Evaluation of Ovarian Follicular Health with the Markers of Endothelial and Granulosa Cells

Tossapol Moonmanee[1], Chainarong Navanukraw[1,2]*, Jiratti Thammasiri[1], Suneerat Aiumlamai[3] and Chuchat Kamollirt[4]

[1]Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand

[2]Agricultural Biotechnology Research Center for Sustainable Economy (ABRCSE), Khon Kaen Univeristy, Khon Kaen 40002, Thailand

[3]Department of Surgery and Theriogenology, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

[4]Department of Anatomy, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

**Corresponding author. E-mail: chanav@kku.ac.th*

ABSTRACT

The aim of the study was to determine the quantitative vascularity of the bovine preovulatory follicle using Factor VIII immunolocalization associated with the follicular health. Eight mature Thai native crossbred beef cows received two injections (11 day apart) of a PGF analogue to synchronize estrus. The study was conducted to test the marker of endothelial cell (Factor VIII) and granulosa cell (Pyknotic nucleus) for follicular health evaluation. Bovine preovulatory follicles (n=32) were obtained on day 6 of the estrous cycle from abattoir located in Khon Kaen provine. Follicles were classified by size as 3-6, 7-10, and >10 mm in diameter and by morphology as healthy and unhealthy. Ovarian tissue samples were fixed and paraffin-embedded prior immunohistochemistry. Factor VIII (Sigma, USA) and Pyknotic nuclei were applied as marker of endothelial cell in the theca and granulosa cell respectively. Quantitative and qualitative analysis of follicular health was determined with immunohistological assay with the aid of image analysis (Media Cybernetics, Inc., Silver Spring, USA). Pyknotic nuclei in unhealthy follicles were significantly greater than that of healthy follicles (P<0.05). Average follicular capillary area density, and capillary number density were greater (P<0.01) in healthy follicles (4.60% and 0.0027 no./μm^2) compared with unhealthy follicles (1.76% and 0.0011 no./μm^2). However, area per capillary was not different between healthy (17.32 μm^2/no.) and unhealthy follicles (14.13 μm^2/no.). These studies indicated that ovarian follicular health could be precisely evaluated with Factor VIII and Pyknotic nuclei immunohistochemistry. In addition, the study supported the relationship between the follicular health and vascularity during follicular development of the estrous cycle.

Key words: Bovine, Factor VIII, Follicular health, Pyknotic nuclei, Vascularity

INTRODUCTION

The ovarian preantral follicles represent 90% of the follicular population (Saumande, 1991; Silva et al., 2002). The mammalian ovary contains thousands of follicles at birth, but the vast majority (99.9%) becomes atretic during their growth and maturation (Carroll et al., 1990). Follicular vasculature has been found to play a key role in maintaining follicular health (Redmer et al., 1991; Navanukraw and Guntaporm, 2006). Maintenance of follicular health depends on the presence of angiogenic factors and a functional vasculature (Zeleznik, 2001). It has been demonstrated that angiogenesis and the development of vascularity may critical for maturation of the preovulatory follicle and selection of a dominant ovulatory follicle (Redmer and Reynolds, 1996). In fact, changes

of vascularization and expression of some regulators, including angiogenic factors, are associated with follicular growth and/or atresia (Fraser, 2006). In agreement with previous studies (Grazul-Bilska et al., 2007), the expression of Factor VIII, vascular endothelial growth factor (VEGF), and endothelial nitric oxide synthase (eNOS) have been used as a markers of vascularization and/or angiogenesis. Apoptosis is a marker of atresia in ovarian follicles (Hussein, 2005; Krysko et al., 2008). Therefore, the high rate of apoptosis and appearance of Pyknotic nuclei in follicles from the penultimate wave in the recent studies (Seelallu et al., 2010) indicated that atresia was initiated in these follicles.

Therefore, the objective of the present study was to determine the quantitative vascularity of the bovine preovulatory follicle using Factor VIII immunolocalization associated with the follicular health.

MATERIALS AND METHODS

The experiment was approved by the Animal Ethics Committee of Khon Kaen University (No. AEKKU 34/2551; November 20th, 2008).

Animals

Eight reproductively mature crossbred Thai native beef cattle (60 mo of age; 461.3 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 1-4 days 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 onset of estrus, at the slaughter house located in Khon Kaen province.

Ovary collection

Reproductive tracts with ovaries were collected at slaughter, placed on ice, and immediately transported to the laboratory. Surface diameter of all visible follicles was determined in two axes by using a ruler; diameter of all follicles ≥3 mm was recorded. The location of all follicles within an ovary was diagrammed.

