Transcriptome analysis reveals corresponding genes and key pathways involved in heat stress in Hu sheep

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Abstract

Heat stress (HS) seriously affects animal performance. In view of global warming, it is essential to understand the regulatory mechanisms by which animals adapt to heat stress. In this study, our aim was to explore the genes and pathways involved in heat stress in sheep. To this end, we used transcriptome analysis to understand the molecular responses to heat stress and thereby identify means to protect sheep from heat shock. To obtain an overview of the effects of heat stress on sheep, we used the hypothalamus for transcriptome sequencing and identified differentially expressed genes (DEGs; false discovery rate (FDR) < 0.01; fold change > 2) during heat stress. A total of 1423 DEGs (1122 upregulated and 301 downregulated) were identified and classified into Gene Ontology (GO) categories and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Heat stress triggered dramatic and complex alterations in gene expression in the hypothalamus. We hypothesized that heat stress induced apoptosis and dysfunction in cells and vital organs and affected growth, development, reproduction, and circadian entrainment via the calcium signaling pathway, which influences ribosome assembly and function. Real-time PCR was used to evaluate the expression of the genes regulating important biological functions or whose expression profiles were significantly changed after acute heat stress (FDR < 0.01; fold change > 4), and the results showed that the expression patterns of these genes were consistent with the results of transcriptome sequencing, indicating that the credibility of the sequencing results. Our data indicated that heat stress induced calcium dyshomeostasis, blocked biogenesis, caused ROS accumulation, impaired the antioxidant system and innate defense, and induced apoptosis through the P53 signaling pathway activated by *PEG3*, decreased growth and development, and enhanced organ damage. These data is very important and helpful to elucidate the molecular mechanism of heat stress and finally to find ways to deal with heat stress damage in sheep.

Keywords Hu sheep · Heat stress · Transcriptome sequencing · GO annotation · KEGG pathway

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Abbreviations

DEG	differentially expressed gene
FDR	false discovery rate
FPKM	fragments per kilobase of transcript per million fragment mapped
KEGG	Kyoto Encyclopedia of Genes and Genomes
GO	Gene Ontology
ROS	reactive oxygen species

Background

As global warming increases, heat stress continues to impede animal performance and cause economic losses. Over the past few years, several researchers focused on the adverse effects of heat stress on poultry (Sun et al. 2015; Kim et al. 2017), livestock (Nasiri et al. 2018; Henry et al. 2018), and other

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domestic animals. For example, heat stress was found to reduce milk production in cows by 1.5–2 L/cow/day and increase mortality (Vitali et al. 2009). Heat stress caused renal damage in poultry (Tang et al. 2018). Huang et al. (2015) found that heat stress impaired mitochondrial function and induced oxidative injury in broiler chickens. It also adversely affected oxidative status and physiological responses in sheep (Chauhan et al. 2014).

Researchers have used various indices such as monitoring various physiological indicators and the expression and genetic variants of candidate genes and so on to measure the effects of heat stress on sheep in order to avoid thermal damage. Seixas et al. (Seixas et al. 2017) devised certain indices to assess heat stress in Santa Ines and Morada Nova sheep. Pantoja et al. (2017) found specific breeds were found to overcome thermal challenges effectively and maintain satisfactory production performance due to specific adaptive morphological and physiological characteristics under heat stress (Salces-Ortiz et al. 2015). The polymorphisms of the HSP90AA1 (Hsp90 chaperone) gene detected in the *Ovis aries* species could be as molecular markers to monitor thermal stress (Salces-Ortiz et al. 2015).

However, the effects of heat stress upon the entire transcriptional expression profile of sheep have not been reported to date. In the present study, Hu sheep indigenous to Southern China were selected to assess the effects of heat stress treatment on the hypothalamus, which maintains thermal homeostasis (Lara and Rostagno 2013). The hypothalamus tissue was subjected to process transcriptome analysis to elucidate the molecular mechanisms involved in heat stress response and hope to possibly provide corresponding protective measures to protect sheep from heat stress and reduce economic losses.

