Original Article

Proliferation and Apoptosis of the Bitch Ovary during the Different Stages of the Oestrous Cycle

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Abstract

In order to maintain homeostasis of the ovary, proliferation and apoptosis occur simultaneously in different ovarian cell types. It was shown that steroid hormone which changed during the oestrous cycle, may regulate these mechanisms in normal mammal ovarian tissues. Therefore, the present study was aimed to study cell proliferation and apoptosis during different stages of the oestrous cycle in the bitch ovary by using immunohistochemistry and TUNEL assay. The bitch ovaries were collected at 4 different stages of the oestrous cycle, which were prooestrus, oestrus, dioestrus and anoestrus. Immunohistochemistry and TUNEL assay were applied to detect Ki-67 protein and apoptosis respectively in different cells of the bitch ovary. The results showed that the most prominent staining for both Ki-67 and apoptosis was found in granulosa cells of the ovarian follicles. During the oestrous cycle, high proliferation was observed at prooestrus and oestrus in almost all ovarian cell types while no proliferating cell was found in the bitch corpus luteum. For apoptosis, high apoptotic rate was found at prooestrus in all ovarian cells except for luteal cells, in which apoptosis was significantly higher during dioestrus compared to the other stages. These results indicated that proliferation and apoptosis varied not only among different ovarian cells but also during the stages of the oestrous cycle, which may partly involve with the levels of ovarian steroid hormones. Moreover, the granulosa cells of the ovarian follicles were the most dynamic cell types which undergo proliferation and apoptosis during the oestrous cycle from the present study.

Keywords : apoptosis, bitch, ovary, proliferation

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บทคัดย่อ

การงอกขยายและการตายของเซลล์รังไข่สุนัขในแต่ละระยะของวงรอบการเป็นสัด

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รังไข่ของสัตว์เลี้ยงลูกด้วยนมสามารถคงสมดุลได้ด้วย การเกิดการงอกขยายและการตายอย่างเป็นปกติของเซลล์ต่างๆ ของรังไข่ ซึ่งอาจจะมีส่วนเกี่ยวข้องกับระดับของสเตียรอยด์ฮอร์โมนที่เปลี่ยนแปลงในแต่ละระยะของวงรอบการเป็นสัด การศึกษาในครั้งนี้จึงมีจุดประสงค์ เพื่อศึกษาความแตกต่างของการงอกขยายและการตายของเซลล์รังไข่สุนัขที่ระยะต่างๆ ของวงรอบการเป็นสัดโดยทำการเก็บตัวอย่างรังไข่ที่ระยะต่างๆ ของวงรอบการเป็นสัด คือ ระยะโปรเอสตรัส ระยะเอสตรัส ระยะไดเอสตรัส และระยะแอนเอสตรัส หลังจากนั้นใช้วิธีอิมมูนโนฮิสโตเคมีและ TUNEL assay เพื่อตรวจหาโปรตีน Ki-67 ซึ่งสามารถบ่งบอกถึงการงอกขยาย และการตายของเซลล์ตามลำดับ ผลการศึกษาพบว่าเซลล์ที่การงอกขยายและการตายเกิดอย่างเด่นชัด คือ เซลล์กรานูโลซาของฟอลลิเคิลชนิดต่างๆ ในระหว่างวงรอบการเป็นสัด สามารถตรวจพบการงอกขยายได้สูงในเซลล์เกือบทุกชนิดของเซลล์รังไข่ที่ระยะโปรเอสตรัส และเอสตรัส แต่ไม่สามารถตรวจพบเซลล์ที่ให้ผลบวกต่อโปรตีน Ki-67 ในคอร์ปัส ลูเทียมได้เลย สำหรับการเกิดการตายของเซลล์นั้นพบว่าเซลล์รังไข่เกือบทุกชนิดมีอัตราการตายสูงในระยะโปรเอสตรัส ยกเว้นในคอร์ปัสลูเทียม ซึ่งพบเซลล์ที่เกิดการตายได้มากสุดที่ระยะไดเอสตรัส จากผลการศึกษาสามารถสรุปได้ว่าทั้งการงอกขยายและการตายของเซลล์มีการแปรผันในแต่เซลล์แต่ละชนิดของรังไข่ รวมทั้งมีการแปรผันตามระยะต่างๆ ของวงรอบการเป็นสัดอีกด้วย ทั้งนี้เซลล์กรานูโลซาของรังไข่เป็นเซลล์ที่มีการเปลี่ยนแปลงจากการเกิดการงอกขยายและการตายมากที่สุดเมื่อเทียบกับเซลล์ชนิดอื่นๆ ของรังไข่

