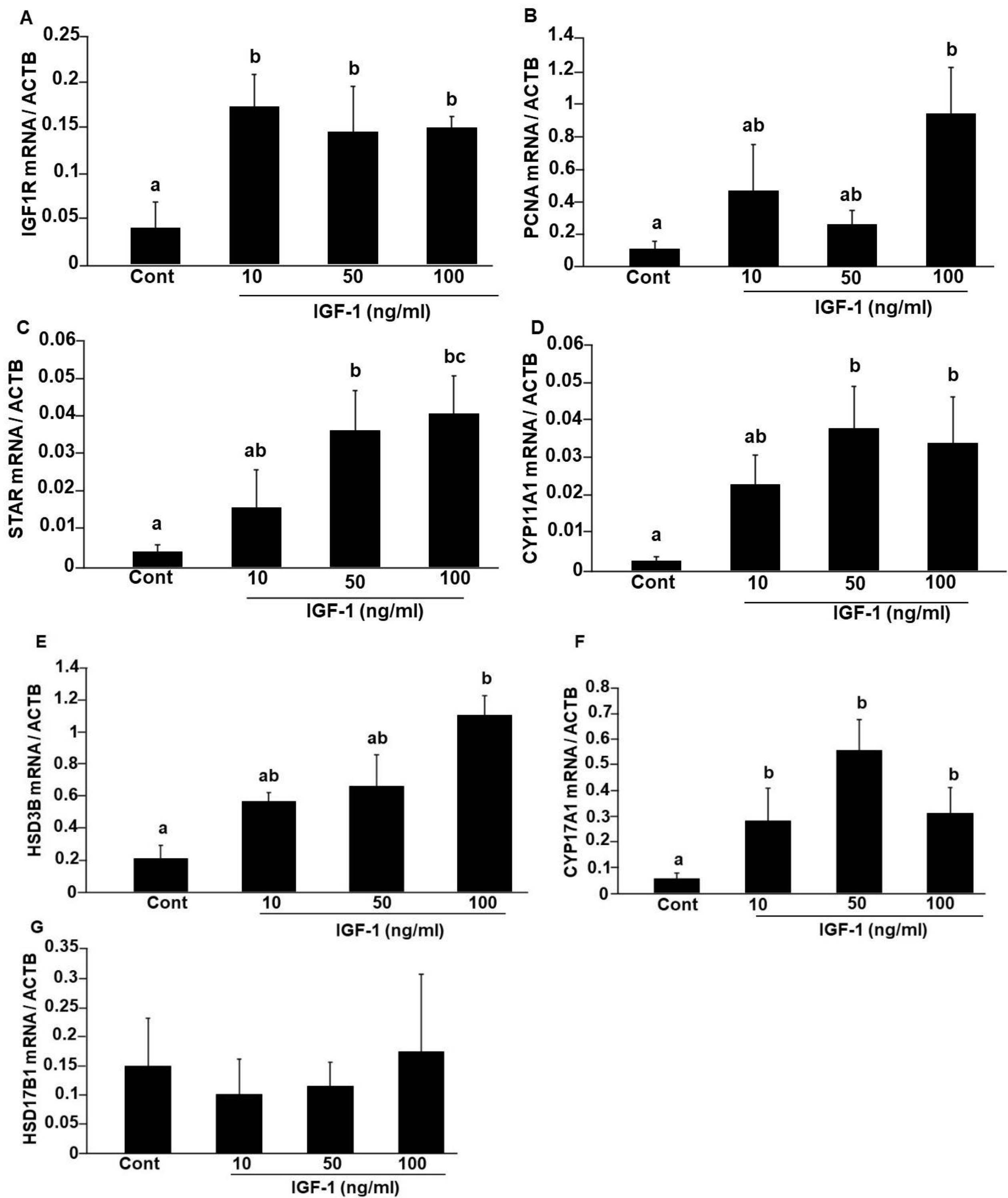
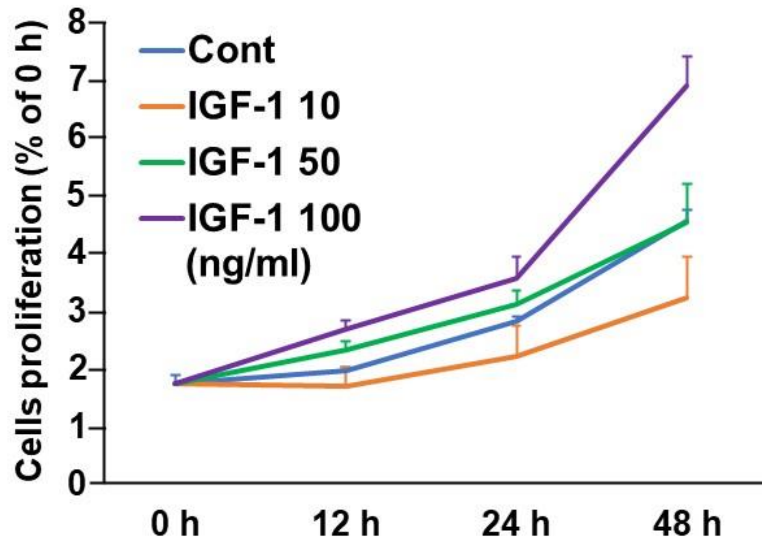

