CHAPTER III
EXPERIMENT I ASSOCIATION AMONG VACULARITY, CELL PROLIFERATION, AND ENDOTHELIAL NITRIC OXIDE SYNTHASE (eNOS) IN BOVINE ANTRAL FOLLICLES OF THE FIRST FOLLICULAR WAVE

3.1 Experiment 1.1: Quantitative vascularity of antral follicle in *Bos indicus* using Factor VIII immunolocalization

3.1.1 Introduction

Productivity in animal livestock is controlled by many factors, including follicular growth and development. Follicular development can be a major determinant of reproductive efficiency in monovular (only one of many available follicles develops into the dominant or ovulatory follicle) farm species (Ginther et al., 2003; Hunter et al., 2004). In monovulatory ruminants, such as cattle, as in other species, primordial follicle growth, once initiated, continues until the follicle either becomes atretic or proceeds to ovulation (Webb et al., 2004; Yang and Rajamahendran, 2000). Although this follicular growth is controlled primarily by gonadotropins and locally produced growth factors, a number of environmental factors, such as nutrition (Webb et al., 2004) and vascularity (Hunter et al., 2004), affect follicular development and oocyte quality, and hence fertility. Therefore, improved understanding of the regulation of vascularity will allow greater control over follicular growth and development (Hunter et al., 2004).

Vascularity of follicle is critical for relevant research in ovarian follicular growth and development (Seekallu et al., 2010; Van Blerkom, 2000). It is known that vasculature is not equally distributed among the population of follicles of the bovine ovary, since only theca cell layers, present in antral follicular stages, have vessels (Bruno et al., 2009). Increase in the blood supply to individual follicles appears to be associated with follicular growth rates and the ability to become the antral follicle, while reduced thecal vascularity appears to be closely associated with follicular atresia (Acosta et al., 2005). A proper blood supply is likely to represent a major regulatory issue for controlling the ovarian function as well as oocyte growth

and maturation (Mattioli et al., 2001). Several angiogenic factors, including VEGF and eNOS, are expressed in ovarian follicles of several species (Redmer and Reynolds, 1996; Grazul-Bilska et al., 2006). Thus, angiogenic factors may be critical for maturation of the antral follicles (Redmer and Reynolds, 1996). Antral follicle is compartmentalized into a highly vascular thecal layer and a nonvascular granulosa layer that are separated by a basement membrane. The follicular growth requires the formation of the associated vascular system to supply the increasing metabolic demands of the proliferating cells (Redmer and Reynolds, 1996).

Methods of vascularity quantitative have been available using immunohistochemistry (Grazul-Bilska et al., 2006) or physiological image of color Doppler ultrasonography (Acosta, 2007). Lectin binding (BS-1 from *Bandeiraea simplicifolia*) and Factor VIII have been used as endothelial markers (Grazul-Bilska et al., 2006; Seekallu et al., 2010). However, Factor VIII with immunofluorescence is more effective than BS-1 lectin due to greater specific to endothelium and greater dilution when Factor VIII was applied (Woywodt et al., 2004). Therefore, our aim was to quantify vascularity of antral follicles in *Bos indicus* using Factor VIII immunohistochemistry in order to investigate the relationship between follicular size, health status and vascularity.

3.1.2 Materials and Methods

1) Animals and Ethics

All experimental beef and procedures were managed according to the guidelines approved by the Animal Ethic Committee of Khon Kaen University (No. AEKKU 34/2551). Prior to experiments, all cows were examined to ensure and absence of reproductive problems and all remained healthy throughout study.

2) Animal and Tissue collection

Eight mature crossbred beef cows (Brahman x Thai-native; 5 year of age; 461 kg of BW) were used for this study. Estrous cycles were synchronized using two injections of $PGF_{2\alpha}$ analogue (Estrumate, Animal Health Crop., Union, NJ) given 11 day apart. All animals were observed for estrus twice daily within 1 to 6 day after the second $PGF_{2\alpha}$ injection. The first day of estrus was designated as day 0 of the subsequent first follicular wave. Ovaries were collected on day 6 after the onset of estrus, at the slaughter house located in Khon Kaen province, Thailand. Thus, day 6

of the first follicular wave represents the transition time of selection and dominance phases (Fortune et al., 2001; Ginther et al., 2003).

3) Tissue preparation and Classification of follicles

For both ovaries from each cow, the number and surface diameter of all visible follicles were recorded in two axes by using a Vernier caliper as previously described (Grazul-Bilska et al., 2007), and antral follicles were classified by size as 3 to 6 (small), 7 to 10 (medium), and >10 mm (large) in diameter (Lucy, 2001) and by morphology as healthy and atretic follicles (Grazul-Bilska et al., 2006). In addition, the location of all visible follicles within an ovary was diagramed so that follicular size before fixation of the ovary was known and could be used for histological analysis of the follicles. All follicles that were subsequently analyzed histologically were accounted for on these surface diagrams. Ovaries then 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 6 μm, and mounted onto glass slides as previously described (Grazul-Bilska et al., 2007). For one section from each of the paraffin-embedded ovarian were stained with haematoxylin-eosin. Tissue sections were positioned under the 40X objective lens so that either the granulosa or theca layer was brought into view and categorized into two groups such as healthy and atretic follicle, following the established morphological criteria. Healthy follicles were characterized as having a thick, continuous granulosa cell layer, and fewer than 10 pyknotic nuclei per cross-section. Atretic follicles were characterized by loosening of granulosa cell layer and by the presence of greater than 10 pyknotic nuclei per cross-section (Grazul-Bilska et al., 2006).

4) Immunofluorescence histochemistry

For each ovarian piece, at least one randomly chosen cross section of each follicle of known surface diameter and follicular health was analyzed. Detection of Factor VIII (a marker of endothelial cells and thus vascularity) was performed as previously described by Grazul-Bilska et al. (2007). Briefly, ovarian tissue sections

were deparaffinized in xylene and rehydrated in ethanol at room temperature. Endogenous peroxidase activity was blocked by immersing the slides in 3% H_2O_2 in methanol for 5 min. The sections were then rinsed several times in PBS containing Triton X-100 (0.3%, v/v) for 10 min. Then, to block non-specific binding of antibodies, the sections were treated for 20 min with PBS containing normal goat serum (2%, v/v; Vector Laboratories, Burlingame, CA, USA). The blocking serum was tapped off, and the sections were covered with primary antibody for Factor VIII, rabbit polyclonal Factor VIII antibody (Sigma, St. Louis, MO, USA) at concentrations of 0.001 mg/ml in PBS containing 2% goat serum for 1 h at room temperature. For the control, the primary antibody was replaced with normal rabbit IgG at concentrations 0.05 mg/ml in PBS containing 2% goat serum. After washing with 10 mM PBS two times (each for 10 min), the slides were incubated with the fluorophores-labeled goat anti-rabbit IgG antibody for Factor VIII (0.002 mg/ml; green-fluorescent Alexa Fluor® 488; Invitrogen Molecular Probes, Eugene, OR, USA) for 45 min at room temperature. Finally, the section prevents rapid photobleaching of fluorescein counterstained by mounting medium (VECTASHIELD® HardSet™ Mounting Medium with DAPI; Vector Laboratories, Burlingame, CA, USA). To mount tissues, dispense three drops of mounting medium were applied onto the section (a 25 µl droplet) and allowed mounting medium to disperse over the entire section. Mounting medium contains 4′,6-diamidino-2-phenylindole (DAPI) and is intended to be used to counterstain DNA. DAPI excites and emits at 360 and 460 nm when bound to DNA, producing a blue fluorescence.

5) Visualization of tissue sections

The tissue sections immunofluorestaining of Factor VIII were visualized using a Zeiss Axiovert 200 fluorescent microscope (Zeiss, Thornwood, NY) equipped with fluorescent filters for fluorophores and DAPI visualization at 20X magnification. Images for the two fluorescent channels were collected separately and combined using the Axiovision version 4.8.1 software package as described by Borowicz et al. (2007).

6) Image analysis

The digital images were then used for quantitative image analysis using Image-Pro Plus, version 4.5.1 software (Media Cybernetics, Inc., Silver Spring, MD, USA). Thereafter, follicular vascularity was quantified according to the procedures of Borowicz et al. (2007). Briefly, images of the sections were taken from individual follicles for each size and health of follicles. Then, we determined the area that exhibited positive staining for Factor VIII protein (occupied by endothelial cells) in the theca layer (green-fluorescent). Total theca layer area (μm^2) was determined, and each theca layer capillary was individually traced to quantify the total vessel area (μm^2), number (no.), and perimeter (μm) per each tissue area. Based on these measurements, capillary area density (CAD; CAD=capillary area/tissue area, %), capillary number density (CND; CND=capillary number/tissue area, no./μm^2), and area per capillary (APC; APC=capillary area/capillary number, μm^2/no.) were calculated for each follicles image. Images were taken from theca layer areas of mapped from individual follicles for each size and health of follicles (Redmer et al., 2009).