คำสำคัญ : การตายของเซลล์ สุนัข รังไข่ การงอกขยาย

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Introduction

The morphology and function of the ovary could represent the potency in mammalian reproduction. In bitches, about half a million of follicles are observed in newborn puppies. Thereafter, the follicles develop and undergo atresia in a certain amount during postnatal life. Thus, the number of follicles decline after birth to about 500 follicles by the end of the reproductive cycle in the bitches (Concannon, 1986; McDougall et al., 1997). It has been proposed that proliferative activities and apoptosis are the mechanism involved in these events in order to maintain ovarian homeostasis in all mammals.

The development of ovarian cells is considered to be regulated by various factors including steroid hormones which change according to the stages of the reproductive cycle (Maruo et al., 1999). There are studies that show that proliferative activities occurring in granulosa and theca cells are the cause of follicular dynamic of the ovary (Monniaux et al., 1997; Isobe and Yoshimura, 2000; Feranil et al., 2004). Ki-67 is a protein which is present during all active phases of the cell cycle, but is absent from resting cells (G0). Therefore, it is an excellent marker for determining the growth fraction of the cell population (Scholzen and Gerdes, 2000). However, little is known about the proliferative status in different cells of the bitch ovary.

In addition to cellular proliferation maintain normal physiology of the ovary, the majority of follicular cells undergo follicular atresia which involve programmed cell death called apoptosis. The role of apoptosis in follicular atresia and luteal regression has been investigated in different species such as in human (Yuan and Giudice, 1997), rat (Tabarowski et al., 2005), pig (Slomczynska et al., 2006), cows (Cushman et al., 2001;

D'Haeseleer et al., 2006), buffalo (Feranil et al., 2005) and dogs (Aiudi et al., 2006; Luz et al., 2006) However, the study of apoptosis in different ovarian cell types, especially during different stages of the oestrous cycle, is still needed. Therefore, the present study was aimed to investigate cellular proliferation and apoptosis in different canine ovarian cells and at different stages of the oestrous cycle by using immunohistochemistry and TUNEL assay.

Materials and Methods

Tissue samples: Twenty four bitches, which were requested for ovariohysterectomy, were used in the present study. All bitches have reached puberty with no pathological signs of the reproductive organs. The ages of the bitches varied from 1 year to 5 years. The stages of the oestrous cycle were determined on the basis of morphological appearance of the ovary and vaginal cytology. Besides, the serum was collected from each bitch prior to the surgery in order to determine oestradiol-17β and progesterone level to confirm the oestrous cycle stages. According to these criteria, the bitches could be classified into 4 groups: prooestrus (n=6), oestrus (n=6), dioestrus (n=6) and anoestrus (n=6).

After surgery, the ovaries were immediately collected and fixed in 4% paraformaldehyde for 24-36 hrs at room temperature. Thereafter, they were histologically processed and embedded in paraffin. Then the 4 μm thick sections were cut and placed on Superfrost Plus® slides (Menzel-Glaser, Freiburg, Germany) for detection of proliferation and apoptosis.

Determination of hormone levels: Serum oestradiol-17β concentrations were measured by chemiluminescent immunoassay system, using IMMULITE® Estradiol kit (Diagnostic Products Corporation USA). The serum progesterone concentrations were measured by using a commercial solid-phase progesterone radioimmunoassay (Coat-A-Count Progesterone kit™, Diagnostic Products Corporation, CA, USA). The progesterone standards used were 0, 0.1, 0.5, 2, 10, 20 and 40 ng/ml. The within-assay coefficients of variation ranged from 7 to 9 %.

