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—Original Article—

Expression and localization of cellular FLICE-like inhibitory protein (cFLIP), an anti-apoptotic factor, in corpora lutea during the estrous cycle and pregnancy in Thai swamp buffalo *(Bubalus bubalis)*

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Abstract. In female mammals, luteal cells rapidly proliferate and form corpora lutea (CLs) after ovulation. The corpus luteum (CL) plays crucial roles in establishing and maintaining pregnancy. To gain further insights into the role of cellular FLICE-like inhibitory protein (cFLIP), an anti-apoptosis factor that is structurally similar to procaspase-8 but lacks proteolytic enzyme activity, we examined the expression in CLs of Thai swamp buffalos (*Bubalus bubalis*) during the early, mid, and late stage of the estrous cycle and pregnancy. cFLIP short form and long form ($cFLIP_S$ and $cFLIP_L$, respectively) mRNA and protein levels were assessed by reverse transcription-polymerase chain reaction and western blotting, respectively. $cFLIP_S$ mRNA levels were low in the mid and late stages of the estrous cycle and increased during pregnancy ($P < 0.05$). $cFLIP_L$ mRNA was highly expressed in CLs during pregnancy and was lower in the mid and late stages of the estrous cycle. The level of $cFLIP_S$ protein was high in CLs during pregnancy and low levels were noted in the mid stage of the estrous cycle ($P < 0.05$). Higher levels of $cFLIP_L$ protein were demonstrated in CLs during pregnancy and lower levels were found in CLs during the early stage of the estrous cycle. Strong positive immunohistochemical staining for $cFLIP_{S/L}$ proteins was observed in luteal cells during pregnancy. The present findings revealed that cFLIP was at the highest level in CLs during pregnancy, and this may act as a dominant survival anti-apoptotic factor by inhibiting intracellular apoptosis signal transduction in luteal cells of CLs during pregnancy.

Key words: Cellular FLICE-like inhibitory protein (cFLIP), Corpora lutea, Thai swamp buffalo (*Bubalus bubalis*)

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The Thai swamp buffalo *(Bubalus bubalis)* is an important farm animal with a long history of involvement with Thai farmers. In the past, buffaloes functioned as a labor source in agriculture and transportation; however, due to increasing demands on agricultural productivity, they have been replaced by farm equipment. This replacement has been one of the major factors that have caused the reduction of the buffalo population in Thailand. Another possible reason for the decrease of the buffalo numbers is their poor reproductive efficiency, which is affected by their own limitations in terms of late maturity as well as poor expression and low detectability of estrous signs. After ovulation, there is a change from the dominant follicle to corpora lutea (CLs); however, only a limited number of follicles can develop to the preovulatory stage and ovulate, after which CLs are generated. More than 99.9% of follicles are eliminated through a degenerative process known as "atresia". The corpus luteum (CL) secretes progesterone (P_4), which causes the thickening of the endometrium and supports the development until embryo implantation. When there is no implanted embryo, the CL rapidly degenerates. During luteolysis, luteal cell apoptosis is a key phenomenon and is closely regulated by the balance of cell death and survival factors [1]. Apoptosis is a form of physiological cell death and has been demonstrated in luteal cells during luteolysis in cows [2–4], humans [5], sows [6], and rats [7]. It is well established that apoptosis is the dominant mechanism regulating apoptosis of granulosa [8–10] and luteal cells [2, 11–15]. Studies on apoptosis signal transduction have focused on cell death ligand- and receptor-dependent intracellular signaling. TNF-related apoptosis-inducing ligand and Fas system, have also been reported [12].

