Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2019.083

RESEARCH ARTICLE

ACTH Induces Oxidative Stress and Decreases DNA Methylation of *Fkbp5* in Suhuai Sow

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ARTICLE HISTORY (17-064)

Received: February 20, 2017
Revised: August 04, 2017
Accepted: August 17, 2017
Published online: June 22, 2019

Key words:
ACTH
Fkbp5
Methylation
Oxidative stress

A B S T R A C T

Hypothalamic-pituitary-adrenal (HPA) axis plays an important role in stress response. However, in Suhuai sows, the relationship between glucocorticoid and oxidative stress is not yet understood. We found that malondialdehyde (MDA) level was increased, whereas the activity of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), were decreased after ACTH treatment in Suhuai sow. Moreover, glutathione (GSH) level and total antioxidant capacity (T-AOC) were also decreased upon ACTH treatment. Moreover, we found that mRNA expression levels of antioxidant enzymes such as *SOD*, *CAT*, and *GPX*, were down-regulated in the ACTH group. We observed that the mRNA expression of glucocorticoid-responsive elements (GRE) such as *CXCR4*, *DUSP1*, *IL7R*, *TXNIP*, and *Fkbp5* were upregulated, whereas DNA methylation in intron 6 of *Fkbp5* was reduced by ACTH administration. Hence, our findings suggest that ACTH induces oxidative stress and increases the expression level of *Fkbp5* by decreasing DNA methylation in the GRE region in Suhuai sow.

To Cite This Article: Weng Q, Li B, Liu Z, Liu K, Wu W and Liu H, 2019. ACTH induces oxidative stress and decreases DNA methylation of *Fkbp5* in suhuai sow. Pak Vet J, 39(3): 401-405. http://dx.doi.org/10.29261/pakvetj/2019.083

INTRODUCTION

The production and secretion adrenocorticotropic hormone (ACTH) from the anterior pituitary and that of the glucocorticoid hormones cortisol or corticosterone (CORT) from the adrenal gland are controlled by the HPA axis of the neuroendocrine system (Spencer and Deak, 2017). The HPA axis stimulates the release of glucocorticoid hormones from the adrenal cortex in response to stress (de Quervain *et al.*, 2016). Glucocorticoid receptors (GRs), one of the widely expressed intracellular proteins, regulate cellular responses by binding to specific glucocorticoid response elements (GREs) in the DNA or by non-genomic mechanisms via protein interactions (Srinivasan and Lahiri, 2016). For example, the chaperones such as heat shock protein 70 and 90 interact with GR protein and influence its assembly and activity (Kirschke *et al.*, 2014). Previous study has shown that glucocorticoid hormones can cause stress (Spiers *et al.*, 2014). For example, dexamethasone (Dex), a synthetic glucocorticoid that mimics the effects of the natural steroid cortisol, induces oxidative stress (Min *et al.*, 2016).

Oxidative stress, which arises from the imbalance of reactive oxygen species (ROS) production and elimination by antioxidant systems, exerts damaging effects on the cellular functions (Newsholme *et al.*, 2016). Oxidative stress is associated with recurrent pregnancy loss, defective embryogenesis, molecular and structural damages in oocytes and granulosa cells, and accelerated oocyte aging (Agarwal *et al.*, 2012). In addition, ROS influences folliculogenesis, oocyte maturation, and corpus luteum and uterine function (Agarwal *et al.*, 2008). H_2O_2 concentrations and antioxidant enzymatic activity are reduced during follicle development in ovary (Basini *et al.*, 2008).

Apart from hormones, several molecules are involved in the regulation of stress response. One such molecule is FK506 binding protein 51 (FKBP51 or FKBP5), a 51-kDa protein, which is a member of family of immunophilins or FK506 binding proteins (FKBPs) (LeMaster *et al.*, 2015). Genetic variation of the gene *Fkbp5* is considered to be associated with some diseases of glucocorticoid abnormalities, including post-traumatic stress disorder and mood disorders (Willour *et al.*, 2009). Fkbp5 acts as a crucial determinant of the adaptive stress response and decreases the binding of cortisol to its receptor (Tatro *et al.*, 2009).

