Inducible Nitric Oxide Synthase Expression and Luteal Cell DNA Fragmentation of Porcine Cyclic Corpora Lutea*

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ABSTRACT : Nitric oxide (NO) derived from inducible nitric oxide synthase (iNOS) is involved in cell apoptosis, which contributes to luteal regression and luteolysis in some species. In large domestic animals, no direct evidence for the relationship between NO and cell apoptosis in the process of corpus luteum regression is reported. The present study was conducted to investigate the localization of iNOS on porcine corpora lutea (CL) during the oestrus cycle and its relation to cell DNA fragmentation and CL regression. According to morphology, four luteal phases throughout the estrous cycle were defined as CL1, CL2, CL3 and CL4. By isoform-specific antibody against iNOS, the immunochemial staining was determined. Luteal cell DNA fragmentation was determined by flow cytometry. The results showed that no positive staining for iNOS was in CL1 and that iNOS was produced but limited to the periphery of CL2, while in the CL3, the spreading immunochemical staining was found inside the CL. No iNOS positive staining was detected in CL4. Meanwhile, DNA fragmentation increased dramatically when CL developed from CL2 to CL3 ($p<0.05$). In CL4, higher proportion of luteal cells still had fragmented DNA than that of luteal cells from CL1 or CL2 ($p<0.05$). These results indicate that iNOS expression is closely related to luteal cell apoptosis and then to luteal regression. *(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 5 : 626-631)*

Key Words : Nitric Oxide Synthase, Corpora Lutea, Apoptosis, Estrous Cycle

INTRODUCTION

In the mammalian ovary, the corporus luteum (CL) is a transitory tissue formed from post-ovulatory follicles. The cyclic regression of this particular endocrine gland is one of its most intriguing features, as it allows repeated opportunities for follicular growth, ovulation and pregnancy. Accumulating evidence has shown that apoptosis is the underlying mechanism of follicle atresia and the regression of CL in many species (Juengel et al., 1993; Bacci et al., 1996; Yoon et al., 2002; Ptak et al., 2004; Takiguchi et al., 2004). Fragmentation of genomic DNA is one of the hallmarks of apoptosis and therefore can be used to evaluate the apoptotic cells (Matsuyama et al., 1996; Tao et al., 2004b).

It is well known that nitric oxide (NO) is an important biological messenger which exhibits a wide range of effects during physiological and pathophysiological processes, such as apoptosis (Kerr et al., 1972; Bacci et al., 1996; Vega et al., 2000). NO is also closely correlated to many reproductive activities in mammalian species (Shukovski

and Tsafriri, 1994; van Voorhis et al., 1994; Chun et al., 1995; Dixit and Parvizi, 2001). Our previous studies also showed evidence that NO was involved in mouse and porcine oocyte meiotic maturation (Bu et al., 2002; Bu et al., 2003; Tao et al., 2004a).

NO is synthesized from L-argentine by nitric oxide synthase (NOS), of which there are 3 isoforms: brain NOS (bNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). By now, no bNOS has been found in mammalian ovaries while eNOS and iNOS can be expressed (Zackrisson et al., 1996; Jablonka-Shariff and Olson, 1997; Takesue et al., 2003). Among the 3 isoforms of NOS, iNOS produces the highest amount of NO, which is related to the apoptotic effect (Dixit and Parvizi, 2001). As for the localization of iNOS, the previous reports were not identical, even in the same animal, such as in rat (Jablonka-Shariff and Olson, 1997; Matsumi et al., 1998).

In swine, Grasselli et al. (2001) found that the expression of iNOS was absent in follicles, but it is still unknown how iNOS expresses in porcine CL. Although it was found that the regression of pig CL was related to apoptosis (Bacci et al., 1996; Wuttke et al., 1997; Ptak et al., 2004; Takiguchi et al., 2004) and the high concentration of NO has a pro-apoptotic effect (Dimmeler and Zeiher, 1997), there is a lack of direct evidence that there is a relationship between iNOS expression and luteal cell development or regression, especially in swine. The aim of the present study was to localize iNOS in 4 phases of pig CL and to test if it is parallel with luteal cell apoptosis.
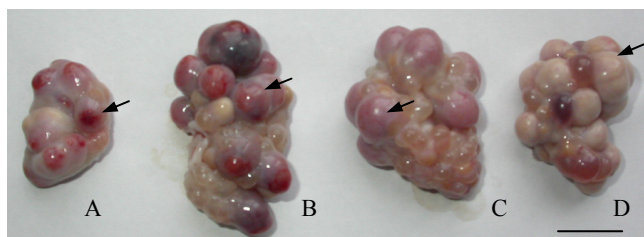
Figure 1. Stereoscopic micrographs of porcine corpora lutea at different stages. A: CL1, newly developed corpora luteum with fresh blood clots inside (arrow); B: CL2, spongy liver-red corpora luteum with noticeable surface blood vessels and traces of blood clots inside (arrow); C: CL3, soft large pink corpora luteum with surface vessels but no clot inside (arrow); D: CL4, pale-pink corpora luteum substantial in structure with vanishing superficial capillaries (arrow). Bar = 10 mm.