Cellular FLICE-like inhibitory protein (cFLIP) is an anti-apoptosis factor, which is structurally similar to procaspase-8 but lacks proteolytic enzyme activity [16]. cFLIP has two splicing variants: short and long forms ($cFLIP_S$ and $cFLIP_L$, respectively). Our study aimed to evaluate the expression and localization of the anti-apoptotic factor cFLIP in buffalo CLs during the estrous cycle and pregnancy. This knowledge will enhance our understanding of the buffalo reproductive system and potentially increase buffalo population levels. To date, our laboratory has investigated a porcine anti-apoptotic protein, cFLIP, which is a dominant regulator of apoptosis in granulosa cells of pig follicles [10]. cFLIP is expressed in porcine granulosa cells and luteal cells, but knowledge about the mechanism of apoptosis regulation in

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luteal cells remains limited. To determine the physiological roles of cFLIP in buffalo CLs, we firstly investigated the changes in expression levels [by reverse transcription-polymerase chain reaction (RT-PCR) and western blotting] and localization (by immunohistochemistry) of cFLIP mRNA in CLs. The CLs were categorized into different stages of the estrous cycle and an additional category was included for pregnant females. Retrospectively, the classification of each CL was confirmed by P_4 production.

Materials and Methods

Animals and classification of CLs

The ovaries were obtained from buffaloes (more than 250 kg in body weight) at a local abattoir in Sakon Nakhon province. Experimental protocols and animal handling procedures were reviewed and approved by the Animal Care and Use Committee of Kasetsart University (ID: ACKU 60-ETC-006). The luteal stage of the estrous cycle was defined by macroscopic observation of the buffalo ovaries. CLs were first classified based on morphological characteristics and P_4 levels, but the ovary weight was not used to determine the stages of CLs. P_4 levels of peripheral blood plasma were measured using an enzyme immunoassay kit (Cayman, Ann Arbor, MI, USA). To classify the stage of the estrous cycle and pregnancy, P_4 levels in isolated peripheral blood plasma were measured. Plasma P_4 levels were used for classifying early, mid, and late stages of the estrous cycle as well as pregnancy. Pregnant buffaloes with fetuses measuring 14–24 cm in length or in buffaloes that were in 60 to 120 days of pregnancy were used for comparing bovine development in this experiment. After classifying these stages, CL tissues were separated from the ovaries, then frozen in liquid nitrogen and stored at –80°C until used for studies of mRNA and protein expression. For immunohistochemistry, CLs were fixed in 10% (v/v) neutral formalin (pH 7.4; Wako Pure Chemicals, Osaka, Japan) for 48–72 h and then embedded in paraffin (Merck, Kenilworth, NJ, USA).

RNA isolation and RT-PCR analyses for cFLIP mRNA expression

Total RNA was extracted from each CL at four different stages of the estrous cycles (N = 15 per stage) and pregnancy (N = 4). cDNA was then synthesized from the total RNA and used for amplification with the following primers: for goat $cFLIP_{S,}$ forward: 5′-TGT CTG CTG AGG TCA TCC ATC-3′ and reverse: 5′-CCA GGA TTC CAC ACA TTT GC-3′ (product size 639 bp); for goat $cFLIP_L$, forward: 5′-CAC AGA CAT TCT TCG GGA CAC-3′ and reverse: 5′-CGC TCC TTA GCA GAC ACT TTG -3′ (product size 690 bp). Cattle glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified with the following primers, forward: 5′-TGG AGG GAC TTA TGA CCA CTG-3′ and reverse: 5′-AGC CTA GAA TGC CCT TGA GAG-3′ (product size 315 bp). PCR products were electrophoresed in 1.5% (w/v) agarose gels and stained with ethidium bromide. A Ready-Load 100-bp DNA ladder (Invitrogen, Carlsbad, CA, USA) was used as a molecular weight marker for electrophoresis. After electrophoresis, the stained gels were visualized under UV light and recorded with a digital fluorescence recorder (LAS-1000; Fuji Film, Tokyo, Japan); mRNA expression levels of each of the genes, which correlated with the fluorescence intensity of each band of PCR product, was quantified using Image-Gauge (Fuji Film). The abundance of cFLIP-specific mRNA was normalized to the relative abundance of GAPDH mRNA.

