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Original Article

Accumulated autophagosomes and excessive apoptosis during the luteal development of pregnant rats

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Abstract: Autophagy plays an important role in the regression of pseudopregnant corpus luteum, whereas the involvement of autophagy in the pregnant luteolysis still remains unknown. Therefore, the present study was designed to investigate the levels and effects of accumulated autophagosomes on excessive apoptosis during the luteal development of 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 clearly showed that autophagy expressed during the pregnant CL and significantly increased at LLP, while the expression changes of apoptosis related proteins cleaved caspase-3 and Bax were similar with LC-3 expression changes, indicating autophagy may be involved in the initiation of pregnant luteolysis through the induction of cellular apoptosis at LLP of pregnant ovaries. The present study was further examined the expressions of other two autophagy-associated proteins p62 and LAMP-2, since the degradation failure of autophagosomes contributed to cellular apoptosis. The results demonstrated p62 protein was accumulated at LLP while its mRNA was maintained during the whole luteal development of pregnant rats. Interestingly, the expressions of LAMP-2 mRNA and premature protein were significantly increased at MLP and LLP, while the expression of mature LAMP-2 increased at MLP and then decreased at LLP, implying autophagosomes were accumulated at LLP. Together, to our knowledge, the present study firstly demonstrated that the insufficient of lysosomal functions contributed to the impaired degradation of autophagosomes and then activated caspase-3 dependent apoptotic pathway during the pregnant luteolysis of rat ovaries, which will provide a new insight into the important mechanism regulating the luteolysis of the pregnant ovaries in mammals.

Keywords: Autophagosome, lysosomal function, cellular apoptosis, corpus luteum, pregnant rat

Introduction

In mammals, corpus luteum (CL) is a unique and ephemeral endocrine gland developed from the remains of ovarian follicle following ovulation, which is essential for the homeostasis of hormones and the establishment of pregnancy [1-4]. When the CL is no longer necessary for the maintenance of pregnant orchestrations in the absence of pregnancy or at the end of pregnancy, it ceases to produce progesterone and thereafter undergoes a process of regression resulting in physical destruction and elimination from the ovary [3, 4]. Therefore, the process of luteolysis is recognized as an essential step for the maintenance of ovarian homeostasis and the re-initiation of next estrous/menstrual cycle [3, 4]. Previous studies have demonstrated that apoptosis is the main event that lead to the death of luteal cells and regression of CL [3, 4]. With pseudopregnant rat model, studies also suggested that autophagy is involved in the regulation of CL regression by promoting the activation of caspase-3 dependent apoptotic pathway [3-5], but the involvement of autophagy in the pregnant luteolysis still remains to be clarified.

Autophagy is an evolutionarily conserved eukaryotic degradation system, involving the

sequestration of cytoplasmic components within double-membraned vesicles termed as autophagosome [6, 7]. Thereafter, autophagosomes fuse with lysosomes to generate so-called autolysosomes, in which the contents of autophagosomes were degraded for the recycle of building blocks [6, 7]. Canonically, the induction of autophagy was originally thought to represent a pro-survival mechanism under diverse cellular stresses. However, the results of many studies also suggested that excessive self-digestion caused by exorbitant autophagy induction or accumulated autophagosomes caused by impaired autophagosome degradation promotes cell death [8-11]. The morphological characteristics of steroidogenic luteal cells in regressing rat and primate CL further suggest the involvement of autophagy during CL regression [12-14]. However, to date, few studies have investigated the involvement and mechanism of autophagy during the initial stage of pregnant luteolysis in rat ovaries.

Therefore, the present study was designed to investigate the expression of autophagy and the effects of accumulated autophagosomes and excessive apoptosis during the luteal development of the pregnant rats. The expression of autophagy was examined by autophagic marker protein LC3, while cellular apoptosis was detected by apoptosis related proteins cleaved caspase-3 and Bax and anti-apoptotic protein Bcl-2 for estimating the relationship between autophagy induction and the occurrence of cellular apoptosis. Furthermore, the mRNA and protein expressions of p62 and LAMP-2 were also examined in order to evaluate the degradation of autophagosomes and the implication of lysosomes during the luteal development of 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). The rats were anesthetized with atropine (0.05 mg/kg body weight i.p., 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× 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.

