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Expression and localization of cFLIP anti-apoptotic protein in the porcine corpus luteum and corpora albicans during the estrous cycle and pregnancy

H.Z. Jin[1], W.S. Shi[1], Y. Tian[1], Y. Liu[1], Y. Jin[1] and N. Manabe[2]

[1]Department of Animal Science, College of Agriculture, Yanbian University, Yanji, China
[2]Animal Resource Science Center, University of Tokyo, Kasama, Japan

Corresponding author: Y. Jin / N. Manabe
E-mail: yijin@ybu.edu.cn / amanabe@mail.ecc.u-tokyo.ac.jp

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ABSTRACT. We determined expression and localization of the anti-apoptotic cellular FLICE inhibitory protein (cFLIP) in the porcine corpora lutea (CL) and corpus albicans (CA) during estrous and pregnancy. The CL and CA were collected at different stages of estrous to determine cFLIP immunolocalization, and mRNA and protein expression. The mRNA expression of the short cFLIP isoform ($cFLIP_S$) was higher at the early and mid CL stages, and lower by the late CL stage ($P < 0.01$); mRNA expression of the long cFLIP isoform ($cFLIP_L$) was higher at the mid CL stage, and lower at the early and late CL stages ($P < 0.01$). Levels of $cFLIP_S$ and $cFLIP_L$ were steady and high during the early and mid CL stages, and had significantly decreased ($P < 0.01$) by the late stage. The cFLIP protein was highly expressed in the early and mid CL stages of estrous, but weakly expressed in the late stage. Expression of $cFLIP_S$ showed no significant difference between preovulatory corpus albicans (CA1) and corpus

albicans (CA2) coexistent with the CL from the previous estrus, but $cFLIP_L$ mRNA expression was higher during CA1 than CA2. The expression of $cFLIP_S$ showed no significant difference between CA1 and CA2, but $cFLIP_L$ was not detected. The cFLIP protein was weakly expressed in the CA. Expression of $cFLIP_S$ and $cFLIP_L$ mRNA and proteins was observed in the CL, and the cFLIP protein was highly expressed during pregnancy. We propose that $cFLIP_{S/L}$ acts as a survival factor, and performs an anti-apoptotic function in the porcine CL.

Key words: Porcine; Corpus luteum; Corpus albicans; Apoptosis; cFLIP

INTRODUCTION

Mammals have evolved a signaling mechanism that actively directs cells to die by apoptosis. Apoptosis is critical, particularly in the immune system, and is induced by death ligands. It can be initiated through engagement of certain members of the tumor necrosis factor (TNF) receptor super-family, such as Fas, Fas ligand (FasL), TNF-α, and TNF-related apoptosis-inducing ligand. When a death ligand binds to a death receptor located on the cell membrane, an apoptotic signal is transduced into the cell. A Fas-mediated apoptosis signaling pathway has been suggested as follows: FasL binds to the extracellular domain of Fas, and Fas is activated; the Fas-associated death domain (FADD) binds to the intracellular domain of activated Fas through the DD; procaspase-8 binds to the FADD through the death effector domain (DED), and is activated; activated caspase-8 causes the activation of procaspase-3, activated caspase-3 activates nucleases, and apoptosis is induced (Nagata, 1997).

Cellular FLICE inhibitory protein (cFLIP; also called CASH, Casper, CLARP, FLAME, or IFLICE) has two isoforms, designated cFLIP short form ($cFLIP_S$) and cFLIP long form ($cFLIP_L$). $cFLIP_S$ consists of two DEDs, while $cFLIP_L$ contains a pseudo-enzyme domain in addition to the two DEDs (Hojo et al., 2010). Both cFLIP isoforms inhibit apoptosis, and mainly act on Fas-mediated apoptosis by competing with procaspase-8 and blocking its function (Krueger et al., 2001a,b); they interact with the FADD and procaspase-8 to potently inhibit cell death receptor-mediated apoptosis signal transduction (Irmler et al., 1997).

It was recently reported that apoptosis is a key mechanism in luteal regression in mammalian ovaries. It is generally accepted that apoptosis is the main type of cell death during structural luteolysis in many species (Davis and Rueda, 2002). Apoptosis is a highly regulated process and various factors are known to be involved in apoptosis in the corpus luteum (CL). A good understanding of anti-apoptotic factors can improve manipulation techniques in luteolysis, which is a crucial element of estrous cycle control in mammals.