MATERIALS AND METHODS

Animals, treatment and blood samples: All animal procedures were performed according to the guidelines of the Nanjing Agricultural University Animal Care and Ethics Committee. The experimental and control groups

consisted of four Suhuai sows each. They were all age-matched and had similar body weights at the time of weaning. Starting from the day after weaning, each animal was administered ACTH (1 IU/Kg) intravenously (IV) thrice a day for 7 days and the control group received saline. Blood samples were collected 2 h after ACTH injection. Each time, animals were gently restrained on their backs to minimize stress due to handling. Blood samples were collected in heparinized tubes and centrifuged for 10 min at 1500 × *g*. Plasma was separated and frozen at -20°C until further analysis.

Measurements of oxidant and antioxidant indices in plasma: The levels of MDA and GSH, and activities of SOD, CAT, GSH-Px, and T-AOC in plasma were determined using commercial analysis kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. Plasma SOD, CAT, GSH-Px, and T-AOC activities were expressed in U/mL, and MDA and GSH concentrations were expressed in nM and μM, respectively.

RNA extraction and Quantitative real-time PCR (qRT-PCR): Total blood RNA was isolated using a blood RNA extraction kit (Nanjing Jiancheng Bioengineering Institute, China) according to manufacturer's instructions and reverse transcription was conducted using Prime Script RT Master Mix Kit (Takara, China). Quantitative real-time PCR was performed with AceQ qPCR SYBR Green Master Mix (Vazyme, China) in a reaction volume of 20 μl. The cycling parameter is as follows: 95°C for 5 min followed by 40 amplification cycles each comprising of 95°C for 10 s and 60°C for 30 s. Primer sequences are listed in Table 1. *GAPDH* served as an internal control and was used to normalize the relative expression level of gene in each sample. Gene expression levels were calculated according to ΔΔCt method.

DNA bisulfite treatment and Pyrosequencing: Blood genomic DNA was isolated using standard phenol-chloroform method. Subsequently, 500 ng of each blood DNA sample was treated by bisulfite and purified with DNA purification Kit (QIAGEN, Germany). PCR was performed with 2 μl of bisulfite-treated DNA, 1 μl each of forward and reverse primers, 1 μl of dNTP, 10 μl of 5 × buffer and 34.8 μl of H_2O. PCR amplification parameters consisted of 3 min at 95°C, followed by 40 cycles of 30 s at 94°C, 30 s at 52°C and 1 min at 72°C. The PCR products were used to perform pyrosequencing using PyroMark Q96 ID system (QIAGEN, Germany). Pyro Q-CpG software (QIAGEN, Germany) was used to quantify the methylation level of each CpG site. The primer sequences used for PCR and pyrosequencing are as follows: forward primer: 5′-GTTTTTTGGTTTTTGTATT TTAGTTTTG-3′; reverse primer: 5′-AACACCCTATTC TAAATATAACTAACAC-3′; sequencing primer: 5′-CCTAT TCTAAATATA ACTAACACAT-3′.

Statistical analysis: Statistical analysis was conducted with SPSS v20.0 software (SPSS Inc., Chicago, USA). Student's *t*-test was used to analyze differences between two groups. All results are presented as mean ± SEM. $P<0.05$ was considered statistically significant.

RESULTS

Effect of ACTH on the oxidant levels and activity of antioxidant enzymes in plasma: In order to determine whether ACTH induces oxidative stress, we examined oxidant indicators such as MDA, GSH, and T-AOC, and antioxidant enzymes such as SOD, CAT, and GSH-Px levels in the plasma sample of Suhuai pigs. Plasma levels of SOD, CAT, GSH-Px, MDA, GSH, and T-AOC did not change significantly on day 1 of ACTH treatment. As shown in Fig. 1A, SOD activity was decreased on days 4, 5, and 7 in ACTH treated group compared with control group. CAT activity declined in the treatment group with time and decreased significantly on days 3, 6, and 7 (Fig. 1B). The activity of GSH-Px significantly decreased on days 3, 4, and 6 after ACTH treatment (Fig. 1C). Plasma MDA concentration increased significantly in the treatment group on several days, whereas the concentrations on days 1, 4 and 7 were not significantly different from that of control group (Fig. 1D). GSH level in ACTH group significantly decreased compared with that in the control group on days 2, 6 and 7 (Fig. 1E). A significant decrease of T-AOC activity was observed on days 2, 4, 5, 6 and 7 in the ACTH-treatment group compared to the control group (Fig. 1F).