Immunohistochemistry: Proliferative activity was studied by detection of Ki-67 protein using avidin-biotin-peroxidase complex (ABC) method as described by Srisuwatanasagul et al. (2006). In brief, the antigen retrieval technique was done by a microwave oven technique at high power (750 W) in 0.01M citrate buffer, pH 6. The primary antibody used was a mouse monoclonal antibody to Ki-67 protein (MIB-1, DakoCytomation, Denmark) in a humidified chamber at room temperature (RT). The color was developed with 3,3'-diaminobenzidine hydrochloride (DAB kit, Vector Lab, Inc., USA). All sections were counterstained with Mayer's hematoxylin. Sections treated with normal mouse immunoglobulin G (IgG) (sc-2025, Santa Cruz Biotechnology Inc., USA) instead of the primary antibody, were used as negative controls. Normal canine intestine was served as positive control for Ki-67 immunostaining.

Detection of apoptosis by TUNEL assay: After being deparaffinized with xylene, the sections were rehydrated with graded alcohol and were washed in phosphate buffer saline (PBS, pH 7.4). The sections were pretreated by the microwave oven at 700 W, 3 min 3 times with the interval of 5 min after each heating. The procedure for staining apoptotic nuclei was performed using an ApopTag Peroxidase Kit (Chemicon International, Inc., CA, USA). Endogenous peroxidase activity was quenched with an aqueous solution of 3% hydrogen peroxide for 5 min. After washing the sections in PBS, they were treated with equilibration buffer for 5 min at RT and then incubated with TdT enzyme (diluted in a labeling reaction mix) for 1 hr at 37°C using parafilm cover. Negative control sections were incubated with PBS instead of TdT working enzyme. Thereafter, a stop buffer was added for 10 min at RT, followed by washing in PBS and incubating with an anti-digoxigenin conjugate for 30 min at RT. In order to visualize the labeled 3'-OH ends of DNA fragments, 3,3'-diaminobenzidine was used as a chromogen for 4 min. The slides were then

rinsed in distilled water, counterstained with Mayer's hematoxylin and mounted with glycerine gelatin.

Microscopic evaluation: In each ovarian sample, the follicles, corpora lutea, surface epithelium were evaluated. Follicles were divided into primary, secondary, tertiary and atretic follicles. The positive of Ki-67 as well as positive TUNEL cells assay were determined by counting the cells presenting a brown nuclear staining or brown apoptotic bodies. At least 100 cells of each cell types were counted at the magnification of 400x. If too many cells were present, such as in the tertiary follicles and corpora lutea, 100 cells of five random areas (100x5 fields) were counted. The results of apoptosis in the bitch ovary were presented as percentage of positive cells per each cell type.

Statistic analyses: The difference of each ovarian cell types at different stages of the oestrous cycle were analyzed by using general linear model (GLM) (version 9, SAS Institute, Inc., 2002, Cary, NC, USA). The data were subjected to Duncan's test of multicomparison among means. Results were considered to be significantly different when $p<0.05$.

Results

The results of serum levels of oestradiol-17β and progesterone were showed in figure 1. The level of oestradiol-17β was high during prooestrus and oestrus, while the level of progesterone was high during dioestrus.

Proliferation: The Ki-67 positive cells were exclusively found in the nuclei of different ovarian cells (Figure 4A). The results of all ovarian cell types at different oestrous stages were summarized in Figure 2. Regarding different ovarian cells, high proliferation was observed in the granulosa cells of various growing follicles while no Ki-67 positive cells were found in the corpus luteum nor in the follicle (germ cell). During the different stages of the oestrous cycle, high proliferation was found during proestrus and oestrus with low proliferative activity during dioestrus in almost all ovarian cells.

Apoptosis: In all bitches examined, apoptotic cell detected by TUNEL assay were completely absent in the follicles, but were obvious in the granulosa cells of the ovarian follicles (Figures 3 and 4B). Comparing between stages of the oestrous cycle, the difference was observed only in secondary, tertiary and corpus lutea. The highest apoptotic rate was significantly found in the granulosa cells of the secondary and the tertiary follicle at prooestrus, while the highest apoptotic cells in the corpora lutea were observed during dioestrus. Moreover, in some cell types and at some stages of the oestrous cycle such as in the primary follicles at oestrus, dioestrus and anoestrus, no apoptotic cell were detected by TUNEL assay from the present study.

