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Original Article

Expression and contribution of autophagy to the luteal development and function in the pregnant rats

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Abstract: Autophagy has been demonstrated as an important regulatory mechanism in the apoptosis of luteal cells during the regression of corpus luteum (CL) in the pseudopregnant rat ovary. However, the role of autophagy during the rat pregnancy still remains unknown. Therefore, the present study was designed to investigate the expression and contribution of autophagy to the luteal development and function in the pregnant rats. Ovaries were obtained from the female rats at the early, middle or late phase of the pregnancy, which correspondingly had three groups, including the early (ELP), middle (MLP) and late luteal phase (LLP). The results of autophagy-associated protein LC-3 showed that autophagy expressed during the pregnant CL and significantly increased during the late luteal phase, which was further confirmed by the mRNA and protein expression changes of the other two autophagy related proteins, Beclin-1 and Atg-5. These results indicated autophagy may play an important role in the luteolysis. Further examinations of cellular apoptosis marker protein cleaved caspse-3 and ovarian prostaglandin$_{2\alpha}$ ($PGF_{2\alpha}$) levels found that cleaved caspse-3 dramatically increased during the late luteal phase which consistent with the changes of autophagy, while ovarian $PGF_{2\alpha}$ level significantly increased during the middle luteal phase and dramatically decreased during the late luteal phase. Together, these results suggested that autophagy may be induced by $PGF_{2\alpha}$ and then contributed to the luteolysis through cellular apoptosis during the late luteal phase of pregnant ovaries. To our knowledge, this will provide a new insight into the important mechanism regulating the luteolysis of the pregnant ovaries in mammals.

Keywords: Autophagy, apoptosis, $PGF_{2\alpha}$, corpus luteum, pregnant rat

Introduction

Autophagy is an evolutionary conserved cellular bulk degradation system in cells, by which cell may envelop part of cytoplasm in double membrane-bound structures called autophagosomes for the recycle of building blocks [1, 2]. Cell usually maintains a basal autophagy level under physiological conditions, whereas adverse survival environments such as short of growth factors or nutrients can lead to the up-regulation of cellular autophagy levels so as to bridge the shortage of energy [3, 4]. Accordingly, autophagy was originally thought as a pro-survival mechanism to nutrient deprivation, hypoxia and other types of cellular stress [5, 6]. However, many studies suggested that excessive self-digestion or the degradation of essential cellular components also promoted the apoptosis of cell by disrupting the normal regulation of cell physiology and provoke the activation of caspase dependent apoptosis pathways [7-9]. In addition, the morphological changes of steroidogenic cells during corpus luteum (CL) regression, which were characterized by the vacuolization of cytoplasmic, and lysosomes, the accumulation of autophagosomes, further confirmed that the apoptosis of steroidogenic luteal cell was induced by autophagy [10-12]. Indeed, previous studies have investigated the relationship between autophagy and apoptosis during the regression of CL in pseudopregnant rat [13], but to date, few studies have evaluated the involvement and induction of autophagy during the process of rat pregnancy.

The CL is an important hormone-responsive and hormone-productive reproductive organ in mammals, and the function of which is required for the success of early pregnancy from embryo implantation to development of the conceptus [14]. The main function of CL is to synthesis and secret progesterone [15], which is maintained during the early phase of pregnancy, and significantly increased at the middle and late stages of pregnancy in mammal [14]. However, the absence of pregnancy or the ending of pregnancy resulted in the removal of CL from ovary, which is mainly caused by the increased level of ovarian prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) [16]. Therefore, the present study was designed to evaluate the role of autophagy during the development of corpus luteum in the pregnant rats. The changes of autophagy related protein expressions and related hormones were examined to explore the expression and contribution of autophagy to the luteal development and function in the pregnant rats.