Immunohistochemistry

Generally, the methods for immunohistochemical staining of LC-3 were done according to the manufacturer's recommendations and reported studies. 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 antibodies

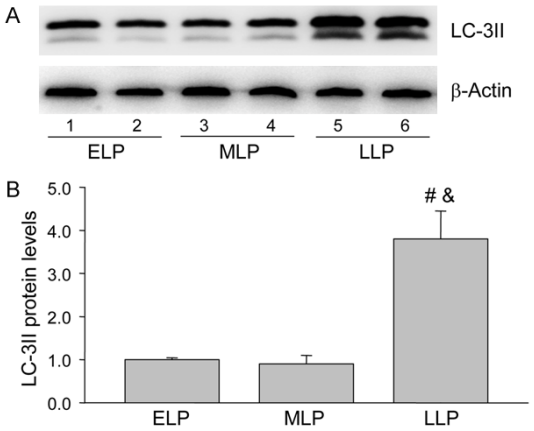

Figure 1. Expression of LC-3II during the luteal development in the ovaries of pregnant rats. A: Representative ECL gel documents of Western blot analyses depicting the protein levels of LC-3II. B: Summarized intensities of LC-3II blots normalized to the control. Each value represents the mean ± SE, 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.

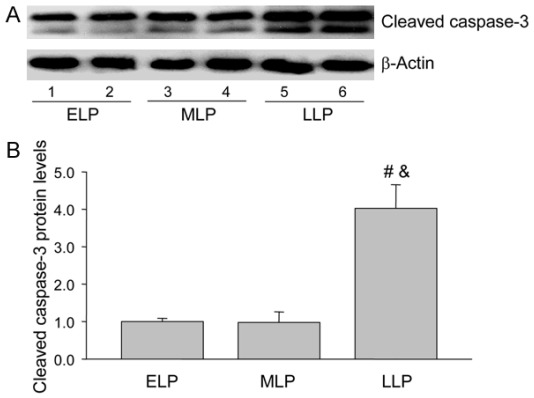

Figure 2. Expression of cleaved caspase-3 during the luteal development in the ovaries of pregnant rats. A: Representative ECL gel documents of Western blot analyses depicting the protein levels of cleaved caspase-3. B: Summarized intensities of cleaved caspase-3 blots normalized to the control. Each 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.

used were anti-p62 rabbit monoclonal antibody (diluted 1:500, Abcam, Cambridge, MA, USA) and LAMP-2 rabbit polyclonal antibody (diluted 1:500, protein tech, Wuhan, China). 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 blotting analysis

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-cleaved caspase-3 antibody (1:1000 dilution; Cell Signaling Technology, Boston, MA, USA), anti-Bax antibody (1:2000 dilution, Protein Tech Group, Wuhan, China), anti-Bcl-2 antibody (1:1000 dilution, Protein Tech Group, Wuhan, China), anti-p62 antibody (1:1000 dilution, Abcam, Cambridge, MA, USA), anti-LAMP-2 antibody (1:1500 dilution, Protein Tech Group, Wuhan, China) and anti-β-actin antibody (1:4000 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

Total RNA was extracted from the ovaries using TRIzol solution (Invitrogen Life Technologies,

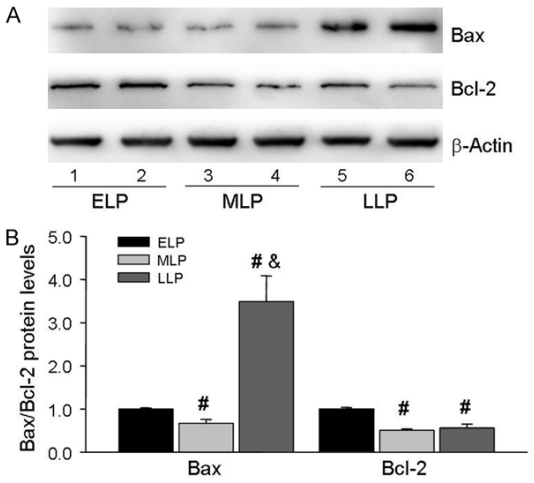

Figure 3. Expression of Bax and Bcl-2 during the luteal development in the ovaries of pregnant rats. A: Representative ECL gel documents of Western blot analyses depicting the protein levels of Bax and Bcl-2. B: Summarized intensities of Bax and Bcl-2 blots normalized to the control. Each value represents 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.

