CHAPTER IV
EXPERIMENT II INDUCTION OF FOLLICULAR GROWTH AND ATRESIA: GOAT MODEL

4.1 Introduction

During female reproductive life, most ovarian follicles (70 to 99.9%) at some stage of their development undergo a degenerative process called atresia, and only few follicles reach the ovulatory stage (Jablonka-Shariff et al., 1996; Yu et al., 2003). The preovulatory phase of the estrous cycle in goats is characterized by selection of one to several dominant follicles and atresia of subordinate follicles coincident with decreasing FSH concentrations in peripheral plasma, which is negatively correlated with the E2 produced by the largest follicle of the wave (Fatet et al., 2011). Early studies showed that FSH and LH could prevent follicular atresia (Yu et al., 2003), and in fact, early atretic follicles can be rescued by the administration of exogenous gonadotropins (Amiridis and Cseh, 2012).

On a cellular level, successful development of preovulatory goat follicles requires both proliferation and differentiation of follicular cells (Silva et al., 2009). Injection of FSH preparations has been shown to stimulate of cell proliferation by FSH preparations has been observed in vitro development of follicles (Faustino et al., 2010). Exogenous FSH is now a common practice to use exogenous gonadotropins to rescue more follicles and achieve superovulation (Paramio, 2010). Improvement of superovulation results necessitates further study of the mechanisms of follicular growth and atresia (Riesenberg et al., 2001). In recent studies indicate that developmental competence of goat oocytes form superovulation is acquired progressively during follicular growth and that only a high proportion of oocytes, those isolated from healthy follicles, have the capacity to progress to the blastocyst stage following in vitro maturation, fertilization and culture (Ayres et al., 2012).

In vivo, during its growth phase, the oocyte accumulates mRNAs and proteins important for maturation, fertilization and the early embryo cleavages (Lonergan et al., 2003a; Nemcova et al., 2006). It plays a key role in supporting embryonic development until the switch from maternal to zygotic gene expression control occurs

(Lonergan et al., 2003b). The oocyte prematuration and maturation conditions affect gene expression not only in the oocyte but also in the blastocyst stages (Rizos et al, 2002). Communication between most cells in animal tissues is executed via unique intercellular cytoplasmic channels which span two cell membranes, known as gap junctions (Gershon et al., 2008). The fundamental unit of gap junction channels is the connexin and each connexon is a hexamer of protein subunits called connexins (Cx) (Kidder et al., 2002). Cx43 had been identified in the ovaries of several species (Grazul-Bilska et al., 1997) and the expression of Cx43 seemed to play important roles in mediating ovarian functions as oocyte maturation (Nemcova et al., 2006). Bcl-2 family proteins are major regulators of cell death and survival (Boumela et al., 2011). Several members of the Bcl-2 family have been found to be expressed in mammalian oocytes and early embryos (Guillemin et al., 2009), and some recent reports provide a global view of transcript levels of Bcl-2 family members (Anguita et al., 2009).

Ovarian follicle growth and atresia also serves as a useful model for hormonal regulation of apoptosis (Yu et al., 2003). Several models for atresia have been developed in an attempt to define when atresia begins and to identify the mechanism(s) by which FSH modulate the incidence of atresia (Greenwald, 1989). However, despite these and other studies, the mechanisms by which FSH influences follicular growth and atresia, quality of oocytes, and embryo development in goats are still unclear (Jablonka-Shariff et al., 1996). Therefore, our aim was to determine the effects of FSH treatment and withdrawal on follicular growth, mitotic activity, oocyte quality, and development of embryo in goat model.

4.2 Materials and Methods

4.2.1 Animals ethics

All experimental goats and procedures were managed according to the guidelines approved by the Animal Ethic Committee of Khon Kaen University (No. AEKKU 53/2555). Prior to experiments, all goats were examined to ensure an absence of reproductive problems and all remained healthy throughout study.

4.2.2 Animals and welfare

This experiment was carried out at the small ruminant unit, Department of Animal Science, Faculty of Agriculture, Khon Kaen University, located at 16° 26' N latitude and 102° 50' E longitude, Thailand. Goats were fed a maintenance diet (NRC, 1981) with ad libitum feeding of fresh ruzi grass. Clean water and mineral block were provided ad libitum. Animals were vaccinated against foot and mouth disease (FMD), hemorrhagic septicemia (HS) and brucellosis according to the standard farm requirement of the Department of Livestock Development, Ministry of Agriculture and Cooperatives, Thailand.

4.2.3 Animals treatment

Forty nulliparous Thai-native goats (10 to 12 months of age and 20 kg of BW) that had exhibited an estrous cycle of normal duration (20 to 21 days) immediately preceding the treatment cycle were used for this study. Day 0 of the estrous cycle (standing estrus) was determined by using vasectomized buck. Beginning on day 17 after estrus, goats received no hormone or twice daily (morning and evening) intramuscular injections of normal saline or FSH-P (a pituitary extration; potency of one Armour Unit/mg; Sioux Biochemical, Inc., IA, USA) and subsequent withdrawal (W; intramuscular injection of normal saline). For ovarian stimulation, goats were allocated randomly to five treatment groups as follows: control (day 17; n=8), control (day 20; n=8), a day FSH-P + two days withdrawal (1 d FSH + 2 d W; n=8), two days FSH-P + a day withdrawal (2 d FSH + 1 d W; n=8), and three days FSH-P (3 d FSH; n=8).

The control (day 17) group received no hormone and control (day 20) group received twice daily injections of normal saline (1 ml) beginning on days 17, 18, and 19. The 3 d FSH group received twice daily injections of FSH-P beginning on days 17, 18, and 19 in decreasing doses (5.0-5.0-4.0-4.0-3.0-3.0; 24 mg total), whereas the 2 d FSH + 1 d W group received on days 17 and 18 (5.0-5.0-4.0-4.0; 18 mg total) followed by normal saline (1 ml) injection on day 19. The 1 d FSH + 2 d W group received FSH-P on day 17 (5.0-5.0; 10 mg total) followed by normal saline (1 ml) injection on days 18 and 19 (Figure 4.1).