Methods

Animal selection and experimental design

Six healthy 1-y Hu ewes were chosen (weight $\sim 33 \pm 1.58$ kg) from Xilaiyuan farm in Jiangsu Province and divided into control and acute heat stress groups. Three ewes were used as control (C) group and maintained at 22 ± 1.2 °C. The other three were designated the acute heat stress (H) group (42 ± 2.4 °C over 4 h), as previously reported (Mahjoubi et al. 2015). Ewes were slaughtered, and their hypothalami were excised and frozen in liquid nitrogen. Jiangsu Academy of Agricultural Sciences, Research Ethics Committee approved this research proposal regarding the source of the animals, health status, inclusion criteria, exclusion criteria, caging, comfort, and the detailed experimental design and procedure.

RNA extraction and preparation

Each whole hypothalamus was ground together and its total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quality, quantity, and integrity were evaluated with a NanoPhotometer spectrophotometer (Implen, Inc., Westlake Village, CA, USA), a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA) and an Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA).

mRNA-Seq library preparation and sequencing

Three micrograms of total RNA was used per sample to construct Illumina sequencing libraries according to the manufacturer's instructions. A cDNA library was sequenced on an Illumina HiSeq 2500 platform (Biomarker Technologies, Emeryville, CA, USA) and paired-end reads were generated. Raw data (raw reads) in fastq format were processed through in-house perl scripts. Clean data (clean reads) were obtained by removing the reads containing adaptor. Q30, GC content, and sequence duplication level were calculated for the clean data. All downstream analyses were based on high-quality clean data. Adaptor sequences and low-quality sequence reads were removed from the datasets. After data processing, the raw sequences were transformed into clean reads, which were then mapped to the reference genome sequence with Tophat 2.

Differential gene expression analysis

Gene expression levels were estimated by FPKM (fragments per kilobase of transcript per million fragment mapped). Differential expression analysis of two conditions/groups was performed using the DESeq R package (v. 1.10.1) (<http://www.bioconductor.org/packages/release/bioc/html/DESeq.html>). *p* values were adjusted using the Benjamini-Hochberg procedure for controlling the false discovery rate (FDR). Genes found by DESeq to have an adjusted *p* < 0.01 were designated as differentially expressed.

Differential gene functional analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes databases (KEGG) analyses were conducted using differential genes as the foreground and all genes as the background for the differential transcripts identified. GO enrichment analysis of the DEGs was implemented using the Goseq R packages (Young et al. 2010), which adjusts for gene length bias in the DEGs. KEGG (Kanehisa et al. 2008) is a database resource that explains

high-level functions and utilities in biological systems (<http://www.genome.jp/keg/>). In the present study, KOBAS was applied (Mao et al. 2005) to test for statistical enrichment of the DEGs in the KEGG pathways.

qRT-PCR analysis for the validation of RNA-Seq data

Quantitative real-time PCR (qRT-PCR) by ABI7500 was used to validate the transcriptome sequencing data. The total RNA isolated for RNA sequencing was used in the qRT-PCR. All the primers worked well checked by Agarose gel electrophoresis. Reverse transcription was performed with 1 µg total RNA and a PrimeScript RT Reagent Kit (Invitrogen, Carlsbad, CA, USA). Individual reactions were prepared with 100 ng cDNA and a SYBR Green PCR Master Mix (Takara, Dalian, China) in a final volume of 20 µL. All reactions were repeated triplicate for each sample. Cycle threshold (Ct) values were normalized to β -actin and comparative mRNA quantification was performed by the $2^{-\Delta\Delta C_t}$ method (Fan et al. 2018). The primer sequences and efficiency used in the qRT-PCR assay are listed in Table 2, and Ct values of each primer at different concentration were shown in supplementary document (Supplementary 1).