ACTH down-regulated the mRNA level of antioxidant enzyme genes: To further understand the effect of ACTH on the expression of antioxidant enzymes, the mRNA expression level of the antioxidant enzyme genes in the blood was investigated. Quantitative real-time PCR analysis showed that the mRNA levels of *SOD*, *CAT*, and *GPX* in the blood decreased significantly after treatment with ACTH (Fig. 2A-C).

ACTH activated the expression of glucocorticoid-responsive genes: Five genes, namely *CXCR4*, *DUSP1*, *IL7R*, *TXNIP*, and *Fkbp5*, have been proposed as markers of differential regulation of glucocorticoid-responsive elements (Donn *et al.*, 2007; Sautron *et al.*, 2015). In order to study whether ACTH activates GR-related pathways and affects downstream gene expression, we examined the mRNA levels of these genes. The results showed that *CXCR4*, *DUSP1*, *IL7R*, *TXNIP*, and *Fkbp5* mRNA levels were significantly increased upon ACTH treatment (Fig. 3A-E).

ACTH decreased the DNA methylation level in the intron 6 region of *Fkbp*: Lee *et al.* (2010) reported that corticosterone increases the expression of *Fkbp5* and decreases the methylation level in intron 1 and 5 regions that contained GRE in mice. In the present study, we also used the website (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) to predict GRE in *Fkbp5* gene and found that a similar region contained GRE in intron 6 of pig *Fkbp5* (Fig. 4A). To further investigate whether the intron 6 region of *Fkbp6* undergoes demethylation after ACTH treatment, we used a pyrosequencing method to detect the methylation status in *Fkbp5*, which revealed that the methylation level of CpG2 site was significantly decreased in the ACTH treatment group compared with control group, while CpG1 site was not significantly different (Fig. 4B).

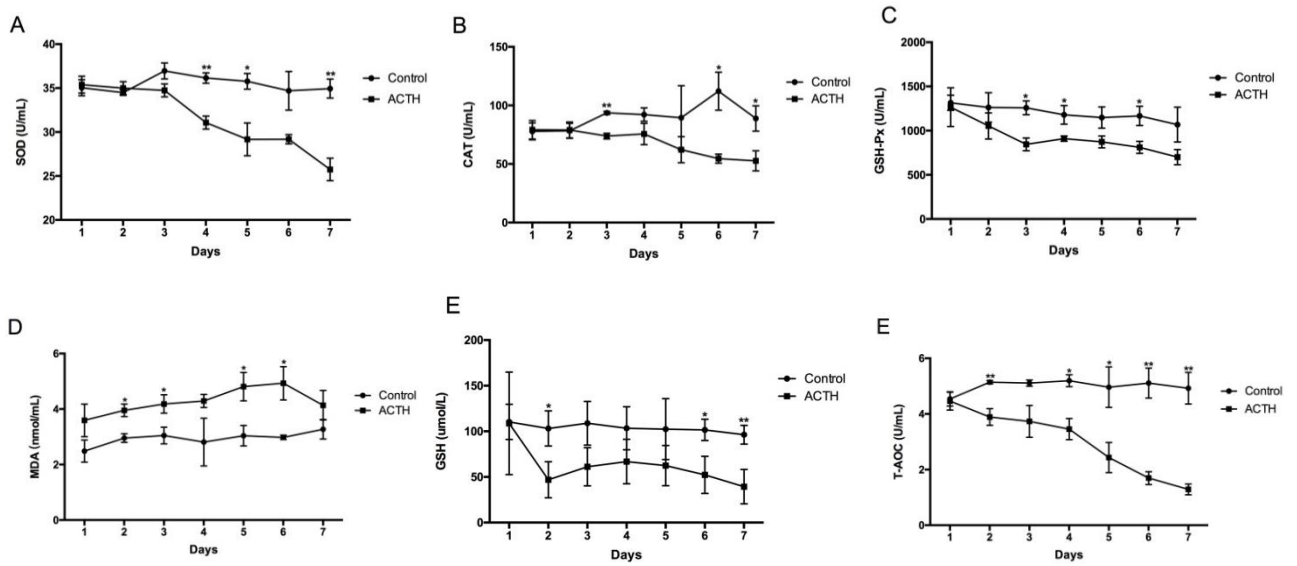

Fig. 1: Effect of ACTH injection for 7 days on contents of oxidant indictors in plasma. Sows were intravenously injected with ACTH (1 IU/Kg) for 7 days. A, B, C, D, E and F) The effect of ACTH on SOD (U/mL), CAT (U/mL), GSH-Px (U/mL), MDA (nM), GSH (uM) and T-AOC (U/mL) in plasma, respectively. Data are expressed as the mean±SEM (n=4); **P<0.01; *P<0.05.