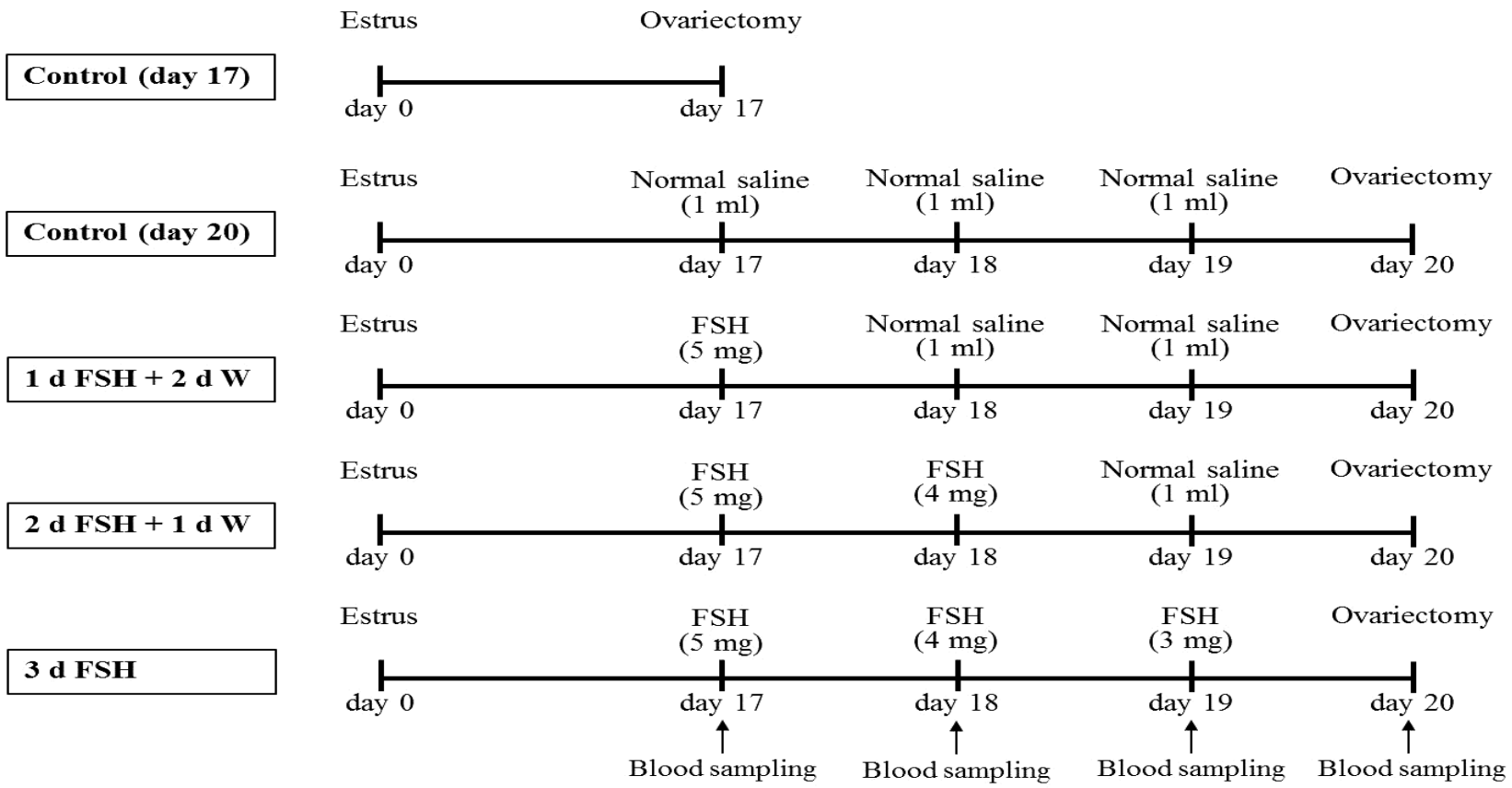

Figure 4.1 Description of timing of injections for the five treatment protocols. 1 d FSH + 2 d W, a day FSH-P + two days withdrawal; 2 d FSH + 1 d W, two days FSH-P + a day withdrawal; 3 d FSH, three days FSH-P.

4.2.4 Ovariectomy and classification of ovarian follicles

Female goats in the control (day 17) group were ovariectomized on day 17 after estrus to obtain ovaries. Goats in the FSH treatments and the control (day 20) group were ovariectomized on day 20 after estrus to obtain ovaries. Goats were injected with 0.075 mg xylazine (Rompun®; L.B.S. Laboratory ,Thailand) and 100 mg ketamine hydrochloride (Ketaset®; Wyeth Animal Health, Canada), and ovariectomy was performed to determine numbers of follicle as previously described for ewes (Luther et al., 2007). Ovaries were collected and swiftly transported to the laboratory.

For both ovaries from each female goat, the number and surface diameter of all visible follicles were recorded in two axes by using a Vernier caliper as previously describes (Grazul-Bilska et al., 2007), and all visible follicles were then classified by diameter into large (≥7 mm), medium (4-6 mm) or small (≤ 3 mm) as described (Gonzalez et al., 2001). In addition, the location of all visible follicles within an ovary was diagrammed so that follicular size before fixation of the ovary was known and could be used for histological analysis of the follicles.

4.2.5 Preparation of ovaries samples

After aspiration of follicles, the remaining ovarian tissue were fixed by perfusion with Carnoy's solution (ethanol: chloroform: acetic acid, 6:3:1), cut longitudinally into two pieces (2 mm thick), and immersed in Carnoy's solution for 2 h at room temperature. Ovarian pieces were then stored in 70% ethanol until further processing. Fixed ovarian pieces were dehydrated by using a graded series of ethanol, cleared with a histological clearing agent (Histo-Clear, National Diagnostics, Atlanta, GA), embedded in paraffin, sectioned at 5 μm, and mounted onto glass slides as previously described (Grazul-Bilska et al., 2007).

4.2.6 PCNA immunohistochemistry

Detection of PCNA was performed to determine the rate of cell proliferation of antral follicles, expressed as a LI. Additional paraffin-embedded sections (6 μm) from each ovary were used for detection of PCNA using a specific monoclonal antibody as described previously (Grazul-Bilska et al., 2007). The tissue sections were deparafinized with 3% xylene for 3 min, rehydrated in ethanol, and quenched in 0.5% hydrogen peroxide in methanol for 30 min to block endogenous

peroxidase activity. Slides were placed on a plastic container filled with 0.01 M sodium citrate buffer and heated in a domestic pressure cooker for 5 min after it reached the highest pressure. Once cooled, the heat-treated slides were washed three times for 3 min each with PBS. Before staining, endogenous peroxidase was inhibited by 0.5% hydrogen peroxide in methanol for 30 min. After washes with water and PBS, 10% normal horse serum in PBS was added for 20 min at room temperature to prevent nonspecific binding.

PCNA, monoclonal mouse antibody (1:100 dilution; MAB424, Millipore, CA, USA) were applied overnight at 4 ºC. After three washes of 10 min with PBS, sections were incubated with a horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:200 dilution; Cell Signaling Technology, CA, USA) for 30 min. After extensive washing with PBS, 0.6 mg/ml of 3,3-diaminobenzidine tetrahydrochloride substrate (Sigma, St. Louis, MO, USA) in PBS containing 0.03% hydrogen peroxide was also applied to develop color of positive staining for 5 min. The sections were then washed in distilled water, counterstained with Mayer's hematoxylin, gradually dehydrated with graded alcohols and mounted with permount (Sigma, St. Louis, MO, USA). For control, the PCNA antibody was replaced with normal mouse IgG (4 µg/ml; Cell Signaling Technology, CA, USA).