Materials and methods

Animals

Sprague-Dawley rats were purchased from Wushi Experimental Animal Supply Co. Ltd. (Fuzhou, China). The animals were maintained under a 14-h light/10-h dark schedule with continuous supply of chow and water. The experimental protocol was approved by the Institutional Animal Care and Use Committee and the Ethics Committee on Animal Experimentation, Fujian Normal University.

Experimental design

The rats were allowed to accommodate for 1~2 week prior to mating with males, which occurred at 2~3 months of age (200-250 g body weight). Previously unmated female rats (three per cage) were mated with a fertile male (one per cage) and were examined every morning for the presence of a vaginal plug. Day 1 of pregnancy was defined as the day when a vaginal plug was recovered. The pregnant females were removed and used in the subsequent experiments.

To further confirm the pregnant rat model, serum levels of progesterone were measured at different phases of pregnancy corresponding to the early, middle and late phase of rat pregnancy, which correspondingly had three groups in the present study, including the early luteal phase (ELP, Day 1 to 4), the middle luteal phase (MLP, Day 5 to 16) and the late luteal phase (LLP, Day 17 to 21) [14]. The rats were anesthetized with atropine (0.05 mg/kg body weight ip, Sigma-Aldrich, St. Louis, MO, USA) on the days of sample collection prior to opening of the abdomen, and ≥3.0 ml blood was collected from the abdominal aorta, and centrifuged at 1,000 x g at 4°C for 10 min. The ovaries were rapidly excised and chilled in ice-cold 0.154 M NaCl with 14.0 μM indomethacin (Sigma-Aldrich) immediately following perfusion for measuring the expression levels of autophagy-related proteins.

For histological analysis, one ovary from each rat was fixed in 4% paraformaldehyde (Sigma-Aldrich), and the other ovary was snap-frozen and used for the remaining experiments.

Ovarian perfusion

To avoid the effects of the vascular system, ovarian perfusion was performed before the collection of the ovaries. The female rats were perfused *in vivo* through the abdominal aorta with 0.154 M NaCl. After euthanasia, the abdomen was opened via a mid-ventral incision and an intravenous cannula was inserted via the aortic bifurcation. About 40 μl of the perfusion solution was perfused at ambient temperature through the lower abdominal vascular system for ~5 min at constant pressure using a hand-held syringe. Perfusion was suspended when the corpora lutea become completely pale. The ovaries were then rapidly removed for subsequent analysis of gene expression.

Immunohistochemistry of LC-3

Generally, the methods for immunohistochemical staining of LC-3 were done according to the manufacturer's recommendations and reported studies [13]. In briefly, paraffin-embedded tissue sections were dewaxed and rehydarated regularly. Hereafter, the sections were subjected to antigen microwave antigen retrieval by 0.01 M citric acid buffer for 10 min. Endogenous peroxide was inhibited by incubation of the sections in 3% H_2O_2 for 30 min. The sections were then incubated in 5% BSA for approximately 20 min to block non-specific conjunctions. Thereafter, 50 μl primary antibodies were applied overnight at 4°C overnight. The specific