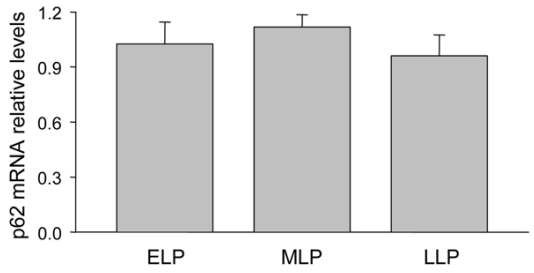

Figure 4. Expression changes of p62 mRNA during the luteal development in the ovaries of pregnant rats. The relative mRNA levels of p62 were examined by real-time RT-PCR analysis. Each value represents the mean ± SE. n=6, One-way analysis of variance (ANOVA) was used to analyze the data. ELP: the early luteal phase, MLP: the middle phase, LLP: the late luteal phase.

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), p62 primer (forward primer 5-AAG TTC CAG CAC AGG CAC AG-3', reverse primer 5'-AGC AGT TAT CCG ACT CCA TCA G-3') and LAMP-2 primer (forward primer 5'-CTC GTC CTG CTC TTT CTG TTC-3', reverse primer 5'-ACT GCT TCC ATT GTA TGT CAC C-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 2× Go Taq qPCR Master Mix (Promega corporation), 0.2 μl CXR reference Dye, 2.0 μl cDNA template, 7.0 μl RNase-free water and 0.8 primer (containing 0.4 μl forward and 0.4 μl 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 [15-17].

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

In order to evaluate the role of autophagy during the luteal development in the ovaries of pregnant rats, the expression of LC-3II, a marker protein of autophagy, was detected in ovarian CL and then found LC-3II expressed during pregnant luteal development and significantly upregulated at LLP compared with ELP and MLP (P<0.05, **Figure 1A** and **1B**), implying autophagy may be involved in the luteolysis of pregnant rats.

Activation of cellular apoptosis during the luteolysis in the ovaries of pregnant rats

Given the important role of cell apoptosis in the luteolysis at LLP of pregnancy, the present study detected the expression of cleaved caspase-3, a marker protein for the occurrence of cell apoptosis, at different pregnant stages and