4.2.7 Visualization of tissue sections and image analysis

The tissue sections immunostaining of PCNA were visualized by using an Olympus IX71 inverted microscope (Olympus America Inc., USA) and a 20X objective. Images were taken from thecal layers of mapped individual follicles. For each follicle, 6 to 10 randomly chosen fields (0.025 mm^2 per field) were evaluated separately for granulosa or thecal layers.

The digital images were then used for quantitative image analysis using Motic Images Plus, version 2.0 software (Olympus, JA). To determine changes in LI, the percentage of the total area that exhibited positive staining for the number of PCNA-positive (proliferating) cells, and the total number of cells per area were determined quantitatively by using an image analysis system. The LI was calculated as a percentage (%) of proliferating cells out of the total number of cells within each tissue area. The LI for granulosa and theca cells included all labeled cells, and no

attempt was made to distinguish between theca cell types within theca layer (Grazul-Bilska et al., 2007).

4.2.8 Oocyte collection and grading

The ovarian were washed three times with prewarmed (30 ºC) saline solution and the oocytes were recovered from small, medium, and large follicles. The oocytes from visible follicles were aspirated using a 21G needle and consisted TCM 199 (Sigma, St. Louis, M.O., USA) as an oocyte aspiration medium. The cumulus-oocyte complexes (COCs) from ≤ 2 mm follicles was not used for in vitro fertilization (IVF). Then, COCs were evaluated based on morphology and categorized as healthy and nonhealthy as previous described (Rahman et al., 2008). The COCs were visually assessed and graded according to their cumulus-corona cell investments and morphology of the ooplasm. Healthy oocytes with finely granulated and homogeneous ooplasm (as viewed with a stereomicroscope) were selected for in vitro maturation (TVM).

4.2.9 In vitro maturation (IVM)

Healthy COCs from follicles were washed three times in maturation media (TCM-199 containing 10% fetal calf serum, 2 mM glutamine, 0.25 mM sodium pyruvate, and 100 µg/ml streptomycin). Cumulus cells were removed using stripping pipette and oocytes were incubated in maturation media for 24 h at 39 °C in 5% CO_2 and 95% air. After IVM, healthy oocytes with a clear first polar body (PB) were considered as mature and meiotically competent (Abdullah et al., 2008).

4.2.10 In vitro fertilization (IVF)

Healthy oocyte only was used for IVF and was transferred to fertilization media incubated overnight under mineral oil as described (Grazul-Bilska et al., 2003). After IVM, frozen buck semen (0.25 ml/mini straw) from the Toggenberg breed (TOG17/50; Department of Livestock Development, Thailand) was thawed at 37 ºC for 30 s and washed medium for a 1 h swim-up procedure. After swim-up, spermatozoa were further washed by centrifuging at 700 *g* twice for 5 min to remove any remaining semen extender. Before transfer to fertilization drops, healthy oocytes were then transferred into 50 µl droplets of fertilization medium (Quinn's Advantage^{TM} Fertilization Medium, SAGE Assisted Reproduction Product, a Cooper Surgical Inc., Trumbull, CT) under 10 ml mineral oil. The samples of

spermatozoa, frozen-thawed, were then added to the mature oocytes in fertilization wells for a final concentration of 2.0 X 10^6 spermatozoa/ml of fertilization medium. Gametes were co-incubated together for 24 h at 38.5 ºC in an environment of 5% CO_2 in air.

4.2.11 In vitro culture (IVC)

After 24 h of fertilization, the zygotes were washed three times in 0.5 ml HEPES-HTF with 0.11 mg hyaluronidas (SAGE Assisted Reproduction Product, CooperSurgical Inc., Trumbull, CT) to remove corona cells and attached spermatozoa, then transferred into 50 µl droplets of culture medium (Quinn's Advantage™ Cleavage Medium, SAGE Assisted Reproduction Product, Cooper Surgical Inc., Trumbull, CT) under mineral oil. Embryos were cultured at 38.5 ºC in an environment of 5% CO_2 in air for 24 to 96 h. After 96 h of culture, embryos were transferred into a second step medium (Quinn's Advantage™ Blastocyst Medium, SAGE Assisted Reproduction Product, Cooper Surgical Inc., Trumbull, CT) for blastocyst development until 24 h of culture, as described by Mortimer (2001). Evaluation of numbers of cleaved oocyte was performed before embryo was transferred to culture media. After 24 h, embryonic development (number of morula and blastocyst embryos) was evaluated every day during the 5 days culture (24, 48, 72, 96, and 120 h of culture).

4.2.12 RNA extraction and cDNA synthesis

Total RNA was extracted from morula embryos (5 embryos/treatment) and blastocyst embryos (3 embryos/treatment) using the High Pure RNA Tissue Kit® (Roche Applied Science, Germany) according to the manufacturer's instruction. The RNA concentration was estimated by reading the absorbance at 260 nm and was checked for purity at 280 nm in a spectrophometer. For each sample, the RNA concentrations were adjusted and 4 µg/ml was used to synthesize cDNA. The reverse transcription (RT) was performed in a total volume of 20 µl composed of 2 µl of sample RNA, 4 µl of reverse transcriptase buffer (Transcriptor High Fidelity Reaction Buffer), 20 units Protector RNase Inhibitor, 10 units of Transcriptor High Fidelity Reverse Transcriptase, 60 µM random hexamer primer, 5 µM DDT, 8 µM $MgCl_2$ and 1 µM of each dNTP. Tubes were heated at 70 ^oC for 5 min to denature the secondary

RNA structure before the addition of RT enzyme. The RT reaction was then incubated at room temperature for 10 min and then at 50 ^{o}C for 30 min to allow the RT of RNA, followed by 85 ^{o}C for 10 min to denature the RT enzyme. The negative control is prepared under the same conditions, but without addition of reverse transcriptase.

4.2.13 Quantitative RT-PCR

The quantification of all gene transcripts was carried out by real-time quantitative RT-PCR. Experiments were conducted to contrast relative levels of each transcript and *β*-actin in every sample. PCR was performed by adding a 5 μl cDNA of each sample to the PCR mix containing the specific primers to amplify Bcl-2, Cx43, and *β*-actin. Primer sequences and the GeneBank accession number are shown in Table 4.1. For quantification, PCR was performed using a Chromo4^{TM} Four-Color Real-Time Detector (Bio-Rad, Laboratories Inc., USA) and SYBR Green Master Mix. The PCR reaction mixture (50 μl) contained 25 μl of Faststart SYBR Green Master, 18 μl of water (PCR grade), 5 μl of cDNA, and 0.33 μM of each primer. The PCR protocol included an initial step of 95 ^{o}C (10 min), followed by 40 cycles of 95 ^{o}C (20 sec), 55 ^{o}C (20 sec), and 72 ^{o}C (45 sec). As negative controls, tubes were always prepared in the RNA or reverse transcriptase that was omitted during the RT reaction.