Statistical analysis

All data are means $\pm$ standard deviation (SD) which were compared by ANOVA in SPSS 16.0. $p < 0.05$ was designated as the cut-off for statistical significance.

Results

Transcriptome analysis and DEGs identification

Illumina sequencing generated > 6.2 Gb clean data per sample, all data were submitted to NCBI and the SRA accession was PRJNA533887. The GC content in the six samples ranged from 50.69 to 54.57%. The Q30(%) for these samples were almost 90% which means that the data

were reliable for the subsequent analysis (Table 1). Approximately 66.30% of the total reads were uniquely mapped to the *Ovis aries* genome, 52.11% were located within an exon, 34.04% were located within intergenic regions, and 13.85% were located within introns (Fig. 1a). After the acute heat stress treatment, gene expression was compared between the control and heat stress groups. A total of 1423 DEGs were identified, of which 1122 were upregulated and 301 were downregulated (Fig. 1b).

Functional annotation and pathway enrichment analysis of DEGs

To elucidate the molecular mechanism of heat stress, the DEGs in this study were subjected to GO annotation analysis. Fifty-six GO classifications were identified, including 22 biological processes, 17 cellular components, and 17 molecular functions. Among the cellular component terms, the cell part category was significantly enriched in both comparisons. For molecular function, the binding term was the abundant category. In the biological process, cellular process covered 1046 genes. Developmental process, growth, hormone secretion, immune system process, metabolic process, reproduction, reproductive process, response to stimulus, signaling, rhythmic process, and others were involved in responding to heat stress and influencing the corresponding biological functions (Fig. 2).

To identify the biological pathways involved in heat stress in sheep, the DEGs from both groups were mapped to the KEGG pathway database. Pathways with $p < 0.05$ were considered significantly enriched. After acute heat stress, the calcium signaling pathway (39 genes; $Q = 1.38E-06$), axon guidance (27 genes; $Q = 8.83E-06$), circadian entrainment (22 genes; $p = 0.000465389$), cholinergic synapse (23 genes; $Q = 0.000482454$), and thyroid hormone signaling pathway (20 genes; $Q = 0.02079$) were significantly enriched (Fig. 3). Therefore, these processes could be influenced by heat stress and participate in acute heat stress regulation in sheep.

Five KEGG categories (cellular processes, environmental information processing, genetic information processing, metabolism, and organismal systems) were analyzed in the present study. Organismal systems (202 genes) and environmental information processing (176 genes) had the highest changes in gene expression profile (Fig. 4). Signal transduction was included in the environmental information process. Several signaling pathways were involved in heat stress regulation in Hu sheep, including the calcium signaling pathway (39 genes), the MAPK signaling pathway (30 genes), and others. In the metabolism process, the expression levels of eight genes related to oxidative phosphorylation were altered after acute heat stress in sheep. Heat stress also induced changes in

Table 1 Statistics of clean reads, GC content, and the percentage of Q30

Groups	Clean reads	Clean bases	GC content (%)	% > Q30
C1	24,718,365	7,375,212,674	50.69	91.83
C2	20,802,846	6,196,056,534	51.52	90.98
C3	29,355,906	8,763,412,178	51.45	89.79
H1	29,079,562	8,439,513,452	54.57	93.01
H2	28,376,446	8,204,479,310	55.56	93.09
H3	22,757,611	6,694,966,316	54.26	92.08

C control group; H heat stress group

Fig. 1 Reads distribution in genome and differentially expression gene (DEG) identification

genetic information processes including folding, sorting, degradation, replication, repair, transcription, and translation (Supplementary 2). Thirty-eight gene

modifications were found at the ribosome. Heat stress also induced changes in the genes regulating the circulatory, digestive, endocrine, excretory, immune, nervous, and sensory