Immunohistochemical analyses demonstrated that the cFLIP protein is highly expressed in the granulosa cells of healthy follicles but weakly expressed in those of the atretic follicles (Matsuda-Minehata et al., 2005). Luteal tissue formation is characterized by the differentiation and proliferation of cells derived from granulosa, theca, and vascular endothelium in the post-ovulatory follicle (Jablonka-Shariff et al., 1993; Murphy, 2000). Lutein cells are derived from granulosa cells and theca cells. The present study was conducted to further investigate the effect of cFLIP on lutein cells derived from granulosa cells.

MATERIAL AND METHODS

Collection of the porcine CL

Ovaries were collected from parity sows at a local slaughterhouse within 40-60 min of slaughter. As described in a previous study (Miyamoto et al., 2000), the luteal stage of the estrous cycle was defined by macroscopic observation of the ovaries (corpora lutea and follicles). The CLs were classified into early (days 3-5 after ovulation), mid (days 8-12), and late (days 15-18) CL stages (Table 1). The CL was taken from the ovaries of a pregnant sow (with a fetus ~20 cm in length). The pre-ovulatory corpus albicans (CA1), and CL coexisted with the corpus albicans (CA2). After determination of the stages, the CLs and CAs were immediately separated from the ovaries, frozen rapidly in liquid nitrogen, and stored at -80°C; portions of the same CL were stored until required for studies of mRNA and protein expression. For immunohistochemistry, portions of the CLs and CAs were fixed in 10% (v/v) neutral formalin, pH 7.4, for 48-72 h and then embedded in paraffin.

Table 1. Classification criteria of the corpus luteum (CL) in the porcine ovary.

Classification	Morphological characteristics
Early CL	No follicle in the cortex, ovulation fossae or intensive blood vessels (5-10 mm in diameter)
Mid CL	No or few follicles in the cortex, CL 10-15 mm in diameter, intensive blood vessels, pink functional CL
Late CL	Several big follicles in the cortex; CL with diameter of 8-10 mm, fewer surface blood vessels, degenerate CL with a fleshy or whitish color

Based on previous reports (Miyamoto et al., 2000).

Preparation of RNA and reverse transcription-polymerase chain reaction (RT-PCR) analyses for *cFLIP* mRNA

Total RNA samples were extracted from the CLs and CAs with the RNeasy mini kit (Qiagen, Chatsworth, CA, USA), and then cDNA was synthesized from 1 µg total RNA using a T-primed first-strand kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to manufacturer directions. For PCR, the primers were as follows: 5'-ATGTC GGCTG AGGTC ATCCA TCA-3' as the $cFLIP_{S/L}$ forward primer, 5'-TCATG CTGGG ATTCC GCACA CTT-3' as the $cFLIP_S$ reverse primer, and 5'-TCCTA GGGGC TTGCT CTGCA-3' as the $cFLIP_L$ reverse primer. The expression level of porcine glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; GenBank accession No. AF017079, used as an intrinsic control) in each sample was also determined using 5'-GGACT CATGA CCACA GTCCA T-3' and 5'-TCAGG TCCAC AACCG ACACG T-3' as forward and reverse primers, respectively. The expected PCR product sizes of $cFLIP_S$, $cFLIP_L$, and *GAPDH* were 645, 762, and 220 bp, respectively. PCR amplification was performed as follows: Platinum Taq DNA Polymerase (Amersham Pharmacia Biotech) was added to the cDNA mixture (each cDNA sample was mixed with PCR mix containing 1X PCR buffer, 0.1 mM dNTP mixture, 1.5 mM $MgCl_2$, and 0.25 µM of each primer). The mixture was denatured, and then subjected to PCR in a thermal cycler (GeneAmp PCR Systems 9700; PE Applied Biosystems; Animal Resource Science Center, University of Tokyo, Kasama, Japan). The PCR cycles for $cFLIP_S$, $cFLIP_L$, and *GAPDH* were as follows: 94°C for 5 min; 40 cycles of 94°C for 30 s; 55°C for 30 s; and 72°C for 1 min, followed by a final extension period at 72°C for 7 min. PCR products were electrophoresed

on 1.5% (w/v) agarose gels (Cambrex Bio Science, Rockland, ME USA) and stained with ethidium bromide (Wako, Osaka, Japan). A Ready-Load 100-bp DNA ladder (Invitrogen) was used as a molecular weight marker for electrophoresis. After electrophoresis, the stained gels were recorded with a digital fluorescence recorder (LAS-1000; Fuji Film, Tokyo, Japan), and each mRNA expression level, which correlated with the fluorescence intensity of each band of PCR product, was quantified using Image-Gauge (Fuji Film) on a Macintosh computer. The abundance of *cFLIP*-specific mRNA was normalized to the relative abundance of *GAPDH* mRNA.