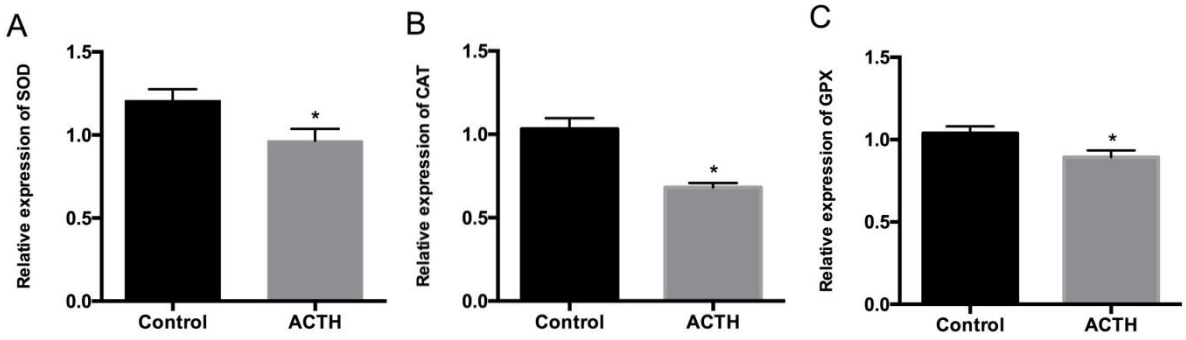

Fig. 2: Effect of ACTH injection on mRNA levels of oxidant enzyme genes in plasma. Relative expression levels of *SOD* (A), *CAT* (B) and *GPX* (C). The *GAPDH* gene is used to normalize these genes mRNA level. Data are expressed as the mean±SEM (n=4); *P<0.05.

