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Functional and morphological maturation of the full-sized and mini-pig corpus luteum by programmed cell death mechanism

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Abstract

Introduction: The formation and function of the corpus luteum (CL) increase the likelihood of pregnancy and efficiently manage implantation. Apoptosis must occur at an appropriate time in the formation of the CL. This also affects its function. However, it is still unclear if the type of apoptosis affects the function. **Material and Methods:** We conducted morphological analysis of the CL collected on day 15 between the middle and late oestrous phases of Yorkshire pigs and mini-pigs, and measured the difference in hormone expression and apoptosis using an immunoassay method and messenger RNA level. **Results:** The CL cells were more uniform in the Yorkshire pigs than in the mini-pigs, and the composition of the CL was also fuller. The expression of luteinising hormone was higher in the Yorkshire pigs. Apoptosis and the rate of action of matrix metalloproteinases (MMPs) were different between the two pig types. Expression of MMPs was higher in the Yorkshire pigs than in the mini-pigs. However, the expression of caspase 3 and 20alpha-hydroxysteroid dehydrogenase, a progesterone inhibitor, was potentiated in the mini-pigs. **Conclusion:** Autophagy throughout the CL was more extensive in the Yorkshire pigs than in the mini-pigs, suggesting that autophagy and cell reorganisation by MMPs were highly correlated. The occurrence of autophagy in the formation and function of the CL may affect the action of hormones and expression of cell reconstitution factors.

Keywords: apoptosis, autophagy, matrix metalloproteinases, corpus luteum, porcine.

Introduction

The mini-pig is an important animal model for biomedical research, including studies on cardiovascular dysfunction, gastric function, oncology, and tissue transplantation (7, 11). They are particularly useful experimental animals because of the ease of utilisation of cloning and genetic manipulation technologies. Despite these advantages, there are very few studies on the reproductive physiology of mini-pigs. According to Kim *et al*. (15), the development of follicles in mini-pigs is slower, immature development is frequent compared to normal pigs, and the offspring production rate is very low (12, 15). The reproduction of so few offspring tends to occur through functional problems of the corpus luteum (CL), and it is thought that there will be problems with hormone production in the mini-pig. In particular, the functional CL is involved in maintaining pregnancy from conception to delivery and plays an essential role in the development and implantation of fertilised eggs by changing the endocrine environment of the uterus through the secretion of progesterone (P4) (19, 23). Persistence of luteal function generally maintains progesterone stimulation, which substantially modulates uterine function and promotes embryo survival and implantation (21, 27). A fall in the concentration of P4 detected in the CL tissue and serum in early pregnancy due to the deterioration of the corpus luteum impairs the development of the endometrium and affects the maternal effect, which is an embryonic interaction (21). Additionally, at the peak of the oestrous cycle, the CL is essential in maximising the development of the endometrium and fertilised embryo. In general, the formation of the CL is determined by an appropriate balance of luteinising factors and apoptotic factors in the process of reconstituting corpus luteum cells from granulosa cells of follicles. With this balance maintained, the resulting porcine CL can perform its normal functions (2, 3). In other words, paradoxically,

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apoptosis is thought to affect the production and function control of the corpus luteum. Control of the CL by apoptosis is dual: the first aspect is functional control, and the second is control by corpus luteolysis. During the oestrous cycle and certain periods of pregnancy, the CL in pigs undergoes a regression characterised by cessation of P4 synthesis (functional luteinisation) and morphological degeneration (structural luteolysis) (6, 18). The representative action of structural CL lysis is induced by uterine prostaglandin F2a (PGF2a) (5, 19). It is generally accepted that structural changes in the CL play an essential role in fertility and the maintenance of pregnancy. Ultimately, apoptosis-induced CL cell death is the basis of the structural degeneration process (25). Importantly, recent studies in rodents (4) and cattle (20) indicated that programmed cell death participates in CL regression (13). Taken together, the results of previous studies suggest that there may be differences in the apoptosis process depending on the pig breed, which may differentiate the structural changes of the CL during oestrus and gestation in pigs. In other words, the difference in the structural change of the CL may be a determinant of the difference in the breeding ability of mini pigs from full-sized breeds. Therefore, this study aimed to investigate whether differences in such structural changes during the oestrous phase of full-sized pigs and mini-pigs could affect the function of the CL. We focused on the luteal phase (when the CL is mature with high steroidogenic activity) to analyse these differences.