Protein detection by western blot

The CL and CA tissues were homogenized on ice in UTD buffer [9 M urea (Wako), 2% (v/v) Triton X-100 (Sigma), 1% (w/v) (±)-dithiothreitol (Wako)], and stored at -80°C. The protein concentrations were quantified based on the Bradford method using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were then solubilized in sample buffer solution (Wako), and heated at 95°C for 5 min. For western blotting, a protein fraction (30 µg per lane) was subjected to electrophoresis on a 10-20% gradient sodium dodecyl sulfate-polyacrylamide electrophoresis gel (ATTO, Tokyo, Japan) for 80 min at 200 V. The separated proteins were electrophoretically transblotted to a polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA, USA) at 25 V for 45 min. The membranes were stained with 0.2% (w/v) Ponceau S solution (Serva Electrophoresis, Heidelberg, Germany), and then immersed in blocking solution [10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1% (v/v) Tween 20 (Sigma), and 2.5% (w/v) bovine serum albumin (BSA; Sigma)] for 1 h at room temperature. After blocking, each membrane was incubated at 4°C for 18 h with rabbit polyclonal anti-human $cFLIP_{S/L}$ antibody (diluted with blocking buffer at 1:100; sc-8347, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for checking $cFLIP_L$ expression, and with Anti-FLIP, NT rabbit polyclonal IgG (human and mouse) (diluted with blocking buffer at 1:100; Catalog 06-697) for checking $cFLIP_S$ expression. After incubation, the membrane was washed 6X for 5 min each in wash buffer [10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% (v/v) Tween 20] and then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (diluted with wash buffer at 1:2000; Dako) for 1 h at room temperature. The expression levels of cFLIP protein were visualized with an ECL western blot detection reagent hydrogen peroxide kit (Amersham Pharmacia Biotech). The chemiluminescence was recorded with a digital fluorescence recorder (LAS-1000; Fuji Film). Using the same PVDF membrane, the β-actin protein level was also examined as an internal control. Rabbit anti-human β-actin antibody (diluted with blocking buffer at 1:2000; Abcam, Cambridge, UK) and HRP-conjugated anti-rabbit IgG (diluted with wash buffer at 1:2000; Dako) were used as the primary and secondary antibodies, respectively. Each protein expression level, as indicated by the chemiluminescence intensity of each protein band, was quantified using the Image-Gauge program (Fuji Film) on a Macintosh computer; cFLIP protein levels were normalized according to the amount of β-actin protein.

Immunohistochemistry

The ovaries obtained at a local slaughterhouse were fixed in 10% (v/v) phosphate-buffered formalin, pH 7.4 (Wako), dehydrated through a graded ethanol series, and embedded

in Histosec-paraffin (Merck, Darmstadt, Germany). Serial paraffin-embedded sections (4 µm thick) were cut, and then mounted on glass slides pre-coated with 3-aminopropyltrimethoxysilane (silane; Sigma Aldrich Chemicals, St. Louis, MO, USA). They were stained with hematoxylin and eosin (H&E), immunostained for cFLIP and the proliferating cell nuclear antigen (PCNA), and subjected to terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphate nick end-labeling (TUNEL).