Table 2 qRT-PCR primers used in this study

Gene	Primers (5'-3')	Primer efficiency (%)
RPS3A	F: ACCTCTTACGCCCAGCAC R:GGAATCAGTTTATTGACCACCT	94.21
RPL23A	F: CCAAATATCCTCGGAAGAGC R: TGGCTGACTCGGTGGTGA	92.40
RPL5	F: AAGTCGAGGTGACTGGTGAT R:CGTACTATGAGGGATAGACAGG	97.92
PKD1	F: TTCTGGCGAAGGAGGAAGC R: CATGGTTGTGGCACGAGGTAT	91.81
PRDX1	F: TTACCCTCTTGACTTCACCTT R: ACTTGGCAGTTCAGTTTCTTA	93.85
BCL2	F: CCCTGTGGATGACCGAGTA R: ACACCCAGACGAGCACCTAC	95.22
NOS1	F: CAACATTGAGAAGGCGAACA R: CCACATAGGTGAGACGGAAC	93.32
RYR1	F: CAAGGCAACATCCACAGC R: AAGCAGCCAATGACAAGGT	94.16
CAMK2A	F: GTCCAGTTCACGCTTCA R: TCCGCACTTTGGTGTCTT	93.72
PEG3	F: CTGAGGATGACGGCAACT R: GTGGGCTGACTTCTTGGT	103.51
FOXA3	F: CTGGCAGAACTCCATCCG R: CTTCTGGCGGCGTAGGTA	103.67
PITX2	F: GCAGGGAAAGAATGAGGA R: GCTGGCTGGTAAAGTGAGT	89.29
MYL2	F: AGAGCAGAGGGAGCCAATT R: CCCAAGAGCAGCAAAGGT	99.02
MPV17	F: CCAACTTCTACCTGGTTCCT R: GGAGTGAAGCCGACTTAGAG	105.15
DAO	F: ACAGTCGATGAATGTGATGG R: TTGGGAGAACCGATGAGG	101.37
β-actin	F: AGCCTTCCTTCCTGGGCATGGA R:GGACAGCACCGTGTGGCGT AGA	97.68

Fig. 2 GO annotation of differentially expression genes in this study

Fig. 3 KEGG enrichment scatter plot of differentially expression genes

Fig. 4 KEGG categories involved in acute heat stress in Hu sheep

organismal systems. All of these were involved in heat stress regulation in Hu sheep (Fig. 4; Supplementary 2).

Key gene selection and validation of real-time PCR results

We selected DEGs that regulated representative functions or whose expression profiles were extremely altered in

response to acute heat stress (Table 3). We used real-time PCR to check their expression patterns after heat stress in order to verify our RNA sequencing analysis. PKD1, RYR1, and CAMK2 were in the calcium signaling pathway. RPL23A, RPL5, and RPS3A were associated with ribosomal protein analysis. PEG3 and BCL2 were related to apoptosis. PRDX1, MPV17, and DAO were correlated to the antioxidant system and amino acid metabolism. PITX2, FOXA1, MYL2,

Table 3 The information of genes selected to verify RNA sequencing analysis by real-time PCR

Gene	Functions	Expression*	Gene	Functions	Expression*
RPS3A	Ribosomal structure and biogenesis	Down	CAMK2A	Calcium ion binding	Up
RPL23A	Ribosomal structure and biogenesis	Down	PEG3	Cell death effector	Up
RPL5	Ribosomal structure and biogenesis	Down	FOXA3	Animal reproduction	Down
PKD1	Calcium influx	Up	PITX2	Cell proliferation and differentiation	Down
PRDX	Antioxidant system	Down	MYL2	Organ damage	Down
BCL2	Apoptosis	Up	MPV17	ROS metabolism	Down
NOS1	Mammalian biological rhythms	Up	DAO	Amino acid metabolism	Down
RYR1	Calcium-release channel	Up			

*This expression indicated the expression changes after acute heat stress by RNA sequencing

Fig. 5 Real-time PCR verification of the expression levels of 15 genes

and NOS1 corresponded to growth, development, reproduction, and circadian entrainment. After heat stress, PRDX and NOS1 were downregulated and upregulated, respectively, but their expression levels did not significantly differ from those of the control ($p > 0.05$) (Fig. 5). The expression profiles of the other genes chosen for real-time PCR verification were entirely consistent with the transcriptome results, which indicate that the transcriptome data were validation.