Material and Methods

All procedures involving the use of animals were conducted following the National Guidelines for Agricultural Animal Care and approved by the Hankyong National University Animal Experimental Ethics Committee, Korea. All immunological experiments were performed as described previously (14).

Full-sized pig and mini-pig sample preparation. The ovarian tissue samples used in the experiment were prepared as previously described (15). The ovaries of five normal Yorkshire pigs aged 10 months and weighing 110 ± 5.1 kg (Farm Animal, Anseong, Korea) and five miniature pigs aged 10 months and weighing 26 ± 2.1 kg (Medicinetics Co., Ltd., Pyeongtaek, Korea) were collected on the 15th day of the oestrous cycle from a local slaughterhouse in Dodram, Anseong, Korea (10). The samples were placed in a freezer box containing liquid nitrogen and transferred to the laboratory within 2 h.

Haematoxylin and eosin staining. All corpus luteum tissue was fixed in 70% diethylpyrocarbonate-ethanol, embedded in paraffin, and cut into 5-μm-thick sections, and then paraffin was removed to hydrate the tissues. Next, sufficient haematoxylin and eosin (H&E; Sigma-Aldrich, St. Louis, MO, USA) was applied to cover the sections completely and the sections were incubated for 5 min. The slides were washed in distilled water twice to stop excessive staining. Then, all tissue sections were subjected to a differentiation step in 1% acidic alcohol for 30 s, followed by washing in water for 1 min. Sufficient bluing reagent (0.2% aqueous ammonia) was applied to cover the tissue sections completely and the sections were incubated for 10–15 s. Afterwards, the slides were rinsed twice with distilled water, and then immersed in 95% alcohol to remove excess stain. Sufficient modified alcoholic eosin Y solution was applied to cover the tissue sections and the sections were incubated for 2 min. Finally, to dehydrate the slide and remove excess pigment, the slide was treated twice with 95% alcohol for 5 min each time. Then xylene was added for 5 min and exchanged twice to remove the alcohol. After all steps, Immu-Mount (Thermo Fisher Scientific, Princeton, NJ, USA) was added, the tissue section was sealed with a cover slide, and the CL was observed under a microscope.

Immunohistochemistry. To analyse the expression patterns and localisation of caspase (Casp)-3 and microtubule-associated protein 1 light chain 3A (MAP1LC3A) in tissues, deparaffinisation and hydration were performed as described in haematoxylin and eosin staining. Antigen retrieval was performed by heating tissue slides at 95°C with 10 mM sodium citrate (pH 6.0). The tissues were treated with endogenous peroxidase and 0.3% hydrogen peroxide in 1× phosphate-buffered saline (PBS) for 5 min at room temperature, following which, the slides were washed with 1× PBS and non-specific binding sites were blocked with 5% normal horse serum as a buffer. Sample slides were reacted with anti-Casp-3 (cat. No. ab208161; Abcam, Cambridge, UK) and anti-MAP1LC3A (cat. No. ab205439; Abcam, San Francisco, CA, USA) antibodies diluted with blocking buffer at room temperature for 2 h. Horseradish peroxidase (HRP)–conjugated anti-rabbit IgG (sc-2054; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-mouse IgG-HRP (sc-2005, Santa Cruz Biotechnology, CA) were used as secondary antibodies for antigen detection and allowed to react for 1 h at room temperature. After the reaction, the sections were visualised using an avidin-biotin-peroxidase complex for 30 min (Vectastain Elite ABC Reagent; Vector Laboratories, Newark, CA, USA) and 3,30-diaminobenzidine (Vector Laboratories). The sections were stained with a haematoxylin solution containing periodic acid–Schiff reagent and 4% acetic acid. Tissues were dehydrated, cleared, and covered with Permount solution (Thermo Fisher Scientific).

Western blotting. Protein extracts with mass of 30 μg from the pig corpus luteum tissues were separated in duplicate using 13% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA).

