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Impact of exogenous adrenocorticotropic hormone on gelatinase expression and steroidogenesis in the newly formed corpus luteum in sows

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ABSTRACT

The development and sustained function of the corpus luteum (CL) after ovulation are important for embryo implantation and early pregnancy maintenance in mammals. Sows raised in commercial group-housing systems are vulnerable to stress and have elevated blood cortisol levels; therefore, it is pivotal to study the influence of increased cortisol levels in circulation on the reproduction of sows. In this study, we aimed to investigate whether stress induced by adrenocorticotropic hormone (ACTH) administration before estrus affected either the development or the functions of the newly formed CL in sows. The results showed that the gene expression levels of the P450 cholesterol side chain cleavage (P450scc) and 3β-hydroxysteroid dehydrogenase (3β-HSD) proteins of newly formed CLs were lower in the ACTH-treated sows than in the controls, whereas the expression and activity of matrix metalloproteinases (MMPs) were significantly downregulated ($P < 0.05$). Moreover, the vascular endothelial growth factor $(VEGF)_{164}$ gene expression levels were significantly lower in the ACTH group than in the controls ($P < 0.05$). These findings indicate that ACTH-induced stress impairs vascularization, and affects the steroidogenesis of newly developed CLs in sows.

1. Introduction

Stress is an important factor that negatively influences reproduction and fertility in livestock throughout the estrous cycle (Rabin et al., 1988). Commercial group-housing systems give rise to stressful situations such as intragroup competition, environmental changes, nutrition, weaning and transport, and can result in elevated plasma cortisol or corticosterone levels in animals (Whirledge and Cidlowski, 2010), which has been commonly acknowledged as an indicator of stress (Turner et al., 2002). Stress activates the hypothalamic-pituitary-adrenal axis, causing the release of corticotropin-releasing hormone from the hypothalamus and adrenocorticotropic hormone (ACTH) from the anterior pituitary. Generally, ACTH reaches the adrenal cortex *via* circulation to stimulate cortisol production, and the produced cortisol is then secreted into the blood and acts on the target tissues (Dobson and Smith, 2000). A previous study found that stress reduced implantation and impaired steroidogenesis and embryo development in pigs (Einarsson et al., 2008). However, it remains unclear how the ACTH-induced elevation in cortisol levels affects reproduction in sows.

The ovulated follicle develops into the corpus luteum (CL) by a complex process, and the CL functions as a transient endocrine gland during the estrous cycle (Stocco et al., 2007). CL provides uninterrupted synthesis and release of progesterone to stimulate the proliferation of endometrial cells and stabilize the uterus for embryo implantation and pregnancy maintenance (de Ziegler et al., 1998). The functional integrity of the CL may be impaired when its maturation is altered during early development (Brown et al., 2014). The developing CL is generally vulnerable to the disturbances of steroidogenesis, capillary development, and oxidative status (Agarwal et al., 2005). It is believed that the integrity and stability of these biological processes are not only essential to the normal function of CL (Niswender et al., 2000), but might also influence the reproduction process because the balance of steroidogenic activity at different periods of the estrous cycle is indispensible for reproduction.

Reproduction in sows can be regulated by administering adrenal-derived hormones. ACTH administration to follicles during the preovulatory period impairs steroidogenesis and angiogenesis in association with ovulation failure in lactating cows (Biran et al., 2015). In addition, cortisol or dexamethasone have been proven to inhibit steroidogenesis in rat luteinized granulosa cells in vitro (Huang and Li, 2001; Duong et al., 2012). Importantly, exogenous or stress-induced glucocorticoids have been found to directly inhibit the transcription of genes encoding testosterone biosynthetic enzymes such as P450 cholesterol side chain cleavage (P450scc), steroidogenetic acute regulatory

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protein (StAR), and cytochrome P450 17A1 (CYP17A1) in men (Hales and Payne, 1989; Whirledge and Cidlowski, 2010). The expression levels of P450scc and 3β-hydroxysteroid dehydrogenase (3β-HSD), which are enzymes necessary for conversion of cholesterol to progesterone, were found to be increased in differentiated cells (follicle cells and luteal cells) for the conversion of cholesterol to progesterone (Bachelot and Binart, 2005a). Cholesterol molecules are transferred to the inner mitochondrial membrane by the StAR protein, and then converted into pregnenolone by P450scc (Bachelot and Binart, 2005b). Progesterone synthesis then occurs in the smooth endoplasmic reticulum catalyzed by 3β-HSD (Peng et al., 2002).

Although glucocorticoids have been reported to act directly on ovarian cells to inhibit steroid biosynthesis and LH action (Huang and Li, 2001; Whirledge and Cidlowski, 2010; Biran et al., 2015), how ACTH-induced stress affects CL function *in vivo* remains to be determined. Therefore, in this study, we established an ACTH-induced stress pig model and measured the gene and protein expression levels of reproduction-related genes in order to evaluate how cortisol affects CL function. The results proved that ACTH-induced stress impairs reproduction by preventing vascularization, disturbing the oxidative stress balance, and affecting the steroidogenesis of newly developed CLs.

2. Material and methods

2.1. Ethical guidelines

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Agricultural University. The study protocol was reviewed and approved by the university (Project number: 090600542). The animal sacrifice and sampling procedures were strictly in accordance with the “Guidelines on Ethical Treatment of Experimental Animals” (2006) No. 398 set by the Ministry of Science and Technology, China, and the “Regulation regarding the Management and Treatment of Experimental Animals” (2008) No. 45 set by the Jiangsu Provincial People's Government.