7) Statistical analysis

Data are presented as mean ± SEM. Vascularity in the thecal layer was analyzed using the general linear model procedure of SAS (SAS Inst. Inc., Cary, NC). Effect of follicle size on vascularity (CAD, CND, and APC) was analyzed using one-way ANOVA (SAS Inst. Inc., Cary, NC). Differences between specific means were evaluated by Duncan's New Multiple Range Test (Steel et al., 1997). Simple liner correlations between size and vascularity were determined by using PROC CORR of SAS. Interactions between follicular health and size were analyzed by two-way ANOVA. The model contained effects of health status (healthy and atretic), follicle size (small, medium, and large), and health status x follicle size interactions. 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).

3.1.3 Results

1) Immunofluorescence histochemistry

Factor VIII binding was localized exclusively in the thecal layer and stroma of the antral follicles (Figure 3.1a, green-fluorescence), but was not found in the granulosa layer. No immunoreactive signal was detected when tissue section was incubated without the primary antibody for Factor VIII (Figure 3.1b, the control). In healthy, small (Figure 3.1c), medium (Figure 3.1e), and large follicles (Figure 3.1g), Factor VIII binding was greater in the blood vessels in the theca layer compared with the atretic, small (Figure 3.1d), medium (Figure 3.1f), and large follicles (Figure 3.1h).

2) Quantitative vascularity

The morphological evaluations to classify follicles as healthy or atretic were followed by evaluation of Factor VIII staining in five follicles per ovary. For all follicles evaluated, CAD and CND were greater ($P<0.05$) in the small and medium follicles compared to the large follicle (Table 3.1). However, APC was greatest in small follicles compared with large follicles but was not different when compared with medium follicles (Table 3.1). In addition, follicular size was negatively correlated with CAD (r^2=-0.766; $P<0.001$), CND (r^2=-0.606; $P<0.001$), and APC (r^2=-0.517; $P<0.001$).

Table 3.1 Means ± SEM of capillary area density (CAD), capillary number density (CND), and area per capillary (APC) of small, medium, and large follicles

Follicle size	Vascularity		
	CAD (%)	CND (no./μm^2)	APC (μm^2/no.)
Small (n=15)	5.04 ± 0.39^a	0.0029 ± 0.0003^a	20.10 ± 2.31^a
Medium (n=9)	4.72 ± 0.48^a	0.0025 ± 0.0002^a	19.40 ± 1.32^{ab}
Large (n=8)	1.87 ± 0.57^b	0.0013 ± 0.0002^b	12.37 ± 2.85^b

Note: Different letters (a, b) in the same column are different ($P < 0.05$). Note: The number of small (n=15; healthy = 12; atretic = 3), medium (n=9; healthy = 7; atretic = 2), and large follicles (n=8; healthy = 4; atretic = 4).

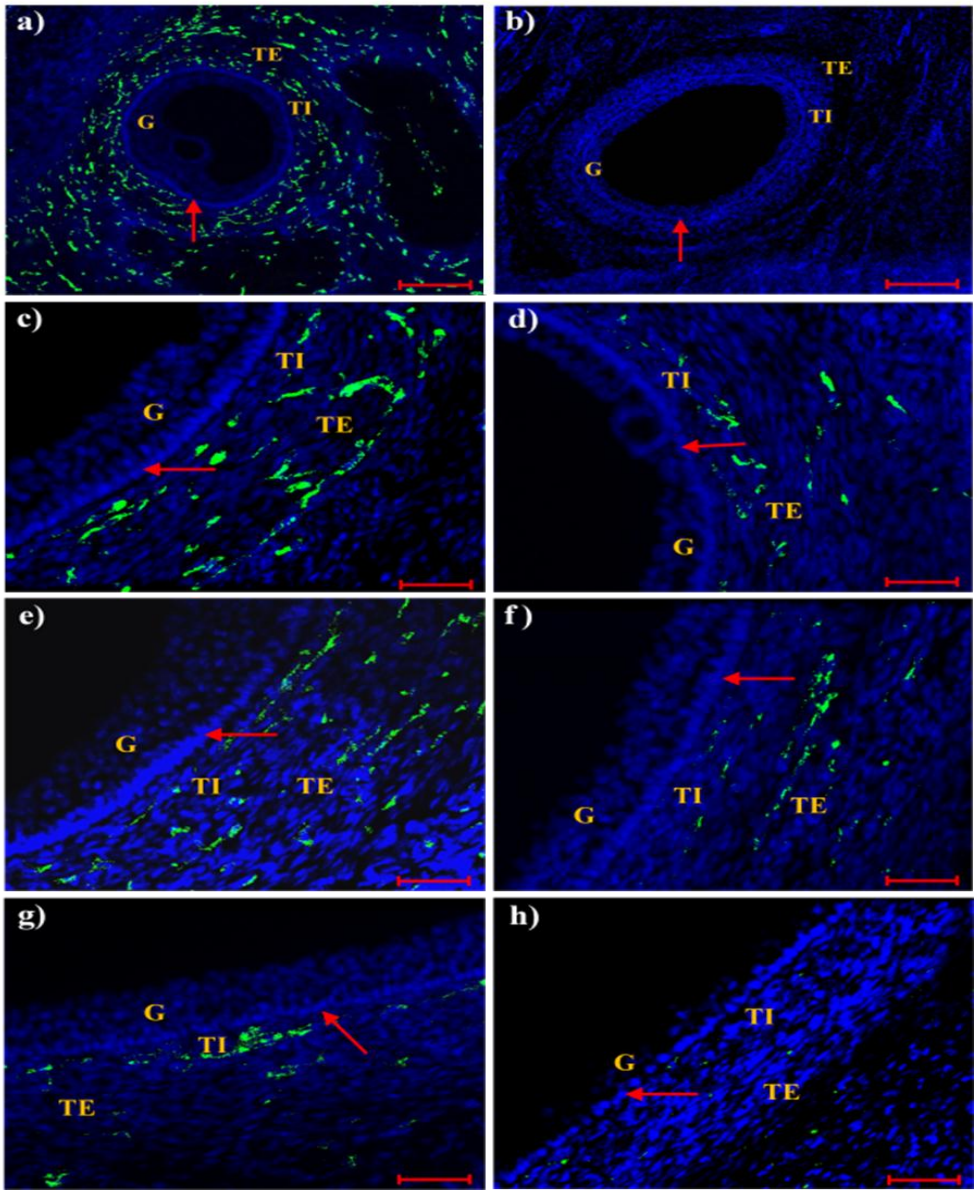

Figure 3.1 Representative fluorescent micrographs of Factor VIII positive staining (green) in antral follicles (a) of bovine ovaries. Control sections did not exhibit any positive staining (b). Immunolocalization of Factor VIII in a follicular section of healthy small (c), atretic small (d), healthy medium (e), atretic medium (f), healthy large (g), and atretic large follicle (h) (objective magnification of a, b = 5 x, c-h = 20 x). Arrows identify the basement membrane. G, granulosa layer; TI, theca interna layer; TE, theca externa layer. Size of bar = 50 μm.

Health status ($P<0.01$) and size ($P<0.01$) of antral follicles affect vascularity. However, no interactions between health status and follicle size on CAD, CND, and APC of antral follicles were observed ($P>0.05$). The average CAD of the healthy follicles was greater ($P<0.05$) than that of the atretic follicles (Table 3.2). Overall, the CAD was greater ($P<0.05$) in the healthy follicle (small, medium, and large) compared to the atretic follicle (small, medium, and large; Table 3.2). The average CND of the healthy follicles was greater ($P<0.05$) than that of the atretic follicles. CND was greatest ($P<0.05$) in theca layer of healthy small follicle and was lowest in atretic large follicle. The atretic follicle showed a significantly lower ($P<0.05$) average APC of theca layer than healthy follicle. Overall, APC was the least ($P<0.05$) in theca layer of the atretic large follicle.