MATERIALS AND METHODS

Tissue collection

Ovaries were collected from a local slaughterhouse from healthy and sexually mature pigs. The phase of CL was determined by morphological criteria previously described (Gebarowska et al., 1997). Briefly, there are 4 stage of CL included. (1) Newly developed CL, with fresh blood clots inside, 1-3 d after ovulation (CL1); (2) Spongy liver-red CL with noticeable surface blood vessels and traces of blood clots inside, 5-7 d after ovulation (CL2); (3) Soft large pink CL with surface vessels but no clot inside, 8-10 d after ovulation (CL3) and (4) Pale-pink CL substantial in structure with vanishing superficial capillaries, 12-14 d after ovulation (CL4) (Figure 1). Three or four ovaries of each phase were collected to conduct the experiment.

Immunohistochemical analysis of iNOS

Pig ovaries were cut into 3-4 mm pieces, fixed in 4% paraformaldehyde at 4°C overnight, dehydrated in ethanol, cleared in toluene and embedded in paraffin wax. Five μm sections made by microtome (Leica, Germany) were deparaffinized, rehydrated, immersed in 5% (v/v) H_2O_2 for 30 min and washed in Tris-buffered saline (TBS). Then, the sections were treated by 0.25% (v/v) Triton ×100 for 10 minutes and incubated in 20% (v/v) normal goat serum for 1 h. Then they were incubated with the primary antibody against iNOS (rabbit polycolonal antibody, SA200, Biomol, USA) or normal rabbit serum at a dilution of 1:500 at 4°C overnight. After being washed in TBS, the sections were incubated with biotinylated goat antiserum to rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:400 for 1 h at room temperature, and washed in TBS. After incubation of the slides in avidin-streptavidin-biotin horseradish peroxidase (HRP) complex (ZB-2404, Lot#N0907, Zhongshan Biotechnology, Zhongshan, CHN) for 1 h at room temperature (dilution 1:150 in PBS-1%
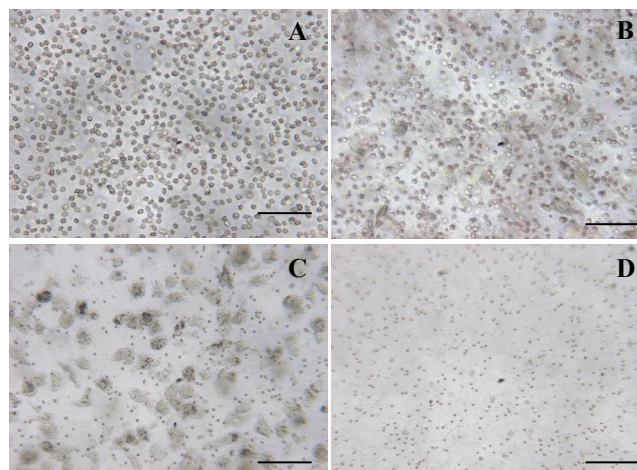
Figure 2. Luteal cells isolated from porcine corpora luteum at different stages. Luteal cells at different stages were different in size. A: luteal cells isoloated from CL1; B: luteal cells isoloated from CL2; C: luteal cells isoloated from CL3; D: luteal cells isoloated from CL4. All bars=400 μM.

BSA), the iNOS immunoreactivity was visualized by exposing the specimens to Tris-buffered 0.05% 3, 3'-diaminobenzidine (DAB) (Zhongshan Biotechnology, CHN) activated with 0.03% H_2O_2 (Beijing Chemical Plant, CHN) for several seconds. Peroxidase activity was revealed by diaminobenidine and H_2O_2.

The specimens were dehydrated by three changes of ethanol and then three changes of xylene, and mounted in Mount Quick (Daido Sangyo Co., Japan) and observed under a microscope. To appreciate negative nuclei better, all sections were counterstained with hematoxylin.

Three repeated trials were undertaken.