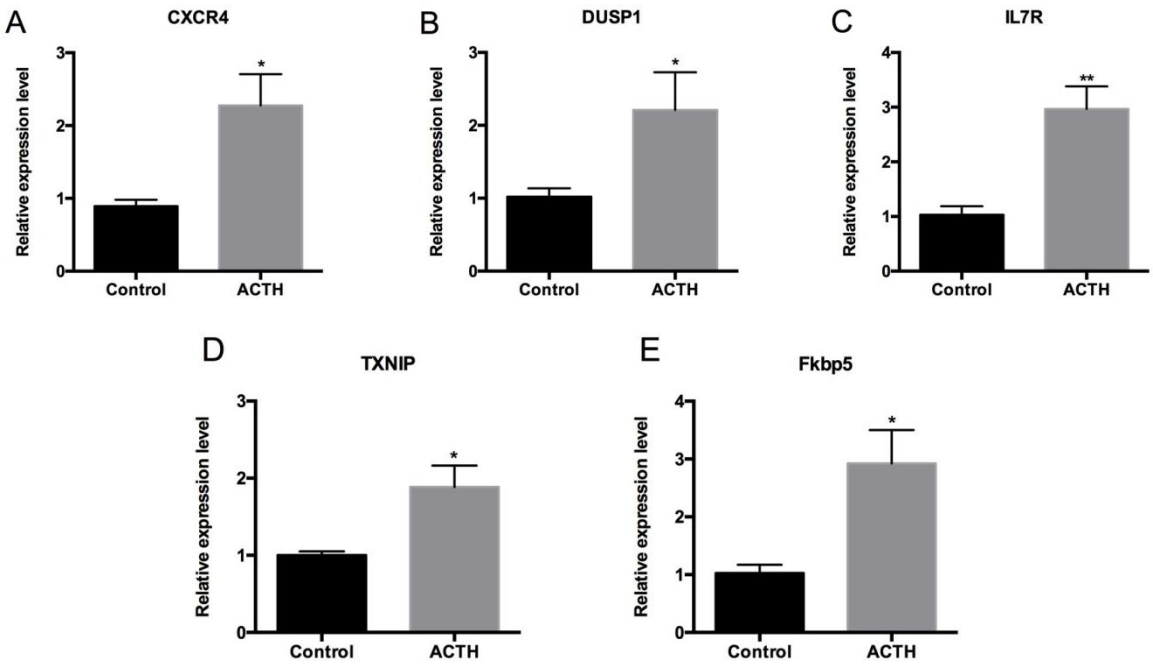

Fig. 3: ACTH up-regulates expression of glucocorticoid-responsive sensitivity genes. Relative expression levels of *CXCR4* (A), *DUSP1* (B), *IL7R* (C), *TXNIP* (D), *Fkbp5* (E). The *GAPDH* gene is used to normalize these genes mRNA level. Data are expressed as the mean±SEM (n=4); **P<0.01; *P<0.05.

Table 1: Primers used in this study

Gene name	GenBank No.	Primer squence (5'-3')	Annealing temp (°C)	Product size (bp)
GAPDH	NM_001206359.1	F: GGTCGGAGTGAACGGATTT R: ATTTGATGTTGGCGGGAT	60	245
Fkbp5	NM_001315611.1	F: GAACCGTTTGTCTTTAGTC R: TTCGTTGGGATTTGAGTA	60	270
CXCR4	NM_213773.1	F: CCTGCCCTCCTGTTGACT R: CGATGCTGATCCCGATGT	60	298
DUSP1	NM_001256075.1	F: ACCACAAGGCGGACATCA R: TGCTCCTCCTCTGCTTCACA	60	198
IL7R	XM_013990620.1	F: CTGTCCCGATTTCTACTGCC R: TTTACTTTCCCTGCTGCCTC	60	286
TXNIP	NM_001044614.2	F: GCCCTATCTTTATGTATGCTCC R: CACAACATCTCACTGGCTGA	60	298
SOD	NM_214127.2	F: ACGGCGGCGGCTATGTCCTTG R: ACGGCAGGTCGGGGAGGGTGT	60	204
CAT	NM_214301.2	F: TGGGAATCCGATAGGAGACAA R: CGCCTTCGAGAATCTGGTAAT	60	199
GPX	NM_214407.1	F: GGCAAGACGGAGGTAAACTAC R: CTTTCATCCACTTCCACAGAG	60	226

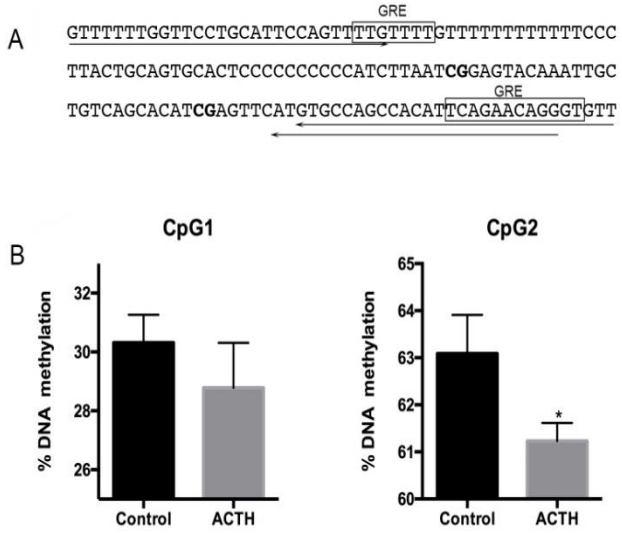

Fig. 4: Analysis of DNA methylation level in the intron 6 regions of Fkbp5. A) Schematic representation of the analyzed methylation region in *Fkbp5* gene. Rectangles indicate the GR binding sequences. The bold "CG" indicates the CpG sites. Arrow indicates PCR and pyrosequencing primers. B) Percent methylation at two CpG positions is shown for *Fkbp5* intron 6 region. Each CpG site was assayed for both ACTH and control groups. Data are expressed as the mean±SEM (n=4); *P<0.05.