2.2. Animals and treatment

Six Suhuai sows in their second to fourth parity were provided by the Huai’An pig breeding farm. Sows were gathered in a separate hog house in the farm and transferred to a farrowing pen on the day of weaning. The animals were fed as normal in the farm and water was provided *ad libitum*.

The sows were randomly allocated into two groups: ACTH and control. On the day of weaning, the right jugular vein of each pig was cannulated by surgery to collect blood. The sows were allowed to acclimate for 1 day, and injected with antibiotics (penicillin and streptomycin) two times daily for the first 3 days after surgery to avoid infection. From day 2–8 after the surgery, sows in the ACTH group were intramuscularly administered 1 IU ACTH/kg (Madej et al., 2005) body weight (extracted from porcine pituitary glands; Sigma, SLBK8723V) every 8 h (Liptrap, 1970; Barb et al., 1982; Einarsson et al., 2007), while sows in the control group were administered normal saline. All sows were slaughtered on day 4 after estrous.

Ovaries were recovered from each animal after slaughter and fixed in 10% neutral formalin liquid. Meanwhile, the number of CLs was counted and 3 new CLs were separated from each ovary and stored at − 80 °C.

2.3. Detection of estrus and hormone analysis

Sows were checked for estrus every 6 h using the back-pressure test with a boar nearby and observed (Langendijk et al., 2000). Blood samples were then collected from the cannulae in a disposable syringe and immediately transferred into vacuum blood collection tubes with heparin. The plasma was separated from the blood by centrifugation and stored at − 20 °C. The cortisol content was determined using a commercial cortisol enzyme-linked immune sorbent assay (ELISA) kit (Institute of Biological Engineering, Jiancheng, Nanjing, China), according to the manufacturer's instructions. According to the manufacturer's instructions, the CV of QC (coefficient of variation of quality control) was determined to be 10%. The concentration was calculated according to the standard curve of the standard cortisol solution.

2.4. Quantitative real-time PCR

Total RNA was extracted from the CLs from each ovary by RNAiso Plus (TaKaRa, cat # 9108, Japan) and dissolved in RNase-free water, according to the manufacturer's directions. Total RNA (1000 ng) was subjected to reverse transcription to complementary DNA (cDNA) in a total volume of 20 μL, using a Hiscrip QRT SuperMix for qPCR kit (VazymeBiotech, NanJing, China). The RNA was incubated at 42 °C with 4 μL gDNA wiper mix and RNase-free double distilled water (ddH_2O) at a total volume of 16 μL for 2 min. Then, 4 μL of qRT Super MIX was added to the PCR tube, and reverse transcription was carried out under the following PCR condition: 25 °C for 10 min, 50 °C for 30 min, and 85 °C for 5 min, according to the manufacturer's instructions.

The resultant cDNA was used for real-time PCR in a 25-μL reaction volume. Gene primers for real-time PCR was designed using Primer premier 5 software (Premier Biosoft International, America) according to the published mRNA sequences from the NCBI PubMed Database and synthesized by Invitrogen (Invitrogen, Shanghai, China). The sequences of all the primers used are presented in Table 1. The target gene mRNA expression levels were normalized to glyceraldehyde-phosphate dehydrogenase (GAPDH) expression using the following formula:

Relative quantity of target gene mRNA $= 2^{-\Delta\Delta Ct}$

ΔΔCt = [Ct (target gene mRNA) – Ct (GAPDH mRNA)] test group – [(Ct (target gene mRNA) – Ct (GAPDH mRNA)] control group.

2.5. Morphology of the CL

Formalin-fixed ovaries were cut into 3-mm thick pieces and fixed in 10% neutral formalin solution for 24–48 h, dehydrated, embedded in paraffin, and sectioned at 5 μm. The slices were dewaxed in xylene and rehydrated for staining with hematoxylin and eosin to study the morphology of CL in the two groups.

The sections were warmed at 60 °C for 30 min, deparaffinized and rehydrated, treated with Tris-EDTA solution (pH 9.0) as antigen retrieval buffer, and heated in a microwave at 98 °C for 10 min. After the sections naturally cooled to room temperature, the slides were rinsed

Table 1
Sequences of the primers used for the quantitative real-time PCR assay.

Gene	Primer sequence	NCBI accession NO.
StAR	For: AGCCCTTTCGTGTCTACCT Rev: ATTCTGACTGCTGCTCTGG	NM_213755
P450scc	For: TGAACACGGAGGTAATGG Rev: TGGTGATGGACTCAAAGG	NM_214427
3β-HSD	For: CGTGGATGTGGGTGTGAG Rev: GGAAAGGGAATGAAGAGG	NM_001004049
MMP-2	For: CGACCACCGCCAACTACGAT Rev: CACTGTCCGCCAGATGAACC	NM_214192
MMP-9	For: GCCCTGCGTGTTTCCATT Rev: AGGTCCCCCGCCGAGTTG	NM_001038004
TIMP-1	For: CCCAGAGTTCAACCAGACCG Rev: TGTGGAAGTATCCGCAGACG	NM_213857
TIMP-2	For: CGACCACCGCCAACTACGAT Rev: CACTGTCCGCCAGATGAACC	FJ394384
$VEGFa_{165}$	For: GAACTTTCTGCTCTCTTGGG Rev: GTTTCTGGTCTCCTTCTGCC	NM_214084.1