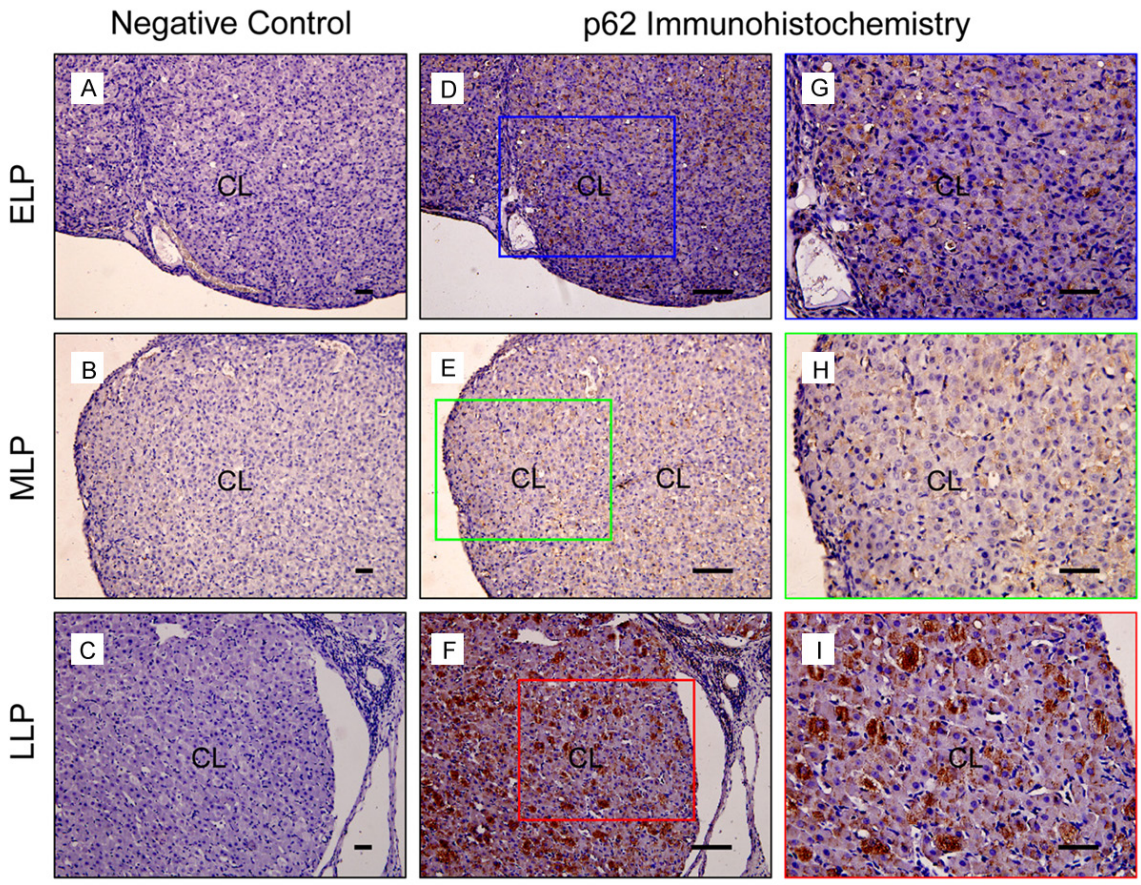

Figure 5. Immunohistochemical analysis of p62 protein expressions during the luteal development in the ovaries of pregnant rats. p62 immunohistochemical signals appear brown and the counterstaining background appears blue in color (D-I). Negative controls remained unstained lacking primary antibody instead of serum (A-C). (A, D and G) ELP group. (B, E and H) MLP group. (C, F and I) LLP group. CL: corpus luteum, ELP: the early luteal phase, MLP: the middle phase, LLP: the late luteal phase, bar =100 um.

then found the expression of cleaved caspase-3 significantly increased at LLP compared with ELP and MLP ($P<0.05$, **Figure 2**), 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.

To clarify the mechanism for the induction of apoptosis, the expression levels of Bax and Bcl-2 were detected and found the levels of Bax expression decreased at MLP ($P<0.05$, **Figure 3A** and **3B**) and then significantly increased at LLP compared with ELP ($P<0.05$, **Figure 3A** and **3B**), while the levels of Bcl-2 expression obviously decreased at MLP and LLP compared with ELP ($P<0.05$, **Figure 3A** and **3B**). These results revealed the upregulation of Bax expression led to the activation of caspase-3 dependent apoptotic pathway in luteal cells at LLP of pregnant rats.

Accumulated autophagosomes during the luteolysis in the ovaries of pregnant rats

Since the excessive induction of autophagy or the impairment of autophagy flux led to cellular apoptosis, the present study also examined the changes of p62 expression for evaluating whether autopahgosomes were normally degraded during the luteal development in the ovaries of pregnant rats. The results found no obvious changes of p62 mRNA expression by RT-PCR at different pregnant stages (**Figure 4**). Interestingly, the results of P62 immunohistochemistry indicated the levels of p62 protein were significantly increased at LLP compared with ELP and MLP ($P<0.05$, **Figure 5**). Therefore, the expression of p62 protein was further examined by western blotting and found its expression decreased at MLP ($P<0.05$, **Figure 6A** and **6B**) and then significantly increased at