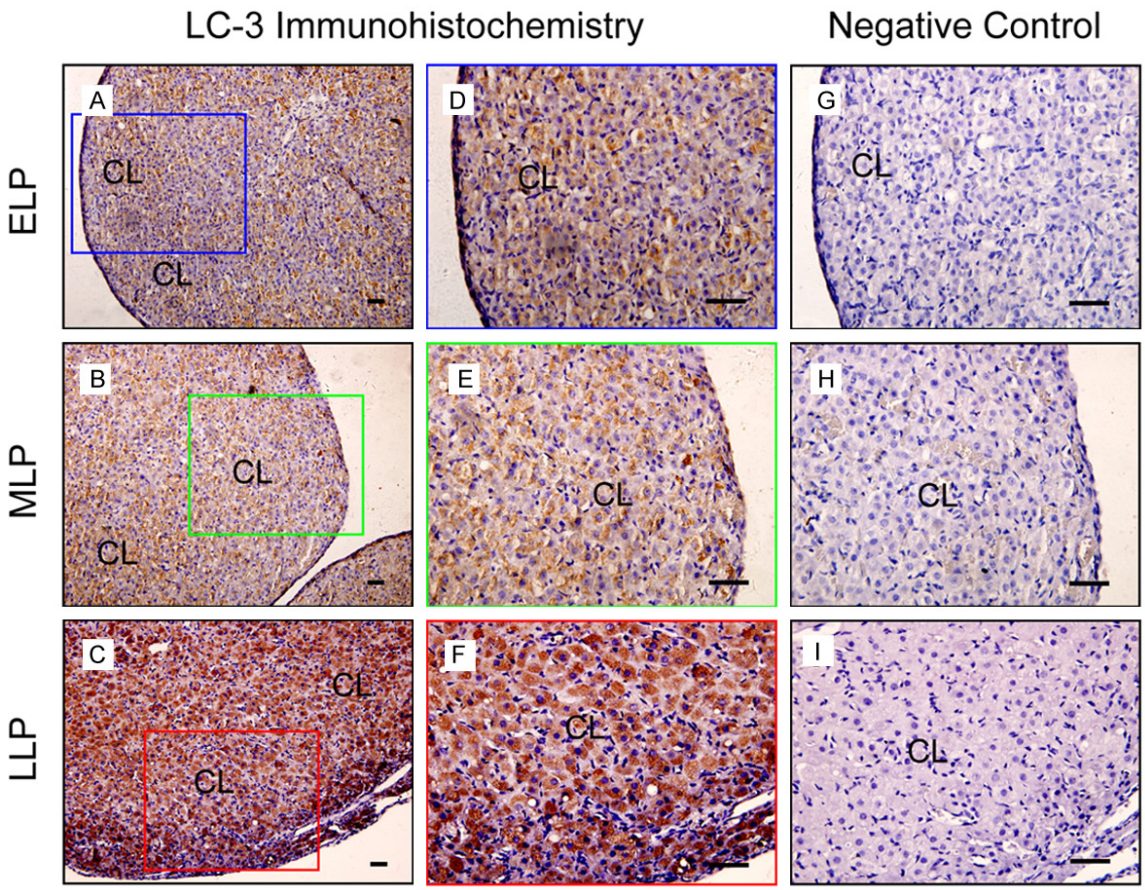

Figure 1. Expression and localization of LC-3 during the luteal development in the ovaries of pregnant rats. LC-3 immunohistochemical signals appear brown and the counterstaining background appears blue in color (A-F). Negative controls remained unstained lacking primary antibody instead of serum (G-I). (A, D and G) ELP group. (B, E and H) MLP group. (C, F and I) LLP group. GC: granulosa cell, Oo: oocyte, ELP: the early luteal phase, MLP: the middle phase, LLP: the late luteal phase, bar=100 um.

antibodies used were anti-LC-3 antibody (diluted 1:500, Abcam, Cambridge, MA, USA). After washing with PBS, slides were incubated with the secondary antibodies at room temperature for 20 min. For visualizing, Diaminobenzidine tetrahydrochloride chromogen staining was applied. All section were counterstained with hematoxylin, dehydrated and mounted lastly.