Western blotting analyses

The protein fraction was prepared from CLs of buffaloes during the estrous cycle (N = 15) and pregnancy (N = 4) and homogenized in ice-cold lysis buffer [9 M urea (Wako Pure Chemicals), 2% (v/v) Triton X-100 (Sigma Aldrich, St. Louis, MO, USA) and 1% (w/v) dithiothreitol (DTT; Wako Pure Chemicals)], and then centrifugation at 15,000 *g* for 2 min at 4ºC. For protein measurement, bovine serum albumin (BSA; Sigma Aldrich) was used as a standard and stored at –80ºC until use. Protein samples (50 µg) were separated on 10% (w/v) SDS-PAGE gels (Atto, Tokyo, Japan) and then transferred to PVDF membranes (Immobilon Transfer Membrane; Millipore, Marlborough, MA, USA). The membranes were stained with 0.2% (w/v) Ponceau S solution (Serva Electrophoresis, Heidelberg, Germany) and immersed in blocking solution [10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1% (v/v) Tween 20 (Sigma Aldrich) and 2.5% (w/v) BSA (Sigma Aldrich)] for 1 h at room temperature (26ºC). The membranes were incubated overnight at 4ºC with anti-FLIP rabbit polyclonal IgG (corresponding to amino acid position 2–17 of human FLIP; diluted 1:100 in blocking solution) for 16–18 h. After washing with the washing solution, membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Dako, Glostrup, Denmark; diluted 1:2,000 with washing solution) for 1 h at room temperature (26ºC) and then washed six further times. The membranes were visualized using an ECL system (GE Healthcare, Little Chalfront, UK) following the manufacturer's instruction. The chemiluminescence was recorded with a digital fluorescence recorder (LAS-1000; Fuji Film). GAPDH was detected on the same membrane following overnight incubation at 4ºC with goat monoclonal anti-GAPDH antibody (Dako; diluted 1:100) and then incubation with HRP-conjugated anti-goat IgG antibody (Dako; diluted 1:2,000) for 1 h at room temperature (26ºC).

Immunohistochemical staining

Dissected CLs that were fixed in 10% (v/v) phosphate-buffered formalin and embedded in paraffin (Merck) were used for preparing sections. Sections (10 µm) were cut from the medial part of each CL, then mounted on glass slides coated with 3-aminapropyltrimethoxylane (silane; Sigma Aldrich). The sections were incubated with rabbit anti-human $cFLIP_{S/L}$ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100 with PBS containing 1% (w/v) BSA overnight at 4ºC. Next, the sections were washed with PBS and incubated with biotinylated anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA, USA) for 30 min, washed three times in PBS, and then incubated using an VectaStain Elite ABC kit (Vector Laboratories) for 1 h at room temperature (26ºC). Peroxidase substrate-chromogen solution (Dako) was added to the sample for color development by observation under a light microscope (BX50, Olympus, Tokyo, Japan). The sections were then washed with distilled water, counter-stained with methylene green, dehydrated, mounted with Entellan (Merck), and then examined using a light microscope.

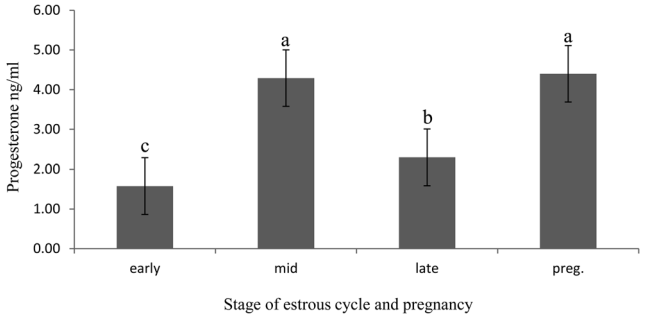

Fig. 1. Changes in progesterone concentration in the plasma during the estrous cycle (early, mid, and late) and pregnancy (preg.) in buffaloes. Different letters above bars indicate significant difference at $P < 0.05$.

Statistical analyses

Statistical analyses were performed using the Stat View-4.5 program (Abacas Concepts, Berkeley, CA, USA) and data are represented as mean ± SE. Briefly, ANOVA with Fisher's least significant differences test for biochemical data and Wilcoxon's signed-rank tests for histological estimation were conducted. Differences at $P < 0.05$ were considered significant.

