High-fat diet and chronic stress aggravate adrenal function abnormality induced by prenatal caffeine exposure in male offspring rats

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We previously demonstrated that prenatal caffeine exposure (PCE) suppressed fetal adrenal steroidogenesis and resulted in developmental programming changes in offspring rats. However, whether these changes play a role in adrenal corticosterone synthesis under high-fat diet (HFD) and unpredictable chronic stress (UCS) remains unknown. In present study, rat model was established by PCE (120 mg/kg.d), and male offspring were provided normal diet or HFD after weaning. At postnatal week 21, several rats fed HFD were exposed to UCS for 3 weeks and sacrificed. The results showed that compared with the corresponding control group, the serum corticosterone levels and adrenal steroid synthetase expression of the PCE offspring without UCS were reduced. Moreover, the glucocorticoid (GC)-activation system was inhibited, and insulin-like growth factor 1 (IGF1) signaling pathway expression was increased. With UCS exposure in the PCE offspring, serum corticosterone levels and adrenal steroid synthetase expression were increased, the activity of GC-activation system was enhanced, and adrenal IGF1 signaling pathway expression was decreased. Based on these findings, PCE induced adrenal hypersensitivity in adult male offspring rats, as shown by the reduced corticosterone levels under HFD conditions but significantly enhanced corticosterone levels with UCS, in which GC-IGF1 axis programming alteration may play an important role.

Caffeine is a methylxanthine alkaloid widely found in coffee, tea, soft drinks, food and certain analgesic drugs¹–⁴. Worldwide, 50% of all adults consume coffee, and in the US, the average daily caffeine intake during pregnancy is 110 mg/d⁵–⁶. Epidemiological and animal studies have shown that prenatal caffeine exposure (PCE) has adverse effects on reproductive and embryo toxicity, such as intrauterine growth retardation (IUGR)⁷–⁸. Another report found that the incidence of adult metabolic syndrome (MS) is 2.53-fold higher in IUGR-affected infants than in those not affected by IUGR⁹. Our previous studies demonstrated that PCE in rodents could lead to overexposure to maternal glucocorticoids (GCs) in IUGR-affected rodent fetuses and hypothalamic-pituitary-adrenal (HPA) axis-associated neuroendocrine metabolic programming alteration. The fetuses showed low basal activity of the HPA axis, enhanced sensitivity of the HPA axis to chronic stress, GC-dependent alterations of blood glucose and lipid, and an increased risk of MS after birth¹⁰–¹³.

The adrenal gland is the earliest and fastest developing organ of the HPA axis, which is responsible for the synthesis of GC¹⁴–¹⁵. GC have key roles in altering fetal tissue morphology and function, and steroidogenic acute regulatory protein (StAR) and cholesterol side-chain lyase (CYP11A1) are the key rate-limiting enzymes in steroid hormone synthesis¹⁶–¹⁷. A previous report showed that 11β-hydroxysteroid dehydrogenase (11βHSDs) is distributed widely in the adrenal gland and other tissues in the body. Moreover, 11βHSD can effectively control the

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The present study sought to explore the adrenal changes in IUGR-affected male offspring rats caused by PCE by detecting alterations in the steroidal synthetase system, IGF1 signaling pathway and GC-activation system (HSDs/CR levels) of the adrenal axis with normal diet, HFD and HFD/chronic stress conditions after birth. Furthermore, based on the two types of intrauterine programming of adrenal developmental toxicity, we elucidated the mechanisms of adrenal functional change, and below, we discuss its significance.

Results

Serum corticosterone levels. Using ELISA technique, we determined serum corticosterone concentrations in adult male offspring in the normal diet, HFD and HFD/chronic stress groups. As shown in Fig. 1, compared with their respective control groups, the serum corticosterone concentrations in the PCE groups showed decreases with a normal diet or HFD and increases (P < 0.01) with UCS exposure under HFD conditions.