Table 3.2 Means ± SEM of capillary area density (CAD), capillary number density (CND), and area per capillary (APC) in antral follicle according to health status and follicle size

Health status	Follicle size	CAD (%)	CND (no./μm^2)	APC (μm^2/no.)
Healthy	Small (n=12)	5.61 ± 0.31^{a}	0.0032 ± 0.0003^{a}	20.24 ± 2.75^{a}
	Medium (n=7)	5.25 ± 0.44^{a}	0.0027 ± 0.0002^{ab}	19.57 ± 1.53^{a}
	Large (n=4)	3.30 ± 0.32^{a}	0.0018 ± 0.0002^{bc}	19.43 ± 2.13^{a}
	Mean	5.10 ± 0.23^{A}	0.0028 ± 0.0002^{A}	19.89 ± 1.51^{A}
Atretic	Small (n=3)	2.76 ± 0.09^{b}	0.0016 ± 0.0004^{bc}	19.56 ± 4.53^{a}
	Medium (n=2)	2.89 ± 0.06^{b}	0.0016 ± 0.0003^{bc}	18.82 ± 3.56^{a}
	Large (n=4)	0.43 ± 0.12^{c}	0.0008 ± 0.0002^{c}	5.43 ± 0.48^{b}
	Mean	1.76 ± 0.42^{B}	0.0012 ± 0.0002^{B}	13.07 ± 2.85^{B}

Note: Means with different letters (A, B) within the same column are different ($P<0.05$) among healthy and atretic follicles. Note: Means with different letters (a, b, c) within the same column are different ($P<0.05$) among follicles of different size and health status. Note: The numbers of healthy and atretic follicles used for analysis were 23 and 9, respectively.

3.1.4 Discussion

This study is the first report of vascularity quantitation in *B. indicus* using Factor VIII immunolocalization. Factor VIII (a marker of endothelial cells and thus vasculature) was localized primarily at endothelial cells of capillary (Seekallu et al., 2010). Thus, the following vascularity measurements were determined in antral follicles. An increase of capillary area in antral follicle was determined by CAD (total area occupied by capillaries expressed as a proportion per unit of follicular tissue) and also determined by CND (total number of capillary per unit of follicular area). The capillary beds of antral follicles grow primarily via an increase in capillary size as determined by APC (average cross sectional area per capillary). Regardless of health status, CAD and CND of small and medium follicles were greater than that of large follicles. It is strongly implied that theca layer of small and medium size follicles has more active angiogenesis than large follicles (Irving-Rodger et al., 2001). These data are supported by previous studies, which demonstrated that the single-layered capillary wreath in the small-sized follicles becomes a multilayered structure in the thickened theca layers as the follicles develop into larger and more mature preovulatory follicles (Yamada et al., 1995). These morphological changes in follicular microvasculature support the need for an increase in blood supply as antral follicles develops (Shimizu and Sato, 2005).

The present study highlight that the decreased follicular vascularity observed in ovaries may be the reason for a decreased developmental competence of the large antral follicles (Shimizu et al., 2003). These data are in agreement with report on swamp buffalo (Feranil et al., 2004). The majority of large follicle, obtained from d 6 of the first follicular wave, was as functional atretic follicles (Valdez et al., 2005). Large follicle had been already selected and most follicles might start to be regressed (Ginther, 2000; Beg et al., 2002; Mihm and Austin, 2002). In addition, Fortune et al. (2001) indicated that the difference in size was not always predictive of future dominance. Size of the follicle at d 6 of the estrous cycle may not always be the distinguishing factor between dominant and subordinate follicles during the first follicular wave in cows. Atresia is mainly induced during the follicle dominance phase and affects follicles of all sizes (Faes et al., 2007). Eighty five percent of the follicles from ovaries taken at any time during the estrous cycle are atretic (Hagemann

et al., 1999). In this study, eight of 32 follicles (25%) were in the large size. Of these, two of four follicles (50%) were morphological classified as atretic follicle and the remaining were as healthy follicles. As for the small follicles (15 of 32 follicles or 47%), three of 15 follicles (20%) were as atretic follicles and 12 of 15 follicles (80%) were as healthy follicles (data not shown). The proportions of atretic follicles from the present study suggest a possible involvement of the vascularity in follicular atresia of large follicles (Robinson et al., 2009).

The results of this study demonstrate that both health status and follicle size affect vascularity in the theca layer of antral follicles in *B. indicus*. No significant interaction was observed in vascularity between health status and follicle size. The significant reductions of vascularity were observed in the atretic follicles compared with healthy follicles suggesting that vascularity of theca layers decline in the atretic follicles of *B. indicus* (Grazul-Bilska et al., 2007). Changes in density of thecal vasculature are observed in the healthy and atretic follicles in farm animal species (Feranil et al., 2004; Grazul-Bilska et al., 2006). We found that CAD was not significantly different among size of healthy follicle, but was different ($P<0.05$) among size of atretic follicle. Large atretic follicles or estrogen-inactive (EI) follicles are regressed whereas large healthy follicle are continued to grow (one is continued to grow but remaining are started to regress) after selection of the dominant follicle (Fortune et al., 2001). Significant difference of vascularity was observed in atretic large follicle compared to medium and small follicle was partly due to less mitogenic activity of theca and granulosa cells (Redmer et al., 1991; Redmer et al., 2001). A greater CAD, CND, and APC in the healthy antral follicles, as observed in our study, indicates a greater surface area of vasculature to supply nutrients, growth factors, and gonadotropins for maintenance of healthy status of antral follicle. Shortly after selection, days 6 to 8 of the estrous cycle, there is a rapid degeneration of thecal vascularity, once atresia has been initiated in the non-dominant follicle (Macchiarelli et al., 2006).

We also found that there are two phenomena of thecal vascularity: one in which reduction of CAD, CND, and APC occurs initially in the atretic large follicle and another in which increasing of CAD, CND, and APC occurs initially in the small healthy follicle. Follicular atresia affects all stages of follicular development, but the

proportion of follicles that become atretic is enhanced by increased follicle size (Feranil et al., 2004; Isobe et al., 2001). A close interrelationship between follicular diameter and follicular vascularity suggests that follicular vasculature and blood supply to individual follicle play a crucial role in the events leading to growing follicle in cattle (Acosta, 2007). In the antral follicles, capillaries in the atretic large follicle with significantly lesser CAD, CND, and APC are sparse and show signs of poor follicular development. In contrast, the healthy small follicle was characterized by the presence of numerous CAD and CND, and presented in antral follicles depend on the stage of development (Feranil et al., 2004). These findings support the concept that antral follicular development and atresia are accompanied by significant thecal vascularity quantitative and functional changes (Reynolds et al., 2002; Robinson et al., 2009).

Neovascularization is crucial for antral follicle growth with a large increase in total vasculature and vascular density during preantral and antral follicle stages (Robinson et al., 2009). The vascular development of the follicle is probably controlled by angiogenic factors, including VEGF, which is a potent angiogenic factor that is selectively mitogenic for vascular endothelial cells (Redmer et al., 2001). A number of studies indicate that one of the primary mechanisms for controlling the onset of atresia may be a disruption of the local blood supply to the follicle (Webb and Armstrong, 1998; Grazul-Bilska et al., 2007). Zeleznik et al. (1981) demonstrated that the density of capillaries surrounding a healthy maturing follicle is greater than that of other smaller follicles, and the density of capillaries is associated with increased delivery of gonadotropins to the maturing follicle, suggesting that angiogenesis may play a role in the development of the growth follicle. In addition, thus, it has been demonstrated that vascularity in the theca layer may serve as an indicator of follicle health in several farm animals (Seekallu et al., 2010). Recent advances in measuring ovarian vascularity and blood flow in *B. indicus* enable us to increase understanding of regulation of antral follicle growth and follicular selection which is primarily based on vascular supply (Acosta et al., 2005; Acosta, 2007; Grazul-Bilska et al., 2007).

3.1.5 Conclusions

The current study showed that immunofluorescence detection of Factor VIII can be used as a reliable marker of endothelium to quantify vascularity of antral follicles in *B. indicus*. Effect of follicular health and size on vascularity was demonstrated by which the healthy follicles exhibited greater vascularity than the atretic follicle whereas the small and medium follicles have greater vasculature than large follicle. Taken together, these data provide insight of the importance of vascularity of growing antral follicle in *B. indicus*.

3.2 Experiment 1.2: Relationships among vasculature, mitotic activity, and endothelial nitric oxide synthase (eNOS) in bovine antral follicles of the first follicular wave

3.2.1 Introduction

Bovine follicular growth and atresia are characterized by two or three follicular waves (Fortune et al., 2004). At each follicular wave, a cohort of follicles is initiated to grow, but only the dominant follicle continues to grow and ovulate or the follicle will be atretic (Beg and Ginther, 2006). Since the development and regression of follicles are associated with major structural and functional changes, it is important to classify follicles accurately as healthy or atretic at all stages of development (Irving-Rodgers et al., 2009; Rodgers and Irving-Rodgers, 2010). Maintenance of follicular health depends on the presence of angiogenic factors and a functional vasculature (Jiang et al., 2003). Changes of vascularization and expression of some regulators, including angiogenic factors, are associated with follicular growth and/or atresia. Vascular network around developing follicles may be rate limiting for the selection of the dominant follicle(s), i.e., the follicle(s) that is/are destined to ovulate. In turn, insufficient vascular supply could act as the trigger that leads to follicular atresia (Augustin, 2001). Thus, vascular supply or blood flow is significantly increased after selection of the bovine dominant follicle (Fraser, 2006; Grazul-Bilska et al., 2007; Garside et al., 2010).