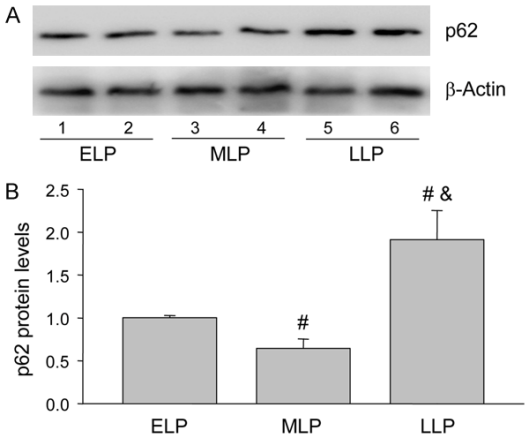

Figure 6. Blotting analysis of p62 protein expressions during the luteal development in the ovaries of pregnant rats. A: Representative ECL gel documents of Western blot analyses depicting the protein levels of p62. B: Summarized intensities of p62 blots normalized to the control. Each value represents 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.

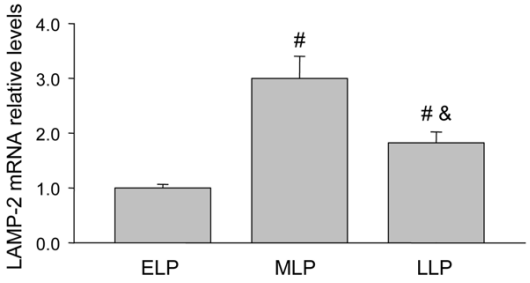

Figure 7. Expression changes of LAMP-2 mRNA during the luteal development in the ovaries of pregnant rats. The relative mRNA levels of LAMP-2 were examined by real-time RT-PCR analysis. Each value represents the mean ± SE. n=6, One-way analysis of variance (ANOVA) was used to analyze the data. ELP: the early luteal phase, MLP: the middle phase, LLP: the late luteal phase, #: P<0.05, vs. ELP group, &: P<0.05, vs. MLP group.

LLP compared with ELP (P<0.05, **Figure 6A** and **6B**). These results indicated that the accumulation of autophagsosomes contributed to the apoptosis of luteal cells at LLP of pregnant rats.

Impairment of lysosome functions during the luteolysis in the ovaries of pregnant rats

Given lysosome is an executor of the autophagic process, the present study further examined the changes of a lysosomal marker protein LAMP-2 (lysosomal associated membrane protein 2) expression for clarifying whether the impairment of lysosome functions contributed to the accumulation of luteal autophagosomes during the luteolysis in the ovaries of pregnant rats. The results showed the expression of LAMP-2 mRNA obviously increased at MLP (P<0.05, **Figure 7**) and then significantly decreased at LLP compared with ELP (P<0.05, **Figure 7**). The immunostaining results of LAMP-2 were consistent with LAMP-2 mRNA expression (**Figure 8**). Furthermore, LAMP-2 expression was also examined by western blotting and found premature LAMP-2 expression significantly increased at MLP and LLP compared with ELP (P<0.05, **Figure 9A** and **9B**), while mature LAMP-2 expression increased at MLP compared with ELP (P<0.05, **Figure 9A** and **9B**) and then significantly decreased at LLP compared with MLP (P<0.05, **Figure 9A** and **9B**). These results suggested that insufficient lysosomes were high related with the impairment of autophagy flux.

Discussion

The present study clearly demonstrated that autophagy was significantly induced at the late stage of ovarian corpus luteum in pregnant rats, which led to the turnover of Bcl-2/Bax ratio and the activation of caspase-3 dependent apoptotic pathway. These results indicated autophagy played an important physiological role in the ovarian luteolysis of pregnant rats.

In mammals, corpus luteum (CL) is evolved from the remains of ruptured follicles, which is essential for the hormone homeostasis as well as the successful implantation and the development of embryos [1-5]. The CL must be regularly eliminated to allow normal reproductive cycle, since the prolonged existence of CL may lead to the disruption of hormonal orchestrations [3-5]. Thus, the existence of CL is high regulated according to the requirement of mammalian physiologies and the regression of CL will be occurred in the absence of pregnancy or at the end of pregnancy [18]. As a steroid secretion gland, CL is involved in the dynamic balance of hormonal secretion, which ensured the successful implantation of embryos during the early pregnant stage [3-5, 19]. In the middle stage of pregnancy, luteal functions are