A
IGF1R mRNA / ACTB
0.25
0.2
0.15
0.1
0.05
0
a
b
b
b
Cont
10
50
100
IGF-1 (ng/ml)
B
PCNA mRNA / ACTB
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
ab
ab
b
Cont
10
50
100
IGF-1 (ng/ml)
C
STAR mRNA / ACTB
0.06
0.05
0.04
0.03
0.02
0.01
0
a
ab
b
bc
Cont
10
50
100
IGF-1 (ng/ml)
D
CYP11A1 mRNA / ACTB
0.06
0.05
0.04
0.03
0.02
0.01
0
a
ab
b
b
Cont
10
50
100
IGF-1 (ng/ml)
E
HSD3B mRNA / ACTB
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
ab
ab
b
Cont
10
50
100
IGF-1 (ng/ml)
F
CYP17A1 mRNA / ACTB
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
b
b
Cont
10
50
100
IGF-1 (ng/ml)
G
HSD17B1 mRNA / ACTB
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Cont
10
50
100
IGF-1 (ng/ml)

Fig. 2. Effects of IGF-1 on mRNA expression in granulosa cells. The granulosa cells from small follicles were treated with various concentrations of IGF-1 (10, 50, and 100 ng/ml) for 24 hours. The gene expression of *IGF1R* (A), *PCNA* (B), *STAR* (C), *CYP11A1* (D), *HSD3B* (E), *CYP17A1* (F), *HSD17B1* (G) mRNA, relative to the amount of *ACTB* in cultured granulosa cells from small follicles (Cont, control; IGF-1, insulin-like growth factor-1). Expression values are presented as means ± SEM, n=3-5. Different superscript letters indicate significant differences ($p < 0.05$; ANOVA) followed by Tukey's multiple comparison test.



	F-Value
IGF-1	36.450 *
Time	83.870 *
IGF-1 x Time	6.287 *

* $P < 0.01$

Fig. 3. The cells proliferation of granulosa cells of small follicles. Granulosa cells were treated with various concentrations of IGF (50, and 100 ng/ml) for 0, 12, 24, and 48 hours. All values are presented as means ± SEM, n=5. Asterisk indicate significant differences (p<0.01; two-way ANOVA) followed by Tukey's multiple comparison test.