Several angiogenic factors, including eNOS and VEGF, are expressed in ovarian follicles (Grazul-Bilska et al., 2006). The eNOS is involved in follicular and luteal angiogenesis through the production of NO, a free radical gas (Redmer and

Reynolds, 1996; Grazul-Bilska et al., 2006). One of the major regulators of NO and eNOS expression is VEGF which, in turn, is regulated by NO (Redmer et al., 2001; Beckman et al., 2006). Although NO may not be a major regulator of follicular growth and angiogenesis, the greater expression of eNOS in blood capillaries of healthy follicles compared with atretic follicles supports a concept that NO and eNOS have a role in maintaining vascularization during follicular growth and selection of follicles for complete ovulation (Grazul-Bilska et al., 2006; Seekallu et al., 2010). All these studies have been conducted in sheep and cattle (*Bos taurus* breeds of European origin); there is little information in *Bos indicus* regarding vasculature, cell proliferation, and eNOS expression of antral follicles during the follicular wave.

We hypothesized that in *Bos indicus*, differences between the healthy and atretic follicles and size of antral follicles could be partly explained by differences in vascularization, mitotic activity, and expression of eNOS. Therefore, the objectives of the present study were to 1) characterize vascularization (Factor VIII protein; a marker of endothelial cells and thus vasculature), expression of eNOS proteins, and PCNA labeling index (LI; an index of the percentage of cells proliferating); and 2) evaluate the relationships among these variables in antral follicles of the first follicular wave.

3.2.2 Materials and Methods

1) Animal assurance

All experimental beef and procedures were managed according to the guidelines approved by the Animal Ethic Committee of Khon Kaen University (AEKKU 54/2555). Prior to experiments, all cows were examined to ensure an absence of reproductive problems and all remained healthy throughout study.

2) Animal and tissue collection

Ten mature crossbred beef cows (Brahman X Thai-native; 4 year of age; 470 kg of BW) were used for this study. Estrous cycles were synchronized using two injections of $PGF_{2\alpha}$ analogue (Estrumate®, Animal Health Crop., Union, NJ) given 11 days apart. All animals were observed for estrus twice daily within 1 to 6 days after the second $PGF_{2\alpha}$ injection. Observation for estrus commenced 1 day (24 h) after the second $PGF_{2\alpha}$. Estrus was defined as standing to be mounted at least once for the visually observed group. Estrus was detected twice daily for a minimum of 30

min per observation using androgenized cow for 6 days or until the last cow exhibited estrus as previously described (Grazul-Bilska et al., 2007). Day of first detection of estrous behavior was designated as day 0 of the subsequent estrous cycle (Austin et al., 2001). Ovaries were collected on day 6 after the onset of estrus, which corresponded to a time before or during the selection of dominant follicle of the first follicular wave (Fortune et al., 2001).

3) Tissue preparation and classification of follicles

After slaughter, ovaries were removed and placed in ice-cold 0.05 M PBS for transport to the laboratory. For both ovaries from each cow, the number and surface diameter of all visible follicles were determined in two axes by using a Vernier caliper, the diameter of all follicles ≥3 mm was recorded. The visible follicles were classified by size as follows: small, 3 to 6 mm; medium, 7 to 10 mm; large, >10 mm (Kaneko et al., 1992). Location of all follicles (n=82) within an ovary was mapped so that follicular size before aspiration and fixation of ovary was known. The FF was gently aspirated from mapped follicles ≥3 mm. The FF was centrifuged at 1,000 X *g* for 10 min at 4°C and stored at -20°C until assayed for E2 and P4.

Ovaries were sectioned into several pieces (1 to 2 mm/piece) and were fixed in Carnoy's solution (ethanol:chloroform:acetic acid, 6:3:1) 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, USA), paraffin embedded, sectioned at 6 µm, and mounted onto glass slides as previously described (Grazul-Bilska et al., 2007). For one section from each of the paraffin-embedded ovarian tissues were stained with haematoxylin-eosin. Follicles were classified as healthy or atretic based on microscopic examination of the granulosa layer by using the following morphological criteria: healthy follicles were characterized as having a thick, continuous granulosa cell layer, and fewer than 10 pyknotic nuclei per cross-section. Atretic follicles were characterized by loosening of granulosa cell layer and by the presence of greater than 10 pyknotic nuclei per cross-section (Grazul-Bilska et al., 2007). In addition, the follicles were classified morphologically as healthy and atretic and classified by the ratio of E2:P4 concentrations in FF as EA/healthy (E2:P4 ≥1) or EI/atretic (E2:P4 <1) according to

Grazul-Bilaka et al. (2007). For each of the ten cows, selected follicles from visible antral follicles (≥ 3 mm) were chosen. Thus, a total of eighty-two follicles were evaluated (Table 3.3) in this study. Based on the morphology and concentrations of E2:P4 ratio and size of follicle, a total of forty-nine healthy (thirty-eight small, seven medium, and four large follicles) and thirty-three atretic follicles (twenty-five small, two medium, and six large follicles) were evaluated across all cow.

4) Hormone assays

Concentrations of P4 and E2 were determined with commercial ELISA kits (DRG Instruments, GmbH, Germany; Pancarci et al., 2011) in unextracted follicular fluid (FF) diluted (1:100 and 1:250 for P4 and E2) with PBS. Sensitivities of these assays were 0.025 ng/ml for P4 and 10.0 pg/ml for E2. Intraassay coefficient of variation (CV) was 8.75% for P4 and 5.91% for E2. Following determination of P4 and E2 concentrations (ng/ml) in FF, aspirated follicles were confirmed as healthy (E2:P4 ≥1) and atretic (E2:P4 <1), as described (Grazul-Bilska et al., 2007).

5) Immunofluorescence localization of Factor VIII and eNOS

Detection of Factor VIII (a marker of endothelial cells and thus vasculature) and eNOS was performed as previously described by Grazul-Bilska et al. (2007) and Seekallu et al. (2010), respectively. Briefly, ovarian tissue sections were deparaffinized in xylene and rehydrated in ethanol at room temperature. Endogenous peroxidase activity was blocked by immersing the slides in 3% H_2O_2 in methanol for 5 min. The sections were then rinsed several times in PBS containing Triton X-100 (0.3%, vol/vol) for 10 min. Then, to block non-specific binding of antibodies, the sections were treated for 20 min with PBS containing normal goat serum (2%, vol/vol; Vector Laboratories, Burlingame, CA, USA).

The blocking serum was tapped off, and the sections were covered with primary antibody for Factor VIII, rabbit polyclonal Factor VIII antibody (Sigma, St. Louis, MO, USA) at dilutions of 1:100 in PBS containing 2% goat serum for 1 h at room temperature or the sections were incubated overnight at 4°C in 2% PBS containing a primary antibody for eNOS, mouse monoclonal eNOS/NOS Type III antibody (1:500 dilution; Transduction Laboratories, Lexington, KY, USA). For controls, the primary antibody for Factor VIII and eNOS were replaced with normal

rabbit IgG and normal mouse IgG respectively (diluted 1:100). After washing with 10 mM PBS two times (each for 10 min), the slides were incubated with the fluorophores-labeled goat anti-rabbit IgG antibody for Factor VIII (1:250 dilution; green-fluorescent Alexa Fluor® 488; Invitrogen Molecular Probes, Eugene, OR, USA) or the fluorophores-labeled goat anti-mouse IgG antibody for eNOS/NOS Type III (1:500 dilution) for 45 min at room temperature. Coverslips were placed on all sections using Vectashield HardSet mounting medium with DAPI in order to visualize all nuclei (Vector Laboratories, Burlingame, CA, USA).

6) Proliferating cell nuclear antigen (PCNA) immunohistochemistry

Detection of PCNA was performed to determine the rate of cell proliferation of antral follicles, expressed as a labeling index (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 and quenched in 3% hydrogen peroxide for 5 min to block endogenous peroxidase activity. After washes with water and PBS, normal horse serum was added for 20 min at 4°C to prevent nonspecific binding. PCNA, monoclonal mouse antibody (1:100 dilution, MAB 2586, Cell Signaling Technology, CA, USA) was added as primary antibody, and incubated 24 h at 4°C as previously described (Grazul-Bilska et al., 2007). Primary antibody was detected by a biotinylated second antibody (antimouse antibody-IgG; 1:100 dilution, Vector Laboratories, Burlingame, CA, USA). Then, specimens were incubated for 60 min at 4°C. Diaminobenzadine (DAB) substrate was also applied to develop color of positive staining. After immunostaining, the sections were counterstained with hematoxylin. For control, the PCNA antibody was replaced with normal mouse IgG (4 µg/ml).