The sections were deparaffinized, rehydrated, and washed in distilled water for 15 min. They were then immersed in antigen retrieval buffer (0.01 M citric acid, pH 6) at 95°C for 10 min and at room temperature for 20 min, and treated with 0.3% (v/v) hydrogen peroxide (H_2O_2) in methanol for 30 min to inhibit endogenous peroxidases. After three washes in phosphate-buffered saline (PBS), the sections were incubated with normal goat serum (Sigma) and diluted in PBS to block non-specific protein binding. Sections were incubated overnight at 4°C with rabbit anti-human $cFLIP_{S/L}$ antibody (sc-8347, Santa Cruz Biotechnology) diluted 1:100 with PBS containing 1% (w/v) BSA (Sigma), while sections for the negative control were incubated with PBS containing 1% BSA. Subsequently, the sections were washed with PBS three times and incubated with 1:200 biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) in PBS for 30 min, followed by three incubations in PBS, and a 60-min incubation at room temperature with VectaStain avidin-biotin-peroxidase kit (Vector Laboratories) according to manufacturer instructions. The sections were washed well with PBS and Dako Envision Kit/HRP (DAB, 3,3'-diaminobenzidine; Dako, USA) was applied to the sample for color development by examining the slide under the microscope. Then, they were washed with distilled water, counter-stained with methyl green, dehydrated, mounted with Entellan (Merck), and examined by light microscope.

Immunohistochemical staining for PCNA was conducted as follows. Serial paraffin sections were deparaffinized, treated with 3% (v/v) H_2O_2 in methanol for 5 min to inhibit endogenous peroxidases, washed with Tris-buffered saline, pH 7.4, and incubated with HRP-conjugated anti-PCNA antibody (Dako, Glostrup, Denmark) for 1 h at room temperature. They were then washed with Tris-buffered saline and incubated with DAB solution for 30 s to 1 min. After washing with distilled water, they were counter-stained with methyl green, dehydrated, mounted with Entellan (Merck), and examined by light microscopy. In each experimental run, adjacent sections were incubated without antibody as a negative control.

To visualize the apoptotic cells, sections were stained by the TUNEL method using an apoptosis *in situ* detection kit (ApopTag; Intergen, Manhattan, NY, USA) according to the manufacturer protocol. Serial paraffin sections were deparaffinized and washed in PBS for 10 min. The sections were treated with proteinase K solution for 20 min at room temperature. They were washed in distilled water and treated with 3% (v/v) H_2O_2 in methanol for 5 min to inhibit endogenous peroxidases. After washing with PBS, they were incubated in equilibration buffer for 10-15 min at room temperature, covered with terminal deoxynucleotidyl transferase in a chamber for 60 min at 37°C, and placed in stop/wash buffer for 10 min at room temperature. After three PBS washes, the sections were incubated using anti-digoxigenin conjugate for 30 min and washed with PBS. They were washed with 0.05 M Tris-HCl for 5 min and incubated with DAB solution for 10-30 s. After washing with distilled water, they were counter-stained with methyl green, dehydrated, mounted with Entellan (Merck), and examined by light microscopy.

Statistical analysis

Before analysis of variance processing, the homogeneity of variance was assessed using the StatView 4.5 program (Abacus Concepts, Berkeley, CA, USA) on a Macintosh computer. Analysis of variance with the Fisher least significant differences test for biochemical data and Wilcoxon signed-rank tests for histological estimation were carried out using the StatView 4.5 program. Differences of $P < 0.05$ were considered to be significant.

RESULTS

cFLIP mRNA expression

The levels of $cFLIP_S$ and $cFLIP_L$ mRNAs in the porcine CLs and CAs were analyzed by RT-PCR. Both mRNAs were expressed in CLs of the estrous cycle and pregnancy, as well as in the CAs (Figure 1A). The normalized expression levels are shown in Figure 1B. The expression levels of $cFLIP_S$ mRNA were higher during the early and mid CL stages, and had significantly decreased at the late CL stage ($P < 0.05$). The expression of $cFLIP_L$ mRNA was higher at the mid CL stage, and lower at the early and late CL stages ($P < 0.05$). The $cFLIP_S$ and $cFLIP_L$ mRNAs were expressed in the CL during pregnancy. The expression of $cFLIP_S$ mRNA showed no significant difference between the CA1 and CA2, but the expression of $cFLIP_L$ mRNA was higher in the CA1.