Adrenal steroid synthesis function. To determine whether the changes in serum corticosterone levels were due to the adrenal steroid synthetase system, we tested the relevant indexes. As shown in Fig. 2A, compared with the respective control, the mRNA expression levels of StAR were decreased and of CYP21 were increased in the PCE group fed a normal diet (P < 0.05, P < 0.01). Adrenal steroid synthetase levels in the PCE group were significantly reduced or presented a decreasing trend under HFD condition, including the levels of StAR, CYP11A1, 3β-HSD and CYP11B1 (P < 0.01). With HFD/UCS exposure, the adrenal steroid synthetase levels in the PCE group were significantly enhanced, including the levels of StAR, CYP11A1 and CYP11B1 (P < 0.05, P < 0.01). Adrenal immunohistochemical analyses are shown in Fig. 2B–D. Compared with their respective controls, the protein expression levels of StAR and CYP11A1 were decreased in the PCE groups under normal diet and HFD conditions (P < 0.05) and were increased with UCS (P < 0.05, P < 0.01). These results suggested that the low adrenal steroid synthetase expression continued into adulthood under HFD conditions and was enhanced with UCS exposure in offspring with IUGR induced by PCE.

Figure 1. Effects of prenatal caffeine (PCE, 120 mg/kg.d) exposure on serum corticosterone (CORT) with normal and high-fat diets without and with unpredictable chronic stress (UCS) in adult male rat offspring. Mean ± S.E.M., n = 10. **P < 0.01 vs. respective control.
Adrenal IGF1 signaling pathway. As shown in Fig. 3, compared with the respective control, only the mRNA expression level of IGF1 was increased in the PCE group fed a normal diet ($P < 0.05$). The mRNA levels of all the IGF1 signal pathway components were increased or showed an increasing trend under HFD conditions, such as the levels for IGF1, IGF1R, AKT1 and steroidogenic factor 1 (SF1) ($P < 0.05$, $P < 0.01$). With UCS exposure, the mRNA levels of IGF1 and IGF1R were decreased compared with the levels in the controls ($P < 0.05$, $P < 0.01$). These results suggested that the expression of the adrenal IGF1 signal pathway was enhanced without UCS exposure but decreased with UCS in male offspring with IUGR induced by PCE.

Figure 2. Effects of prenatal caffeine (PCE, 120 mg/kg, d) exposure on the mRNA and protein expression of adrenal steroidogenic enzymes with normal and high-fat diets without and with unpredictable chronic stress in adult male rat offspring. Mean ± S.E.M., n = 5 (the adrenal samples from two litters were counted as one sample for adrenal mRNA). *$P < 0.05$, **$P < 0.01$ vs respective control. (A) The mRNA expression of adrenal steroidogenic enzymes; (B) The mean optical density (MOD) of steroidogenic acute regulatory (StAR) or cytochrome P450 cholesterol side chain cleavage (CYP11A1) in the adrenal cortex; (C) The protein expression of StAR (Immumohistochemical staining, ×200); (D) The protein expression of CYP11A1 (Immumohistochemical staining, ×200). 3β-HSD: 3β-hydroxysteroid dehydrogenase; CYP21: steroid 21-hydroxylase; CYP11B1: steroid 11β-hydroxylase.
Adrenal \(11\beta\)HSD/CR system expression. As shown in Fig. 4, compared with their respective controls, the PCE group fed a normal diet exhibited increased expression of \(11\beta\)HSD2 (\(P < 0.01\)), which resulted in a decreased \(11\beta\)HSD1/\(11\beta\)HSD2 ratio (\(P < 0.01\)), and the PCE group fed an HFD exhibited increased expression of \(11\beta\)HSD2 (\(P < 0.01\)) as well as decreased expression of \(11\beta\)HSD1, a decreased \(11\beta\)HSD1/\(11\beta\)HSD2 expression ratio and decreased MR expression (\(P < 0.05\), \(P < 0.01\)). With UCS exposure, the PCE group exhibited decreased expression of \(11\beta\)HSD2 (\(P < 0.01\)) as well as increased expression of \(11\beta\)HSD1, an increased \(11\beta\)HSD1/\(11\beta\)HSD2 expression ratio and increased MR expression (\(P < 0.05\), \(P < 0.01\)). These results demonstrated that the adrenal \(11\beta\)HSD/CR system was suppressed under HFD conditions and activated with UCS conditions in male offspring with IUGR induced by PCE.