The method used for quantification of expression was the relative standard curve method. The quantification was normalized to an endogenous control (*β*-actin), and standard curves were prepared for each target and the endogenous reference. For each experimental sample, the amount of mRNA of each transcript and *β*-actin was determined from the appropriate standard curve. Subsequently, the quantity of each transcript was divided by *β*-actin to obtain a normalized value for each transcript. The normalized target values were divided by the calibrator normalized target values to generate the relative expression levels (Lonergan et al., 2003a).

4.2.14 Blood sampling and P4 concentrations

Blood samples (7 ml) were collected on day 17 to day 20 (Figure 4.1) via jugular venipuncture into an EDTA solution, then immediately centrifuged at 1500 x *g* for 15 min. Blood plasma samples were harvested and frozen stored at -20 °C until assayed. P4 concentrations were determined by Enzyme-linked

Immunosorbant Assay or ELISA (Cushwa et al., 1992). The intra-assay coefficient of variation was 5.3%, and assay sensitivity was 0.025 ng/ml.

Table 4.1 Quantitative RT-PCR primers

Tart gene	Primer sequence (5'-3')	Sense (s) Anti-sense (as)	GenBank accession no.	References
Bcl-2	CTGCACCTGACGCC CTTCAC	s	XM_586976	Li et al. (2009)
	GCGTCCCAGCCTCC GTTGT	as		
Cx43	ATTGTACTTTTCTT GTTGCTTG	s	AF028611	van der Velden et al. (2000)
	ACTTTATTGAATAA AGAACACTC	as		
β-actin	ACCACTGGCATTGT CATGGACTCT	s	GI:28628620	Frota et al. (2011)
	TCCTTGATGTCACG GACGATTTCC	as		

4.2.15 Statistical analysis

Data are presented as mean ± SEM. Data on number of visible follicles, number of oocytes, healthy oocytes, cleaved oocytes, morula embryos, blastocyst embryos, relative expression of Bcl-2 and Cx43 in embryos, and also LI of granulosa and theca cells were analyzed using the general linear model (GLM) procedure of SAS (SAS Inst. Inc., Cary, NC). Concentrations of P4 were analyzed with a nested analysis of variance including treatment, animal (treatment), and day in the model as describe before (Navanukraw et al., 2004). When the *F*-test was significant ($P<0.05$), differences among means were evaluated by using the Duncan's New Multiple Range Test (Steel et al., 1997).

4.3 Results

4.3.1 Number of visible follicles

To study the effect of FSH on follicular development (Table 4.2), goats receiving only one day of FSH treatment (1 d FSH + 2 d W) have numerous number of small follicles compared with their of other groups ($P<0.05$). The 3 d FSH and 2 d FSH + 1 d W groups demonstrated a significantly greater ($P<0.05$) mean number of medium follicles than 1 d FSH + 2 d W, control (day 17 and day 20) groups. Interestingly, the mean number of large follicles was greatest in goats receiving continuous FSH (3 d FSH) and was significantly greater ($P<0.05$) than that of other groups. Moreover, although goats of 3 d FSH group had a similar number of medium follicles, they had greater total follicles compared with 2 d FSH + 1 d W and was significantly greater ($P<0.05$) than that of other groups.

4.3.2 Number of healthy and cleavage oocytes

Total number of oocytes recovered was less ($P<0.05$) in the control (day 17 and day 20) groups but increased dramatically ($P<0.05$) in the FSH groups (Table 4.2). The number of healthy oocytes was greater ($P<0.05$) in the 3 d FSH treated goats than in the 2 d FSH + 1 d W, 1 d FSH + 2 d W, and the control (day 17 and day 20) groups (Table 4.2). The number of cleaved oocytes was greater ($P<0.05$) in the 3 d FSH and the 2 d FSH + 1 d W groups compared to the 1 d FSH + 2 d W and the control groups.

4.3.3 Number of embryonic development

The number of morula embryos was greater ($P<0.05$) in the 3 d FSH and 2 d FSH + 1 d W groups than in the 1 d FSH + 2 d W and the control groups. The number of blastocyst embryos in the 3 d FSH and 2 d FSH + 1 d W groups was higher ($P<0.05$) than those in the 1 d FSH + 2 d W and control groups (Table 4.2).

4.3.4 Immunohistochemical localization of PCNA

Positive PCNA staining was detected in the granulosa and theca layers of ovarian follicles in the control day 17 (Figure 4.2a), control day 20 (Figure. 4.2b), 1 d FSH + 2 d W (Figure 4.2c), 2 d FSH + 1 d W (Figure 4.2d), and 3 d FSH (Figure 4.2e) groups. Controls staining (no primary antibody) did not show any positive staining (Figure 4.2f).

Table 4.2 Number of visible follicles, total oocyte, healthy oocytes, cleaved oocytes, morula embryos, and blastocyst embryos of goats received no hormone (control day 17) or normal saline (control day 20) or FSH-P and subsequent withdrawal (1 d FSH + 2 d W, 2 d FSH + 1 d W, and 3 d FSH).

Measurement	Treatments				
	Control (day 17) n=8	Control (day 20) n=8	1 d FSH + 2 d W n=8	2 d FSH + 1 d W n=8	3 d FSH n=8
Small follicle ≤3 mm (n)	9.6 ± 0.7^{a}	9.9 ± 1.2^{a}	15.4 ± 0.3^{b}	12.5 ± 0.4^{c}	4.8 ± 0.6^{d}
Medium follicle 4-6 mm (n)	2.1 ± 0.4^{a}	2.8 ± 0.5^{a}	8.1 ± 1.0^{b}	13.3 ± 0.4^{c}	13.5 ± 0.5^{c}
Large follicle ≥7 mm (n)	1.3 ± 0.2^{a}	1.9 ± 0.2^{a}	2.3 ± 0.4^{a}	4.0 ± 0.7^{b}	16.0 ± 0.3^{c}
Total number of follicles (n)	13.0 ± 0.5^{a}	14.5 ± 0.6^{a}	25.8 ± 0.4^{b}	29.8 ± 0.6^{c}	34.3 ± 0.5^{d}
Total number of oocytes (n)	12.0 ± 0.9^{a}	11.6 ± 0.7^{a}	23.0 ± 0.8^{b}	26.8 ± 0.4^{c}	32.6 ± 0.3^{d}
Healthy oocytes (n)	11.3 ± 0.7^{a}	10.3 ± 0.7^{a}	15.8 ± 0.6^{b}	23.9 ± 0.6^{c}	29.3 ± 0.6^{d}
Cleaved oocytes (n)	8.0 ± 0.6^{a}	7.8 ± 0.6^{a}	9.6 ± 0.5^{a}	18.9 ± 0.7^{b}	20.8 ± 0.9^{b}
Morula embryos (n)	5.5 ± 0.4^{a}	5.4 ± 0.6^{a}	6.1 ± 0.7^{b}	12.3 ± 0.9^{c}	14.3 ± 0.8^{c}
Blastocyst embryos (n)	3.0 ± 0.4^{a}	3.1 ± 0.4^{a}	3.3 ± 0.8^{a}	7.0 ± 0.9^{b}	8.3 ± 0.8^{b}

Note: Means ± SEM are expressed per goat. Different letters (a, b, c, d) in the same row are different ($P<0.05$).