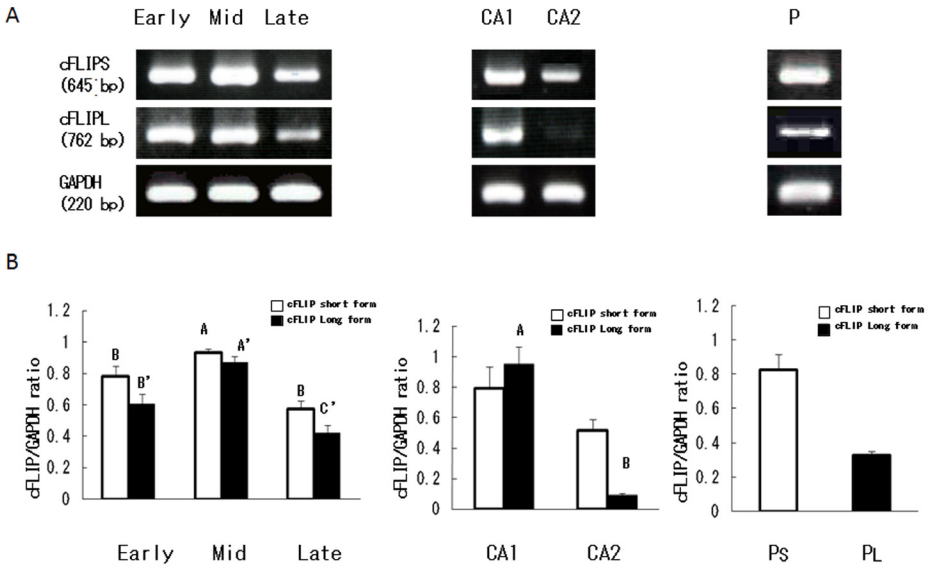

Figure 1. Changes in mRNA expression levels of $cFLIP_S$ and $cFLIP_L$ in the porcine corpora lutea (CL) during the estrous cycle and pregnancy were examined by reverse transcription-polymerase chain reaction. Representative photographs of the electrophoresis gel are shown in **A**. Each band was normalized to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA level (**B**). All data are reported as means ± standard error of the mean (SE) in the porcine CL during the estrous cycle; different letters above bars indicate a significant difference at $P < 0.05$. CL: early, mid, and late corpus luteum; CA1: preovulatory corpus albicans; CA2: corpus luteum coexistence with corpus albicans; P: corpus luteum during pregnancy.

Expression of cFLIP proteins

The protein levels of cFLIP were assessed by western blotting. To recognize both $cFLIP_S$ and $cFLIP_L$, two antibodies were used. The bands of $cFLIP_S$ and $cFLIP_L$ protein were seen at 28 and 55 kDa, respectively (Figure 2A). Protein levels of $cFLIP_S$ and $cFLIP_L$ showed steady higher levels during the early and mid CL stages, and had significantly decreased ($P < 0.05$) by the late CL stage (Figure 2B). The $cFLIP_S$ and $cFLIP_L$ proteins were also detected in the CL of pregnancy (Figure 2B). The expression of $cFLIP_S$ protein showed no significant difference between the CA1 and CA2; however, the expression of $cFLIP_L$ protein was not detected (Figure 2B).