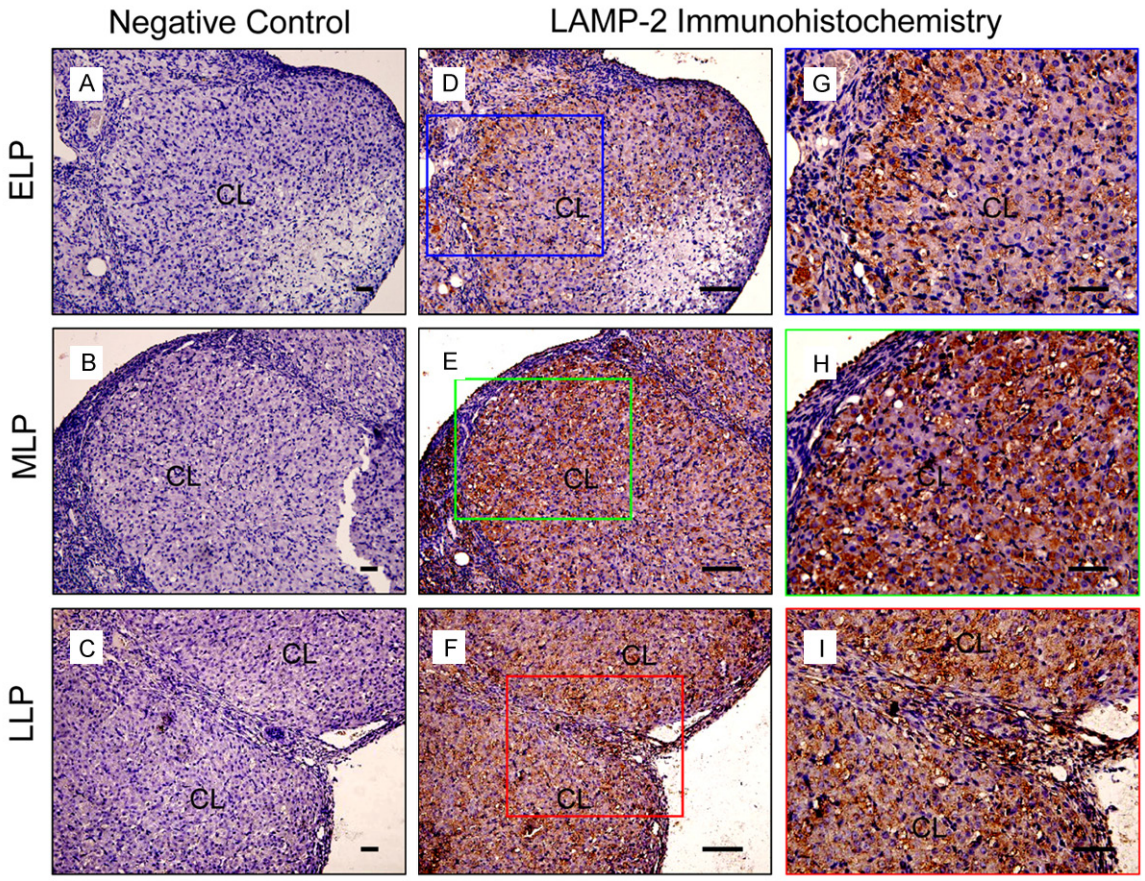

Figure 8. Immunohistochemical analysis of LAMP-2 protein expressions during the luteal development in the ovaries of pregnant rats. LAMP-2 immunohistochemical signals appear brown and the counterstaining background appears blue in color (D-I). Negative controls remained unstained lacking primary antibody instead of serum (A-C). (A, D and G) ELP group. (B, E and H) MLP group. (C, F and I) LLP group. CL: corpus luteum, ELP: the early luteal phase, MLP: the middle phase, LLP: the late luteal phase, bar =100 um.