The membranes were blocked with 5% non-fat dry milk overnight at 4°C and then washed for 10 min with 50 mM Tris–HCl, 200 mM NaCl and 0.1% Tween 20 (TBS-T) buffer. The membranes were incubated for 2 h with primary antibodies at 1:1,000 dilution) to recognise the active forms of 20alpha-hydroxysteroid dehydrogenase (20α-HSD) (16), Casp-3 (cat. No. ab208161; Abcam, San Francisco, CA, USA), mammalian target of rapamycin (mTOR, cat. No. sc-8319), β-actin (cat. No. sc-47778; both from Santa Cruz Biotechnology, Santa Cruz, CA, USA), and B-cell lymphoma 2 (BCL-2, cat. No. ab117115, Abcam, Cambridge, UK). Following primary antibody binding, the membranes were washed thrice with TBS-T buffer for 15 min each time and then incubated for 2 h with HRP-conjugated secondary anti-rabbit (cat. No. sc-2054), anti-mouse (cat. No. sc-2031, all/both from Santa Cruz Biotechnology, CA, USA), or anti-goat (cat. No. sc-2354; diluted 1:5,000 in blocking buffer) antibodies. The membranes were incubated with enhanced chemiluminscence detection reagent (ECL Western Substrate; Thermo scientific, IL, USA) for 5 min in the dark and then exposed to X-rays for 10 min. Finally, the relative protein expression was normalised to that of β-actin, which served as an internal control, using AlphaImager ver. 4.0.0 (Alpha Innotech, San Leandro, CA, USA).

ELISA. Total protein was extracted from CL tissues using a Pro-Prep solution (Intron, Seoul, South Korea) according to the manufacturer's instructions and was quantified using a Bradford protein assay kit (Bio-Rad). For quantification of specific proteins from the culture medium and cellular proteins, samples diluted in assay buffer were used to coat a 96-well ELISA plate overnight at 4°C. Insulin like growth factor 1 receptor (IGF1-r, cat. No. ab182408; Abcam, Cambridge, UK), mammalian target of rapamycin (mTOR, cat. No. ab109268; Abcam, Singapore), progesterone-receptor (P4-r, cat. No. ab16661; Abcam, Cambridge, UK), follicle-stimulating hormone (FSH, cat. No. ab231672; Abcam, Singapore), luteinising hormone (LH, cat. No. ab130750; Abcam, Cambridge, UK), tumour necrosis factor alpha (TNF-α, cat. No. ab6671; Abcam, Cambridge, UK), matrix metalloproteinase 2 (MMP-2, cat. No. ab78796-100; Abcam, Cambridge, UK), MMP-9 (cat. No. sc-13520 cat. No. Santa Cruz Biotechnology, Dallas, TX, USA), tissue inhibitor of metalloproteinases 2 (TIMP-2, cat. No. ab230511; Abcam, Cambridge, UK), and TIMP-3 (cat. No. ab85926; Abcam, Cambridge, UK) primary antibodies were added to 96-well ELISA plates to analyse the expression levels of these proteins in the sample and culture media. The samples were incubated at 4°C for 24 h. After washing twice with a buffer solution (1× PBS containing 2.5% Triton X-100), the well contents were blocked for 24 h at 4°C using a 1% skimmed milk bsolution. After washing with buffer, anti-rabbit (cat. No. sc-2054; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-mouse (cat. No. sc-2031; Santa Cruz Biotechnology, Santa Cruz, CA, USA) secondary antibodies were added. Each well and plate was incubated for 2 h with detection or substrate solution (R&D Systems, Minneapolis, MN, USA). The reaction was stopped with 1 M NH_2SO_4 and the absorbance was measured at 450 nm.

Zymography. Gelatin zymography analysis was performed according to the method described by Kim and Yoon (17). *In vitro* culture medium was added to fast of zymography (Foz) loading buffer (0.06% bromophenol, 10% SDS, and 2% glycerol), left on ice for 5 min, and then electrophoresed at 150V for 1 h 30 min. A 12% SDS-PAGE gel containing 100 mg/mL gelatin A/B was used. The gel was washed twice with reconstruction buffer (2.5% Triton X-100) for 20 min, then placed in a zymography reaction buffer and incubated at 37°C for 18 h. The gels were then stained with 0.5% Coomassie blue R250 (Bio-Rad) to measure the activity of MMP-2 and MMP-9 in the white bands. The average fold values for all hormones were measured and expressed as the mean ± standard deviation.