Western blot analysis of LC-3, Beclin-1 and Atg-5 expression levels

Ovarian tissues from each group were homogenized in ice cold RIPA lysate buffer (Beyotime Institute of Biotechnology, Haimen, China) and centrifuged at 15000 g for 15 min at 4°C, and then the supernatant was collected. Protein concentrations were thereafter determined using BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Haimen, China). Protein samples were diluted into the equal concentration and then 20 μg of the protein samples were subjected to 10% SDS-PAGE gel electrophoresis and then electrophoretically transferred onto a polyvinylidene difluoride membrane (Pall Life Sciences, Port Washington, NY, USA). The membrane was washed with TBS with 0.2% Tween 20 (TBST; Sigma-Aldrich). Nonspecific binding to the membrane was blocked with 5% nonfat milk in Tris buffered saline-Tween 20 (TBST, pH 7.4) for 1 h at room temperature. After that, the membranes were incubated overnight at 4°C with anti-LC-3 antibody (1:1000 dilution, Abcam, Cambridge, MA, USA), anti-Beclin-1 antibody (1:2000 dilution, Protein Tech Group, Wuhan, China), anti-Atg5 antibody (1:1000 dilution, Protein Tech Group, Wuhan, China) and anti-β-actin antibody (1:5000 dilution, Protein Tech Group, Wuhan,

China). After washing with TBST for three times, the membrane were incubated in horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG (1:5000 dilution, Beyotime Institute of Biotechnology, Haimen, China) for 1 h at room temperature. After that, bands were visualized by using the enhanced chemiluminescence star (ECL, Beyotime Institute of Biotechnology, Haimen, China). The blots were quantified using ImageJ 1.49 software (National Institutes of Health, Bethesda, MD, USA).

RNA extraction and reverse transcription-quantitative polymerase chain reaction analysis of Beclin-1 and Atg-5 mRNA

Total RNA was extracted from the ovaries using TRIzol solution (Invitrogen Life Technologies, Carlsbad, CA, USA) and then reverse-transcribed using a cDNA Synthesis kit (Promega, Biotech Co., Ltd). The reverse-transcribed products were amplified using a FasQuant RT Kit (TIANGEN BIOTECH CO., LTD, Beijing, China), with Go Taq qPCR Master Mix (Promega corporation, Lot 0000209928), Beclin1 primer (forward primer 5-ATG CTG TCC TTT CCC TCT TCC-3', reverse primer 5'-ACC TTT ACC TCT TGT CCC TTC C-3') and Atg-5 primer (forward primer 5'-AGA AGA AGA GCC AGG TGA TGA-3', reverse primer 5'-AAT GCT GAT GTG AAG GAA GTT GT-3'). A kit for detecting the levels of 18S ribosomal RNA (Hs99999901_s1) was used as an endogenous control. The 20 μl PCR reaction mix contained 10.0 μl 2X Go Taq qPCR Master Mix (Promega corporation), 0.2 ul CXR reference Dye, 2.0 μl cDNA template, 7.0 μl RNase-free water and 0.8 primer (containing 0.4 ul forward and 0.4 ul reverse). The PCR conditions of the RT-qPCR system (Applied Biosystems Life Technologies), were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. The relative gene expression levels were calculated in accordance with the ΔΔCt method, and relative mRNA levels were expressed as $2^{-\Delta\Delta Ct}$ values [17, 18].

Radioimmunoassay of progesterone and prostaglandin $F_{2\alpha}$

The levels of serum progesterone and ovarian prostaglandin $F_{2\alpha}$ were determined using specific radioimmunoassay kits, according to the manufacturer's instructions. The progesterone RIA kit [intra-assay coefficient of variation (CV) < 4.3%; inter-assay CV < 7.1%] and the prostaglandin F_{2a} radioimmunoassay kit (intra-assay CV < 10.0% and interassay CV < 15.0%) were from Atomic Gaoke Co., Ltd., Department of Isotope, China Institute of Atomic Energy (Beijing, China). Protein concentrations were determined using a BCA kit (Beyotime Institute of Biotechnology, Haimen, China), with bovine serum albumin standards.

Statistics

Data are presented as means ± SE. The significance of differences in mean values within and between multiple groups was evaluated using a one-way ANOVA, followed by a Tukey's multiple range test. $P < 0.05$ was considered statistically significance. #: $P < 0.05$, vs. the early lutral phase (ELP), and &: $P < 0.05$, vs. the middle lutral phase (MLP).