Luteal cell Collection and DNA fragmentation analysis

In order to find the relationship between luteal regression and luteal cell apoptosis, we detected the luteal cell DNA fragmentation since it is closely related to apoptosis (Tao et al., 2004b). Luteal cells were obtained from pools of freshly excised corpora lutea of three animals in the same phase. In brief, the corpora lutea at different stages were isolated from porcine ovaries freshly collected from a local abattoir. The corpora lutea were rinsed with PBS and then the stromal tissues were removed by watch forceps and an eye scalpel. The corpora lutea were minced mechanically to very small pieces. Then the mixture was filtered by 300×cell sieve and then cells were treated with 0.9% prewarmed ammonium chloride at 37°C for 1 min to remove red blood cells. The cells were re-suspended in PBS supplemented with 2% (v/v) new born calf serum (NBCS) and the ammonium chloride was removed. The cells were processed to produce a single-cell suspension (Figure 2). According to our previous report (Tao et al., 2004b), flow cytometry (FCM) analysis was performed. In brief, luteal
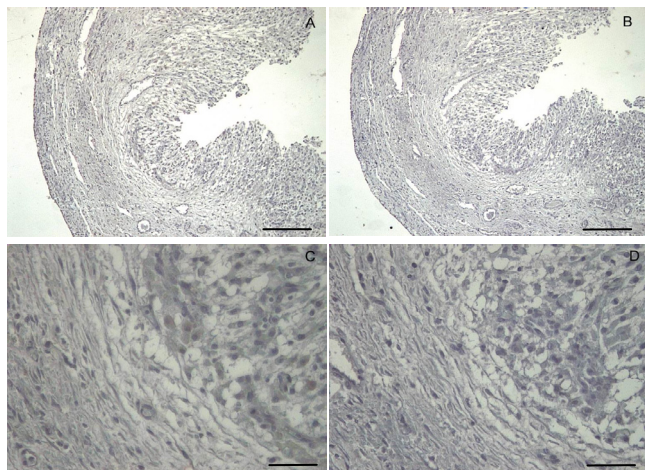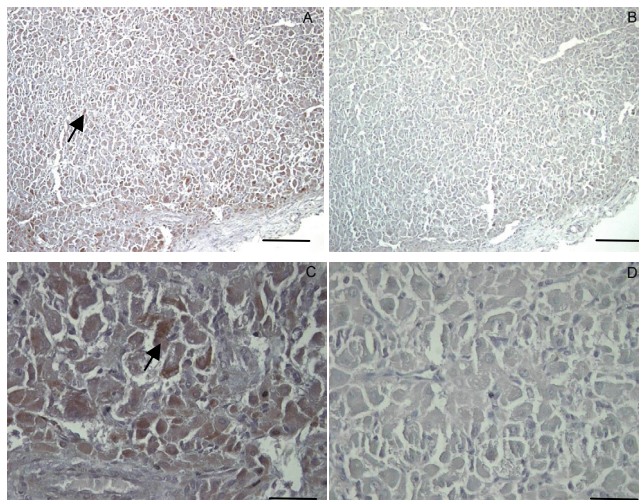
Figure 3. Immunohistochemistal staining of iNOS in pig CL1. Blue staining show the nuclei by hematoxylin. A, C: primary antibody against iNOS; B, D: normal rabbit serum instead of primary antibody. No positive cells were detected in this stage of CL. A, B: Bars=400 μM; C, D: Bars=100 μM.
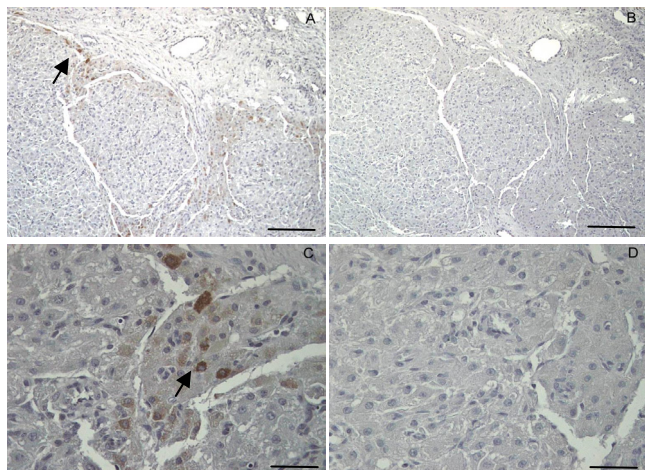
Figure 4. Immunohistochemistal staining (brown) of iNOS in pig CL2. Blue staining shows the nuclei by hematoxylin. A, C: primary antibody against iNOS; B, D: normal rabbit serum instead of primary antibody. iNOS was localizes in the periphery (arrow in A) and in the cytoplasm of luteal cells (arrow in C). A, B: Bars=400 μM; C, D: Bars=100 μM.