4.3.5 Labeling index (LI)

The granulosa cell LI of follicles from the 3 d FSH goats was greater (P<0.05) than for follicles from the 2 d FSH + 1 d W, 1 d FSH + 2 d W, and control goats (Table 4.3). The theca cell LI was greater (P< 0.05) for follicles from the 3 d FSH and 2 d FSH + 1 d W goats than for those from the 1 d FSH + 2 d W and control goats (Table 4.3).

Table 4.3 Labeling index (LI) of granulosa and theca cells of follicles in goats received no hormone (control day 17) or normal saline (control day 20) or FSH-P and subsequent withdrawal (1 d FSH + 2 d W, 2 d FSH + 1 d W, and 3 d FSH).

Treatments	LI (%)	
	Granulosa	Theca
Control (day 17)	32.45 ± 1.0^{a}	29.62 ± 0.7^{a}
Control (day 20)	32.07 ± 0.8^{a}	30.35 ± 0.8^{a}
1 d FSH + 2 d W	33.10 ± 0.5^{a}	30.57 ± 0.8^{a}
2 d FSH + 1 d W	38.85 ± 0.9^{b}	35.41 ± 0.5^{b}
3 d FSH	41.78 ± 0.9^{c}	36.94 ± 0.7^{b}

Note: Different letters (a, b, c) in the same column are different (P<0.05). The number of follicles used for analysis was 80. Labeling index (LI) = number of proliferating cells expressed as a percentage of total number of cells per area.

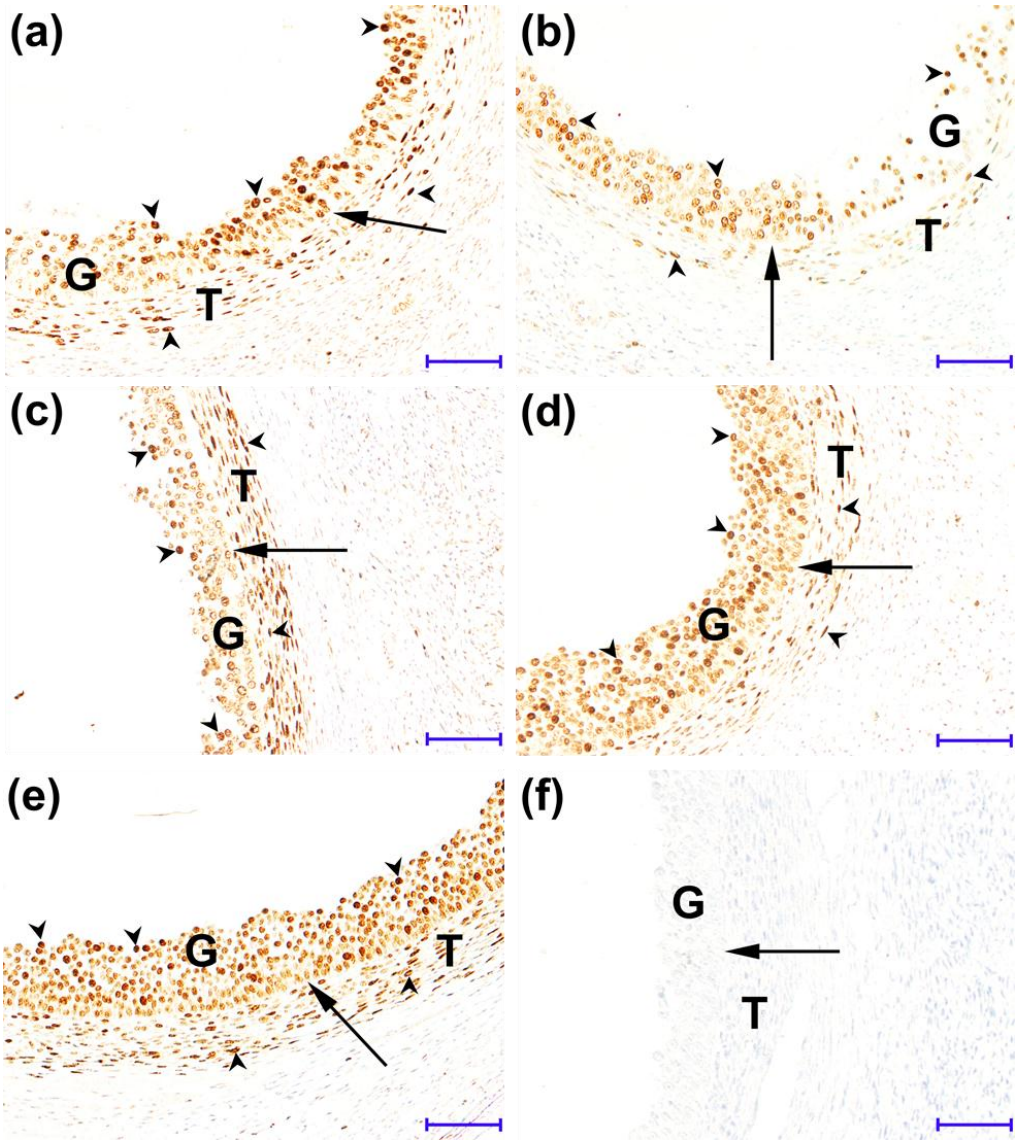

Figure 4.2 Representative micrographs of positive staining for PCNA in the section of follicles collected from the control (day 17; a), control (day 20; b), 1 d FSH + 2 d W (c), 2 d FSH + 1 d W (d), and 3 d FSH (e) groups. The brownish color indicates positive PCNA staining (arrowheads). The large whitish area on each image is the follicular antrum. Control section (no primary antibody) did not exhibit any positive staining (f). Abbreviations: G, granulosa cell layers; T, theca cell layers. Arrows identify the basement membrane. Magnification using 20X objective lens. Scale bars represent 50 µm.