7) Image analysis

Images were taken from granulosa and thecal layers of mapped small, medium, large, healthy, and atretic follicles. Thereafter, follicular vasculature and eNOS expression were quantified according to the procedures described by Borowicz et al. (2007) using an Image-Pro Plus, version 4.5.1 software (Media Cybernetics, Inc., Silver Spring, MD). Briefly, images of the sections were taken from individual follicles classified by size and health status (Rodgers and Irving-Rodgers, 2010).

Then, we determined the area that exhibited positive staining for Factor VIII protein (occupied by endothelial cells) in the theca layer (green-fluorescent). Total theca layer area (μm^2) was determined, and each theca layer capillary was individually traced to quantify the total vessel area (μm^2), and number per each tissue area. Based on these measurements, capillary area density (CAD; capillary area/tissue area; %), capillary number density (CND; capillary number/tissue area; number/μm^2), and area per capillary (APC; capillary area/capillary number; μm^2/number) were calculated for each follicles image (Moonmanee et al., 2012). For each follicle, six to ten randomly chosen fields (0.025 mm^2 per field) were evaluated only for thecal layers. The data were expressed as the mean percentage ± SEM of the total area that exhibited positive staining of Factor VIII and eNOS (green-fluorescent) within each field.

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).

8) Statistical analysis

Data are presented as mean ± SEM. Follicles were classified into two groups (healthy and atretic follicle) and into three different sizes (small, medium, and large). Vasculature, LI, and eNOS were analyzed using ANOVA with the general linear model procedure of SAS (SAS Inst. Inc., Cary, NC). In present study, percentage data did not follow a normal distribution and thus, are transformed to arcsine before analysis (Sokal and Rohlf, 1995). Differences between means were evaluated by Student *t*-test and Duncan's New Multiple Range Test (Steel et al., 1997). Means were considered significantly different if $P<0.05$, unless otherwise stated. Simple liner correlations between specific variables were determined by using PROC CORR of SAS.

Table 3.3 Size and status of the follicles evaluated[a].

Animal no.	Follicles selected for evaluation																				
	Follicle 1			Follicle 2			Follicle 3			Follicle 4			Follicle 5			Follicle 6			Follicle 7		
	Diameter (mm)	Status[b] EA	EI	Diameter (mm)	Status[b] EA	EI	Diameter (mm)	Status[b] EA	EI	Diameter (mm)	Status[b] EA	EI	Diameter (mm)	Status[b] EA	EI	Diameter (mm)	Status[b] EA	EI	Diameter (mm)	Status[b] EA	EI
1	13 X 14	-	1	6 X 7	-	1	4 X 4	1	-	-	-	-	-	-	-	-	-	-	-	-	-
2	11 X 12	1	-	3 X 4	1	-	3 X 3	1	-	-	-	-	-	-	-	-	-	-	-	-	-
3	12 X 13	1	-	7 X 8	1	-	6 X 7	1	-	5 X 5	-	1	4 X 5	2	1	4 X 4	1	-	-	-	-
4	11 X 12	1	-	5 X 5	3	-	4 X 5	3	1	4 X 4	1	1	-	-	-	-	-	-	-	-	-
5	15 X 15	-	1	7 X 7	2	-	5 X 6	-	1	5 X 5	-	1	4 X 5	1	1	4 X 4	2	-	3 X 3	2	-
6	11 X 11	1	-	6 X 7	-	1	5 X 5	2	2	4 X 5	1	-	4 X 4	1	-	3 X 4	1	1	-	-	-
7	15 X 16	-	1	5 X 5	3	-	4 X 5	1	2	4 X 4	-	1	3 X 3	-	2	-	-	-	-	-	-
8	14 X 15	-	1	6 X 7	1	-	5 X 6	1	-	5 X 5	-	2	4 X 5	-	1	4 X 4	2	1	-	-	-
9	15 X 16	-	1	6 X 7	1	-	5 X 5	1	2	4 X 5	2	1	4 X 4	2	-	-	-	-	-	-	-
10	13 X 14	-	1	7 X 8	1	-	5 X 5	-	1	4 X 5	3	-	4 X 4	-	1	3 X 3	-	1	-	-	-

Abbreviations: EA, estrogen-active; EI, estrogen-inactive.

[a] n = eighty-two follicles.

[b] EA = E2:P4 ratio ≥1; EI = E2:P4 ratio <1.

3.2.3 Results

1) Follicular steroid concentrations

Data for concentrations of E2, P4 and E2:P4 ratio of healthy and atretic follicles are shown (Table 3.4). Mean concentrations of E2 and E2:P4 ratio in FF were greater ($P<0.05$), but P4 concentrations were less ($P<0.05$), in the healthy follicles compared with the atretic follicles.

The concentrations of E2, P4 and E2:P4 ratio in FF of healthy follicles are demonstrated (Table 3.5). The concentrations of E2 and P4 of FF in large and medium follicles were higher than those of small follicles ($P<0.05$). The E2:P4 ratio did not differ ($P>0.05$) among small, medium and large follicles.

2) Immunofluorescence localization and image analysis of FactorVIII and eNOS

Factor VIII (occupied by endothelial cells) and eNOS were detected in the thecal layer of healthy small (Figure 3.2a and b), medium (Figure 3.2c and d), and large (Figure 3.2e and f) follicles, whereas Factor VIII and eNOS staining were scarce in atretic small (Figure 3.3a and b), medium (Figure 3.3c and d), and large (Figure 3.3e and f) follicles. In the healthy small, medium, and large follicles, Factor VIII (Figure 3.2a, c, and e) and eNOS (Figure 3.2b, d, and f) were located exclusively in the blood vessels of the theca layers. Overall, for the small, medium, and large follicles combined, vasculature and expression of eNOS were greater significantly ($P<0.05$) in the healthy follicles than those in the atretic follicles (Table 3.4). In Table 3.5, vasculature and expression of eNOS in the theca layer were greater ($P<0.05$) in the healthy small and medium follicles compared with the large follicles.

3) Immunohistochemical localization of PCNA and labeling index

Positive PCNA staining was detected in the granulosa and theca layers of healthy (Figure 3.4a, c, and e) and atretic follicles (Figure 3.4b, d, and f). Across all follicular size classes, the LI of granulosa and theca cells of the atretic follicles were less ($P<0.05$) than those of the healthy follicles (Table 3.4). In Table 3.5, the LI of granulosa and theca layers were greater ($P<0.05$) in the healthy small and medium follicles than the large follicles. In addition, for healthy follicles across all groups, the LI of granulosa and theca decreased ($P<0.05$) as follicular size increased.

Table 3.4 Least squares means (±SEM) of follicular diameter, follicular fluid E2 and P4 concentrations, E2:P4 ratio, vasculature, eNOS expression, and labeling index (LI) of granulosa and theca cells of total antral follicles (healthy and atretic; n=82).

Follicle status	n	Diameter (mm)	E2 (ng/ml)	P4 (ng/ml)	E2:P4 ratio	Vasculature (%)[a]	eNOS (%)[b]	LI (%)[c]	
								Granulosa	Theca
Healthy	49	5.35 ± 0.3^{x}	152.60 ± 14.6^{x}	61.64 ± 3.8^{x}	3.51 ± 0.7^{x}	5.10 ± 0.2^{x}	1.26 ± 0.2^{x}	38.65 ± 1.2^{x}	32.60 ± 1.6^{x}
Atretic	33	6.51 ± 0.7^{y}	31.26 ± 10.6^{y}	218.87 ± 25.4^{y}	0.25 ± 0.2^{y}	1.92 ± 0.4^{y}	0.35 ± 0.1^{y}	15.36 ± 2.2^{y}	13.30 ± 1.4^{y}

Abbreviations: E_2, estradiol; P_4, progesterone.

[a] Area of positive staining of Factor VIII (a marker of endothelial cells) expressed as a percentage of total tissue area.

[b] Area of positive staining of eNOS expressed as a percentage of total tissue area.

[c] Labeling index (LI) = number of proliferating cells expressed as a percentage of total number of granulosa and theca cells per area.

[x,y] Different superscripts within a column indicate differences among values ($P<0.05$).

Table 3.5 Least squares means (±SEM) of follicular diameter, follicular fluid E2 and P4 concentrations, E2:P4 ratio, vasculature, eNOS expression, and labeling index (LI) of granulosa and theca cells of the healthy small (3 to 6 mm), medium (7 to 10 mm), and large follicles (>10 mm).