Results

Expression and localization of LC-3 during the luteal development in the ovaries of pregnant rats

During the pregnancy, corpus luteum is the main part in the ovary. The present results showed LC-3 expressed during this period and mainly localized in steroidogenic cells (**Figure 1**). Notably, the immunoreactivity of LC-3 maintained a weak level at ELP (**Figure 1A** and **1D**) and MLP (**Figure 1B** and **1E**) compared with the negative control (**Figure 1G-I**), LC-3 expressions significantly increased at LLP (**Figure 1C** and **1F**). These results indicated that LC-3 mediated-autophagy expressed during the luteal development of pregnant rats, especially luteolysis at LLP.

LC-3II expression increased during the luteal development in the ovaries of pregnant rats

For further confirming the results of LC-3 immunohistochemistry, the present study also detected LC-3 protein expression by western blotting and found LC-3II expression significantly increased at LLP compared with those at ELP and MLP (**Figure 2**), further implying LC-3II mediated-autophagy was induced at LLP and involved in the luteolysis of the pregnant ovaries.

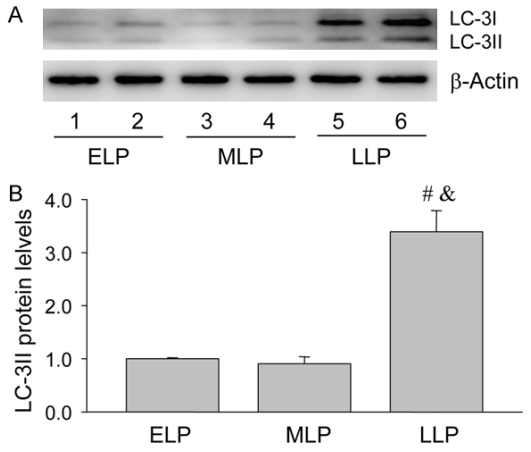

Figure 2. Blotting analysis of LC-3I and LC-3II during the luteal development in the ovaries of pregnant rats. A: Representative ECL gel documents of Western blot analyses depicting the protein levels of LC-3I and LC-3II. B: Summarized intensities of LC-3II blots normalized to the control. Each value represents the mean ± SE, n=6. One-way analysis of variance (ANOVA) was used to analyze the data. ELP: the early luteal phase, MLP: the middle phase, LLP: the late luteal phase, #: $P < 0.05$, vs. ELP group, &: $P < 0.05$, vs. MLP group.

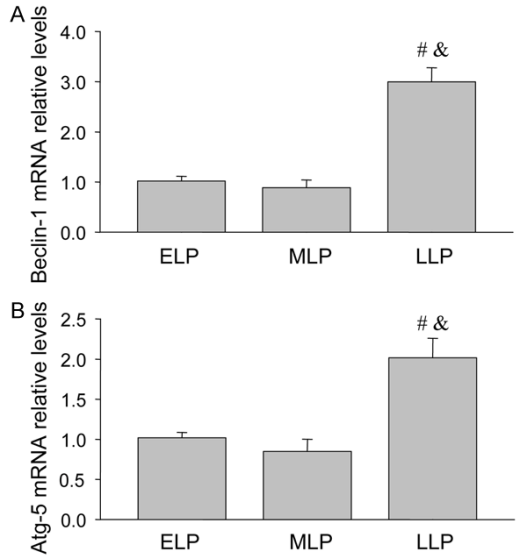

Figure 3. Expression changes of Beclin-1 and Atg-5 mRNA during the luteal development in the ovaries of pregnant rats. A: The relative mRNA levels of Beclin-1 by real-time RT-PCR analysis. B: The relative mRNA levels of Atg-5 by real-time RT-PCR analysis. Each value represents the mean ± SE. n=6, One-way analysis of variance (ANOVA) was used to analyze the data. ELP: the early luteal phase, MLP: the middle phase, LLP: the late luteal phase, #: $P < 0.05$, vs. ELP group, &: $P < 0.05$, vs. MLP group.