Discussion
Developmental toxicity refers to permanent changes in morphology and function caused by damage in the early developmental stages\(^34\). The alteration of intrauterine programming is a permanent change in morphology and function caused by damage \textit{in utero}, which is usually maintained from the developmental period into adulthood and even throughout the individual’s lifetime, leading to multiple effects on the adults\(^35\). Research has shown that high concentration of GC (dexamethasone) exposure during pregnancy can affect the sensitivity of the adrenal gland, mainly by increasing the mRNA expression of the ACTH receptor\(^36\). Our previous studies confirmed that PCE led to a low birthweight in fetal offspring rats and a high IUGR rate, and that offspring rats with PCE could undergo a period of catch-up growth after birth\(^11–13\). Furthermore, in the PCE-induced IUGR models, we found that the expression of STAR and CYP11A1 were reduced in the fetal adrenals due to overexposure to the maternal GCs\(^22\). Furthermore, when the PCE male offspring rats were fed a normal diet after birth, serum corticosterone
levels presented a decreasing trend, the adrenal GC-activation system was inactivated, the expression of adrenal 
IGF1 signal pathway was enhanced, and the expression of the steroid synthetase system was increased. Hence, 
we had proposed a “two-programming” mechanism for PCE-induced adrenal developmental abnormality in the 
offspring rats, which included the low-function programming of adrenal de novo steroidogenesis and GC-IGF1 
axis programming-mediated compensatory changes of steroidogenesis before and after birth49.

Studies have shown that an HFD can also affect the basic activity and stress sensitivity of the HPA axis47,88, 
and HFD exposure during pregnancy or lactation increased stress reactivity and serum corticosterone levels in 
spontaneously hypertensive adult rats89. Our previous study demonstrated that PCE induced a low expression 
of adrenal GC synthetase in the fetal rats and low basal activity of the HPA axis after birth1,23. Moreover, we 
had demonstrated an interaction between HFD after birth and PCE, which could affect the serum corticoste-
one levels35. In the present study, we found that the mRNA expression of the adrenal steroid synthetase system 
(including STAR, CYP11A1, 3β-HSD and CYP11B1) was reduced under HFD conditions. The immunohisto-
chemical results showed that the protein levels of STAR and CYP11A1 were consistent with the gene expression, 
and the serum corticosterone concentration also showed a decreasing trend. These results suggested that the 
inhibited adrenal function in male offspring with PCE under HFD conditions may be the effect of programming, 
which was caused by the low basal activity of the HPA axis and the adrenal function inhibition. At the same 
time, the adrenal GC-activation system showed significant inactivation (reflected by effects such as decreased 
expression of 11β-HSD1 and increased expression of 11β-HSD2 as well as a decreased 11β-HSD1/11β-HSD2 
ratio and decreased MR expression). Furthermore, the expression of the adrenal IGF1 signaling pathway compo-
nents (including IGF1, IGF1R and AKT1) was notably increased, and the expression of the transcription factor 
for steroid synthetase, SF1, also showed an increasing trend. These results indicated that under HFD conditions, 
the inhibition of the GC-activation system may be related to the low serum GC level and may participate in the 
enhancement of the activity of the adrenal IGF1 signaling pathway (including SF1 expression) and steroid 
synthesis function. Hence, we hypothesized that the inactivation of GC-activation system increased adrenal steroidogenesis by enhancing the expression of the adrenal IGF1 signaling pathway, which then promoted local adrenal 
corticosterone synthesis and increased the serum corticosterone levels.

Studies have shown that chronic stress can affect the body’s homeostasis, such as by interfering with the syn-
thesis and secretion of adrenal GCs42, and chronic stress has a long-term impact on the activity of the HPA axis41. 
Additionally, chronic stress can seriously affect the body’s endocrine and metabolic balance, increasing suscepti-
bility to a variety of diseases, including cardiovascular disease and type 2 diabetes92–94. Our previous study found 
that ethanol exposure during pregnancy could change HPA axis activity in offspring under HFD and UCS condi-
tions, which increased the susceptibility of the offspring to MS95. Caffeine exposure during pregnancy also could 
result in high stress sensitivity of the HPA axis in offspring rats after birth under normal diet conditions96. In 
the present study, we found that compared with the HFD control, the serum GC level and adrenal synthetase system 
expression were increased in the PCE male offspring with UCS, which suggested that UCS could improve adrenal 
steroid synthesis, leading to an increased serum GC level. Furthermore, the adrenal GC-activation system was 
triggered and the IGF1 signaling pathway was inhibited with UCS. These results showed that the UCS-induced 
high serum GC level could inhibit the adrenal IGF1 signaling pathway via negative feedback by activating the 
GC-activation system, which then decreased local corticosterone synthesis to maintain homeostasis97.