Follicle size	n	Diameter (mm)	E2 (ng/ml)	P4 (ng/ml)	E2:P4 ratio	Vasculature (%)[a]	eNOS (%)[b]	LI (%)[c]	
								Granulosa	Theca
Small	38	4.4 ± 0.1^{x}	115.76 ± 15.5^{x}	55.08 ± 4.5^{x}	3.48 ± 0.9	5.61 ± 0.3^{x}	1.43 ± 0.1^{x}	41.38 ± 1.4^{x}	33.75 ± 0.5^{x}
Medium	7	6.9 ± 0.2^{y}	280.99 ± 4.2^{y}	83.72 ± 8.1^{y}	3.60 ± 0.4	5.05 ± 0.4^{x}	1.33 ± 0.1^{x}	38.66 ± 1.5^{x}	32.71 ± 1.1^{x}
Large	4	11.6 ± 0.3^{z}	254.70 ± 4.9^{y}	89.31 ± 4.2^{y}	3.12 ± 0.2	3.30 ± 0.3^{y}	0.63 ± 0.2^{y}	30.44 ± 0.7^{y}	28.92 ± 0.8^{y}

Abbreviations: E_2, estradiol; P_4, progesterone.

[a] Area of positive staining of Factor VIII (a marker of endothelial cells) expressed as a percentage of total tissue area.

[b] Area of positive staining of eNOS expressed as a percentage of total tissue area.

[c] Labeling index (LI) = number of proliferating cells expressed as a percentage of total number of cells per area.

[x,y,z] Different superscripts within a column indicate differences among values ($P<0.05$).

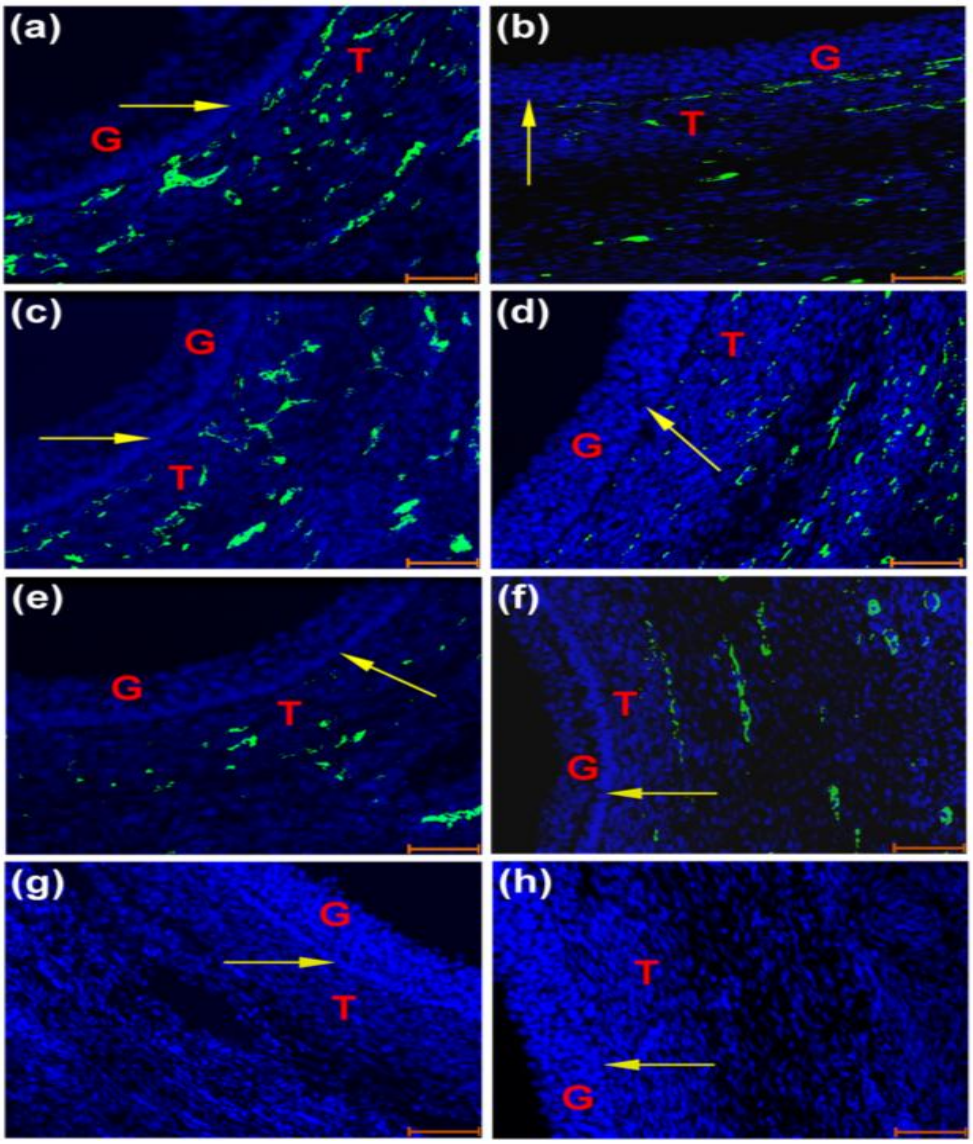

Figure 3.2 Representative fluorescent micrographs of positive staining for Factor VIII and eNOS (green) in the section of healthy small (a and b), healthy medium (c and d), and healthy large follicles (e and f) in cattle ovaries from the first follicular wave. Note the relatively stronger (a and c for Factor VIII and b and d for eNOS protein) and weaker (e and f for Factor VIII and eNOS protein, respectively) positive staining in blood vessels of the theca layers. Controls staining (no primary antibody) did not show any positive staining (g and h). Abbreviations: G, granulosa cell layers; T, theca cell layers. Arrows identify the basement membrane. Magnification using 20X objective lens. Scale bars represent 50 µm.

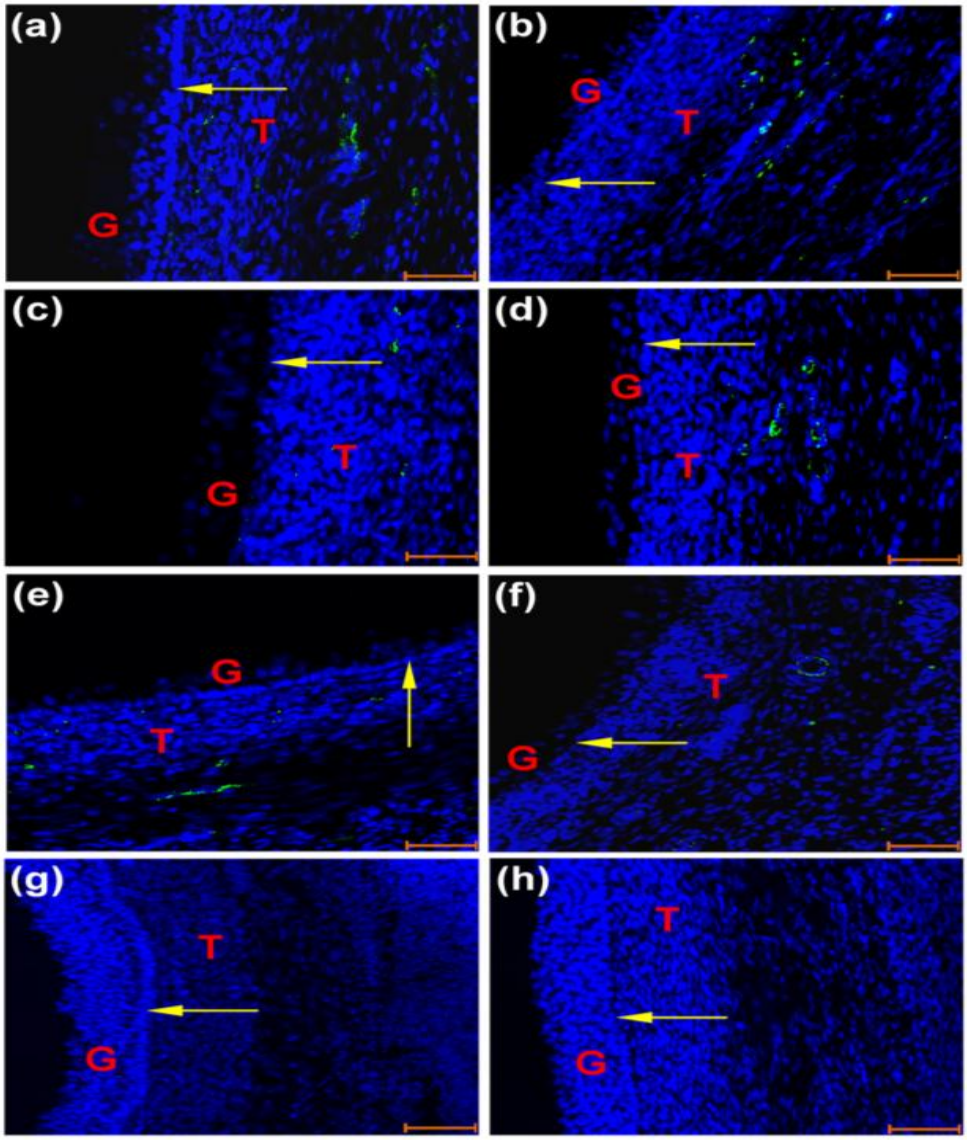

Figure 3.3 Representative fluorescent micrographs of positive staining for Factor VIII and eNOS (green) in the section of atretic small (a and b), atretic medium (c and d), and atretic large follicles (e and f) in cattle ovaries from the first follicular wave. In the theca layer of atretic follicles, fluorescent staining for Factor VIII (a, c, and e) and eNOS (b, d, and f) was weak. Controls staining (no primary antibody) did not show any positive staining (g and h). Abbreviations: G, granulosa cell layers; T, theca cell layers. Arrows identify the basement membrane. Magnification using 20X objective lens. Scale bars represent 50 µm.