Ovary preparation and classification of follicles

Ovaries were cut cross sectionally into several pieces (1-2 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), paraffin embedded, sectioned at 6 µm, and mounted onto glass slides as previously described (Grazul-Bilska et al., 2007).

Follicles were histologically classified into two groups: healthy and unhealthy follicle. Healthy follicles had an intact granulosa layer with a compact and well organized arrangement. On the other hand, unhealthy follicles had granulosa layer with incompact and had numbers of Pyknotic cells along the antral border of the granulosa layer (Feranil et al., 2004).

Histological evaluation of Pyknotic nuclei

Tissue ovarian were dehydrated, embedded in paraffin, sectioned serially at a thickness of 6 µm and stained with haematoxylin-eosin. Tissue sections were positioned under the 40X objective lens so that either the granulosa layer was brought into view. For one section from each of the paraffin-embedded ovarian the following characteristic were evaluated the for Pyknotic index, defined as the percentage of Pyknotic granulosa cell nuclei per total number of granulosa cells (Jablonka-Shariff et al., 1996). Tissue sections of follicles were categorized into two groups, healthy and atretic, following the established morphological criteria. All follicles with more than 5% Pyknotic granulosa cell nuclei and loosely attached granulosa cells were classified as atretic. Alternatively,

healthy follicles were those with intact membrane granulosa cells and few Pyknotic nuclei (<5% Pyknotic granulosa cell nuclei) (Bedaiwy and Hussein, 2004)

Quantification of vascularity

To evaluate the expression of Factor VIII, a marker of endothelial cells and thus vascularization, ovarian tissue sections were deparaffinized, rehydrated, and incubated with 3% H_2O_2 in methanol to eliminate endogenous peroxidase activity. The sections were then rinsed several times in PBS containing Triton X-100 (0.3%, v/v) for 10 min. 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 sections were incubated with a primary antibody against Factor VIII (1:100; rabbit polyclonal; Sigma) in PBS containing 2% goat serum for 1 h at room temperature. At the end of incubation, the sections were washed two times with 10 mM PBS (each for 5 minutes) and incubated in a secondary antibody (goat anti-rabbit IgG with Alexa Fluor Green 488; Invitrogen Molecular Probes, Eugene, OR, USA) diluted in PBS containing 2% goat serum (diluted 1:250) for 45 min at room temperature. For controls, the primary antibody was replaced with normal rabbit IgG (diluted 1:100). After immunofluorestaining of Factor VIII, the tissue sections were coverslip with mounting medium (Vectashield Hard Set with DAPI; Vector Laboratories, Burlingame, CA, USA).

Image analysis

The tissue sections immunofluorestaining of Factor VIII were visualized using a Zeiss Axiovert 200 fluorescent microscope (Zeiss, Thornwood, NY) equipped with fluorescent filters for fluorescein isothiocyanate and DAPI visualization under the 20x objective lens. Images for the two fluorescent channels were collected separately and combined using the Axiovision version 4.8.1 software package as described by Gumienny et al. (1999).

For all the follicles, images of the sections (0.025 mm^2 per field) were taken from individual follicles for each size of follicles. 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). Then, we determined the area that exhibited positive staining for Factor VIII (occupied by endothelial cells) in the theca layer. 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 (%; capillary area/tissue area), capillary number density (number of capillaries per μm^2; capillary number/tissue area), and area per capillary (μm^2; capillary area/capillary number) were calculated for each follicle image (Redmer et al., 2009).

Statistical analyses

Data are presented as mean±SEM. Follicles were classified into two groups (healthy and unhealthy follicle), vascularity in the theca layer, and Pyknotic index were analyzed using a one-way analysis of variance with the general linear model procedure of SAS. Differences between means were evaluated by a Student t-test (SAS, 2001). Means were considered significantly different if $P<0.05$.

RESULTS

In total 23 and 9 healthy and unhealthy follicles were examined, respectively. The occurrence and distribution of Pyknotic granulosa cell nuclei index was 4.39 and 12.86%, respectively (Table 1). This study demonstrates the occurrence of Pyknotic nuclei in unhealthy follicles were significantly greater than that of healthy follicles ($P<0.05$).

Table 1. Number of follicle and Pyknotic granulosa cell nuclei index between healthy and unhealthy follicle.

Follicular type	Number of follicle	Pyknotic index (%)
Healthy follicle	23	4.39 ± 0.37^a
Unhealthy follicle	9	12.86 ± 1.05^b

^{a,b}Within a column, means with different superscripts differ (P<0.05).