Numerous experiments have shown that high blood GC levels increased the blood glucose level and increased 
the risk of insulin resistance, diabetes and other metabolic diseases98–100. Our previous additional study found 
that blood glucose and lipid levels and liver and pancreatic function were all damaged by PCE under normal 
diet conditions98. Meanwhile, an HFD could change HPA axis function in offspring rats with PCE, leading to 
a GC-associated blood glucose and lipid changes, which increased the susceptibility of the offspring to MS in a 
gender-specific manner97. In the present study, regardless of HFD or UCS conditions, the greater adrenal sensitiv-
ity of male offspring rats caused by PCE led to abnormal changes in adrenal synthesis, in which the GC-IGF1 axis 
played an important role by maintaining the stability of the serum GC level, serving a possible endocrine negative 
feedback axis. Furthermore, the serum GC level in the PCE group with HFD and UCS exposure was higher than 
in any other group (such as the normal diet group and the HFD group), which suggested that the compensatory 
effect maintained adrenal synthesis, and it could have easily caused the depletion and decompensation of adrenal 
function as well. Once decompensated, the glucose and lipid metabolic functions were affected, resulting in an 
enhanced susceptibility to MS and related metabolic diseases.

It is worth mentioning that gender differences in intrauterine programming of fetal development related 
research is commonly reported92,93,98. Meanwhile, another study indicated that prenatal corticosterone exposure 
induced offspring adrenal function abnormality in male but not female offspring97. Although only the male off-
spring rats were included in the present study, we tested the female rats at the same time. We were surprised to see 
some obvious gender differences in the GC-activation system and IGF1 signaling pathway of the PCE offspring 
rats under normal diet and HFD conditions, especially in the females, there was even disorder after UCS (data 
not shown). In view of the importance of gender differences in fetal origin adult diseases, we will strengthen the 
gender difference and its potential mechanism-associated research in the future.

One limitation of the present study is that the adrenal steroid synthetase system changes only were measured 
in gene expression not protein expression based on the limited amount of samples, but the immunohistochem-
istry of StAR and P450scc were included in the results. The other limitations are intragastric administration and 
litter size correction after birth. As a major confounding factor, caffeine administration by gavage is a stimulus 
and it can affect the corticosterone secretion of the pregnant rats, although the animals of the control group were 
given equal dose of saline also by gavage, which could relatively eliminate the gavage influence. Additionally, the 
litter size probably changed the offspring growth profile and metabolic health, therefore, we had corrected the lit-
ter size in both the experimental and control groups after birth, which would minimize the individual difference.
Conclusions
PCE induced greater adrenal sensitivity in adult male offspring rats, as shown by the reduced corticosterone synthesis under HFD conditions and the significantly enhanced corticosterone levels with UCS exposure. Adrenal GC-IGF1 axis programming changes may play an important role in these alterations by maintaining the stability of the blood GC level via regulation of local corticosterone synthesis through negative feedback. The adaptive changes resulting from the GC-IGF1 axis programming may maintain blood GC level within a certain range. This study provides an experimental basis for clarifying PCE-caused adrenal dysfunction and abnormal circulating GC levels, which would be beneficial in addressing GC-associated diseases in the IUGR-affected offspring.

Materials and Methods
Chemicals and reagents. Caffeine (CAS #58-08-2, N99% purity) was purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). Isoflurane was purchased from Baxter Healthcare Co. (Deerfield, IL, USA). A rat corticosterone ELISA kit was obtained from Assay-pro LLC. (Saint Charles Missouri, USA). Reverse transcription and real-time quantitative PCR (RT-qPCR) kits were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). The oligonucleotide primers for rat genes were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). All other chemicals and agents were of analytical grade.

Animals and treatment. Specific pathogen-free (SPF) Wistar rats (with weights of 200–240 g for females and 260–300 g for males) were obtained from the Experimental Center of Hubei Medical Scientific Academy (No. 2008–0005, Hubei, China). The animal experiments were performed at the Center for Animal Experiment of Wuhan University (Wuhan, China), which has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). All animal experimental procedures were approved by and performed in accordance with the Guidelines for the Care and Use of the Animal Welfare Committee (AWC) of Wuhan University and the International Council on Research Animal Care; the AWC specifically oversees the university's animal programs, equipment and procedures.

Animals were held under temperature-controlled conditions on a 12 h light: dark cycle and ad libitum access to standard chow and tap water at all times. After one week of acclimation, two females were mated with one male for one night. Upon confirmation of mating by the appearance of sperm in a vaginal smear, the day was defined as gestational day (GD) 0. Pregnant females were then transferred to individual cages.