cells were counted and 1×10^6 cells were desirable for flow cytometry analysis. The cells were then fixed in 70% cold ethanol overnight and the analysis was undertaken in 2 weeks. Before analysis, the solution was washed with PBS to remove the ethanol and the cells were re-suspended in PBS. RNase (50 μg/ml) was introduced to the solution at 37°C for 30 min and then was washed with PBS and removed from the suspension by centrifugation at 1,000×g 5 min. Cells were resuspended in 100 μl PBS and then stained with 50 μg/ml propidium iodide (PI) supplemented with Triton for 30 min in dark. Filtration of the solution was conducted with 200×cell sieve before flow cytometry

Figure 5. Immunohistochemistal staining (brown) of iNOS in pig CL3. Blue staining shows the nuclei by hematoxylin. A, C: primary antibody against iNOS; B, D: normal rabbit serum instead of primary antibody. iNOS spreads all over CL in CL3 (arrows in A and C). A, B: Bars=400 μM; C, D: Bars=100 μM.

analysis (BD LSR). The coefficient of variation was controlled to 3%.

The experiment was repeated 3 times.

Statistical analysis

The data for luteal cell DNA fragmentation are presented as mean±SEM. The results were analysed using an unpaired Student's t-test. p<0.05 was considered statistically significant.

RESULTS

Immunochemical localization of iNOS

With an isoform-specific antibody against iNOS we examined the expression of iNOS in pig CL throughout the estrous cycle. The results showed that no iNOS positive staining was found in CL1 (Figure 3A, 3C). With the development of CL, iNOS was found to be expressed in CL2 (Figure 4A, 4C). In this stage, iNOS staining was limited to the periphery (Figure 4A) and some, not all, of parenchymal cells were iNOS positive, especially in the larger cells. Specifically, the positive staining was limited in the cytoplasm (Figure 4C). Then the positive staining spread to the deep area in CL3 and almost all the parenchymal cells showed strong iNOS staining (Figure 5A, 5C). Moreover, iNOS immunoreactive staining was limited in the cytoplasm. In CL4, all the luteal cells showed no iNOS immunochemial staining and only the nuclei of the luteal cells showed clearly stained with hematoxylin (Figure 6A, 6C). In the meantime, the sections of each CL phase were incubated with normal rabbit serum instead of antibody (Control). They showed no positive staining.
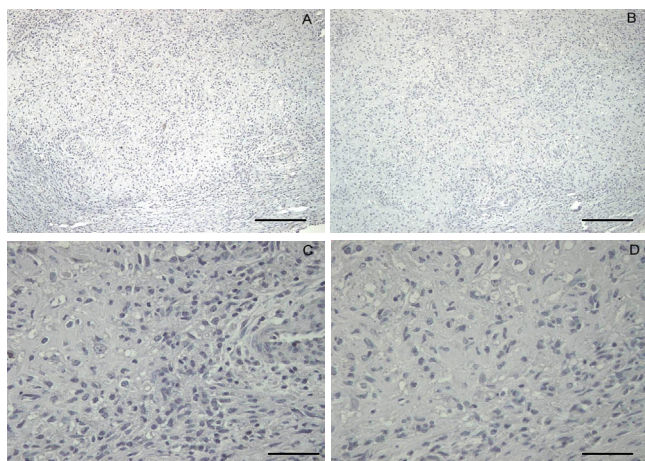
Figure 6. Immunohistochemistal staining (brown) of iNOS in pig CL4. Blue staining shows the nuclei by hematoxylin. A, C: primary antibody against iNOS; B, D: normal rabbit serum instead of primary antibody. No iNOS positive cells were detected in CL4. A, B: Bars=400 μM; C, D: Bars=100 μM.

DNA fragmentation of CL cells

By flow cytometry, DNA fragmentation of CL cells was determined. The results were listed with statistical analysis in Table 1. Approximately, 4% of CL1 cells had been fragmented. This proportion increased to 5% in CL2 but there was no statistical difference ($p > 0.05$). CL3 cells had a high rate of luteal cells with fragmented DNA (30%). Still, the rate was 24% in CL4, significantly higher than that of CL1 or CL2 ($p < 0.05$) but not significantly different from that of CL3 ($p > 0.05$).

DISCUSSION

The objective of this study is to test if iNOS expression in porcine CL is parallel with luteal cell apoptosis and CL regression. We find that luteal cell DNA fragmentation was closely related to iNOS secretion and that luteal development and regression is parallel with luteal cell DNA fragmentation, and iNOS expression is prior to luteal cell DNA fragmentation. The evidence indicates that iNOS is functionally responsible, at least partly, for CL regression by mediating luteal apoptosis.