Real-time polymerase chain reaction. Real-time PCR was performed using the One-Step SYBR RT-PCR Kit (TaKaRa, Kusatsu, Japan) according to the manufacturer's instructions. The primers used for PCR are listed in Table 1. The real-time PCR reaction was performed once at 42°C for 15 min and at 95°C for 2 min, and subsequently at 95°C for 40 s, 58°C for 15 s, and 60°C for 32 s for 40 cycles. The final annealing step was performed once at 72°C for 5 min. Gene amplification was quantified based on the threshold cycle (Ct) value obtained from a semi-log amplification plot of the geometric region.

Statistical analysis. Ratio analysis for protein expression in Western blotting experiments was quantified using Excel 2016 (Microsoft, Redmond, WA, USA). Real-time RT-PCR data were analysed using the Welch's *t*-test and the generalised linear model (GLM) method in IBM SPSS Statistics 20 (IBM, Seoul, Korea). Western blotting and ELISA data were analysed by using Welch's *t*-test, fold change, and GLM in SAS (Cary, NC, USA). All data are presented as the mean ± standard error of the mean. Differences between groups were considered significant at $P < 0.05$. All experiments were repeated three or more times.

Results

Comparative analysis of the morphology of the corpus luteum. A comparative analysis of CL formation in the ovaries of the Yorkshire pigs and mini-pigs showed that there was a difference in the development and morphology of luteal cells. In the Yorkshire pigs, the CL was composed of large lutein cells and had a very wide cytoplasmic distribution. However, in the mini-pigs, the distribution of small lutein cells was wider than that in the Yorkshire pigs,

and the development of large lutein cells was sparser. Additionally, the angiogenesis around the CL was more pronounced and the distribution of theca lutein cells was wider in the full-sized pigs than in the mini-pigs (Fig 1).

Table 1. Primers used for real-time PCR analysis of apoptosis-associated genes

Primer name	Sequence	GenBank gene ID
Porcine GAPDH F	5′ CCCGTTCGACAGACAGCCGTG 3′	NM_001206359.1
Porcine GAPDH R	5′ CCGCCTTGACTGTGCCGTGG 3′	
Porcine BCL-2 F	5′ AGCATGCGGCCTCTATTTGA 3′	XM_021099593.1
Porcine BCL-2 R	5′ GGCCCGTGGACTTCACTTAT 3′	
Porcine Casp-3 F	5′ CATGGTCAGGCCTTGTGAAGCTGAC 3′	NM_214131.1
Porcine Casp-3 R	5′ TCTTCTTCATGACCTCACCGTCGGG 3′	
Porcine BAX F	5′ TGCTTCAGGGTTTCATCCAG 3′	AM233489.1
Porcine BAX R	5′ GTGTCCCAAAGTAGGAGAGG 3′	
Porcine 20α-HSD F	5′ GCCATTGCCAAAAAGCACAAG 3′	NM_001044618.1
Porcine 20α-HSD R	5′ GGAAAGCGGATAGTCAGGGTGATC 3′	
Porcine ATG1 F	5′ CAGCGCATTGAGCAGAACCTCAGT 3′	NM_140344.3
Porcine ATG1 R	5′ GATGGTTCCGACTTGGGGAGATGGT 3′	
Porcine ATG13 F	5′ ATGGAAGCTGATCTCAATTCCCAGG 3′	XM_005660951.3
Porcine ATG13 R	5′ TTACTGCAGGGTTTCCACAAAGGCA 3′	
Porcine ATG5 F	5′ AGAGAAGTCTGTCCTTCCGCAGTCG 3′	NM_001037152.2
Porcine ATG5 R	5′ AAGCAGAAGGGTGACATGCTCTGGT 3′	
Porcine MAP1LC3A F	5′ AGAAGCAGCTGCCAGTCCTGGACA 3′	GU272221.1
Porcine MAP1LC3A R	5′ CAGGCAGGCCTGAGCAATCTTTATT 3′	
Porcine Beclin-1 F	5′ TGGCGGAAAATCTCGAGAAGGTCCA 3′	CX061365.1
Porcine Beclin-1 R	5′ TGTGCCAAATTGTCCACTGTGCCAA 3′	

F – forward; R – reverse; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; BCL-2 – B cell lymphoma 2 protein; Casp-3 – caspase 3 protein; BAX – Bcl-2-associated X protein; 20α-HSD – 20alpha-hydroxysteroid dehydrogenase; ATG – autophagy-related protein; MAP1LC3A – microtubule-associated protein 1 light chain 3A