4.3.6 Plasma P4

Plasma P4 concentrations were monitored throughout the experiment (Figure 4.3). There were not significantly different between treatment groups ($P > 0.05$) at any time during the experiment. For all treatments, plasma P4 concentrations were high on day 17 then decreased on day 20 (Figure 4.3). On day 17, the average P4 concentrations were similar for control (day 17 and day 20), 1 d FSH + 2 d W, 2 d FSH + 1 d W, and 3 d FSH groups (3.04 ± 0.2, 3.09 ± 0.2, 3.00 ± 0.1, 3.10 ± 0.4, and 3.16 ± 0.3 ng/ml, respectively). On day 20, the average P4 concentrations were similar for control (day 20), 1 d FSH + 2 d W, 2 d FSH + 1 d W, and 3 d FSH groups (0.26 ± 0.03, 0.25 ± 0.07, 0.23 ± 0.06, and 0.29 ± 0.09 ng/ml, respectively)

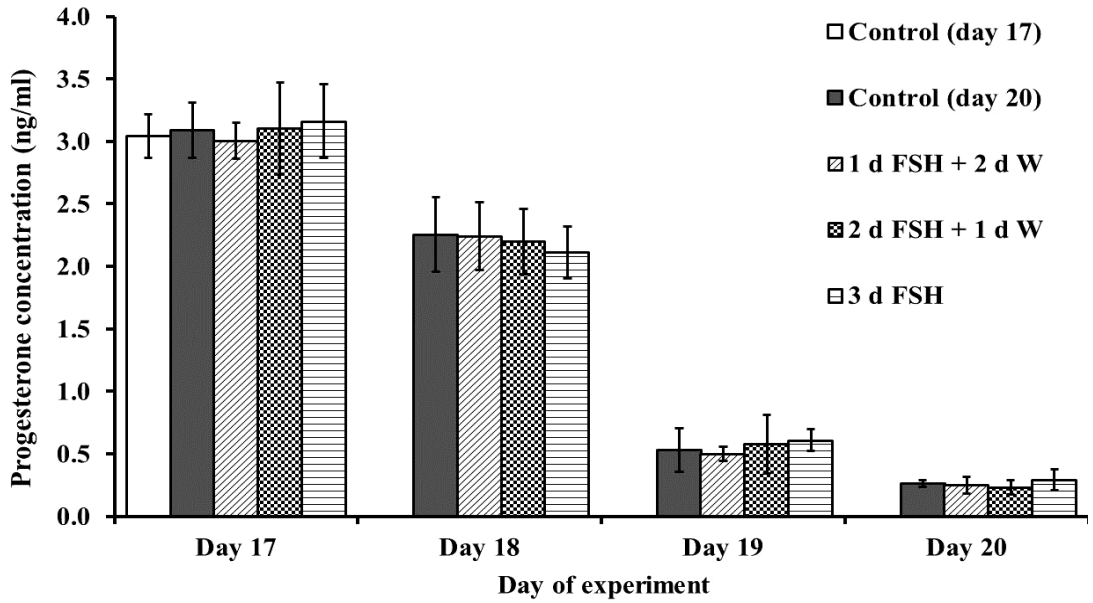

Figure 4.3 Mean (± SEM) plasma progesterone (P4) concentration in goats received no hormone (control day 17) or normal saline (control day 20) or FSH-P and subsequent withdrawal (1 d FSH + 2 d W, 2 d FSH + 1 d W, and 3 d FSH). Note: day 0 = day of estrus; day 17 to day 20 = day of FSH-P or normal saline injection.

4.3.7 Quantitative RT- PCR

The transcripts for Cx43 and Bcl-2 were detected in morula and blastocysts embryos originating from oocytes collected from follicles in the control, 1 d FSH + 2 d W, 2 d FSH + 1 d W, and 3 d FSH groups (Figure 4.4). The level of transcript for Cx43 was higher ($P<0.05$) in morula and blastocysts embryos of the 3 d FSH and the 2 d FSH + 1 d W groups than in morula and blastocysts embryos of the 1 d FSH + 2 d W and control groups (Figure 4.4).

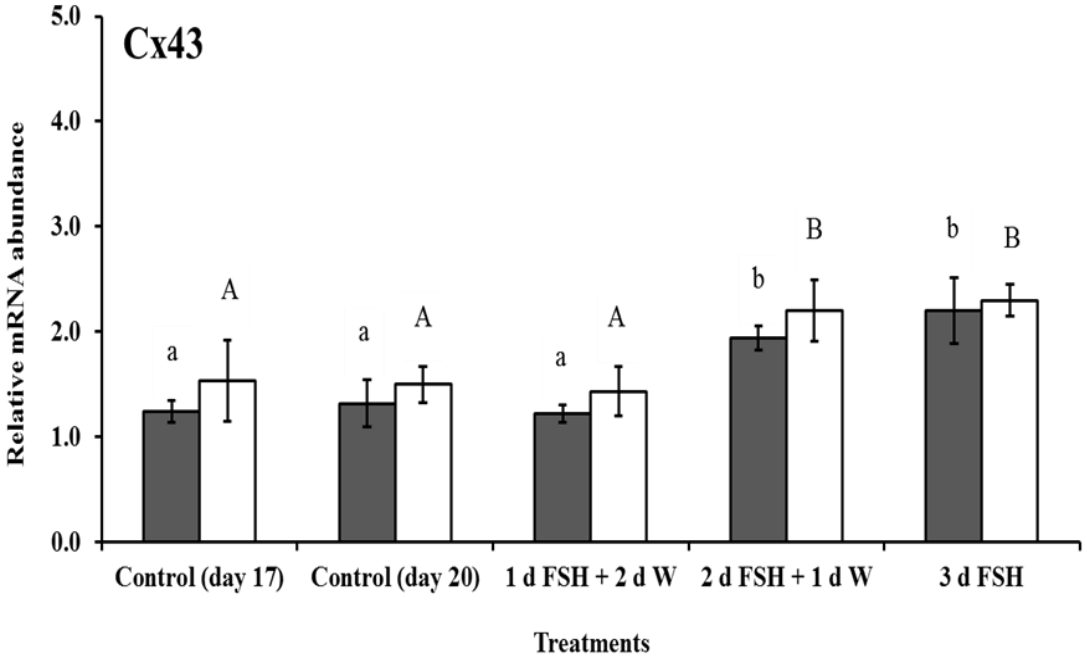

Figure 4.4 Relative abundance of Cx43 in morula embryos (black bars) and blastocyst embryos (white bars) developed from oocytes collected from follicles in goats received no hormone (control day 17) or normal saline (control day 20) or FSH-P and subsequent withdrawal (1 d FSH + 2 d W, 2 d FSH + 1 d W, and 3 d FSH). Different letters (a, b) refers to significant differences in relative transcript abundance among in morula embryos of different treatments; ABC refers to significant differences in relative transcript abundance among in blastocyst embryos of different treatments.

The relative abundance of Bcl-2 mRNA increased significantly (P< 0.05) in morula embryos originating from oocytes collected from follicles in the 3 d FSH and 2 d FSH + 1 d W in comparison with morula embryos originating from oocytes collected from follicles in the 1 d FSH + 2 d W and control groups (Figure 4.5). The relative abundance of Bcl-2 transcript was greater (P<0.05) in blastocysts embryos of the 3 d FSH than in blastocysts embryos of the 2 d FSH + 1 d W, 1 d FSH + 2 d W, and control groups (Figure 4.5).