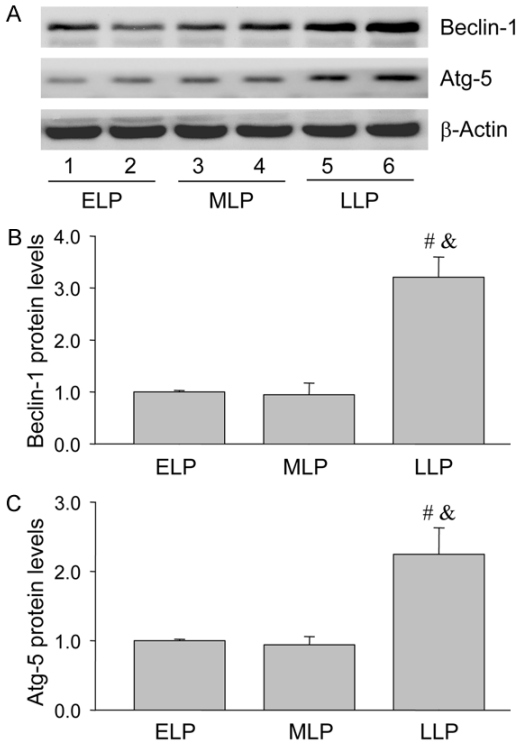

Figure 4. Blotting analysis of Beclin-1 and Atg-5 during the luteal development in the ovaries of pregnant rats. A: Representative ECL gel documents of Western blot analyses depicting the protein levels of Beclin-1 and Atg-5. B: Summarized intensities of Beclin-1 blots normalized to the control. C: Summarized intensities of Atg-5 blots normalized to the control. Each value represents the mean ± SE, n=6. One-way analysis of variance (ANOVA) was used to analyze the data. ELP: the early luteal phase, MLP: the middle phase, LLP: the late luteal phase, #: $P < 0.05$, vs. ELP group, &: $P < 0.05$, vs. MLP group.

Autophagy-related proteins Beclin-1 and Atg-5 increased during the luteal development in the ovaries of pregnant rats

Beclin1 and Atg-5 are two essential factors that involved in the initiation of autophagy in mammalian cells though autophagy could also be induced via Beclin-1 independent pathways [19]. The present study firstly examined the mRNA expression levels of Beclin1 and Atg-5 and found their expressions dramatically increased at LLP compared with ELP and MLP (**Figure 3**) and then detected the protein expression levels of these two proteins and found the similar results with mRNA expressions (**Figure 4**). These results demonstrated the expression levels of both proteins were upregulated and the induction of autophagy was occurred in a

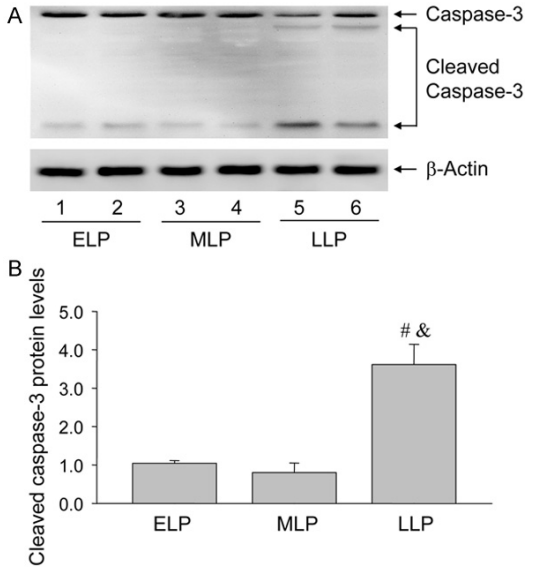

Figure 5. Blotting analysis of caspase-3 and cleaved caspase-3 during the luteal development in the ovaries of pregnant rats. A: Representative ECL gel documents of Western blot analyses depicting the protein levels of caspase-3 and cleaved caspase-3. B: Summarized intensities of cleaved caspase-3 blots normalized to the control. Each value represents the mean ± SE, n=6. One-way analysis of variance (ANOVA) was used to analyze the data. ELP: the early luteal phase, MLP: the middle phase, LLP: the late luteal phase, #: P < 0.05, vs. ELP group, &: P < 0.05, vs. MLP group.