Results

Progesterone analyses

CLs were classified in each stage based on morphological characteristics based on color. Vascularity was also used to classify stages,

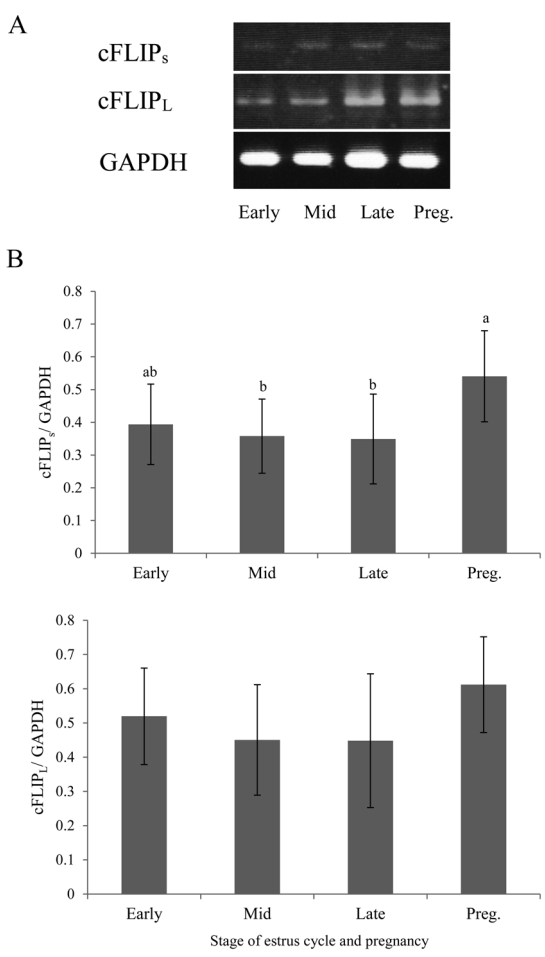

Fig. 2. Changes in the mRNA expression levels of $cFLIP_S$ and $cFLIP_L$ in buffalo corpus lutea (CLs) were examined by reverse transcription-polymerase chain reaction. Representative photographs of the electrophoresis gel are shown in (A). Each band was normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level (B). All data are reported as mean ± standard error of the mean (SE) in the buffalo corpus luteum (CL) during the estrous cycle and pregnancy; different letters above bars indicate significant difference at $P < 0.05$. CL: early, mid, late stages during the estrous cycle and pregnancy (preg.).

