Effects of N-Acetylcysteine on the reproductive performance, oxidative stress and RNA sequencing of Nubian goats

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Abstract

N-acetylcysteine (NAC) has been found to enhance the protective ability of cells to counter balance oxidative stress and inflammation. To investigate the effects of dietary NAC supplementation on the reproductive performance of goats, the reproductive performance and endometrial transcriptome of goats fed with diets with NAC (NAC group) and without NAC supplementation (control group) were compared. Results showed that the goats fed with 0.03% and 0.05% NAC had similar litter size, birth weight, nitric oxide (NO), sex hormones and amino acids levels compared with the goats of the control group. However, feeding with 0.07% NAC supplementation from day 0 to day 30 of gestation remarkably increased the litter size of goats. The goats of the 0.07% NAC group presented increased levels of NO relative to the control group, but their sex hormones and amino acids showed no differences. Comparative transcriptome analysis identified 207 differentially expressed genes (DEGs) in the endometrium between the control and the 0.07% NAC groups. These DEGs included 146 upregulated genes and 61 downregulated genes in the 0.07% NAC group. They were primarily involved in the cellular response to toxic substances, oxidoreductase activity, immune receptor activity, signalling receptor binding, cytokine–cytokine receptor interactions, PI3K-Akt signalling pathway and PPAR signalling pathway. In conclusion, results showed that dietary 0.07% NAC supplementation exerted a beneficial effect on the survival of goat embryos at the early pregnancy stage. Such positive outcome might be due to the increased NO production and affected expression of genes involved in the anti-inflammation pathways of the endometrium.

KEYWORDS

embryo survival, goats, N-acetylcysteine, oestrous synchronization, RNA-Seq

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1 | INTRODUCTION

Reproductive performance exerts a considerable effect on the economic efficiency of the livestock industry. At present, oestrous synchronization and timed artificial insemination protocols have been applied routinely in reproductive programs for goats to detect oestrus, shorten production cycle, increase conception rate and facilitate management (Baruselli et al., 2017; Rodgers et al., 2012). The widely utilized procedures for oestrous synchronization in goats are treatment with an intravaginal sponge impregnated by flurogestone acetate and intramuscular injection of equine chorionic gonadotropin with prostaglandin F2α (PGF2α) at the end of treatment (Menchaca, Miller, Salveraglio, & Rubianes, 2007; Vilarino, Rubianes, & Menchaca, 2011). However, several studies have shown that the long-term application of this procedure may result in reduced fertility (Sonmez et al., 2009; Vinoles, Forsberg, Banchero, & Rubianes, 2001). Because the drainage of vaginal secretion is blocked by the vaginal sponge, which increases the risk of infection and inflammation with its adherence to the vaginal mucosa. The occurrence and development of inflammation are key components of many pathologies of the reproductive tract that may affect fertilization, embryo survival and placental functions (Arita et al., 2019; Jabbour, Sales, Catalano, & Norman, 2009). Therefore, the anti-inflammation property should be improved to ensure the conception rate of goats under oestrous synchronization.

N-acetylcysteine (NAC), the acetylated variant of L-cysteine, can stimulate glutathione synthesis, promote detoxification and act as free radical scavengers (Rushworth & Megson, 2014). NAC has an optimal thiol redox state to enhance the protective ability of cells to counter balance oxidative stress and inflammation; in addition, NAC affects several signalling pathways involved in apoptosis, angiogenesis, cell growth and arrest, redox-regulated gene expression and inflammatory response (de Andrade et al., 2015; More et al., 2018). NAC also possesses mucolytic properties for breaking disulphide bonds. Research in mares has shown that the administration of NAC can support antibiotic therapy for endometritis by reducing inflammation (Witte et al., 2012). Furthermore, maternal supplement of NAC has a beneficial effect on restoring foetal growth and ovarian function to improve pregnancy outcomes (Gao, Liang, Ma, Dong, & Yan, 2017; Herrera et al., 2017). Recently, a study had shown that NAC administration could improve antioxidant status and reproduction performance of breeder Japanese quail (Coturnix japonica) under heat stress condition (Omid, Amirali, & Ahmad, 2018). Therefore, NAC is a potentially effective treatment for various pregnancy complications, such as recurrent pregnancy loss (Amin, Shaaban, & Bediawy, 2008).