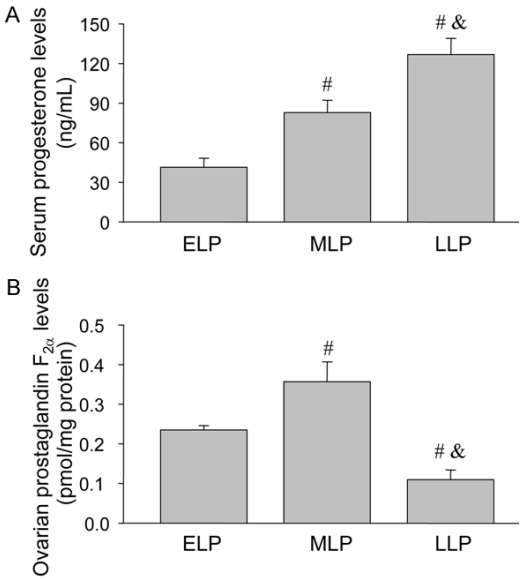

Figure 6. Changes of serum progesterone levels and ovarian prostaglandin F_{2a} during the luteal development in the ovaries of pregnant rats. Pregnant rats were sacrificed by decapitation and trunk blood was collected for progesterone determination. The abdomen was opened and the ovaries were rapidly excised and chilled in ice-cold 0.154 M NaCl containing 14 uM indomethacin just after perfusion for measuring prostaglandin F_{2a} expression. A: Serum progesterone levels, B: Ovarian prostaglandin F_{2a} levels. Each value represents the mean ± SE, n=6. One-way analysis of variance (ANOVA) was used to analyze the data. ELP: the early luteal phase, MLP: the middle phase, LLP: the late luteal phase, #: P < 0.05, vs. ELP group, &: P < 0.05, vs. MLP group.

Beclin-1 dependent manner during the late luteal phase of rat pregnancy.

Activation of luteal cell apoptosis during the luteal development in the ovaries of pregnant rats

Given the important role of cell apoptosis in the luteolysis at the late luteal phase of pregnancy, the present study detected the expression of caspase-3 and found the expression of cleaved caspase-3 significantly increased at LLP compared with ELP and MLP (**Figure 5**), indicating the apoptosis of luteal cells via the activation of caspase-3 may be related with the induction of autophagy during the luteolysis of pregnancy.

Serum progesterone and ovarian prostaglandin F_{2a} during the luteal development in the ovaries of pregnant rats

It is well-known that serum progesterone levels keep increasing during the pregnancy and ovarian prostaglandin F_{2a} contributes to the luteolysis of the ovary. Therefore, the present study measured these two hormone levels and found serum progesterone level was the highest at LLP (**Figure 6A**) and the lowest at ELP (**Figure 6A**) as reported previously. Interestingly, ovarian prostaglandin F_{2a} increased at MLP (**Figure 6B**) and then decreased at LLP (**Figure 6B**), implying the changes of prostaglandin F_{2a} were also involved in the role of autophagy during the luteolysis through cell apoptosis in the ovaries of pregnant rats.

Discussion

The results of the present study clearly demonstrated that autophagy was significantly induced and activated at LLP of pregnant rats, while the expression of cleaved caspase-3 in a similar manner. These results suggested that autophagy may play an important role in the luteal regression of pregnant ovaries *in vivo* in mammals.