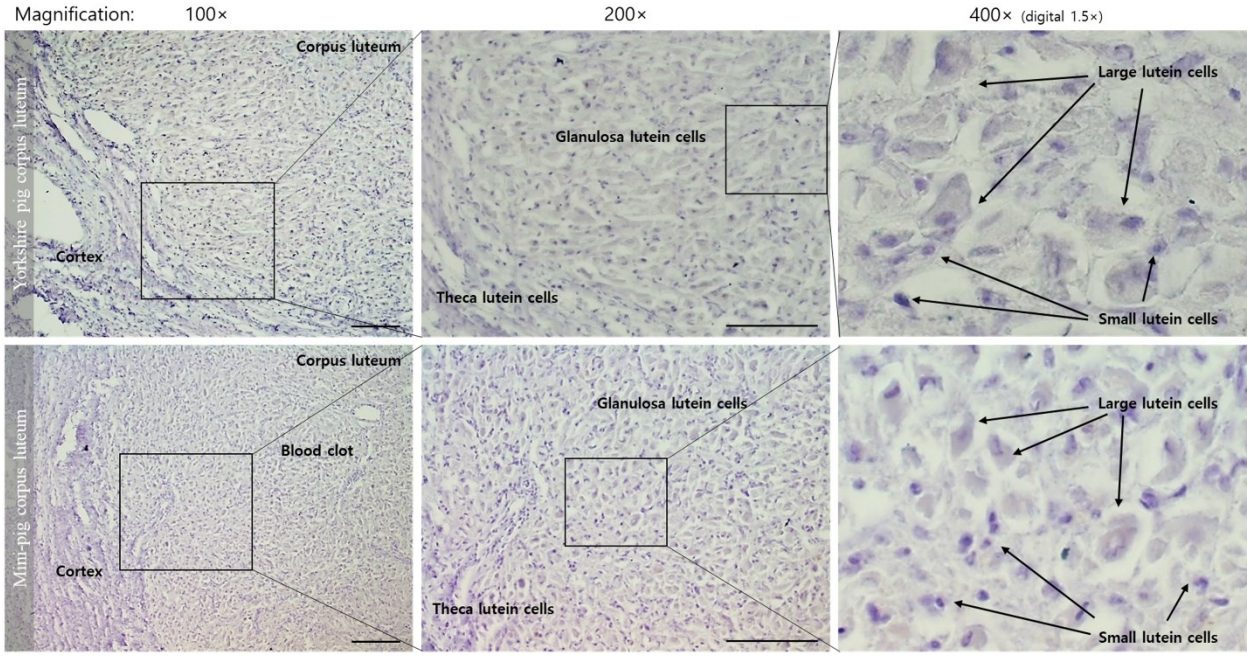

Fig. 1. Morphological analysis of corpus luteum tissues of full-sized Yorkshire pigs and mini-pigs using haematoxlin and eosin staining, showing the distribution of large corpus luteum cells and small corpus luteum cells in both pig species

Apoptosis according to caspase 3 activity in the corpus luteum. Analysis of the expression pattern of genes involved in apoptosis revealed that their expression by the Yorkshire pig CL cells was generally higher than that by the mini-pig cells (Fig. 2). B cell lymphoma 2 protein, which has an apoptosis inhibitory function, was more potently expressed in Yorkshire pigs, while expression of BCL-2 messenger RNA (mRNA) was higher in mini-pigs. Overall, the expression of Casp-3 and 20α-HSD, which are apoptotic proteins, was markedly more intensive in mini-pigs than in Yorkshire pigs, but the expression pattern of TIMP-2, an inhibitory factor of MMP-2, was intense in Yorkshire pigs. The analysis of the expression patterns of major survival factors and hormones revealed that the expression of the mTOR factor was greater in mini-pigs, but LH, which affects luteinising activity, was significantly upregulated in Yorkshire pigs. The action of MMPs appeared similar in the Western blotting results in both species as did the expression levels of IGF, P4, and FSH, although there were slight differences.