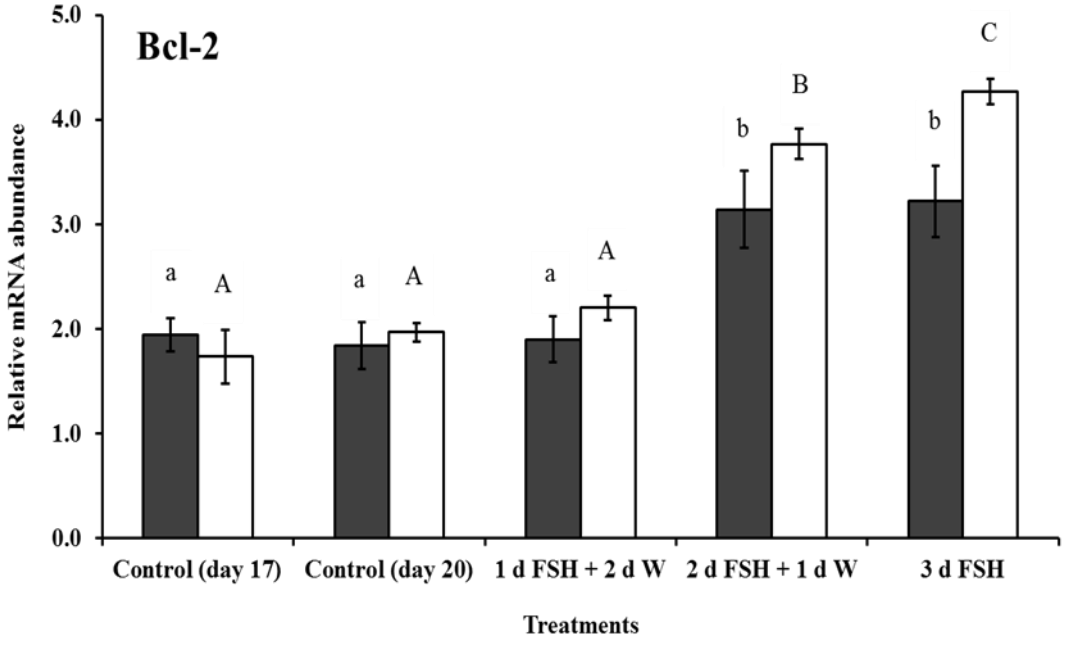

Figure 4.5 Relative abundance of Bcl-2 in morula (black bars) and blastocyst embryos (white bars) developed from oocytes collected from follicles in goats received no hormone (control day 17) or normal saline (control day 20) or FSH-P and subsequent withdrawal (1 d FSH + 2 d W, 2 d FSH + 1 d W, and 3 d FSH). Different letters (a, b) refers to significant differences in relative transcript abundance among in morula embryos of different treatments; ABC refers to significant differences in relative transcript abundance among in blastocyst embryos of different treatments.

4.4 Discussion

In the present model, we found that FSH treatment (3 d FSH) commenced on day 17 to day 19 increased the number of large follicles and total number of follicles, while the number of small follicles decreased slightly. The increase in the number of large follicles of the 3 d FSH treatment was probably due to the continued effect of FSH to stimulate growth of small and medium follicles, respectively, into medium and large follicles (Greenwald and Terranova, 1988; Jablonka-Shariff et al., 1996). Additionally, the 1 d FSH + 2 d W treatment decreased the number of large follicles compared with continuous 3 d FSH treatment. The decrease in the number of large follicles after FSH withdrawal may have been due to the loss of gonadotropic support necessary to stimulate or maintain the growth of small and medium follicles, as evidenced by the decrease in granulosa and (or) thecal cell proliferation (Jablonka-Shariff et al., 1996). Sufficient FSH concentrations are critical for survival of follicles that have differentiated to the antral stage or beyond (Markstrom et al. 2002; Quirk et al., 2004). It has been hypothesized that larger antral follicles require gonadotropic support to sustain their growth and prevent follicular atresia (Jablonka-Shariff et al., 1994; Rubianes and Menchaca, 2003).

The decrease of mitotic activity in granulosa cells during follicular development, therefore, is associated with granulosa cell differentiation and illustrates the inverse relationship between granulosa cell growth and differentiated function (Jablonka-Shariff et al., 1994; 1996). Numerous studies of the rate of cell proliferation in ovarian tissues have been conducted with ewes (Jablonka-Shariff et al., 1994), and cattle (Fricke et al., 1997; Grazul-Bilska et al., 2007). However, only a few studies have investigated the regulation of follicular cell proliferation by gonadotropins in goats. The administration of continuous FSH (3 d FSH and 2 d FSH + 1 d W groups) to goats in this study increased the LI for both granulosa and thecal cells compared with those of withdrawal treatment (1 d FSH + 2 d W group). The high rate of cell proliferation reported for ovarian tissues (Fricke et al., 1997; Grazul-Bilska et al., 2007) is underscored by the high level of PCNA immunoreactivity present in ovarian follicles in this study. Increased PCNA expression correlates with the earliest signs of granulosa cell growth (Oktay et al., 1995). Because PCNA is most abundant in cell during the S phase of the cell cycle, it is generally considered to be a reliable marker

for proliferating cells (Dietrich, 1993; Grazul-Bilska et al., 2007). In addition, a greater proliferation rate was observed in healthy follicles compared with the atretic follicles in sheep and cattle (Isobe and Yoshimura, 2000; Seekallu et al., 2010).

Previous studies reported in goat that FSH enhanced development of follicles by suppressing the apoptosis of granulosa cells. FSH inhibited granulosa cell apoptosis by increasing production of steroids and insulin-like growth factor-1 (IGF-1) (Behl and Pandey, 2002; Chaves et al., 2012). Recent studies demonstrated that FSH acts synergistically with IGF-1 to increase cell number and expression of steroidogenic enzymes in granulosa cells (Mani et al., 2010). Follicular atresia in cattle, sheep and goat was also characterized by a loss of cytochrome P450 aromatase (P450 arom) in granulosa cells and a decrease in levels of cytochrome P450 17α-hydroxylase (P450c17) in the theca interna (Chaves et al., 2012). Furthermore, the reversal of atresia by administration of gonadotropins has been reported for many domestic livestock species including pigs, ewes, and cows (Jablonka-Shariff et al., 1994; Fricke et al., 1997).