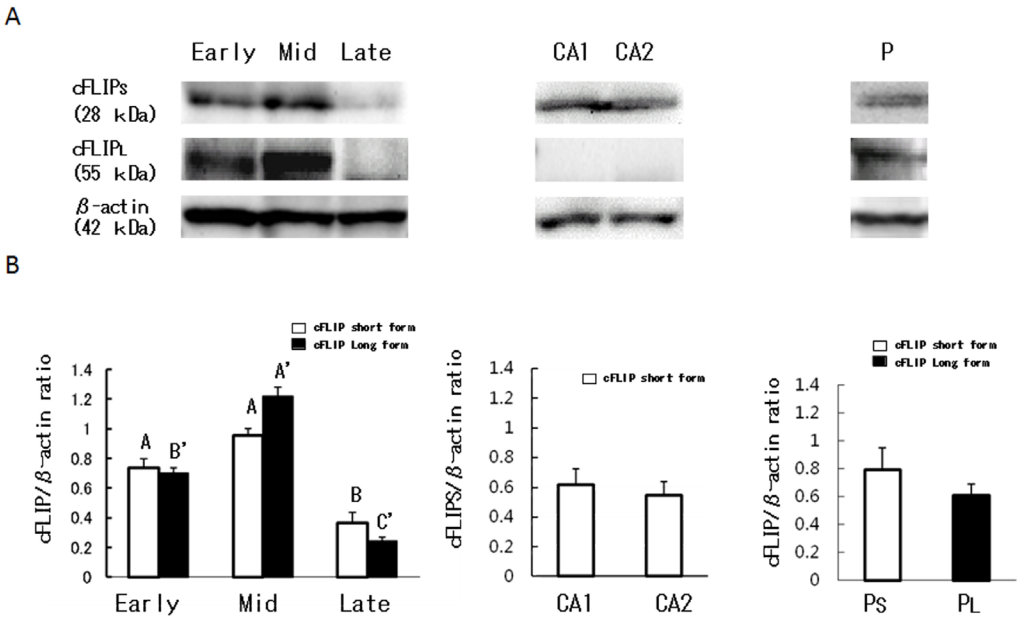

Figure 2. Changes in the relative levels of $cFLIP_S$ and $cFLIP_L$ proteins in the porcine corpora lutea (CL) during the estrous cycle and pregnancy were examined by western blotting. Representative photographs of the electrophoresis gel are shown in **A**. Each band was normalized to the β-actin protein level (**B**). All data are reported as means ± standard error of the mean (SE). The different letters indicate significant difference ($P < 0.05$). CL: early, mid, and late corpus luteum; CA1: preovulatory corpus albicans; CA2: corpus luteum coexistence with corpus albicans. P: corpus luteum during pregnancy.

Histology and immunohistochemistry analysis

For morphological analysis, some sections were also stained with H&E. The morphology of luteal cells was observed in H&E-stained tissue sections using an optical microscope. The H&E stain results indicated that the luteal cells at the early and mid CL stages of the estrous cycle and pregnancy were normal (Figure 3A, E, U), but the luteal cells at the late CL and CA stages of the estrus cycle were atrophied (see Figure 3I, M, Q).