DISCUSSION

The effects of IGF-1 on mRNA expressions of *IGF1R*, *PCNA*, steroidogenic enzymes (*STAR, CYP11A1*, *CYP17A1*, *HSD17B1,* and *HSD3B*), and cell proliferation were studied in GCs isolated of small follicles. *IGF1R* is essential for the formation of preovulatory follicles, the lack of IGF1R signaling in GCs of follicles that develop to the secondary stage fail to respond to FSH (109) and the low FSH receptor expression is the reason for reduced follicle growth in IGF-1 knockout mice (59). These findings show that *IGF1R* is crucial for follicular growth and suggest that the effects of IGF-1 are controlled by *IGF1R* in GCs as previously reported (39). Our results demonstrated that the expression of mRNA *IGF1R* increased with follicle diameter and significantly increased the expression in fresh GCs of large follicles, indicating that *IGF1R* is involved in the growth of all stages of dominant follicles. The expression of mRNA I*GF1R* was lowest in GCs of small follicles. While the GCs of small follicles were treated with IGF-1, the mRNA expression of *IGF1R* was significantly increased in GCs treated with IGF-1 for 24 h. Interestingly, the low dose of IGF-1 (10 ng/ml) increased the mRNA *IGF1R* in GCs. These results align with the previous study, where IGF-1 (50 ng/ml) increased *IGF1R* mRNA in bovine GCs isolated from follicles (2-8 mm) (72). Although the culture system and incubation period differed, taking together the present results support the hypothesis that IGF-1 boosts *IGF1R* mRNA in GCs of small follicles which can mediate the effect of exogenous IGF-1 in bovine follicles.

The success of studies on the initiation of follicular development depends on the availability of sensitive markers to detect the onset of follicular growth. One such marker is PCNA, a protein expressed by growing and proliferating cells (112). During follicular growth, IGF-1 works in synergy with FSH and LH to regulate the proliferation and differentiation of granulosa cells (119, 120). PCNA is an essential protein found in proliferating eukaryotic cells

and plays crucial roles in DNA replication, repair, and control of cell proliferation (121). The present study showed that the mRNA expressions of *PCNA* were lower in GCs of small follicles compared to large follicles. Interestingly, IGF-1 (100 ng/ml) significantly increased *PCNA* mRNA expression in cultured GCs of small follicles. These results are similar to a previous study conducted on human primary follicles, where IGF-1 induced the expression of *PCNA* mRNA in GCs of primary follicles (122). This result indicates that IGF-1 enhances the levels of *PCNA* mRNA in GCs of small follicles, which play a key role in the proliferation of GCs during follicular growth. This implies that the effects of IGF-1 on proliferation may be due to the enhanced mRNA expression of *PCNA*.

IGF-1 is potent and regulates A4 production, the main substrate for E2 essential for follicular growth (59). In the present study, we did not measure the A4 production. Our previous studies showed that IGF-1 increased A4 production in GCs (59) and TCs (118) of small follicles. In the present study, we observed the expression of mRNA for steroidogenic enzymes in fresh granulosa cells isolated from small, medium, and large-sized follicles. We found that the expressions of *STAR, CYP11A1, HSD3B, CYP17A1,* and *HSD17B1* mRNA were lower in the GCs of small follicles than in medium and large-sized follicles. Next, we examined the effect of IGF-1 on the mRNA expression of mentioned steroidogenic enzymes. IGF-1 enhanced the expressions of mRNA for *STAR* in GCs of small follicles and is responsible for cholesterol transport to the inner layer of mitochondria. This result supports the previous study that IGF-1 significantly increased the expression of *STAR* mRNA in cultured follicles of mice (74). IGF-1 (50-100 ng/ml) increased *CYP11A1* mRNA expression in cultured GCs of small follicles, which convert the cholesterol of pregnenolone. This result is also consistent with previous reports that IGF-1 increased the *CYP11A1* mRNA in the GCs of porcine (98). The next step for A4 synthesis is the conversion of pregnenolone to DHEA which is regulated by *CYP17A1*(71). In the present study, IGF-1 increased the expression of mRNA of *CYP17A1* in cultured GCs of small follicles,