DISCUSSION

Superoxide is first dismutated by SOD to H_2O_2 and then catalyzed by two enzymes, CAT and GSH-Px, into H_2O and O_2 (Sun *et al.*, 2014). Therefore, antioxidant systems play an important role in alleviating oxidative stress. It has been demonstrated earlier that administration of a certain dose of carbon disulfide (CS_2) significantly reduces GSH, SOD, CAT, and GSH-Px contents in the rat hippocampus (Wang *et al.*, 2017). Moreover, early weaning in piglets disrupts oxidative balance and causes oxidative injury in piglets, resulting in decreased expression of CuZnSOD, MnSOD, GSH-Px1, and GPx4 (Yin *et al.*, 2014). In the present study, SOD, CAT, GSH-Px, GSH, and T-AOC activity were significantly decreased after ACTH treatment. We further found that *SOD*, *CAT*, and *GPX* mRNA levels were also decreased in ACTH-induced group. These results suggest that elevated ACTH concentration in sows suppresses the antioxidant enzyme expression level, resulting in oxidative stress in Suhuai sow.

Increasing evidence suggests that ACTH affects ovulation, reduces estrogen secretion and delays onset of estrus and shortens the duration of estrus (Brandt *et al.*, 2006). Studies have shown that CAT and SOD activities are high in the human (Sugino *et al.*, 2000) and bovine (Rueda *et al.*, 1995) corpus luteum during early pregnancy. Furthermore, oxidative stress can lead to meiosis, fertilization and embryo development defects, and cause infertility (Keefe and Liu, 2009). Oxidative stress has been linked to endometriosis and polycystic ovarian syndrome (Menezo *et al.*, 2016). In our study, ACTH increased the oxidative damage and decreased the activity of oxidant enzymes. Hence, it is possible that excessive ACTH will induce oxidative stress and play a critical role during estrous cycle in the Suhuai sow.

Glucocorticoid production is induced by stress and it regulates gene expression by binding to its intracellular receptor (Hapgood *et al.*, 2016). It has been demonstrated that glucocorticoids increase the transcription of *DUSP1* gene by binding to GRE region in A549 human lung adenocarcinoma cells (Shipp *et al.*, 2010). *CXCR4*, *IL7R*, and *TXNIP* genes were differentially expressed in response to ACTH in pigs (Sautron *et al.*, 2015). *Fkbp5* is identified as glucocorticoid-responsive genes and is regulated by the binding GR to the sequences corresponding to the GRE in *Fkbp5* gene region (U *et al.*, 2004). In the present study, we demonstrated that mRNA expression level of *CXCR4*, *DUSP1*, *IL7R*, *TXNIP*, and *Fkbp5* were increased after ACTH treatment, which are consistent with previous study. These results showed that ACTH promoted transcription of glucocorticoid sensitive genes.

Fkbp5 protein acts an important co-chaperone in the formation of GR-complex (LeMaster *et al.*, 2015). When Fkbp5 binds to GR-complex, the formation of GR-complex has a lower affinity for glucocorticoid (Wochnik *et al.*, 2005). However, GR activation can promote the transcription of Fkbp5 via activation at GR elements (Kitraki *et al.*, 2015). Therefore, in response to stress, Fkbp5 can inhibit GR affinity for glucocorticoid via negative feedback. It has been shown that the DNA methylation of GRE region is decreased after treatment with corticosterone in mice hypothalamus and

hippocampus (U *et al.*, 2004; Lee *et al.*, 2010). A previous study also showed that Dex treatment induced demethylation in intron 7 in the human hippocampal progenitor cell line (Klengel *et al.*, 2013). In the present study, we found that ACTH induces *Fkbp5* gene expression and decreases the methylation level in GRE region of intron 6 in *Fkbp5* gene. Therefore, it appears that ACTH treatment induces DNA demethylation in *Fkbp5* gene, resulting in increased transcription of *Fkbp5*.

Conclusions: In summary, ACTH treatment induces oxidative stress in Suhuai sow and increases the expression of glucocorticoid-responsive genes. ACTH also regulates *Fkbp5* expression via demethylation in the GRE region. Hence, this study provides novel insights into the regulatory mechanisms between glucocorticoids and oxidative stress in Suhuai sow.

Acknowledgements: We thank Richard S. Lee (Johns Hopkins School of Medicine, Baltimore, MD 21287, USA) for helping to analyze the GRE of *Fkbp5* gene. This project was supported by the 973 Program (2014CB 138502), the National Natural Science Foundation of China (31472073), the State Key Program of National Natural Science Foundation of China (31630072), the National Natural Science Foundation of China (315019 20) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Authors contribution: QW carried out the study and drafted the manuscript. BL and ZL helped to perform the study. KL analyzed the data. WW and HL designed the study and revised the manuscript. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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