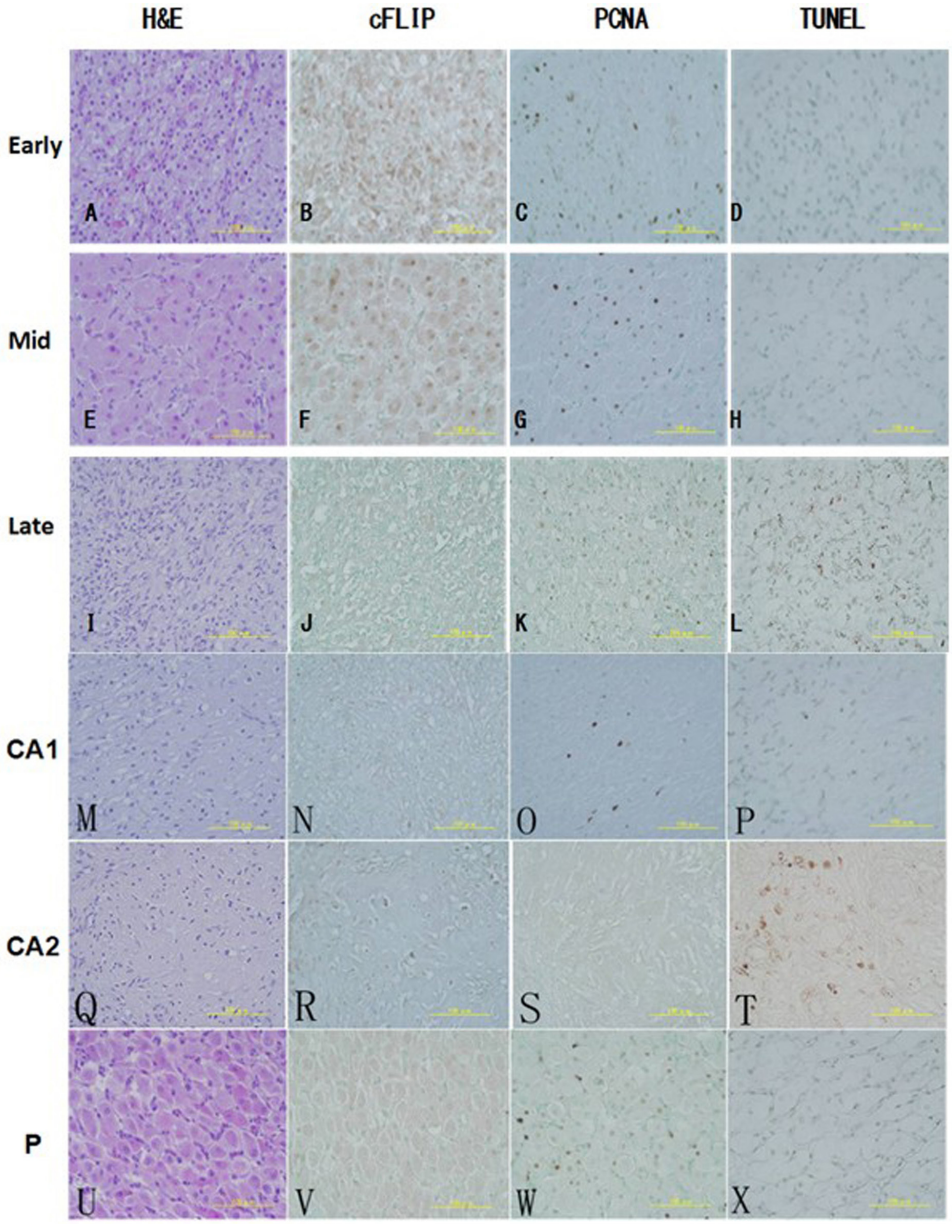

Figure 3. Results of hematoxylin and eosin (H&E) staining, immunoreactions of cFLIP and proliferating cell nuclear antigen (PCNA), and terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphate nick end-labeling (TUNEL) for the corpora lutea (CL) and corpus albicans (CA) stages of the estrous cycle and pregnancy. The cFLIP- and PCNA-positive stains were seen in the early and mid CL stages of the estrous cycle and pregnancy; these luteal cells were normal by H&E staining. In the late CL and CA stages of the estrus cycle, the H&E stain results showed atrophied luteal cells; there was TUNEL-positive staining, and no positive staining of PCNA and cFLIP. CL: early, mid, and late corpus luteum; CA1: preovulatory corpus albicans; CA2: corpus luteum coexistence with corpus albicans. P: corpus luteum during pregnancy. A.-X. Histology and immunohistochemistry analysis, each band (A-X) could not be normalized to which protein level. Scale bar = 100 μm.

Immunohistochemistry analysis of cFLIP

Positive immunostaining for cFLIP was found predominantly in the cytoplasm of luteal cells. Luteal cells were immunostained during the early and mid CL stages during the estrous cycle and pregnancy (Figure 3F, V). Pale immunostaining was found in the late CL and the CA stages (Figure 3N, R).

Immunohistochemistry analysis of PCNA

Proliferating (PCNA-positive) cells were found in the early and mid CL stages of the estrous cycle and pregnancy (Figure 3C, G, W). PCNA-negative reactions were found in the late CL and CA2 stages during the estrous cycle (Figure 3K, S), but PCNA-positive reactions were observed in a few CA1 cells such as endothelial cells and fibroblasts (Figure 3O).

Detection of apoptotic cells in histological sections

Apoptosis (TUNEL-positive) cells were found in many cells of the late CL and CA2 stages (Figure 3L, T), and a few cells of the CA1 stage (Figure 3P). TUNEL-negative reactions were found in the early and mid CL stages during the estrous cycle and pregnancy (Figure 3D, H, X).

DISCUSSION

The CL needs to undergo functional luteolysis to decrease progesterone secretion, allowing the induction of ovulation for the new estrous cycle. Structural luteolysis (destruction and removal of the luteal cells) is also required to keep the ovary in proper proportion to the rest of the reproductive tract (Skarzynski et al., 2013). Luteolysis is defined as the loss of function and subsequent involution of the luteal structure. The luteolytic process is usually subdivided, whereby the decline in progesterone is described as functional luteolysis and the structural involution is described as structural luteolysis (Sugino and Okuda, 2007). Apoptosis plays a key role in the demise of the majority of mammalian gonadal cells (follicular granulosa and luteal cells in ovaries and germ cells in the testes) during the reproductive cycle. In mammalian ovaries, a balance of cell proliferation and apoptosis in granulosa cells is maintained in healthy follicles and any imbalance of the two processes can lead to atresia in the ovarian follicles (Manabe et al., 2004). cFLIP acts as an anti-apoptotic factor that inhibits the intracellular apoptotic signal by competing with its homologue, procaspase-8, which is an upstream inducer of apoptosis activated by TNF family death ligands (TNF-α, FasL, TNF-related apoptosis-inducing ligand, etc.) (Krueger et al., 2001b; Thome and Tschopp, 2001). By inhibiting the recruitment of procaspase-8 to FADD, cFLIP can stop the death receptor-mediated apoptosis. In rat granulosa cells, it was demonstrated that the over-expression of $cFLIP_S$, but not $cFLIP_L$, prevented apoptosis induced by TNF-α (Xiao et al., 2002). TNF-α is an important factor for inducing apoptosis in luteal cells, and it was demonstrated that apoptosis plays an important role in luteolysis during the late stages of the estrous cycle (Okano et al., 2006). *cFLIP* is more efficient than other anti-apoptotic genes, such as *TRAF1*, *TRAF-2*, *cIAP-1*, and *cIAP-2*, in preventing cell death induced by death receptors (Wang et al., 1998).