Pregnant rats were randomly divided into two groups: a control group and a PCE group. Ten dams were in each group. Starting from GD11 until term delivery (GD20), the PCE group was administered 120 mg/kg/d caffeine via oral gavage, as previously reported12. The control group was administered the same volume of vehicle. A schematic of the treatments for maternal and offspring rats is shown in Fig. 5. The pregnant rats were allowed to deliver spontaneously at term.

On postnatal day (PD) 1, the number of pups in each litter was set to 8 (♂:♀ = 1:1) randomly; to ensure adequate and standardized nutrition until weaning26. At postnatal week (PW) 4, 60 male pups from 20 different mothers (3 male pups were chosen from each mother) were selected randomly for each group (30 pups with IUGR from the caffeine group and 30 normal pups from the control group), and all pups were fed a normal diet or HFD ad libitum before being sacrificed. The standard rodent chow purchased from the Experimental Centre of Hubei Medical Scientific Academy contained 21% kcal from protein, 68.5% kcal from carbohydrate and 10.5% kcal from fat. The HFD was previously described by our laboratory27 and contained 88.0% corn flour, 11.5% lard, and 0.5% cholesterol, which provided 18.9% kcal from protein, 61.7% kcal from carbohydrate and 19.4% kcal from fat.

At PW-21, 40 male rats fed a normal diet or HFD were anesthetized with isoflurane and decapitated in a room separate from where the other animals were kept. To minimize the effect of corticosterone circadian rhythm, all the rats were sacrificed within 9:00 am to 11:00 am. Trunk blood was collected, and serum was prepared by centrifugation at 17,205 g for 15 min at 4 °C and stored at −80 °C until use for detection of the corticosterone concentration. The adrenal glands were dissected for RT-qPCR and fixed in 4% formaldehyde for histological examination.
significance was set at \( P < 0.05 \).

Gain rates of serum parameters were transformed if necessary before one-way ANOVA evaluations. Statistical differences between different treatment groups were assessed with one-way analysis of variance (ANOVA). The aldosterone.

The intra-assay and inter-assay coefficients of variation for corticosterone were 5.0% and 7.2%, respectively. The lowing the manufacturer's protocol. The limit of detection for the corticosterone concentration was 0.39 ng/mL.

The concentration for each gene are listed in Table 1; and elongation, 72 °C for 30 s (the elongation step was performed for the 3β-HSD reactions).

For RT-qPCR analysis, single-stranded cDNA was prepared from 2 μg of total RNA according to the protocol of the ExScript RT reagent kit. Primers were designed using Primer Premier 5.0, and their sequences are shown in Table 1. Relative standard curves were constructed for the target genes (Table 1), using the corresponding RT-qPCR products, isolated using a DNA extraction kit, at different concentrations, ranging from 10–10,000 pg per reaction. PCR was performed in 96-well optical reaction plates using the ABI Step One real-time PCR thermal cycler (ABI StepOne, NY, USA) were used for data analysis. All data presented are expressed as the mean ± S.E.M. The significant differences between different treatment groups were assessed with one-way analysis of variance (ANOVA). The gain rates of serum parameters were transformed if necessary before one-way ANOVA evaluations. Statistical significance was set at \( P < 0.05 \).

### Table 1. Oligonucleotide primers and PCR conditions of rat in quantitative real-time PCR. StAR: steroidogenic acute regulatory protein. CYP11A1: cytochrome P450 cholesterol side chain cleavage, 3β-HSD: 3β-hydroxysteroid dehydrogenase, CYP21: steroid 21-hydroxylase, IGF-1: insulin-like growth factor-1, IGF-1R: IGF-1 receptor, AKT1: protein kinase B, SFI: steroidogenic factor 1, 11β-HSD1: 11β-hydroxysteroid dehydrogenase type 1, 11β-HSD2: 11β-hydroxysteroid dehydrogenase type 2, MR: mineralocorticoid receptor, GR: glucocorticoid receptor, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