Discussion

In the present study, both Ki-67 protein and TUNEL-positive cells could be detected in various ovarian cell types. The most prominent staining of Ki-67 and TUNEL assay was observed in the granulosa cells of the different follicles while no positive staining was found in the germ cell. As proliferating cells tend to be susceptible to apoptosis (Quirk et al., 2004), therefore our results on the proliferation and apoptosis in the bitch ovary were in agreement with this finding.

Regarding different stages of the oestrous cycle, high proliferative rate was found during prooestrus and oestrus with low proliferative rate during dioestrus in almost all ovarian cell types. This may suggest that high proliferative rate involved with high levels of oestrogen during prooestrus and oestrus. On the other hand, during dioestrus when the level of progesterone increased, proliferation was lower. These results from the present study were in agreement with other studies on proliferation in different species, which reported the positive effects of oestrogen on cell proliferation (Dorrington et al., 1993; Bai et al., 2000; Medh and Thompson, 2000; Quirk et al., 2004; Zhu and Pollard, 2007; Perniconi et al., 2008) and antiproliferative effect with progesterone (Chaffkin et al., 1993) especially in

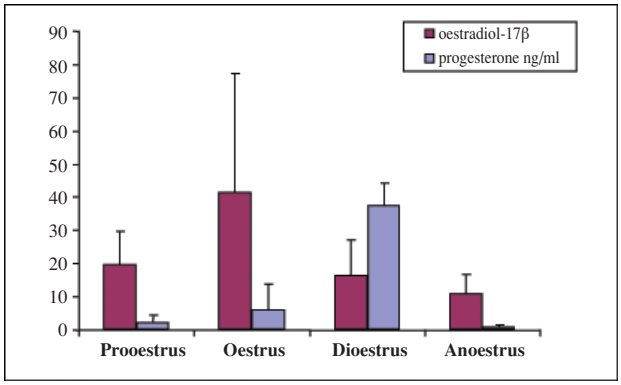

Figure 1 Serum levels of oestradiol-17β and progesterone at the different stages of the oestrous cycle (Mean±SD).

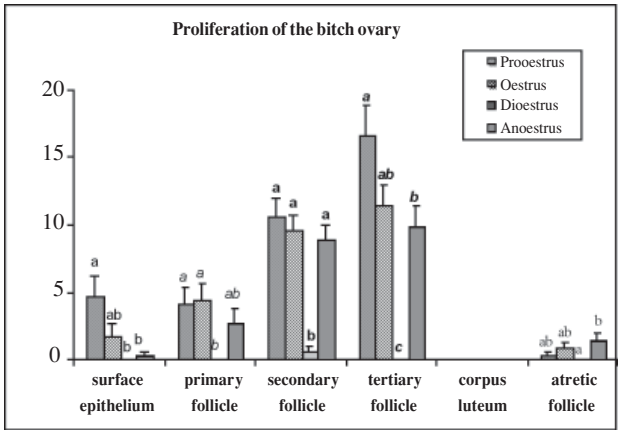

Figure 2 Proliferation of the different ovarian cell types during the stages of the oestrous cycle (Mean±SD). Different letters within the same category represented significantly different.

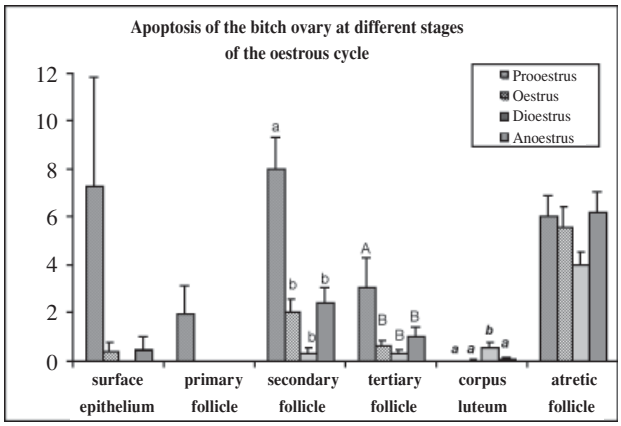

Figure 3 Apoptosis of the different ovarian cell types during the stages of the oestrous cycle (Mean±SD). Different letters within the same category represented significantly different.