Comparison of autophagy marker gene expression in the corpus luteum. The expression of MAP1LC3A, known as an autophagy marker gene, was stronger confirmed in lutein cells of Yorkshire pigs compared to mini-pigs, and it was mainly expressed in large lutein cells (Fig. 3). In mini-pigs, the expression of LC-3 in the corpus luteum tissue was insufficient, and the expression pattern was confirmed in general large lutein cells. Comparison of gene expression patterns revealed that the expression of the autophagy-related ATG13, MAP1LC3A, and beclin-1 genes was higher in the Yorkshire pigs than in the mini-pigs. While there was no significant difference in the expression pattern of the MAP1LC3A protein, the expression of the ATG5 protein was higher in mini-pigs than in Yorkshire pigs.

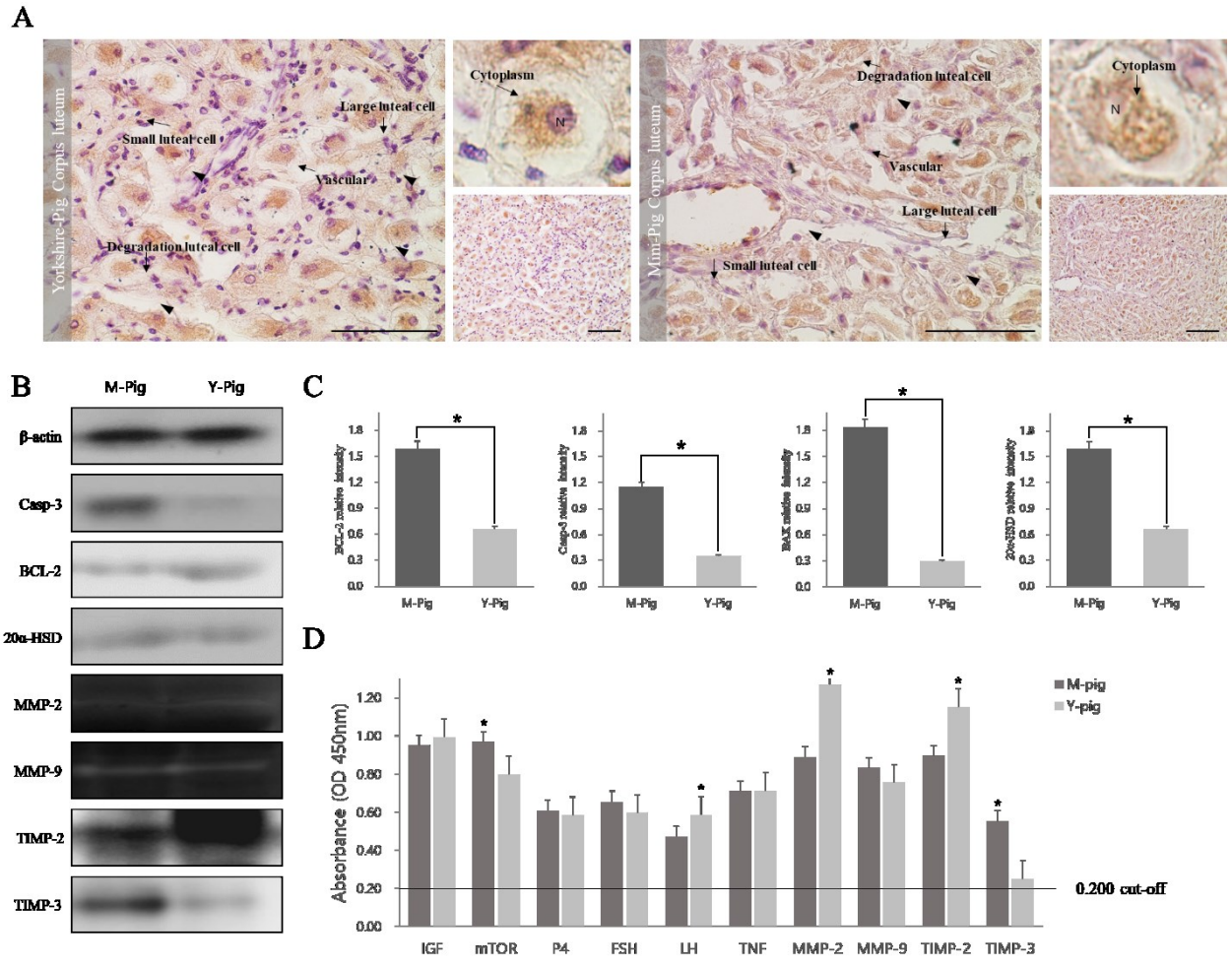

Fig. 2. Genes related to apoptosis, cell reorganisation and hormones in the proteins and mRNAs of the corpus luteum tissues of Yorkshire pigs (Y-pig) and mini-pigs (M-pig). A – immunohistogram of caspase 3 (Casp-3) protein in the