but IGF-1 did not affect the expression of *HSD17B1* mRNA. IGF-1 significantly elevated *HSD3B* in cultured GCs of small follicles which regulate the conversion of DHEA to A4. These results agree with past studies that IGF-1 increased *HSD3B* in TCs of rats (123), in bovine GCs (30), and in bovine TCs (118). The A4 is mainly synthesized in TCs under the stimulation of LH while the GCs also play a role in the synthesis of A4 (35). The GCs produce only a small amount of A4 (124). Previous reports indicate that the bovine follicle wall, containing both TCs and GCs, produced significantly higher quantities of A4 than did isolated TCs (125). The minimal secretion of A4 by GCs suggests that GCs somehow enhance the ability of theca cells to produce A4, the substrate for E2 synthesis (126). Taken together, these findings show that IGF-1 enhances the mRNA expression of steroidogenic enzymes that regulate E2 and A4 production, leading to follicular growth and selection (18, 27).

IGFs comprise a family of peptides that promote cell proliferation, and differentiation and produce insulin-like metabolic effects. In a previous study, the CGs of large follicles were treated with IGF-1 for 2 days to check whether IGF-1 enhances the proliferation of GCs in vitro, and the results showed that IGF-1 (100 ng/ml) significantly increased the number of GCs compared to the control group (73). In the present study, the GCs of small follicles were treated with IGF-1 for 12, 24, and 48 hours. IGF-1 (100 ng/ml) significantly increased the proliferation of GCs at 48-hour incubation. These results are consistent with the above reports and indicate the importance of IGF-1 in follicular growth.

In conclusion, IGF-1 increased the expression of *IGF1R* mRNA which can mediate the bioactivity of IGF-1 in GCs. IGF-1 up-regulated key steroidogenic enzymes such as *STAR, CYP11A1, HSD3B,* and *CYP17A1* mRNA which are essential for A4 synthesis, a crucial substrate for E2 biosynthesis. IGF-1 Enhanced the mRNA expression of *PCNA* and increased the proliferation percentage of GCs of small follicles. These results indicate that IGF-1 has an excellent capacity to enhance factors that are involved in follicular growth. Further investigation

is required to confirm whether IGF-1 directly increases the mRNA expression of *PCNA* or indirectly through the enhanced *IGF1R* mRNA in GCs of bovine follicles.

SUMMARY

Insulin-like growth factor-1 (IGF-1) hormone plays a crucial role in follicular growth, antral formation, and steroidogenesis. IGF-1 is known to play a key role in the acquisition and maintenance of functional dominance. However, the biological effect of IGF-1 is a topic of interest, whether it is managed by IGF-1 receptors (*IGF1R*) or IGF-binding proteins. We examined the expression of *IGF1R, PCNA, STAR, CYP11A1, CYP17A1, HSD17B1* and *HSD3B* mRNA in uncultured granulosa cells (GCs) of the three largest follicles from paired ovaries and the effects of various concentrations (10, 50, and 100 ng/ml) of IGF-1 on the expression of *IGF1R*, *STAR*, *CYP11A1*, *CYP17A1*, *HSD17B1* and *HSD3B* mRNA in cultured GCs. We also investigated the effects of IGF-1 on the proliferation of GCs. Small follicles (<6 mm) expressed significantly lower *IGF1R, PCNA,* and steroidogenic enzymes than large follicles (≥9 mm) (p<0.05). IGF-1 (10-100 ng/ml) significantly increased *IGF1R* mRNA in cultured GCs. IGF-1 (50-100 ng/ml) increased *STAR*, *CYP11A1* mRNA, and IGF-1 (10-100 ng/ml) enhanced *CYP17A1* mRNA in GCs. IGF-1 had no effect on *HSD17B1* mRNA expression in GCs. IGF-1 (100 ng/ml) increased the *HSD3B* mRNA in GCs of small follicles ($p < 0.05$). Interestingly, IGF-1 (100 ng/ml) significantly increased the *PCNA* mRNA expression in cultured GCs and GC number of small follicles. These results showed that IGF-1 increased *IGF1R* mRNA in GCs which may mediate the action of IGF-1 required for the follicular growth. IGF-1 increased the steroidogenic enzyme mRNA which are essential for E2 synthesis and IGF-1 increased the GC number. Overall, these findings indicate that IGF-1 is critical for follicular growth and selection.