Maternal dietary NAC supplementation may improve the pregnancy outcomes of goats by enhancing oxidation resistance and anti-inflammation activity. To test this hypothesis, the present study compared the reproductive performance and endometrial transcriptome of goats fed with a control diet (control group) and those fed with a NAC-supplemented diet (NAC group) from days 0 to 30 of gestation.

2 | MATERIALS AND METHODS

2.1 | Animals and diets

Forty-nine multiparous Nubian goats (Capra hircus) aged 2–4 years with a body weight of 42.4 ± 3.4 kg (mean ± SEM) were obtained from the same farm. The conditions of constant temperature (22°C) and fixed light/dark cycle (12 hr:12 hr) were maintained. The goats were clinically healthy and were synchronized into oestrus using intravaginal progesterone-impregnated vaginal implants for 12 days. After implants were removed, prostaglandin F 2α (PGF2α, 20 mg; LUTALYSE®, Zoetis, Kalamazoo, MI, USA) was intramuscularly administered. The goats were artificially inseminated two times with unfrozen semen obtained from three Nubian bucks. The goats were then assigned randomly to one of the four treatment groups fed with a basal diet (control group, n = 13) or a basal diet supplemented with 0.03% NAC (Zhuhai Fanhai Biotechnology Co., Ltd., Zhuhai, China) (0.03% NAC group, wt:wt, n = 13), 0.05% NAC (0.05% NAC group, wt:wt, n = 13) and 0.07% NAC (0.07% NAC group; wt:wt, n = 13). The goats were individually housed where they were fed 2.5 kg of basal diet daily and had free access to drinking water from mating to day 30 of gestation. Details of the diets are given in Table 1 to meet nutrient requirements for gestating goats recommended by the National Research Council (NRC, 2007). All goats consumed 100% of the feed provided daily.

2.2 | Sample collection

Blood samples from the maternal jugular vein after overnight starvation were collected on day 30 of gestation. Serum was obtained following centrifugation (3,700 rpm, 15 min, 4°C) and was immediately snap-frozen in liquid nitrogen and stored at −80°C until use. At day 30 of gestation, three goats of each group were hysterectomized after induction of anaesthesia. The uteri were rapidly separated from the reproductive tract, transported to the laboratory in an icebox, and then cut off. Pregnancy diagnosis was determined using transabdominal ultrasonography with a Micro-imager 1,000 sector ultrasound scanner equipped with a 3.5 MHz abdominal transducer (Asonics, Sydney, Australia) at gestation day 30. Diagnosis results showed two goats from the control, two goats from the 0.03% NAC group, two goats from the 0.05% NAC group and one goat from the 0.07% NAC group were not pregnant on day 30 of gestation and were therefore removed from the experiment. The endometrium samples were collected at the attachment site in the uterus of the confirmed pregnant goats. All samples were immediately snap-frozen in liquid nitrogen and stored at −80°C until use.

2.3 | Analysis of free amino acids

Quantification of serum amino acids was carried out with an amino acid analyser A300 (MembraPure, GmbH, Germany) as described
previously (Lei et al., 2013). In brief, the serum samples (750 μL) were precipitated by adding 250 μL of 15% (v/v) sulphosalicylic acid solution. Then, the mixtures were incubated for 1 hr at 4°C and centrifuged (15,000 g, 15 min, 4°C). Prior to analysis, the supernatant was filtered through a 0.22 μm cellulose membrane (Amersham Pharmacia Biotech Company, Yancheng, China) according to the manufacturer’s instructions.

### 2.5 Total RNA isolation and RNA sequencing

Total RNA of endometrial tissues was extracted from the control group and the 0.07% NAC group using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and RNeasy RNA purification kit with DNase treatment (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. The RNA quality was assessed using a 2.100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and RNase-free agarose gel electrophoresis; the total RNA concentration was also measured with the 2.100 Bioanalyzer. Equal amounts of RNA from each sampled tissue were combined for subsequent experiments, and RNA purity was assessed at absorbance ratios of OD260/280 and OD260/230. RNA integrity was confirmed by 1% agarose gel electrophoresis. All extractions exhibited an RNA integrity number of > 7.0 and a 28S:18S ratio of > 1.0. The sequencing of single-end 150 bp reads was conducted using the HiSeq 2,500 system (Illumina, San Diego, CA, USA).