Acute heat stress causes calcium dyshomeostasis in the endoplasmic reticulum and ribosomal biogenetic synthesis. It also induces mitochondrial dysfunction by altering ROS metabolism, antioxidant systems, amino acid metabolism, and apoptosis. It may also result in dysfunctional growth, development, reproduction, and circadian entrainment, and produce organ damage in Hu sheep.

Discussion

Heat stress influences animal reproduction and growth. It may induce multi-organ dysfunction and has resulted in huge economic losses (Kim et al. 2017; Sohail et al. 2012). In the present study, we subjected Hu sheep to heat stress. This breed is indigenous to Southern China. We analyzed their hypothalamus (Lara and Rostagno 2013), which regulates body temperature. We performed process transcriptome sequencing to identify changes in various pathways and gene expression levels. We also attempted to elucidate the possible molecular mechanisms involved in heat stress regulation in sheep.

We isolated 1424 DEGs, of which 1124 were upregulated and 301 were downregulated after heat stress. A KEGG pathway analysis was performed, and it predicted that the calcium

Fig. 6 Schematic diagram depicting the probable mechanism and pathway of acute heat stress in Hu sheep

and thyroid signaling pathways among others were significantly altered after acute heat stress in sheep. The calcium signaling pathway regulates numerous biological processes (Verkhatsky and Parpura 2014). Calcium is used by cells as a second messenger to translate extracellular stimuli into intracellular activities necessary for the regulation of cell survival, development, gene expression, and differentiation (Speidel 2010). Ca^{2+} release from internal stores is mediated by two intracellular calcium release channels: IP3Rs and RYRs. These are thought to be vital Ca^{2+} release modulators (Bezprozvanny 2005). Our research indicated that *RYR1* and *RYR3* were upregulated after heat stress whereas there was no apparent change in *IP3R*. Therefore, the influence of heat stress on Ca^{2+} release is mediated by *RYR1/3* upregulation in response to acute heat stress in Hu sheep. PKD1, which encodes polycystin-1 (PC1), may interact with PC2 (polycystin-2), form a PC complex, and act as a mechanosensor controlling mechanically induced calcium influx through the plasma membrane (Chebib et al. 2015). When acute heat stress occurs, PKD1 is upregulated in Hu sheep, promotes calcium release, and regulates gene expression. Intracellular Ca^{2+} overload may be cytotoxic and trigger apoptosis (Jan et al. 2013; Orrenius et al. 2003). Shin et al. (Shin et al. 2018) found that compound K induced apoptosis via endoplasmic reticular Ca^{2+} release through the ryanodine receptor in human lung cancer cells. The P53 signaling pathway induced cell apoptosis in mitochondria (Speidel 2010). *PEG3* was a potential cell death effector of p53 (Deng and Wu 2000). The p53 was upregulated after acute heat stress in the present study. It was thought that cell apoptosis was initiated via p53 signaling pathway activation in Hu sheep. The antiapoptotic *BCL2* (B cell lymphoma-2) was also upregulated after acute heat stress in sheep. This response may help the organism resist apoptosis via the p53 signaling pathway and also inhibit Ca^{2+} release by targeting IP3R (inositol-1,4,5-trisphosphate receptor), maintaining the calcium balance, and recovering the damage caused by acute heat stress (Vervliet et al. 2014). In the present study, the expression profiles of MPV17 (mitochondrial inner membrane protein MPV17), PRDX1 (peroxiredoxin 1), and DAO (*D*-amino acid oxidase), which regulate ROS metabolism (Casalena et al. 2014), the antioxidant system (Shim and Kim 2013), and amino acid metabolism (Du et al. 2018), respectively, were altered after acute heat stress. MPV17 is essential for the maintenance of mitochondrial homeostasis and protects podocytes against oxidative stress (Casalena et al. 2014). In the present study, MPV17 was downregulated after acute heat stress, impeded ROS metabolism, promoted ROS accumulation, and induced apoptosis (Kamiya et al. 2018). Under normal physiological conditions, there is a balance between ROS production and antioxidant enzyme system capacity. PRDX1 is an antioxidant enzyme and a free radical scavenger (Shim and Kim 2013); when sheep were subjected to heat stress, PRDX1 was downregulated and the