Since oocyte developmental competence increased with follicular sizes, an increased expression of these enzymes may be associated with the selection of dominant follicle and oocytes that are more competent (Yu et al., 2003). Previous studies conclude that the morphological quality of COCs appears to be the most important factor that influences the efficiency of in vitro embryo production in goats (Katska-Ksizakiewicz et al., 2007). In the recent study indicated a high correlation between follicle quality or granulosa cells apoptosis and distribution of COCs with different morphology (Gonzalez-Bulnes et al., 2003). In most studies reported an overall positive effect of FSH treatment on the developmental competence of COCs (Pereira et al., 2012). Increased oocyte quality has been associated with increased follicle size and oocyte developmental potential following FSH treatment (Avelar et al., 2012). It has already been reported that FSH requires the use of multiple doses due to its shorter half-life when compared to eCG (Monniaux et al., 1983). FSH is a batter choice of hormone for superovulationg goat as it provides more oocytes than eCG or PMSG (Medan et al., 2003). FSH is usually administered in decreasing doses of 1 to 5 mg, injected in 12 h intervals over a period of 3 to 5 days of the experimental regimes (Rahman et al., 2008). Thus, it was hypothesized that the intervals between

the injections of FSH were too short in the 1 d FSH + 2 d W group (single dose, 10 mg of FSH-P in two injections at 12 h intervals). In this case, it is possible that inadequate gonadrotropin stimulation could decrease the oocyte's meiotic competence in recovered COCs (Graff et al., 2000; Avelar et al., 2012). In the recent study reported oocyte production in goats raised in Northeast Brazil using two FSH protocols for ovarian stimulation (five vs. three injections). The use of the protocol with the higher number of FSH injections resulted in COCs with greater oocyte meiotic competence when compared with the treatment with fewer doses (Almeida et al. 2011). In present study, the number of healthy and cleaved oocytes collected from 3 d FSH group was higher than 1 d FSH + 2 d W group. These data imply that, continuous 3 d FSH treatment enhanced not only number of healthy oocytes, but also improved their ability to develop into embryos.

Traditionally, morphological criteria are used to select good-quality COCs for embryonic IVP (Pereira et al., 2012). Oocytes with a compact cumulus, which is composed of several layers of cells and a homogeneous cytoplasm, are considered healthy (Baldassarre et al., 2003). Differences in the relative abundance of some developmentally important gene transcripts have been reported between in vivo and in vitro produced bovine embryos (Lonergan et al., 2003b). The question remains whether blastocysts developed in vitro from oocytes with different developmental competence exhibit a different relative abundance of developmentally important genes (Nemcova et al., 2006). In this study, we investigated the level of transcript for the gap junctional intercellular communication gene (Cx43) and anti-apoptotic gene (Bcl-2) in morula and blastocyst embryos from goat COCs recovered from follicles after a day, two day, and three day of FSH treatments for ovarian stimulation.

Intercellular communication via gap junction is required to coordinate embryonic development (Nemcova et al., 2006). During bovine preimplantation development functional gap junctions are first observed at compaction at the eight-cell stage (Wrenzycki et al., 1996). In this experiment, the transcripts for Cx43 was detected in morula and blastocysts embryos originating from oocytes collected from visible follicles in different FSH regimes. In agreement, Cx43 mRNA was detected from the four-cell stage onwards in in vitro-cultured embryos (Houghton et al., 2002). These results are consistent with those of Nemcova et al. (2006), who reported that transcript

of Cx43 detected at the morula and blastocyst stages in in vitro-produced bovine embryos. In previous work from Houghton et al. (2002), the level of expression of Cx43 was significantly higher in blastocysts produced following in vivo culture than those produced in vitro (Rizos et al., 2002); in addition culture from the zygote to blastocyst stage in the absence of serum significantly increased the relative transcript abundance for Cx43 transcripts, compared with those in which serum was presented (Rizos et al., 2003). This pattern of expression reflects the quality of these blastocysts measured in terms of cryotolerance. Such results strongly suggested that the relative abundance of a Cx43 transcript correlates with normal blastocysts (Wrenzycki et al., 1996; Rizos et al., 2003). These observations are in agreement with the findings of our study, i.e., the relative abundance of Cx43 transcripts was increased in morula and blastocyst embryos derived from oocytes collected from visible follicles in the 3 d FSH and 2 d FSH + 1 d W groups compared with the 1 d FSH + 2 d W group. Due to the higher abundance of Cx43 mRNA, those blastocysts were able to form stable gap junctions at an adequate developmental stage (Nemcova et al., 2006).

The incidence of apoptosis in blastocysts has been related to embryo quality (Li et al., 2009). Apoptosis plays a very important role during pre- and post-implantation development, removing abnormal cells or cells that are no longer required, and controlling the embryo cell number (Anguita et al., 2009). In our study, Bcl-2 mRNA was detected in in vitro produced goat murulae and blastocysts. It has been found that the Bcl-2 protein prevents apoptosis induced by a variety of stimuli and maintains cell survival by influencing the release of cytochrome c from mitochondria rather than by altering proliferation (Yang et al., 1997). Therefore, the Bcl-2 family of proteins constitutes a critical intracellular checkpoint of apoptosis within a distal common cell death pathway (Yang and Rajamahendran, 2002). In our study, the level of transcript for Bcl-2 was much higher in embryos derived from oocytes collected from visible follicles in the 3 d FSH and 2 d FSH + 1 d W treatments than in embryos derived from oocytes collected from visible follicles in the 1 d FSH + 1 d W treatment. These results suggest that embryos of the 3 d FSH and 2 d FSH + 1 d W groups are in a more advance anti-apoptotic process, which explains why these oocytes have greater developmental competence compared with embryos of the 1 d FSH + 2 d W group. The administration of continuous FSH treatments (3 d FSH and 2 d FSH + 1 d W

groups) to goats in this study demonstrated that sufficient FSH concentrations are critical for growth, development, and survival of follicles. Moreover, each component of the growing follicle contributes to the microenvironment essential for successful oocyte differentiation and subsequent fertilization (Epigg, 2001). Furthermore, in recent study demonstrated that oocyte population obtained from non-treated goats or single doses FSH treatment is very heterogeneous showing different degrees of maturation and atresia (Anguita et al., 2009; Avelar et al., 2012). It is therefore possible that the low blastocyst yield obtained so far indicate that the oocytes of the 1 d FSH + 2 d treatment used are undergoing a process of apoptosis.

4.5 Conclusion

In conclusion, using goat ovarian model, we have demonstrated the administration of continuous FSH-P (3 d FSH and 2 d FSH + 1 d W treatments) dramatically increased the total number of follicles compared with that in the 1 d FSH + 2 d W and control groups, and the regimes of the 3 d FSH and 1 d FSH + 1 d W groups and increased granulosa and thecal cell LI in follicles compared with those of the 1 d FSH + 2 d W and control groups. Moreover, the relative abundance of Cx43 and Bcl-2 transcripts in embryos seems to be associated with different developmental competence. Therefore, the stimulations of donor goat with 3 d FSH and 2 d FSH + 1 d W treatments are an efficient regime to high healthy oocytes able to produce high quality embryos.