In mammals, corpus luteum is an ephemeral endocrine structure formed from a ruptured and ovulated follicle, the function of which is important for the female reproductive cycle [20-23]. At the end of pregnancy, the CL undergoes a process of regression leading to its disappearance from the ovary and allowing the initiation of a new cycle [14]. Previous studies have revealed that autophagy is involved in the regression of mammalian CL, such as the induction of autophagy in the regressing CL of marmoset monkeys [24, 25] and humans [26]. In the pseudopregnant rat, the induction of autophagy was also revealed at the late stage of rat pseudopregnancy [13]. However, there are no studies of the induction and involvement of autophagy in the luteal regression of pregnancy, since the mechanisms regulating the luteal development and function were different physiologically between pseudopregnancy and pregnancy. In the present study, the results of LC-3 immunohistochemistry indicated LC-3 mediated autophagy expressed during the luteal development and significantly increased at LLP of pregnant ovaries. During the induction of autophagy, LC-3 is converted from LC-3I to LC-3II; LC-3II and then localized to isolated membranes and autophagosomes [27, 28]. Accordingly, the amount of LC-3II expressed is positively correlated with the number of autophagosomes [29]. In present study, the expression of LC-3II was measured and showed the same pattern with LC-3 staining intensity in the luteal cells, which implied that luteal cells might also be the primary site for autophagosome formation at LLP of rat pregnancy.

Since Atg-5 is an essential protein that implicated in the initiation of autophagy [30] and Beclin1 is another autophagy related protein that involved in the formation of autophagosome in mammalian cells [31, 32], the present study also examined the expressions of another two autophagy-related proteins, Beclin-1 and Atg-5, during the luteal development of the pregnant ovaries. Consistently, the result showed that the expression of Atg5 was simultaneously increased at LLP of rat pregnancy. However, autophagy could also be induced via a Beclin-1 independent pathway [33, 34]. Previous studies have revealed that Beclin-1 is crucial for the induction of autophagy in mouse [35], but it is still unknown whether Beclin-1 is also involved in the regulation of autophagy in pregnant rat model. Therefore, the present study checked the expression of Beclin-1 at different stages of rat pregnancy and found the expression of Beclin-1 significantly increased at LLP compared with ELP and MLP. These results further demonstrated autophagy may play an important role in the luteal regression of pregnant ovaries *in vivo* in mammals.

At the late stage of pregnancy, part of CL functions is taken over by placenta and pregnant CL thereafter undergoes a process of regression [16, 36, 37]. Given the contribution of cell apoptosis to the luteal regression, the present study further detected the expression of cleaved caspase-3 during the luteal development and found its expression levels obviously increased at LLP, which is consistent with the expressions of autophagy-related proteins. These results together indicated the induction of autophagy contributed to the luteolysis of the pregnant ovaries. Interestingly, the concentrations of ovarian PGF_{2a} significantly increased at MLP and then dramatically decreased at LLP. In most mammalian species, PGF_{2a} is recognized as a decisive factor in the apoptosis of luteal cells during the functional regression of corpus luteum [38]. In addition to canonical apoptosis pathway induced by PGF_{2a} emergence, the induction of autophagy is also identified as one of the main factors that contributed to cell apoptosis. Choi et al studies have also demonstrated that administration of PGF_{2a} is capable of inducing autophagy in luteal cells *in vitro* [39], further indicating that autophagy may be induced by prostaglandin F_{2a} and then contribute to the luteolysis through cell apoptosis in the ovaries of pregnant rats.

In conclusion, the present study is the first time, to our knowledge, to demonstrate the expression and contribution of autophagy to the luteal development and function in the pregnant rats. Based on the previous investigations, the present study demonstrated that autophagy may be induced by prostaglandin F_{2a} and then contribute to the luteolysis through cell apoptosis in the ovaries of pregnant rats, which will help us to further understand the molecular mechanism regulating the luteal development and function of pregnant ovaries, and provide a theoretical basis for the clinical treatment of luteal dysfunctions, like ovarian hyperstimulation syndrome.

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Disclosure of conflict of interest

None.

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