Granulosa cells with the histological features of Pyknotic nuclei were observed in atretic follicles either within the central region of the membrane granulosa layer (Figure 1, arrow heads) or loosely attached to the membrane granulosa near its antral surface or in the antral follicular fluid (Figure 1, circle).

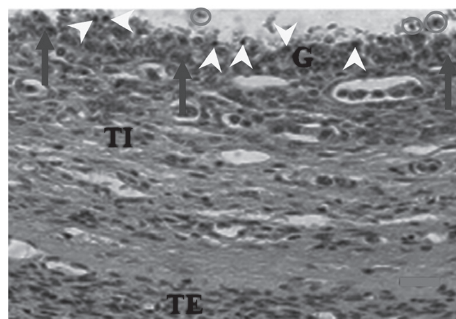

Figure 1. Histological section staining of unhealthy follicle with haematoxylin-eosin. The large white area is the follicular antrum. Small arrows identify the basement membrane. Arrow heads and circle identify Pyknotic nuclei (darkred) in the granulosa layer of unhealthy follicle. G, granulosa cell layer; TI, theca interna; TE, theca externa. Size of bar = 100 µm.

Follicular capillary area density, capillary number density, and capillary surface density were greater ($P<0.01$) in healthy follicles compared with unhealthy. However, area per capillary was not different between healthy and unhealthy follicles ($P>0.05$, Table 2).

Table 2. Follicular vascularity of healthy and unhealthy follicle.

Follicular type	Capillary area density (%)[1]	Capillary number density (no./µm^2)[2]	Area per capillary (µm^2/no.)[3]
Healthy follicle	4.60 ± 0.45^a	0.0027 ± 0.0002^a	17.32±1.93
Unhealthy follicle	1.76 ± 0.42^b	0.0011 ± 0.0002^b	14.13±2.98

^{a,b}Within a column, means with different superscripts differ ($P<0.01$)
[1]Capillary area density = capillary area/tissue area
[2]Capillary number density = capillary number/ tissue area
[3]Area per capillary = capillary area/capillary number

Figure 2 shows fluorescent micrographs of positive staining for Factor VIII in follicles. Factor VIII was predominantly detected in the theca layer of healthy follicles but was negligible in unhealthy follicles (Figure 2C and D) and in blood vessels of ovarian stromal tissues. In theca layer of healthy follicles, Factor VIII immunostaining were strong and restricted to blood vessels (Figure 2C) but were weak in unhealthy follicle and in some sections were not detectable (Figure 2D). However, Factor VIII was not detected in granulosa cells of all follicles. This study demonstrated that vascularity differed between healthy and unhealthy follicles.

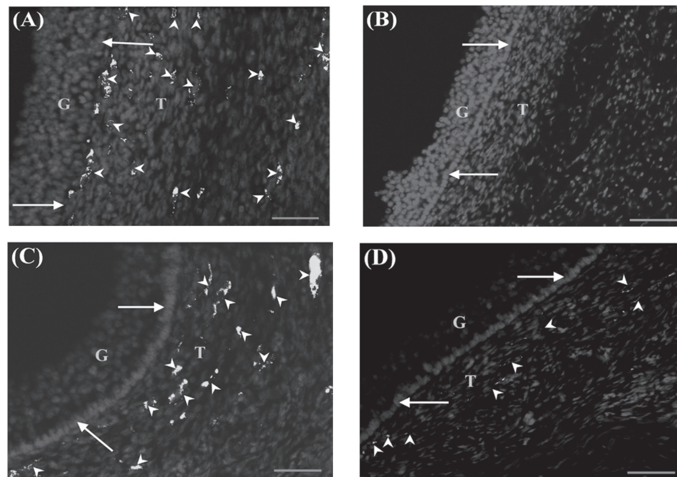

Figure 2. Representatives fluorescent micrographs of staining for Factor VIII (arrow heads) in a section of the positive staining of follicle (A), in a section of the negative control (B), healthy follicle (C), and unhealthy follicle (D). G, granulosa cell layer; T, theca cell layer. Arrows identify the basement membrane. Size of bar = 50 µm.