Recently, our colleagues found that *cFLIP* mRNAs are expressed in the granulosa cells of porcine follicles. They went on to determine the mRNA sequences of porcine $cFLIP_S$

and *cFLIP*$_L$, which have a similar function to those of human and murine systems. They act as an antagonist for Fas and TNF receptors mediating apoptosis in granulosa cells of porcine ovarian follicles (Goto et al. 2004). Furthermore, it was demonstrated that cFLIP may act as anti-apoptotic/survival factor in the granulosa cells of porcine follicles (Matsuda-Minehata et al., 2005).

To determine the role of cFLIP in ovarian luteal tissue, we examined the expression of cFLIP by RT-PCR and western blotting in lutein cells of porcine ovaries. In the present study, the cFLIP$_S$ and cFLIP$_L$ mRNA and protein were highly expressed in the early and mid CL stages of the estrous cycle, and both had significantly decreased expression in the late CL stage. The concentration of progesterone was higher at the early and mid CL stages, and was significantly decreased at the late CL stage. The expression levels of cFLIP$_S$ and cFLIP$_L$ mRNA and protein were also observed in the CL during pregnancy. These results indicate that cFLIP expression levels are related to the stage of CL. We suggest that cFLIP proteins are involved in the function of CL in mammalian ovaries by maintaining the luteal cells.

At the early and mid CL stages of the estrous cycle and pregnancy the H&E stain results showed that the luteal cells were normal, PCNA immunoreactions showed that the luteal cells were at the proliferating stage, TUNEL immunoreactions showed that there were no luteal cells in the apoptotic stage, and cFLIP immunoreactions showed intense localization in the cytoplasm of luteal cells. At the late CL stage and CA stages of the estrus cycle the H&E stain results showed that the luteal cells were atrophied; PCNA immunoreactions were negative for cell proliferation, and PCNA-positive cells were only observed during CA1 in a few cells such as endothelial cells and fibroblasts; TUNEL immunoreactions indicated the apoptotic stage; and cFLIP immunoreactions showed weak expression. Based on the relationship between the morphology of luteal cells and immunohistochemical characteristics, we suggest that cFLIP proteins might be involved in the morphology of CL of mammalian ovaries by blocking apoptosis of luteal cells in the early and mid CL stages.

The expression levels of cFLIP$_S$ mRNA and protein showed no significant differences between the CA1 and CA2. Moreover, expression levels of *cFLIP*$_L$ mRNA in the CA1 were higher than those during CA2, but the expression of cFLIP$_L$ protein was not detected in either the CA1 or CA2. Regarding the lack of expression of cFLIP$_L$ protein in the CA, we speculate that the high expression of *cFLIP*$_L$ mRNA in the CA1 might be derived from the PCNA-positive reactions in a few cells such as endothelial cells and fibroblasts. To determine the role of cFLIP in the CA, further study is needed to clarify the results of the CA studies. Moreover, the functional differences between cFLIP$_S$ and cFLIP$_L$ have not yet been demonstrated, and this will require further elucidation.

In the present study, the expression levels of cFLIP were consistently higher during the early and mid CL stages of the estrus cycle, and had decreased significantly by the late CL stage, as shown by RT-PCR, western blot, and immunohistochemistry analyses. From these results, we propose that cFLIP$_{S/L}$ acts as a survival factor, and plays an anti-apoptotic role in the porcine corpus luteum.

Conflicts of interest

The authors declare no conflict of interest.

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