RNA-seq fastq raw data were preprocessed to obtain clean reads through the removal of adapter reads and low-quality reads. Using Tophat2 (version 2.1.0) and Bowtie2 (version 2.2.5), the trimmed reads from six samples were mapped to the goat (Capra hircus) reference genome to determine the expression levels of the identified genes. The default parameters were selected to analyse the RNA-seq results. Mismatches of no more than two bases were allowed in the alignment, and uniquely mapped reads were obtained and used for downstream analyses. Gene expression was measured with fragments per kilobase million mapped reads (FPKM) in this experiment. DESeq2 was used to normalize the read counts and derive FPKM values to quantify the relative gene expression differences with regard to fold change (log2) and statistical significance (Benjamini Hochberg-corrected p-values). Differentially expressed genes (DEGs) with |log2 Fold change| ≥ 1 and P-adj < 0.05 were selected as significantly differential genes. Gene ontology (GO) enrichment and KEGG pathway analysis were performed on all identified DEGs using KOBAS3.0 (http://kobas.cbii.pku.edu.cn) and were considered significant at p < .05.

### 2.6 Quantitative real-time polymerase chain reaction

All samples utilized for RNA-seq were used to validate gene expression levels by quantitative real-time polymerase chain reaction (qRT-PCR). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the PrimeScript RT Reagent Kit (Takara, Kusatsu, Japan). The primers used for qRT-PCR are listed in Table S1. qPCR reaction was performed with 2 × T5 Fast qPCR Mix (SYBR Green I) Kit (TSINGKE, Beijing, China) on the CFX96 Real-Time PCR detection system (Bio-Rad, Foster City, CA, USA) following the parameters recommended by the manufacturer. The reaction parameters were 1 cycle at 95°C for

### TABLE 1 Ingredients and composition of the diets fed to pregnant goats in early gestation (DM basis)

| Items                  | Nutrient level (%) |
|------------------------|--------------------|
| Corn silage            | 50.00              |
| Chinese wildrye        | 20.00              |
| Corn                   | 15.00              |
| Soybean meal           | 8.02               |
| Wheat bran             | 4.98               |
| Calcium bicarbonate    | 0.50               |
| Sodium chloride        | 0.50               |
| Premix                 | 1.00               |

*Per kilogram of premix of the diet contains vitamin A 55,000 IU, vitamin D 11,500 IU, vitamin E 13,000 IU, MgSO4·H2O 110 g, CuSO4·5H2O 0.7 g, FeSO4·7H2O 3.0 g, MnSO4·H2O 2.5 g, ZnSO4·H2O 5.0 g, Na2SeO3 15 mg, KI 40 mg, CoCl2·6H2O 28 mg, DM, dry matter; NDF, neutral detergent fibre; ADF, acid detergent fibre.

### 2.4 Measurement of nitric oxide, oestradiol and progesterone in serum

Serum NO concentrations were determined using nitrate reductase measurement in accordance with the manufacturer’s instructions (Nanjing Jiangcheng Biotechnology Institute, Nanjing, China). Oestradiol and progesterone levels in serum were measured with commercially available enzyme immunoassay kits (Jiangsu Baolai Biotech Company, Yancheng, China) according to the manufacturer’s instructions.
60 s followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. All gene expression experiments were carried out in triplicate. The specificity of the PCR reaction was confirmed through a single peak in the melting curve. β-Actin was used to normalize gene expression levels, and the relative gene expression levels were calculated using the 2^ΔΔCt method. The amplification efficiency of each pair of primers at different cDNA template concentrations was validated to be > 95% (Table S1) on the basis of a previously reported method (Ao et al., 2019).

2.7 Statistical analysis

Differences in reproductive performance, serum biochemical indexes, levels of uterine gene expression were analysed with Student’s t-test procedures using SPSS 19.0 software (IBM Corp., Armonk, NY). A value of p < .05 was considered statistically significant. Values of reproductive performance and serum biochemical indexes are presented as means ± Standard Deviation (SD).

3 RESULTS

3.1 Reproductive performance of goats fed diets with and without N-acetylcysteine supplementation

To examine the effects of dietary N-acetylcysteine (NAC) supplementation on the reproductive performance of goats, litter size and birth weight of kids were compared among the control, 0.03% NAC, 0.05% NAC and 0.07% NAC groups (Table 2). There were no differences in birth weight of kids among NAC and control groups. The number of kids in the 0.07% NAC group was significantly (p < .05) higher than that in the control group, but the 0.03% NAC, 0.05% NAC and control groups were similar (p > .05).