DISCUSSION AND CONCLUSION

In the present study, we demonstrate that the Pyknotic nuclei index in unhealthy follicles were significantly greater than that of healthy follicles. Similarly with Braw and Tsafriri (1980) reported that Pyknotic index of preantral and antral atretic follicle (unhealthy follicles) was 11.2 and 10.8%, respectively. Using light microscopy, structure that stained with hematoxyline-eosin, Pyknotic nuclei were also present in the antrum, often loosely associated with the antral layer of granulosa cells. Similar to the report of Bedaiwy and Hussein (2004), the positively stained Pyknotic nuclei were observed either in the central layers of the membrane granulosa, at the antral part or floating in the follicular antrum. In the atretic follicles Van Wezel et al. (1999) and Silva et al. (2002) observed globules of DNA and Pyknotic nuclei, frequently in membrane-bound bodies. They were originally described as arising from the swelling of cells at the surface of the membrane granulosa, followed by the dissolution of the cell membrane and later the nuclear membrane (Marion et al., 1968). It is likely that the DNA globules, as they have called them, are derived from aggregates of variable numbers of Pyknotic nuclei after dissolution of the cell membrane. The apoptotic response in the atretic follicles may be due to the activation of endonucleases that cleave genomic DNA into oligo-nucleosomal fragment (Wyllie, 1980). Of note, follicular atresia plays a critical role in recruitment of follicles for ovulation as well as in their depletion during menopause and premature ovarian failure (Gilbert et al., 1983).

The study supported the relationship between the follicular health and vascularity during follicular development of the estrous cycle. Specific changes in vascularity of theca cells layer as indicated by Factor VIII associated with follicular growth, development, and atresia are described in this our research. In the previous studies (Redmer et al., 2001; Grazul-Bilska et al., 2007) demonstrated that follicular growth is influenced by vascularization and angiogenic factors. Several angiogenic factors, including VEGF and eNOS, are expressed in ovarian follicles of several species (Grazul-Bilska et al., 2006). In sheep and cattle, it has been shown that the expression of angiogenic factors such as VEGF and eNOS is associated with enhanced vascularization of large ovarian antral follicles, which likely contributes to maintaining these follicles in a nonatretic state (Redmer and Reynolds, 1996). Both VEGF and eNOS are major regulators of follicular development in sheep (Fraser, 2006). The high expression of angiogenic factors and increased vascular surface area, as

indicated by Factor VIII expression, likely resulted in an enhanced vascular supply and hence enhanced exchange of nutrients, gonadotropins, and growth factors in follicles from the final wave compared with the follicles from the penultimate wave of the cycle (Grazul-Bilska et al., 2006, 2007). In fact, it has been demonstrated that vascularity in the theca layer may serve as an indicator of follicle health in several species. Increased vascularity in healthy follicles, as observed in our study, results in a greater capillary area density, capillary number density, and area per capillary for exchange of transcapillary nutrients, gonadotropins, growth factors, and other factors. In a monkey model, Zeleznik et al. (1981) demonstrated that the density of capillaries surrounding a healthy maturing follicle was greater than that of other smaller follicles, and that this density of capillaries was 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 conclusion, one major area of importance in the female bovine reproductive cycles is initiation of ovarian follicular growth that results in a fertile ovulation. These results indicated that ovarian follicular health could be precisely evaluated with Factor VIII and Pyknotic nuclei immunohistochemistry as markers of endothelial and granulosa cells, respectively. In addition, the knowledge of quality of follicles in each class is importance to understand follicular atresia at the antral stage as well as to provide healthy oocytes for in vitro studies.

ACKNOWLEDGEMENTS

We gratefully acknowledge the financial support from the Office of the Higher Education Commission, Thailand under the program strategic scholarships for frontier research network and the National Research Council of Thailand (NRCT). We thank Drs. Redmer and Reynolds for their help in immunohistochemisty. We would also thank Dr. Borowicz for their help in advance image analysis. We would also like to acknowledge Department of Animal Sciences, North Dakota State University (NDSU), Fargo, North Dakota, USA for the facility during the study.

REFFRENCES

Bedaiwy, M.A., and M.R. Hussein. 2004. Histological evaluation and in situ localization of apoptosis in fresh and cryopreserved ovarian tissue. Middle East. Fertil. Soc. J. 9: 163-170.

Braw, R.H., and A. Tsafriri. 1980. Effect of PMSG on follicular atresia in the inmature rat ovary. J. Reprod. Fert. 59: 267-272.

Carroll, J., D.G. Whittingham, M.J. Wood, E. Telfer, and R.G. Gosden. 1990. Extra-ovarian production of mature viable mouse oocytes from frozen primary follicles. J. Reprod. Fert. 90: 321-327.

Feranil, J.B., N. Isobe, and T. Nakao. 2004. Cell proliferation in the atretic follicle of buffalo and cattle ovary. Reprod. Dom. Anim. 39: 405-409.

Fraser, H.M. 2006. Regulation of the ovarian follicular vasculature. Reprod. Biol. Endocrinol. 4: 18-26.

Gilbert, A.B., M.M. Perry, D. Waddington, and M.A. Hardie. 1983. Role of atresia in establishing the follicular hierarchy in the ovary of the domestic hen (*Gallus domesticus*). J. Reprod. Fertil. 69: 221-227.