CHAPTER 4
CONCLUSION

The present study investigated the effects of IGF-1 on the factors involved in follicular growth and selection in cattle. Both granulosa cells (GCs) and theca cells (TCs) from small and medium-sized follicles were used in the present study. Based on the findings, the first series of experiments demonstrated that IGF-1 plays a significant role in enhancing the expression of mRNA steroidogenic-related enzymes and promoting the steroid hormone (A4 and P4) production in TCs substrate for E2 production. Furthermore, IGF-1 enhanced the *FSHR*, *CYP19A1* and *ESR2* mRNA, suggesting its involvement in mediating the synthesis and bioactivity of E2 in GCs of small follicles. These findings emphasize that IGF-1 plays a crucial role in regulating the complex molecular processes related to steroids and the development of follicles. The second series of experiments using GCs of small follicles indicated that IGF-1 enhanced other factors involved in follicular growth including mRNA expression of *IGF1R, PCNA, STAR*, *SYP11A1*, *CYP17A1*, *HSD3B,* and promoting cell proliferation. These findings underscore the involvement of IGF-1 in fostering the factors crucial for the growth of follicles and indicate how IGF-1 controls the complicated processes involved in follicle development.

Overall results suggested that IGF-1 plays a critical role in promoting various factors essential for follicular growth. In TCs, it enhances the expression of steroidogenic enzymes and hormones, while in GCs, it boosts factors such as *IGF1R*, *PCNA*, steroidogenesis enzymes mRNA, and cell proliferation. These findings highlight that IGF-1 actively regulates important processes in both cell types, contributing to follicular growth and selection.

Abbreviation	**Complete word (s)**
A4	Androstenedione
ANOVA	Analysis of variance
ACTB	β-actin
BSA	Bovine serum albumin
CYP17A1	Cytochrome P450 17A1
CYP19A1	Cytochrome P450 aromatase
CYP11A1	P450 side-chain cleavage
COC	Cumulus-oocyte complexes
CDH1	E-cadherin
CDH2	N-cadherin
CL	Corpus luteum
CO_2	Carbon dioxide
ºC	Degree Celsius
DHEA	dehydroepiandrosterone
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
EIA	Enzyme immune assay
EMT	Epithelial-to-mesenchymal transition
E2	Estradiol 17β
ER	Estrogen receptor
ESR1	Estrogen receptor α
ESR2	Estrogen receptor β
FBS	Fetal bovine serum
FSHR	Follicle-stimulating hormone receptor

FSH	Follicle simulating hormone
Fig	Figure
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCs	Granulosa cell
GnRH	gonadotropin-releasing hormone
HSD17B	17β hydroxysteroid dehydrogenase
HSD3B	3β hydroxysteroid dehydrogenase
H2SO4	Sulphuric acid
HRP	Horseradish peroxidase
IGF-1	Insulin-like growth factor-1
IGFBP	IGF binding protein
IGF1R	Insulin-like growth factor-1 receptor
LH	Luteinizing hormone
LHCGR	Luteinizing hormone receptor
ml	Mili liter
µl	Microliter
µg	Micro gram
mm	Mili meter diameter
mRNA	messenger RNA (ribonucleic acid)
ng	Nanogram
P4	Progesterone
PBS	Phosphate-buffered saline
P450SCC (CYP11A1)	P450 side chain cleavage
PGE2	Prostaglandin E2

PCNA	Proliferative cell nuclear antigen agent
RT-PCR	Reverse Transcription Polymerase Chain Reaction
rpm	Revolution (s) per minute
StAR	Steroidogenic acute regulatory
SEM	Standard error of the mean
SD	Standard deviation
T	Testosterone
TCs	Theca cell

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