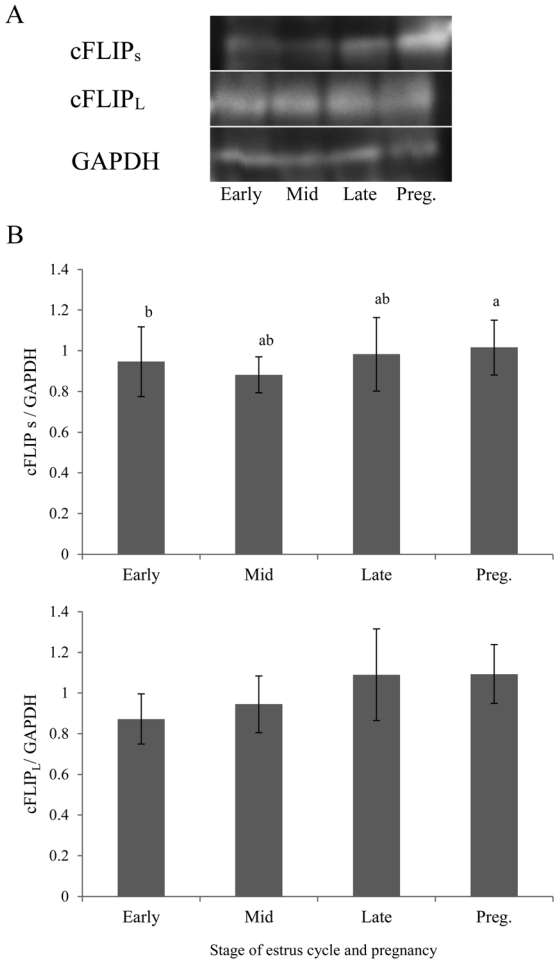

Fig. 3. Changes in the protein expression levels of short form of cellular FLICE-like inhibitory protein ($cFLIP_S$) and long form of cFLIP ($cFLIP_L$) in buffalo corpus lutea (CLs) during the estrous cycle and pregnancy were examined by western blotting. Representative photographs of the electrophoresis gel are shown in (A). Each band was normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein level (B). All data are reported as mean ± standard error of the mean (SE). The different letters indicate significant difference ($P < 0.05$). CL: early, mid, late stages during the estrous cycle and pregnancy (preg.).

whereby color appeared during the early and mid stages of the estrous cycle but was colorless at the late stage. Significant differences in peripheral P4 concentrations were found among different stages of the estrous cycle and pregnancy. Plasma P_4 levels were used for classifying the stages of the estrous cycle as early (0.94–2.20 ng/ml), mid (3.5–5.00 ng/ml) and late (2.50–3.00 ng/ml), as well as pregnancy (4.30–5.29 ng/ml). Higher plasma P_4 levels were found in the mid stage of the estrous cycle and during pregnancy. The results are summarized in Fig. 1.

Changes in expression levels of cFLIP mRNAs in CLs

Changes in expression levels of cFLIP mRNAs prepared from each CL during the estrous cycle and pregnancy are summarized in Fig. 2. Lower levels of $cFLIP_S$ mRNA were seen in the mid and late stages of the estrous cycle, and higher levels were noted during pregnancy. $cFLIP_L$ mRNA expression level was higher during pregnancy, and lower in the mid and late stages of the estrous cycle.

Expression levels of cFLIP proteins in CLs

Western blotting was performed to investigate the changes in the protein levels of $cFLIP_S$ and $cFLIP_L$. $cFLIP_S$ protein (28 kDa) was highly expressed during pregnancy ($P < 0.05$); high levels of $cFLIP_L$ protein in CLs were observed in the late stage of the estrous cycle and pregnancy although there was no significant difference (Fig. 3).

Immunohistochemical localization of cFLIP protein in CLs

Localization of cFLIP protein was immunohistochemically demonstrated, and no difference in immunohistochemical staining of $cFLIP_{S/L}$ was seen in luteal cells of CLs throughout the estrous cycle. However, a strong reaction was found in the luteal cells of CLs during pregnancy (Fig. 4).

Discussion

Apoptosis is regulated by hormones in reproductive tissues such as the prostate after castration [17], uterine epithelia [18], mammary glands [19], bovine luteal cells [20], and rabbit luteal cells [21]. For example, prolactin (PRL) and P_4 regulate luteolysis [22], and PRL is a critical stimulator of CL development and P_4 production [23]. PRL induces apoptosis in luteal cells of CLs by structural luteolysis [24]. P_4 has an important role in controlling the progression of pregnancy. In CLs, P_4 acts as a survival factor by inhibiting luteal cell apoptosis [25]; peripheral P_4 concentrations are minimal on the day of estrus (0.1 ng/ml), and rise to peak concentrations of 1.6–3.6 ng/ml on days 13 to 15 of the cycle in Thai swamp buffaloes [26, 27]. It was reported that P_4 levels continued to increase in Thai swamp buffaloes that conceived but decreased 3 days before the next estrus, and that P_4 levels during the estrous cycle in these individuals gradually increased after estrus with the first significant rise on day 6 with a peak concentration of 2–7 ng/ml, and reached a plateau