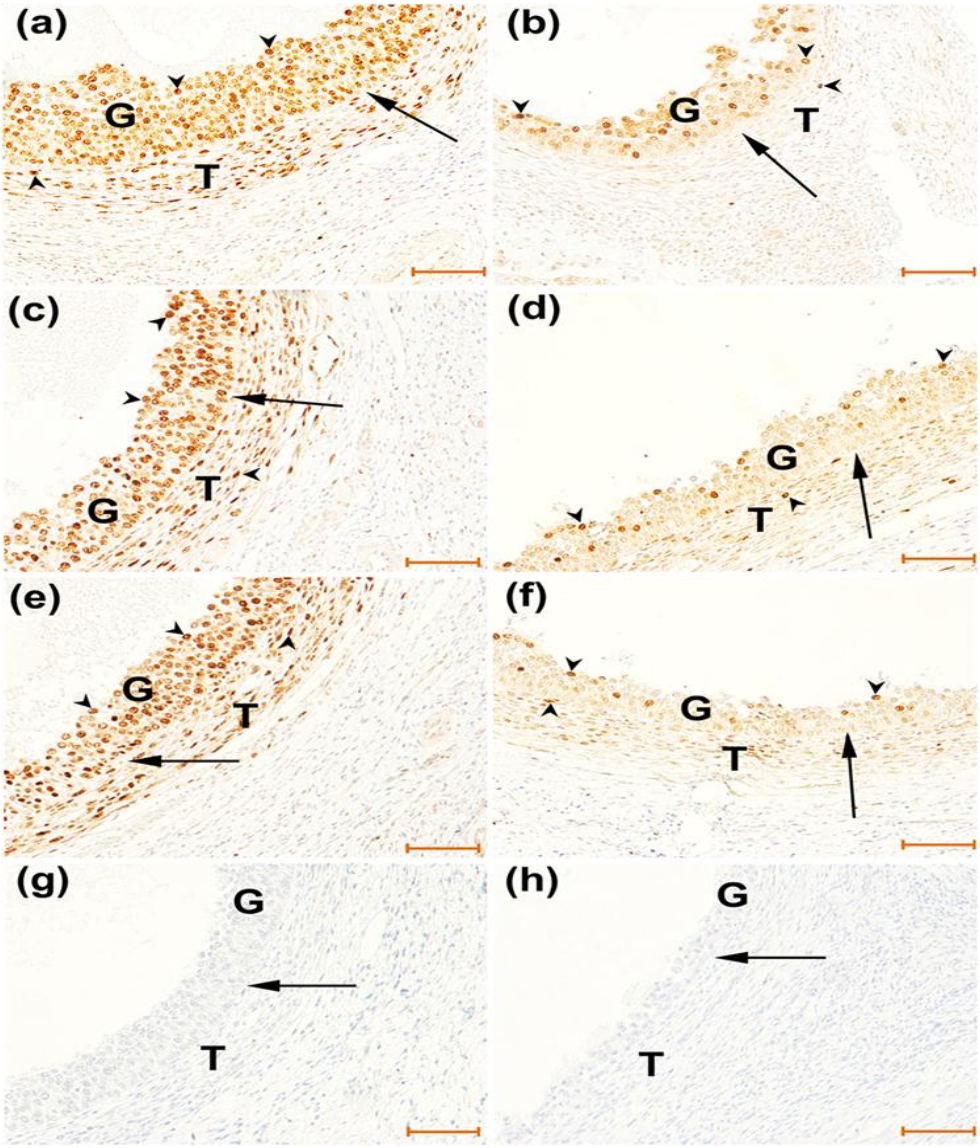

Figure 3.4 Representative micrographs of positive staining for PCNA in the section of healthy small (a), atretic small (b), healthy medium (c), atretic medium (d), healthy large (e), and atretic large follicles (f) in cattle ovaries from the first follicular wave. The brownish color indicates positive PCNA staining (arrowheads). The large whitish area on each image is the follicular antrum. Control sections (no primary antibody) did not exhibit any positive staining (g and h). 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) Correlation coefficients between specific variables of healthy follicles evaluated

For all healthy follicles evaluated, vasculature was positively correlated with eNOS protein expression and with the LI of granulosa and theca layers, but was negatively correlated with the follicular size (Table 3.6). Expression of eNOS protein was positively correlated with vasculature (CAD, CND, and APC) and with the LI of granulosa and theca layers, but was negatively correlated with the follicular size. The LI of granulosa layer was positively correlated with CAD, CND, eNOS protein expression, and with the LI of theca layer, but was negatively correlated with the follicular size. The LI of the theca layer was positively correlated with CAD, CND, APC, eNOS protein expression, and with the LI of granulosa layer, but was negatively correlated with the follicular size (Table 3.6).

Table 3.6 Correlation coefficients among variables evaluated for the healthy antral follicles (E2:P4 ratio ≥1; n=49).

Items	Follicle size (mm)	CAD[a]	CND[b]	APC[c]	eNOS[d]	LI in G[e]	LI in T[e]
CAD	-0.842	-	0.608	0.753	0.825	0.699	0.641
	P<0.001		P<0.003	P<0.001	P<0.001	P<0.001	P<0.002
CND	-0.647	0.608	-	-0.004	0.566	0.677	0.443
	P<0.002	P<0.003		P<0.987	P<0.006	P<0.001	P<0.035
APC	-0.623	0.753	-0.004	-	0.663	0.374	0.497
	P<0.001	P<0.001	P<0.987		P<0.001	P<0.079	P<0.017
eNOS	-0.875	0.825	0.566	0.663	-	0.683	0.599
	P<0.001	P<0.001	P<0.006	P<0.001		P<0.001	P<0.003
LI in G	-0.675	0.699	0.677	0.374	0.683	-	0.732
	P<0.005	P<0.001	P<0.001	P<0.079	P<0.001		P<0.001
LI in T	-0.546	0.641	0.443	0.497	0.599	0.732	-
	P<0.008	P<0.002	P<0.035	P<0.017	P<0.003	P<0.001	

Abbreviations: G, granulosa cell layer; T, theca cell layer; E2, estradiol; P4, progesterone.

[a] Capillary area density (CAD, %) = capillary area per tissue area.

[b] Capillary number density (CND, number/μm^2) = capillary number per tissue area.

[c] Area per capillary (APC, μm^2/number) = capillary area per capillary number.

[d] Area of positive of eNOS expressed as a percentage of total tissue area.

[e] Labeling index (LI) = number of proliferating cells expressed as a percentage of total number of cells per area.

3.2.4 Discussion

In the present study, Factor VIII was immunolocalized to blood vessels in the theca layer of antral follicles. Expression of eNOS was detected in the theca layer of all sizes of follicles, which is in agreement with previous studies in cattle and in sheep (Seekallu et al., 2010). In contrast, Factor VIII and eNOS were not detected in granulosa layer of all follicles, because, the granulosa layer has no blood vessels (Redmer and Reynolds, 1996). Although eNOS mRNA was detected in both theca and granulosa cell layers of ruminant species, the protein was immunolocalized only in the theca, and exclusively in the blood vessels of developing preovulatory and postovulatory healthy follicles (Grazul-Bilska et al., 2006; Tessaro et al., 2011).