corpus luteum tissue; B –Western blot of cell death factors and zymography of the activity of matrix metalloproteinases (MMPs) involved in cell reorganisation; C – real-time PCR analysis of messenger RNA involved in apoptosis; D – detection of hormone receptors and proteins involved in cell reorganisation. BCL-2 – B cell lymphoma 2 protein; BAX – Bcl-2-associated X protein; 20α-HSD – 20alpha-hydroxysteroid dehydrogenase; TIMP – tissue inhibitor of metalloproteinases; OD – optical density; IGF – insulin-like growth factor; mTOR – mammalian target of rapamycin; P4 – progesterone; LH – luteinising hormone; TNF – tumour necrosis factor

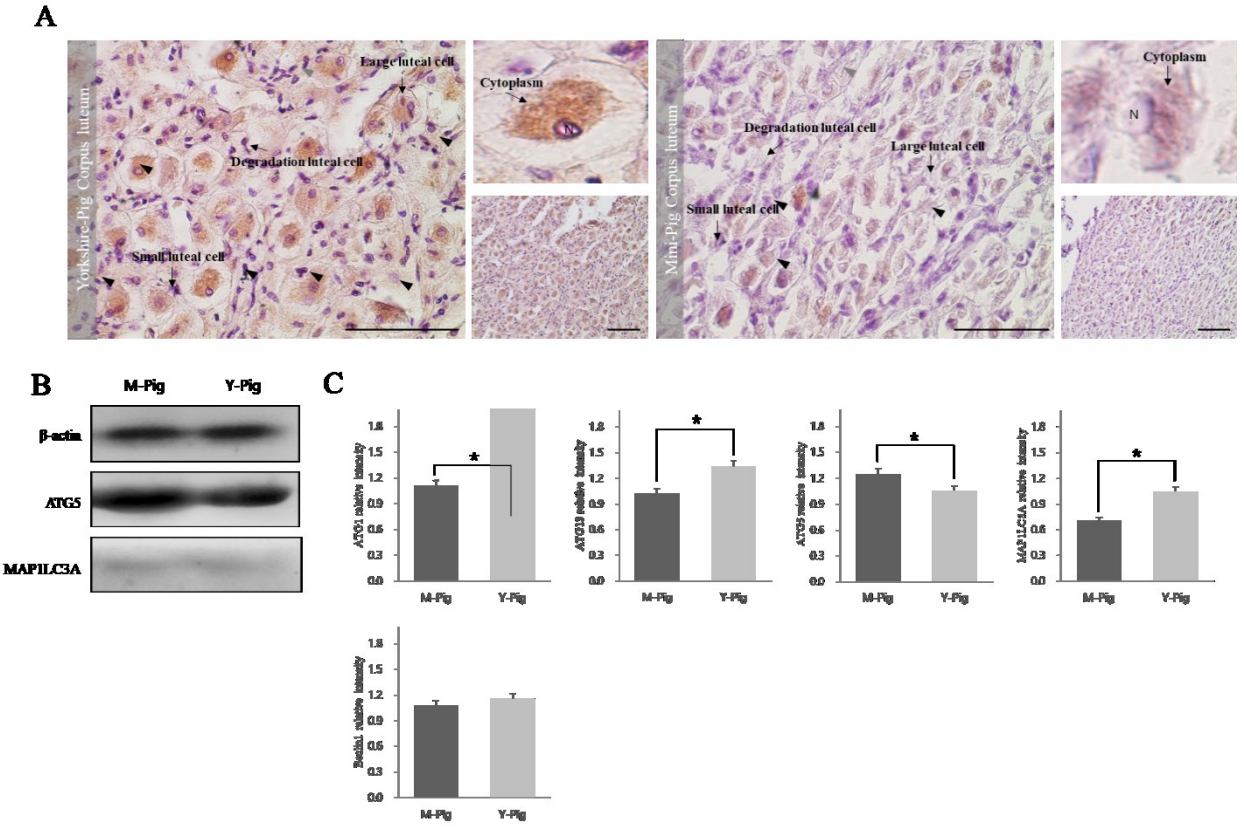

Fig. 3. Comparative analysis of expression patterns of major marker proteins and mRNAs related to the occurrence of autophagy in Yorkshire pigs (Y-pig) and mini-pigs (M-pig)). A – expression pattern of MAP1LC3A, shown byimmunohistochemistry; B – expression of autophagy-related proteins compared by Western blotting; C – analysis of autophagy-related gene expression by real-time PCR