Mammalian CL regression is necessary for the cyclicity of the reproductive process, and occurs as the synchronous loss of cellular function and subsequent cell death of the corpus luteum. CL regression consists of two processes: functional and structural luteolysis. Functional luteolysis refers to the suppression of progesterone synthesis and secretion, while structural luteolysis refers to the physical elimination of CL from the ovaries (Bacci et al., 1996). Apoptosis was the molecular basis of CL regression. NO could act with a dual action (protective and pro-oxidant) in CL development. Specifically, anti-apoptotic effects are

Table 1. DNA fragmentation of porcine luteal cells during different luteal phases

Stage	DNA fragmentation
CL1	4.06±0.97%
CL2	5.12±1.36%
CL3	30.35±4.74%*
CL4	23.69±3.58%*

Luteal cell DNA fragmentation was determined by flow cytometry. Values are expressed as percentage mean±SEM of three experiments.
* $p < 0.05$ versus CL1 or CL2.

mainly mediated by low amounts of NO or stimulation of eNOS while the proapoptotic effects appear to be linked to the production of high concentrations of NO by the activity of iNOS (Dimmeler and Zeiher, 1997; Motta et al., 2001). It has been reported that iNOS was expressed in CL of rats (Jablonka-Shariff and Olson, 1997), rabbits (Boiti et al., 2002) and humans (Vega et al., 2000). The present results showed that iNOS was also expressed in porcine CL. In the meantime, according to the method we reported previously, we examined luteal cell DNA fragmentation, which could, at least partly, reflect the apoptosis (Tao et al., 2004b).

We found that no iNOS expression was observed at the first stage of CL. It is reasonable that comparatively fewer luteal cells undergo apoptosis. When CL progressed to second stage, i.e. 5-8 d after ovulation, a few luteal cells began to produce iNOS. At this time, however, apoptotic cells characterized with fragmented DNA didn't increase significantly. It seems that a small amount of newly secreted iNOS is not enough to immediately activate apoptosis and that iNOS expression is prior to apoptosis. Strongest expression of iNOS was found in CL3. Consequently, DNA fragmented cells dramatically increased. It appeared that much NO had been produced and then induced apoptosis. Finally, the immunostaining for iNOS disappeared again at the late stage of CL. These cells isolated from regressing CL are losing their biochemical functions. But the DNA fragmentation was still serious and contributed to luteolysis. The iNOS was produced prior to luteal cell DNA fragmentation and such fragmentation continued after CL stopped secreting iNOS. These results indicate that iNOS expression is the up-stream event for apoptosis of luteal cells. It is understandable that the protein is synthesized at first and NO is then generated and triggers the apoptosis of luteal cells. This result was basically identical to the previous study. By electrophoretic and histochemical analysis, Matsuyama et al. reported that distinct DNA fragmentation occured in proestrus rat CL while the extent significantly decreased at estrus (Matsuyama et al., 1996). By molecular analysis, Boiti et al. found that iNOS mRNA was barely detectable throughout the late luteal stages during spontaneous luteolysis in pseudopregnant rabbits (Boiti et al., 2004).

Interestingly, we found that iNOS expressed in

parenchymal cells in the periphery of CL2, and the expression pattern changed and positive staining moved to the deep area of CL3. In 1997, Gebarowska et al. reported that LH receptor was located in the periphery of sow CL1. In CL2 and CL3, LH receptor spread to the central area (Gebarowska et al., 1997). This indicated the interrelation between LH and iNOS expression.

NO exerts a physiological role as modulator of luteal demise. Meanwhile, increased evidence shows that luteal cell apoptosis is controlled by a number of regulatory factors in many mammalian species, such as rats, sheep, cattle, rabbits and humans (Moeljono et al., 1976; Boone and Tsang, 1998; Faletti et al., 1999; Gaytan et al., 1998; Goodman et al., 1998; Roughton et al., 1999; Rueda et al., 2000; Ziecik, 2002; Takiguchi et al., 2004). In pig, cytokines, growth factors, peptide hormones and steroids are also related to CL regression (Gregoraszczuk, 1983; Gadsby et al., 1996; Bacci et al., 1996; Nicholson et al., 1999; Ge et al., 2000; Ptak and Gregoraszczuk, 2003; Ptak et al., 2004). These luteal factors may be involved in the up-regulation of NOS activity, while downstream NO may induce the regression of CL more directly.

It is concluded that iNOS expression changes with the development of porcine CL and is closely related to luteal cell apoptosis, which contributes to porcine luteolysis or luteal regression.

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