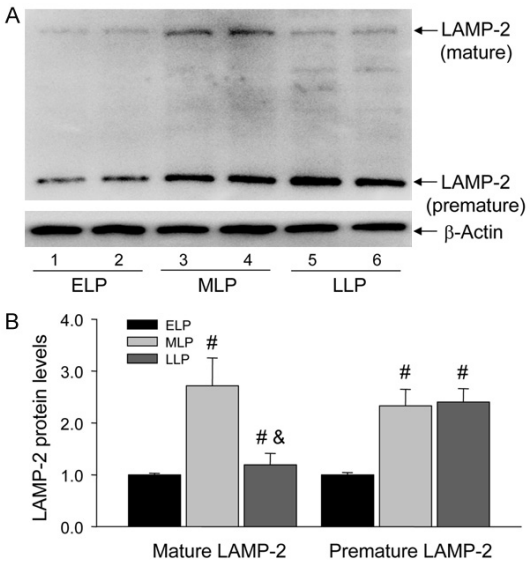

Figure 9. Blotting analysis of LAMP-2 protein expressions during the luteal development in the ovaries of pregnant rats. A: Representative ECL gel documents of Western blot analyses depicting the protein levels of LAMP-2. B: Summarized intensities of mature LAMP-2 and premature LAMP-2 blots normalized 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.

maintained by maturing decidua and placenta and pregnant CL is mainly associated with the production of progesterone to support the development of conceptus [1-5]. Accordingly, the pseudopregnant CL underwent a process of luteolysis without the presence of conceptus [20]. At the late stage of pregnancy, CL is not required for the development of fetus and initiating a process of luteal regression to ensure successful delivery [3-5]. Recently, some studies suggested that autophagy is involved in the

regression of CL in many species [4, 5, 13], but the involvement of autophagy in the pregnant luteolysis still remains unknown.

Previously, cellular apoptosis was recognized as the main mechanism for the elimination of CL [3-5] and the induction of autophagy was highly related with the cellular apoptosis [6-8]. Therefore, the present study detected the expression of autophagy maker protein LC-3II at different stages of rat pregnancy and found autophagy was involved in the pregnant luteolysis. To elucidate the association between autophagy and apoptosis, the present study also detected the expression of apoptosis related protein cleaved caspase-3 and found the changes of cellular apoptosis in pregnant CL were consistent with the changes of autophagy, implying autophagy may play a regulatory role in the initiation of luteolysis through the activation of caspase-3 during the luteal development in pregnant rats. Further investigation found Bax expression was similar with the expression of cleaved caspase-3, which was consistent with previous studies [3-8]. These results indicated the turnover of Bcl-2/Bax caused by the upregulation of autophagy led to the activation of caspase-3 during the ovarian luteolysis of pregnant rats.

It's well-known that autophagy is a building block recycling system for maintaining cellular homeostasis under stressful conditions, the induction of autophagy is essential for cell survival and the deficiency of autophagy lead to the cellular apoptosis [21, 22]. However, the accumulation of autophagsosomes or the impairment of autophagy flux may contribute to the cellular apoptosis [6-8]. Therefore, the present study also examined the expression of autophagic adaptor protein p62 and found its expression significantly increased at the late stage of corpus luteum in pregnant rats, indicating the accumulation of autophagsomes may be responsible for the apoptosis of luteal cells during the luteolysis of pregnant rats. The integrate process of autophagy is orchestrated by several steps and the dysregulation of each step may be associated with the disturbance of cellular functions and physiological catabolism [6-8]. Lysosome is a critical subcellular organelle involved in the execution of autophagy [23], so the present study further detected the expression of Lysosome-associated membrane protein-2 (LAMP-2), a highly glycosylated lysosomal maker protein, during the luteal development in the ovaries of pregnant rats. The results showed premature LAMP-2 expression significantly increased at MLP and LLP, but mature LAMP-2 expression significantly increased at MLP and then obviously decreased at LLP, indicating posttranscriptional glycosylation of LAMP-2 may contribute to the insufficient of lysosomal functions, which led to the impairment of autophagy execution and the accumulation of autophagosomes during the luteolysis of pregnant rats.

In conclusion, it is the first time, to our knowledge, to demonstrate the insufficient of lysosomal functions contributed to the impaired degradation of autophagosomes and the following activation of caspase-3 dependent apoptosis during the pregnant luteolysis of rat ovaries, which will provide a new insight into the important mechanism regulating the luteolysis of the pregnant ovaries in mammals.

Acknowledgements

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Disclosure of conflict of interest

None.

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