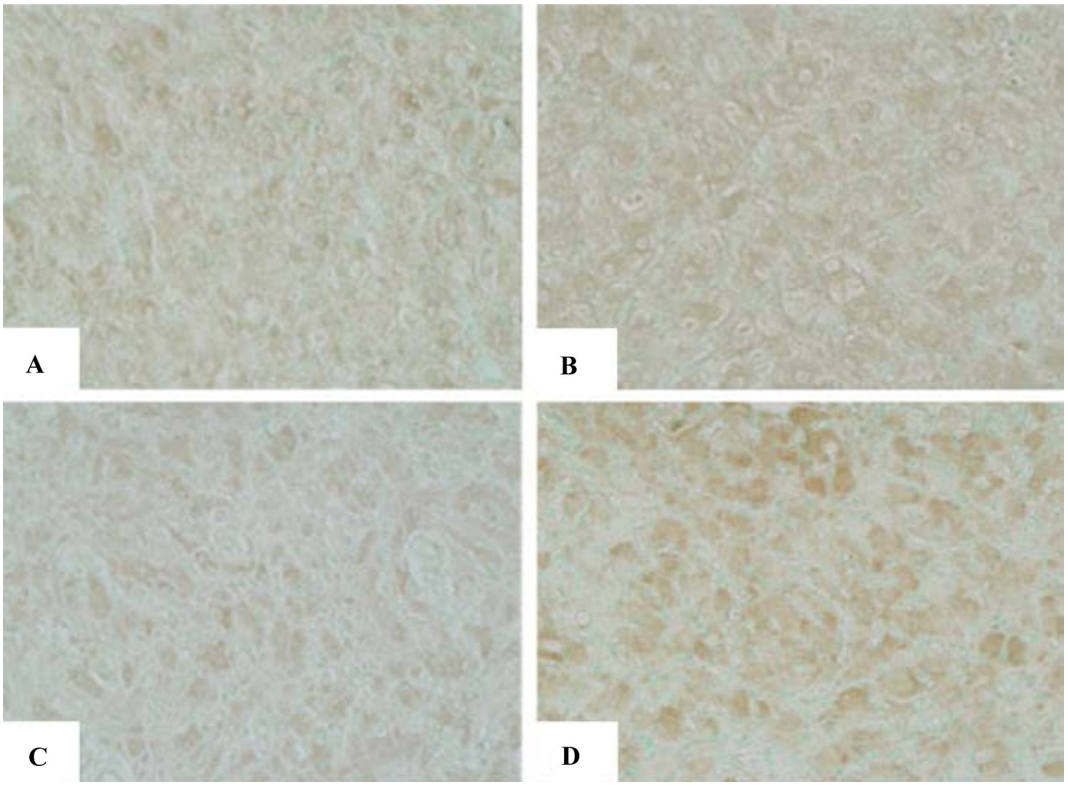

Fig. 4. Representative sections in luteal cells of buffalo corpus lutea (CLs) were stained with immunoreactions of $cFLIP_{S/L}$ during (A) early, (B) mid, and (C) late stages of the estrous cycle, and during (D) pregnancy.

after day 10 [26, 27]. A precipitous decrease in P_4 levels occurred during the 5 days before estrus [28]. Our findings in the present study demonstrated that the P_4 and cFLIP expression levels were high during pregnancy, but the amount of cFLIP declined during CL regression. The structural regression during the mid and late stages of the estrous cycle in buffalo ovaries began later than usual. In pregnant buffalos, both P_4 and cFLIP expression levels were very high. The mRNA expression levels of $cFLIP_S$ and $cFLIP_L$ were high during the pregnancy period, but there was no significant difference between $cFLIP_L$ during the estrous cycle and pregnancy. A recent study reported that high levels of cFLIP mRNA and protein were shown during the mid stages of the estrous cycle and pregnancy in porcine CLs [15]. The present study found significantly high levels of cFLIP mRNA and protein in CLs of Thai swamp buffalos during the early stage of the estrous cycle and during pregnancy ($P < 0.05$). Thus, in buffalo CLs, cFLIP increases at early stages of the estrous cycle as well as pregnancy, indicating that cFLIP acts as a dominant survival factor by inhibiting intracellular apoptosis signals. In addition, cFLIP acts as a dominant survival factor in Thai swamp buffaloes by inhibiting intracellular apoptosis signal transduction in CLs during pregnancy. These findings lead to the hypothesis that cFLIP is a major regulator of the maintenance and regression of CLs in domesticated mammals, although detailed regulating mechanisms are different among species. Further studies are necessary to reveal the regulation mechanism of cFLIP expression in isolated luteal cells of CLs in Thai swamp buffaloes.

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