3.2 Comparison of serum concentrations of nitric oxide, selected hormones and amino acids between control and 0.07% NAC group

As shown in Table 3, the goats fed with 0.07% NAC had significantly (p < .05) higher serum concentrations of nitric oxide (NO) than those of 0.03% NAC and control groups. Whereas serum concentrations of NO of 0.03% NAC, 0.05% NAC and control groups did not differ (p > .05) at days 30 of gestation. In addition, concentrations of amino acids, oestradiol and progesterone levels in serum of goats among NAC and control groups showed no differences (p > .05) (Table 3, Table S2).

3.3 Transcriptomic analysis of endometrial tissues of control and 0.07% NAC groups

Transcriptomic analysis was carried out to examine the differences in the gene expression patterns of endometrial tissues between the control and 0.07% NAC groups. The mean numbers of clean reads were 46,256,180 and 44,613,989; and the mapped reads were 44,933,272 and 43,445,321 in the 0.07% NAC and control groups respectively (Table S3). Moreover, 21,289 and 20,958 genes were expressed by alignment analysis in the 0.07% NAC and control groups respectively. Among them, 20,167 genes were expressed in the two groups of endometrial tissues (Figure S1). Through an analysis of gene expression levels, 207 genes were identified as differentially expressed genes (DEGs) under the thresholds of q-value (P-adjusted) <0.05 and fold change > 2 in endometrial tissues between the control and 0.07% NAC groups. These genes included 146 upregulated genes and 61 downregulated genes in the 0.07% NAC group relative to those in the control group (Figure S1).

Gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of all DEGs were carried out between the two groups. GO analysis revealed that the DEGs primarily involved in the biological processes included cellular processes, response to nitrogen compound, cellular response to toxic substance, positive regulation of embryonic development and negative regulation of response to external stimulus (Figure 1a). According to the GO molecular functions, the DEGs were primarily associated with ion binding, binding, oxidoreductase activity, immune receptor activity and signalling receptor binding (Figure 1b). For the GO cellular components, the genes involved

| TABLE 2 Average number and birth weight of kids delivered from pregnant goats of the control, 0.03% NAC, 0.05% NAC and 0.07% NAC groups |
|-----------------|--------|-----------------|------------------|
| Group           | No. of goats | No. of average kid | Birth weight (g) |
|-----------------|-------------|--------------------|------------------|
| Control         | 8           | 1.87 ± 0.35b       | 1960.1 ± 112.0   |
| 0.03% NAC       | 8           | 2.25 ± 0.46ab      | 1902.2 ± 122.9   |
| 0.05% NAC       | 8           | 2.00 ± 0.53ab      | 1951.1 ± 150.5   |
| 0.07% NAC       | 9           | 2.33 ± 0.50a       | 1887.4 ± 159.2   |

Values labelled with different superscripts within the same column signify different values from each other at p < .05. NAC, N-Acetylcysteine. Values are presented as mean ± SD.

| TABLE 3 Concentrations of nitric oxide and selected hormones in sera of goats fed diets supplemented with 0 and N-acetylcysteine from day 0 to 30 of gestation |
|-----------------|--------|-----------------|------------------|
| Group           | NO (μmol/L) | Progesterone (pmol/L) | Estradiol (ng/L) |
|-----------------|------------|------------------------|-----------------|
| Control (n = 8) | 9.4 ± 2.0b | 1,199.4 ± 222.7 | 116.8 ± 22.8    |
| 0.03% NAC (n = 8)| 10.6 ± 1.9b | 1,201.9 ± 159.9 | 126.3 ± 24.8   |
| 0.05% NAC (n = 8)| 11.0 ± 2.2ab | 1,197.8 ± 169.2 | 129.3 ± 24.3   |
| 0.07% NAC (n = 9) | 12.3 ± 1.0ab | 1,127.3 ± 155.2 | 129 ± 20.9     |