Grazul-Bilska, A.T., C. Navanukraw, M.L. Johnson, D.A. Arnold, and L.P. Reynolds. 2006. Expression of endothelial nitric oxide synthase in the ovine ovary throughout the estrous cycles. Reproduction 132: 579-587.

Grazul-Bilska, A.T., C. Navanukraw, M.L. Johnson, K.A. Vonnahme, S.P. Ford, L.P. Reynolds, and D.A. Redmer. 2007. Vascularity and expression of angiogenic factors in bovine dominant follicles of the first follicular wave. J. Anim. Sci. 85: 1914-1922.

Gumienny, T.L., E. Lambie, E. Hartwieg, H.R. Horvitz, and M.O. Hengartner. 1999. Genetic control of programmed cell death in the *Caenorhabditis elegans* hermaphrodite germline. Dev. 126: 1011-1022.

Hussein, M.R. 2005. Apoptosis in the ovary: molecular mechanisms. Hum. Reprod. Upd. 11: 162-178.

Jablonka-Shariff, A., L.P. Reynolds, and D.A. Redmer. 1996. Effects of gonadotropin treatment and withdrawal on follicular growth, cell proliferation, and atresia in ewes. Biol. Reprod. 55: 693-702.

Krysko, D.V., A. Diez-Fraile, G. Criel, A.A. Svistunov, P. Vandenabeele, and K.D Herde. 2008. Life and death of female gametes during oogenesis and folliculogenesis. Apoptosis 13: 1065-1087.

Marion, G.B., H.T. Gier, and J.B. Choudary. 1968. Micromorphology of the bovine ovarian follicular system. J. Anim. Sci. 27: 451-465.

Navanukraw, C., and S. Guntaprom. 2006. Mitotic activity of bovine preovulatory follicles on day 2 or day 3 of the first follicular wave. J. Sci. Technol. Human 4: 25-30.

Redmer, D.A., J.D. Kirsch, and L.P. Reynolds. 1991. Production of mitogenic factors by cell types of bovine large estrogen-active and estrogen-inactive follicles. J. Anim. Sci. 69: 237-245.

Redmer, D.A., and L.P. Reynolds. 1996. Angiogenesis in the ovary. Rev. Reprod. 1: 182-192.

Redmer, D.A., V. Doraiswamy, B.J. Bortnem, K. Fisher, A. Jablonka-Shariff, A.T. Grazul-Bilska, and L.P. Reynolds. 2001. Evidence for a role of capillary pericytes in vascular growth of the developing ovine corpus luteum. Biol. Reprod. 65: 879-889.

Redmer, D.A., J.S. Luther, J.S. Milne, R.P. Aitken, M.L. Johnson, P.P. Borowicz, M.A. Borowicz, L. P. Reynolds, and J.M. Wallace. 2009. Fetoplacental growth and vascular development in overnourished adolescent sheep at day 50, 90 and 130 of gestation. Reproduction 137: 749-757.

SAS. 2001. SAS System (Release 8.2). SAS Institute Inc., Cary, NC.

Saumande, J. 1991. Folliculogenesis in the ruminants. Rec. Med. Vet. 167: 205-223.

Seekallu, S.V., B.M. Toosi, A.T. Grazul-Bilska, and N.C. Rawlings. 2010. Markers of ovarian antral follicular development in sheep: comparison of follicles destined to ovulate from the final or penultimate follicular wave of the estrous cycle. Reproduction 140: 559-568.

Silva, J.R.V., M.A.L. Ferreira, S.H.F. Costa, R.R. Santos, F.C.A. Carvalho, A.P.R. Rodrigues, C.M. Lucci, S.N. Bao, and J.R. Figueiredo. 2002. Degeneration rate of preantral follicles in the ovaries of goats. Small Rumin. Res. 43: 203-209.

Van Wezel, I.L., A.M. Dharmarjan, T.C. Lavranos, and R.J. Rodgers. 1999. Evidence for alternative pathways of granulosa cell death in healthy and slightly atretic bovine antral follicles. Endocrinology 140: 2602-2612.

Wyllie, A.H. 1980. Glucocorticoig-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284: 555-556.

Zeleznik, A.J. 2001. Follicle selection in primates: many are called but few are chosen. Biol. Reprod. 65: 655-659.

Zeleznik, A.J, H.M. Schuler, and L.E. Reichart. 1981. Gonadotropin-binding sites in the rhesus monkey ovary: role of the vasculature in the selective distribution of human chorionic gonadotropin to the preovulatory follicle. Endocrinology 109: 356-362.

none