| Genes          | Forward primer | Reverse primer | Product (bp) | Annealing |
|---------------|----------------|----------------|--------------|-----------|
| StAR          | GGGAGATGCCTGAGCAAAGC | GCTGGCGAACTCTATCTGGGT 188 | 65°C, 30 s   |
| CYP11A1       | GCTGGCGGCTTGGTGATTTTC | GATGTTGGCTTGGATTTGCTTG 188 | 63°C, 30 s   |
| CYP21         | GAGGTAGCTGGCGACCAACAG | GGAGTACGGTCTTGGCCTC 188 | 63°C, 30 s   |
| CYP11B1       | CCCCTTGGTTTAGTGGTAGT | CACGCTCTCAGTTTGCAGTGTT 188 | 61°C, 30 s   |
| 3β-HSD        | TCTACTCTGACAGCTAGGAC | ATACCTTTATTTTGAGGGG 271 | 58°C, 30 s   |
| IGF1          | GACCAAGGTTGGCTTTATCTCAAC | TTTGAGGCTTGGCGGAGCAC 148 | 60°C, 30 s   |
| IGF1R         | GTCTCTCGGGAGTGTCTCA | TGGGCTTTGGGACTACAC 188 | 62°C, 30 s   |
| Ak1           | ATGAGGGCAGGCTGATGTTGAG | GAGGGCCTCAGCAGCTGCTGAGTTG 156 | 60°C, 30 s   |
| SFI           | CCAGTAGCGGCGAACAGAGA | GAGGGCTGAAAGGAAGAGGAAGGA 188 | 63°C, 30 s   |
| 11β-HSD1      | GAAAGAAGCTGAGGTTCAAC | GAAATCCAGAGTTGGTGTCACT 133 | 63°C, 30 s   |
| 11β-HSD2      | TGGGAACATTGCTCTAGAGAG | TTCAGAATGCTGCAAGT 76 | 63°C, 30 s   |
| MR            | TGGGCTGCTGAGTGAAG | GAGGGCGTACGACAGTCTGGATG 156 | 63°C, 30 s   |
| GR            | CACCCATGACCCCTGTCAGTC | AAAGCCTCCTGCTGCTAAAC 156 | 63°C, 30 s   |
| GAPDH         | GCAAGGTCAATGGCGGACAG | AGTCTCTGCTGGCGGATAT 140 | 63°C, 30 s   |

### Analysis of blood samples. The serum corticosterone concentration was detected using an ELISA kit following the manufacturer's protocol. The limit of detection for the corticosterone concentration was 0.39 ng/mL. The intra-assay and inter-assay coefficients of variation for corticosterone were 5.0% and 7.2%, respectively. The cross-reactivity of the corticosterone ELISA, as defined by the manufacturer, was 2% for progesterone and 2% for aldosterone.

**RT-qPCR detection.** Total RNA was isolated from the rat adrenal glands using TRIzol reagent according to the manufacturer's protocol. The same tissues of littermates were pooled for homogenization. The concentration and purity of the isolated RNA were determined by spectrophotometry and adjusted to 1 μg/μl. Total RNA was stored in diethyl pyrocarbonate-H2O (DEPC-H2O) at –80°C.

Immunohistochemical examination. The adrenal glands were fixed in 4% paraformaldehyde solution overnight and then processed using the parafin-sectioning technique. The immunohistochemical procedures were performed using a streptavidin-peroxidase (SP)-conjugation method according to the manufacturer's instructions. Paraffin-embedded tissues were cut into 5 μm-thick serial sections and stained for StAR and CYP11A1 with a goat monoclonal antibody (1:200 dilution; sc-7846, Santa Cruz Biotechnology). At least five random fields from each section were examined. The signal was visualized using light microscopy and imaged, and the positively stained areas were analyzed using the Photo Imaging System (Nikon H550S, Japan).

**Statistical analysis.** SPSS 17 (SPSS Science Inc., Chicago, IL, USA) and Prism (GraphPad Software, La Jolla, CA, USA) were used for data analysis. All data presented are expressed as the mean ± S.E.M. The significant differences between different treatment groups were assessed with one-way analysis of variance (ANOVA). The gain rates of serum parameters were transformed if necessary before one-way ANOVA evaluations. Statistical significance was set at \( P < 0.05 \).

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Author Contributions
Z.H., F.L., H.H. and C.Z. contributed to the acquisition of animal data. Z.H., F.L., L.L. and H.H. performed the total RNA isolation and participated in the experimental design, interpretation and manuscript writing. Y.D., L.Z. and Y.L. assisted with data analysis and statistical methods. H.W. and C.S. provided the experimental design, interpretation and critical revision of the manuscript for important intellectual content.

Additional Information
Competing Interests: The authors declare that they have no competing interests.

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