cancer cells (Dai et al., 2002; Fauvet et al., 2006). However, the influence of oestrogen and progesterone on proliferation was cell type specific since different ovarian cells showed different proliferative rates in spite of the same level of plasma steroid hormone from the present study.

In the corpus luteum, no proliferating cell was found in this study and only small number of apoptotic cells were observed. This may indicate that the luteal cells may proliferate in a short period and would not be detected by Ki-67 but may enter the apoptotic pathway for luteal regression. In addition, Boos (1998) demonstated that luteal cellular proliferation was restricted mainly to theca interna-derived cells and that Ki-67 positive cells were mainly found in vascular walls and connective tissue of the bovine corpus luteum. This may explain the result of the present study which could not detect Ki-67 protein in the bitch luteal cells at diestrus.

For apoptosis, the TUNEL assay, which detected DNA fragmentation was used in the present study. In comparison with the cascapse-3 assay, which can present the DNA fragmentation only at the onset of the apoptotic process, The TUNEL assay has the advantage of maintaining the DNA fragmentation. (D'Haeseleer et al., 2006). The significantly higher rate of apoptosis was detected in the granulosa cells of the secondary and tertiary follicle at prooestrus while the highest rate of apoptosis was found at dioestrus in the corpora lutea. This indicated that apoptosis varied among different cells and different stages of the oestrous cycle as suggested for cell proliferation. An earlier study in bovine suggested that high progesterone level could trigger apoptosis in non-ovulatory tertiary follicle during the oestrous cycle (Yang and Rajamahendran, 2000). However, the results from the present study were different as higher apoptotic rate was always found during prooestrus and oestrus, when the level of progesterone is low. These may account to the differences in the oestrous cycle as well as ovulation patterns between these two species. In addition, high proliferation was found in the bitches