The expression patterns of Factor VIII and eNOS appear to be different between follicular size and among physiologic status of follicles. In our study, the expression of Factor VIII and eNOS in thecal layers were greater in the healthy follicle compared to the atretic follicle. Thus, the pattern of Factor VIII and eNOS protein expression followed the pattern of vascular development during follicular growth by which these data support the idea of vascularity and angiogenic factors play a crucial role in follicular growth and health (Fraser, 2006). The eNOS expression had a similar pattern, as reported for *Bos taurus* cattle (Grazul-Bilska et al., 2007) and sheep (Grazul-Bilska et al., 2006) ovaries. The greater expression of eNOS seen in blood vessels of healthy follicles (small, medium, and large) compared with all atretic follicles in this study also supports a concept that NO has a role in maintaining or developing vascularization during the follicular growth and development (Austin et al., 2001).

In Brahman heifers, follicular waves of ovarian follicular development were observed with the majority of cows having two (26.5%), three (66.7%), and four (6.8%) waves, respectively (Rhodes et al., 1995b). The average estrous cycle length were 20.3, 20.8, and 24.3 d in cows with two, three, and four follicular waves (Zeitoun et al., 1996). In *Bos indicus* breeds, such as Nelore, emergence of the first follicular wave of the estrous cycle occurred 0.9 d after onset of estrus (Sartorelli et al., 2005). A greater numbers of small follicles were observed in *Bos indicus* during the emergence of the first follicular wave (Buratini et al., 2000). For follicular deviation in Zebu cattle, the deviation occurs with the smaller size (5 to 7 mm) of

largest growing follicle compared with the Holsteins heifer (8.3 mm) and lactating cow (9.8 mm) (Castilho et al., 2007; Gimenes et al., 2008; Sartori and Barros, 2011).

The present study demonstrated a greater E2 concentration in FF of the healthy follicles, compared with the atretic follicles, which indicated that the healthy follicles have a greater ability to produce E2 during a period of relatively low serum gonadotropin concentration (Fraser, 2006). The E2 is recognized as the follicular growth, differentiation, and survival factor, which enhances aromatase activity, promotes expression of LH receptors, and increases sensitivity of granulosa cells to FSH and LH (Ginther et al., 2000; Rosenfeld et al., 2001; Quirk et al., 2004; Mihm and Bleach, 2003). Cessation of growth and reductions in estrogenic activity in first-wave dominant follicle occur simultaneously with the initial decline in LH pulse frequency on day 8 of the bovine estrous cycle (Carriere et al., 1996), which is usually 6 d after emergence of the first follicular wave (Mihm et al., 2006). Although the ratio of E2:P4 did not differ among the size of the healthy follicles (small, medium and large), vasculature, eNOS, and LI were significantly less in healthy large follicles compared with healthy small and medium follicles.

In nonovulatory follicle, during follicular growth from 2 to 5 d after wave emergence, sizes achieved by individual dominant follicle were comparable to those of ovulatory follicles (Ireland and Roche, 1983; Xu et al., 1995), the granulosa cells of dominant follicle acquire more LH receptors based on mRNA encoding gonadotropin receptors (Mihm et al., 2006). LH is therefore a major transcript of angiogenic factors (eNOS). A lesser in E_2 concentrations of large and medium healthy follicles compared with small follicle in this study may influence the eNOS expression involved in mitotic activity of granulosa and theca cells which ultimately controls the physiological fate (health or atresia) of the dominant follicle.

In the present study, healthy antral follicle of *Bos indicus* on day 6 of the first follicular wave showed greater vasculature, cell proliferation, and expression of eNOS than atretic follicles. Similarly, in the *Bos taurus*, vasculature, cell proliferation, and eNOS protein expression were greater in healthy follicles (EA follicles) than in atretic follicles (EI follicles) on day 3 of the bovine estrous cycle (Grazul-Bilska et al., 2007). The vasculature of follicle is thought to be necessary for the delivery of hormones, hormone precursors, oxygen, and nutrients (Reynolds et al.,

2002). It has been suggested that the preferential delivery of gonadotropins via a more highly developed vascular system in individual follicles plays an instrumental role in selection (day 3 to day 6) and growth of the dominant follicle (Wulff et al., 2002). In the rodent model, a previous study indicated that decreased proliferation of thecal capillary endothelial cells was associated with reduced thecal vasculature and was one of the earliest events during follicular atresia (Greenwald, 1989). Based on the previous observations, a cessation in proliferation of thecal endothelial cells and a decrease in thecal vasculature occurred soon after the onset of atresia in bovine, ovine, and porcine follicles (Jablonka-Shariff et al., 1996; Fricke et al., 1997).

Proliferative activity of the granulosa and theca cells, as detected by expression of PCNA, was greater in the healthy small and medium follicles than in the large follicles. In addition, a greater proliferation rate was observed in the healthy follicles compared with the atretic follicles in cattle (Grazul-Bilska et al., 2007), sheep (Jablonka-Shariff et al., 1994), and swamp buffalo (Feranil et al., 2004). On a cellular level, successful development of ovulatory follicles requires both proliferation and differentiation of follicular cells (Jablonka-Shariff et al., 1994). This demonstrated that a significant decrease in granulosa and theca cell proliferation appears in atretic follicles on day 6 of the estrous cycle. Such high proliferation of granulosa and theca cells of healthy follicles may be closely associated with increased vasculature and eNOS protein in theca layers as observed in our study.

Until now, little study has focused on describing the characterization of follicular vasculature, follicular cell proliferation, and expression of angiogenic factors in growing antral follicles during follicle waves, and evaluating the relationships among these variables in *Bos indicus* such as Brahman crossbred beef cattle. The present study provides the first description of an association among vasculature, cell proliferation, and angiogenic expression of *Bos indicus* follicles during the first follicular wave. In this study, we demonstrated that vasculature of healthy follicles was positively correlated with eNOS protein expression, and cell proliferation, but was negatively correlated with follicular size. In addition, the vasculature of theca layers, follicular cell proliferation, and expression of eNOS protein tended to decrease as follicular diameter increases in healthy follicles after deviation. These may probably due to a rapid generation of the thecal vasculature.

Shortly after selection of the dominant follicle, cell proliferation and eNOS expression also reduced, once atresia has been initiated in the subordinate follicle (Grazul-Bilska et al., 2007; Robinson et al., 2009). Further, a novel observation of previous study was enhanced expression of VEGF and eNOS in EA follicles, which likely contributes to formation of a dense capillary network surrounding healthy EA follicles (Redmer and Reynolds, 1996; Reynolds et al., 2002).

In fact, EI follicles exhibited reduced expression of eNOS as well as reduced vasculature and LI of granulosa and theca cells, despite the fact that they were larger than the EA follicles (Grazul-Bilska et al., 2007). Thus, across all healthy follicles, vasculature was positively correlated with eNOS protein expression and LI of granulosa and theca cells, but not with follicular diameter. This result demonstrated a greater vasculature and eNOS expression in theca layers of healthy small and medium follicles, compared with healthy large follicles, which indicates that healthy small and medium follicles have a greater ability to transfer gonadotropins and nutrients during a period of relatively high rates of granulosa and theca cell proliferation. Moreover, the indication of an early decline in the E2 producing capacity during growth of dominant nonovulatory follicles may have important consequences for the regulation of genes involved in survival and apoptosis of granulosa and theca cells, the balance of which ultimately controls the physiological fate (growth or atresia) of dominant follicle (Mihm et al., 2006; Seekallu et al., 2010). Consequently, such an early change in vasculature, mitotic activity, and expression of eNOS may coincide with the beginning decrease in the E2 producing capacity of the healthy largest follicles on day 6 of the estrous cycle or in the early luteal phase (Mihm et al., 2006), however, in the present study medium and large healthy follicle did not differ in follicular fluid E_2 concentrations.

3.2.5 Conclusions

In conclusion, this study demonstrated 1) immunofluorescence localization of Factor VIII and eNOS in blood vessels of the theca layers of healthy small, medium, large, and atretic follicles, 2) greater vascularization and expression of eNOS proteins along with a greater LI in granulosa and theca cells of healthy small and medium follicles than large follicles, and with a greater LI in granulosa and theca cells of healthy than atretic follicles, 3) positive correlations between vasculature

(CAD and CND), eNOS expression in the theca, and the LI of granulosa and theca cells, and between APC, eNOS expression, and the LI of theca cells, 4) negative correlations between follicular size, vasculature (CAD, CND, and APC), eNOS expression in theca layers, and LI of granulosa and theca cells of healthy follicles from the first follicular wave in *Bos indicus*. Taken together, we suggest that differences between the healthy and atretic follicles and size of antral follicles could be explained by differences in vascularization, mitotic activity, and expression of eNOS. In the first follicular wave of the estrous cycle in *Bos indicus*, it appears that vasculature, mitotic activity and eNOS protein play a more dynamic role and are likely to be critical during the selection of dominant follicle.