Values labelled with different superscripts within the same column are significantly different (p < .05). The values are presented as mean ± SD. NAC, N-Acetylcysteine; NO, nitric oxide.
in the intracellular organelle, cytoplasm and intracellular integral component of membranes were most represented (Figure 1c). The KEGG pathway analysis showed that the DEGs of the two groups were primarily involved in the pathways of neuroactive ligand-receptor interaction, protein digestion and absorption, cytokine-cytokine receptor interactions, PI3K-Akt signalling pathway and PPAR signalling pathway (Figure 1d). Most of the DEGs involved in the PI3K-Akt signalling pathway (PGF, IL6R, FLT1) and PPAR signalling pathway (PPARG, FABP3, CD36) had increased expression levels in the 0.07% NAC group relative to those in the control group (Table 4). The qPCR analysis revealed that the transcription levels of PGF, IL6R, FLT1, PPARG, FABP3 and CD36 of the endometrium were significantly higher in the 0.07% NAC group than in the control group (Table 2). The result is consistent with the outcomes of RNA-seq.

4 | DISCUSSION

In mammals, the implantation of the blastocyst into the maternal endometrium is the crucial step for a successful pregnancy (Ashary, Tiwari, & Modi, 2018). In this study, maternal dietary NAC supplementation was performed to explore the effects of NAC on the reproductive performance of goats during early pregnancy. The results showed that 0.07% NAC supplementation in the diets of goats from day 0 to 30 of gestation remarkably increased the number of births, thus suggesting that NAC may enhance embryonic survival and development.

In various studies, NAC is described as an antioxidant against oxidative stress (Elbini Dhouib et al., 2016; Paschalis, Theodorou, Margaritelis, Kyparos, & Nikolaidis, 2018). A developing embryo is susceptible to various stresses, including oxidative stress. Oxidative stress, a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defences, may result in tissue damage (Skvarc et al., 2017). ROS plays a crucial role in the aetiology of abnormal embryo development of mammal species throughout pregnancy (Lan et al., 2019). Extensive research in experimental animals and humans has shown that NAC treatment has protective effects for treating multiple disorders and helps control oxidative stress by increasing NO production (Buhimschi, Buhimschi, & Weiner, 2003; Nogueira et al., 2018). The results in the current work showed that the dietary 0.07% NAC supplementation
Embryo implantation requires the endometrium to be synchronously receptive to allow the establishment of a reciprocal interaction between the conceptus and the endometrium (Moraes et al., 2018). The endometrium undergoes cyclic changes with the proliferation and differentiation of specific uterine cell types that are regulated by ovarian steroids, such as oestrogen and progesterone (P4) (Gomez, Ruiz-Alonso, Miravet, & Simon, 2015). As such, alterations in the morphologies and functions of the endometrium are responsible for embryo loss and foetal dysplasia. In the present study, endometrial transcriptome analysis revealed that the identified DEGs between the control and the 0.07% NAC groups were primarily associated with cellular response to toxic substances, oxidoreductase activity, immune receptor activity, signalling receptor binding, cytokine–cytokine receptor interactions, PI3K-Akt signalling pathway and PPAR signalling pathway.

The PI3K-Akt signalling pathway is activated by varied cellular stimuli or toxic insults and plays a pivotal role in fundamental cellular functions, such as transcription, translation, proliferation, growth and survival (Osaki, Oshimura, & Ito, 2004). Considerable evidence shows that NAC has anti-inflammatory and anti-apoptotic properties (Lasram, Dhouib, Annabi, El Fazaaz, & Gharbi, 2015; Skvarc et al., 2017), which may be related to the anti-oxidant and anti-apoptotic actions of the PI3K-Akt signalling pathway. A previous study reported that NAC attenuates diabetic myocardial ischaemia/reperfusion injury due to the activation of the PI3K/Akt signalling pathway and an increase in NO production (Wang et al., 2013). Long-term low-dose NAC treatment increases the expressions of proinflammatory cytokines through the enhancement of kinase phosphorylation (Ohnish, Bando, Kakimoto, Kusuyama, & Matsuguchi, 2014). Results obtained showed that several genes involved in the PI3K-Akt signalling pathway, such as PGF, IL6R and FLT1, have elevated expression levels. FLT1 binds to vascular endothelial growth factor receptor-A (VEGFR-A), VEGFR-B and PGF, and it plays an important role in angiogenesis and vasculogenesis (Ji, Xin, Li, & Su, 2018; Olofsson et al., 1998). As such, the increased expressions of FLT1 and PGF may improve uterine vascular development. Notably, IL6R is responsible for the signal transduction of IL6 that might have associations with immune diseases and diverse inflammatory diseases (Ferreira et al., 2013). The upregulation of IL6R expression may affect the anti-inflammatory pathways of the endometrium. Furthermore, growing evidence demonstrates that the PPAR signalling pathway participates in the regulation of the immune response, particularly in inflammation (Wahli & Michalik, 2012). Herein, the expression levels of PPARG, FABP3 and CD36 of the PPAR signalling pathway were altered in the endometrium supplied with 0.07% NAC. CD36 belongs to the class B scavenger receptor family and is involved in various physiological and pathological processes, such as angiogenesis, atherosclerosis, inflammation and lipid metabolism (Febbraio, Hajjar, & Silverstein, 2001; Wang & Li, 2019). Many studies have shown that PPARG is an important regulator of CD36, which has been genetically linked to lipid accumulation in macrophages (Chawla et al., 2001; Szanto & Nagy, 2008). Therefore, dietary NAC supplementation contributes to embryo development and survival by regulating certain important