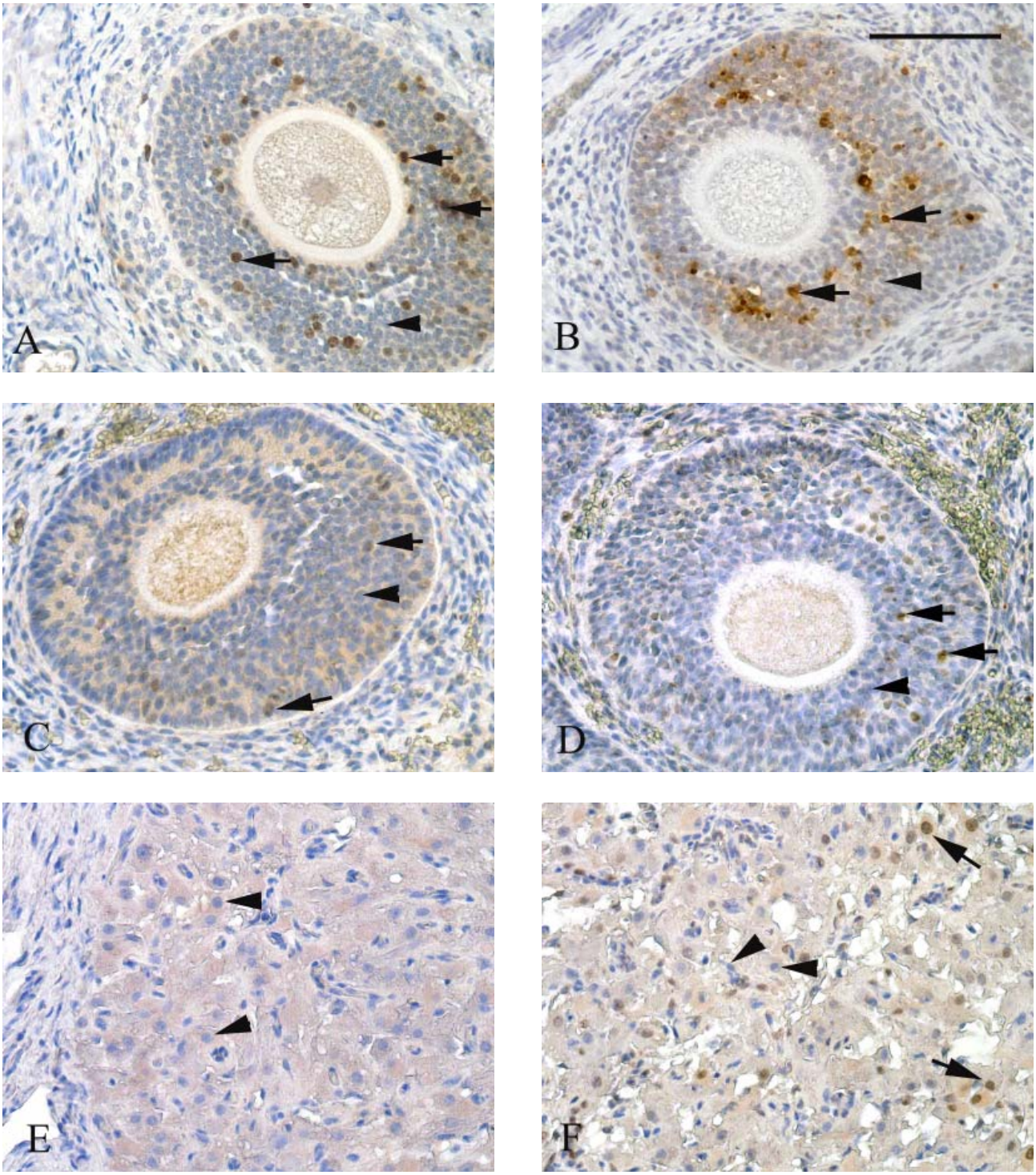

Figure 4 Proliferation (A, C and E) and apoptosis (B, D and F) of the bitch ovary during prooestrus (A-B) and dioestrus (C-F). Arrows represent positive staining cells while arrow heads represent negative staining cells in the granulosa cells of ovarian follicles (A-D) and in the luteal cells (E and F). Scale bar = 100 μm.

during the stage of prooestrus and oestrus which should be the reason why higher apoptotic rate was also needed during these periods.

Regarding corpus luteal regression, the study in the bitch by Luz et al. (2006) suggested that apoptosis may not be the major mechanism involved in this phenomenon as apoptotic cells were rarely detected. Our result was similar. There were only few TUNEL positive cells in the corpora lutea although they were significantly higher during dioestrus compared to the other stages. In contrast, another earlier study reported that apoptosis plays an important role in the bitch luteal regression (Aiudi et al., 2006). The disagreement between these two studies happened due to different methods of apoptosis evaluation in the bitch luteal cells. Moreover, apoptotic changes may occur much earlier before morphological changes of cell death; therefore, less apoptotic cells could be detected in luteal regression mechanism. However, more investigation was needed to clarify the mechanism of luteal regression in the bitch ovary.

In general, apoptosis occurs at the highest frequency in proliferating tissues rather than quiescent tissues in order to maintain the equilibrium between cell growth and cell death (Guo and Hay, 1999; Schutte and Ramaekers, 2000). On the other hand, cells that have exited the cell cycle are resistant to apoptosis. The finding from these earlier studies supported our results that the highest proliferation and apoptosis were prominently found in the granulosa cells of the ovarian follicles compared to luteal and ovarian surface epithelial cell.

In conclusion, the present study showed that proliferation and apoptosis differed not only in different ovarian cell types, but also at different stages of the oestrous cycle, which may depend on the level of steroid hormones. The granulosa cells of the ovarian follicles were the most dynamic cells which undergo proliferation and apoptosis. Moreover, it was not completely clarified that apoptosis was the major mechanism involved in luteal regression in the bitch ovary and therefore, further investigation was needed.

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