Discussion

Structural changes in the corpus luteum can be observed in the rapid reorganisation of tissues during the process of a mammal becoming fertile during the oestrous cycle. In the process of tissue reorganisation, functional changes involve cytoplasmic reorganisation and changes in the role of cells utilising the apoptosis mechanism (14). However, the main functional changes in cells can be observed simultaneously in apoptosis and cytoplasmic changes. This process is initiated by progesterone stimulation (13, 16).

In the cell-death process, programmed death by autophagy is caused by irregular CL formation or physiological abnormalities in many mammalian animals. Thus, it is possible to evaluate the physiological phenomenon caused by hormone action according to the formation of the corpus luteum (1, 20). We analysed the corpus luteum of Yorkshire pigs and mini-pigs in the middle stage of the oestrous cycle, which is considered to have the highest steroid-producing activity and CL function. The overall cell uniformity was higher in Yorkshire pigs than in mini-pigs. Additionally, the programmed cell death action showed different aspects, and tissue reorganisation according to the type of cell death occurred differently. The most important form of apoptosis discussed in cell reorganisation is the apoptosis induced by the p53/Casp-3 gene. It can be viewed as an important cell death process for smooth cell reorganisation (17).

In our study, the expression of Casp-3, a marker of apoptosis, was confirmed in both Yorkshire and mini-pigs. This result confirms that the apoptosis mechanism is functional in most luteal cells (15). However, our results confirmed that the activity of MMPs was different according to the route by which cells were programmed to die (16, 17). Although the expression of Bcl-2, an apoptosis inhibitor, was upregulated in mini-pigs, the expression of Bax, which heterogeneously binds to Bcl-2, could interfere with apoptosis inhibition (9).

In Yorkshire pigs, the overall expression in large luteal cells confirmed the presence of autophagy during the reorganisation of luteal tissue. However, autophagy activity was significantly lower in mini-pigs, and the expression of MAP1LC3A was also lower than that in Yorkshire pigs. It has been noted that the actions of apoptosis and autophagy in luteal cells can be seen simultaneously or in interaction, which can affect cell reorganisation (8, 10). As stated by Grzesiak *et al.* (10), a very important factor in generating a full-sized pig's corpus luteum is the appropriate action of apoptosis and the activity of cell reconstitution factors. Additionally, autophagy is essential for maintaining and extending the lifespan and function of the corpus luteum.

Particularly, as shown by Kim *et al.* (13), low LH expression in mini-pigs and high concentrations of

20α-HSD compared to Yorkshire pigs can affect the apoptotic process. However, it is unclear whether this promotes cell survival and/or death in luteal cells (22). Most studies report that apoptosis reduces CL function during the peak phase of the corpus luteum. Additionally, cell death is regulated by the level of Beclin-1 acting on luteinised follicle cells after ovulation. In a broader sense, it is believed that the mechanism driving a process regulating apoptosis and survival should be a hormonal one (24, 26). In our results, hormone action was different between mini-pigs and Yorkshire pigs. The maintenance of LH and the difference in expression of 20α-HSD may have a certain influence on cell reorganisation because of the difference in expression of MMPs and TIMPs. However, it is not known whether these differences regulate the two processes of apoptosis (13, 14, 16). The regulation of autophagy has not yet been elucidated, and further studies are needed to understand the interaction of several components (15). In particular, autophagy cannot be determined solely by the expression of MAP1LC3, an autophagosome component involved in it. The expression of LC3 in Yorkshire pigs was higher than that in mini-pigs; however, similarly to LC3, the expression of ATG5 constituting the double membrane of the autophagosome was high in mini-pigs.

It can be assumed that the component action of apoptosis in mini-pigs reduces the function of the corpus luteum to a certain level. This also suggests that the involvement of autophagy in CL formation may directly affect the function of the corpus luteum.

*These authors contributed equally to this work.

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