### Table 4

| Gene symbol | Accession no. | FC | p-value | Gene description |
|-------------|---------------|----|---------|------------------|
| PGF         | 102,169,297   | 3.1| 0.004   | Placenta growth factor |
| IL6R        | 102,179,824   | 2.4| 0.006   | Interleukin 6 receptor |
| LPAR3       | 102,186,820   | 0.3| 0.006   | Lyosphosphatidic acid receptor 3 |
| FLT1        | 100,860,837   | 3.2| 0.014   | Fms-related tyrosine kinase 1 |
| PPARG       | 100,861,309   | 2.5| 0.029   | Peroxisome proliferator-activated receptor gamma |
| FABP3       | 100,860,846   | 3.9| 0.008   | Fatty acid binding protein 3 |
| CD36        | 100,860,958   | 4.3| 0.015   | CD36 molecule |

*Fold change between the 0.07% NAC and control groups.

### Figure 2

Validation of differentially expressed genes involved in the PI3K-Akt signalling pathway and PPAR signalling pathway in endometrial tissues between the control and 0.07% NAC groups by q-PCR. Values are presented as mean ± standard error of the mean. Each experiment was performed in triplicate. Values labeled with asterisk (*) indicate significant difference (p < .05).

Remarkably increased the NO levels of the goats relative to NAC supplementation. Hence, NAC supplementation may facilitate embryo survival and improve NO production. NO is an important signalling mediator involved in various physiological and pathological processes, including angiogenesis, apoptosis, cell cycle, invasion and metastasis (Choudhari, Chaudhary, Bagde, Gadbail, & Joshi, 2013; Gupta et al., 2020). Therefore, NO plays a crucial role in maintaining the normal endothelial function of the endometrium by regulating uterine vascular development, which ultimately affects embryonic fate.

*Remarkably increased the NO levels of the goats relative to NAC supplementation.*
signalling pathways associated with the inflammation response of the endometrium.

In conclusion, maternal dietary supplementation with NAC during days 0–30 of gestation exerts beneficial influence on the litter size of Nubian goats. NAC supplementation increases NO production that may contribute to the improvement of uterine vascular development and give rise to the alteration of the endometrial transcriptome primarily involved in the PI3K-Akt signalling pathway and PPAR signalling pathway to modulate the immune and inflammation response of the endometrium. These findings provide useful information for elucidating the potential mechanisms of the effects of NAC supplementation on pregnancy outcomes in goats.

ETHICS STATEMENT

The animal handling procedures were in line with the Chinese Animal Welfare Guidelines and were approved by the Animal Protection and Use Committee of Guizhou University, Guiyang, China (Approval number: EGZU-2017T010). All efforts were made to minimize animal suffering.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest that could inappropriately influence or be perceived to influence the submitted work.

AUTHORS’ CONTRIBUTIONS

Jinhong Luo and Xiang Chen conceived and designed the experiments; Jinhong Luo and Ye Ao performed the experiments; Zheng Ao and Zhiqiang Duan analysed the data; Ye Ao, Shihan Wei, Wei Chen contributed materials; Jinhong Luo, Zheng Ao and Xiang Chen wrote and revised the paper.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms.338.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.