effect was consistent with the function of this enzyme (Lv et al. 2017). DAO catalyzes the oxidative deamination of *D*-amino acids, such as *D*-serine, which then bind to *N*-methyl-*D*-aspartate (NMDA) glutamate receptors. It plays crucial roles in neurophysiology and pathology (Balu et al. 2013). Sasabe and Suzuki (2018) found that *D*-amino acids participated in innate defense in mammals. In the present study, DAO was upregulated after acute heat stress, lowered *D*-amino acid concentrations and, by extension, impaired innate defense in sheep after acute heat stress. In summary, heat stress disrupts normal organismal function, impairs innate defense and antioxidant capacity, increases ROS, and induces apoptosis mainly through the calcium signaling pathway.

Thyroid hormones are important modulators of developmental processes and general metabolism (Vancamp et al. 2019; Duntas and Brenta 2018). Under heat stress, blood T3 and T4 levels decrease along with metabolic rates, feed intake, growth rates, and milk production. Koluman et al. (Koluman and Daskiran 2011) found that the T3 and T4 levels were lower in the afternoon than in the morning. Thyroid hormone influences HSP72 expression during heat acclimation (Maloyan and Horowitz 2002). In the present study, we found that the thyroid signaling pathway was involved in regulating heat stress in Hu sheep.

Ribosomes are abundant, large RNA-protein complexes and the cellular sites of all protein synthesis. Ribosome biosynthesis is extremely expensive from an energetic standpoint. This process has been linked to both human health and disease (Pelava et al. 2016). Ribosome biogenesis is affected by numerous cellular stressors, including DNA damage, oxidative stress, and others (Deisenroth and Zhang 2010). In prokaryotes, ribosomes are sensors for heat and cold shock response networks. They may also have this function in eukaryotes (VanBogelen and Neidhardt 1990). In the present study, we found that nearly all of the genes involved in ribosome assembly were downregulated and encoded ribosomal proteins such as RPL5, RPL23A, and RPS3A. Therefore, acute heat stress reduced ribosome production, which, in turn, activated the P53 signaling pathway, reprogrammed cellular transcription, and initiated apoptosis (Deisenroth and Zhang 2010).

The GO analysis revealed that several genes corresponding to growth, development, reproduction, and circadian rhythm regulated heat stress. Heat stress markedly alters weight gain energetics during growth (Mahjoubi et al. 2015). The average weight of heat-stressed Malpura rams was significantly lower than that of the control (Maurya et al. 2016). After acute heat stress, certain genes regulating growth and development such as PITX2 (pituitary homeobox 2) were significantly downregulated. Previous studies reported that PITX2 participates in growth, vertebrate left-right asymmetry development, cell proliferation and differentiation, and myogenesis via the Wnt/beta-catenin pathway (Hernandez-Torres et al. 2017; Zhang et al. 2018; Jiang et al. 2018). *FOXA3*, also known as

hepatocyte nuclear factor 3 γ , is a transcription factor in the evolutionarily conserved forkhead box protein superfamily. It is expressed mainly in Leydig and ovarian theca cells and regulates animal reproduction (Behr et al. 2007). *Foxa3*^{−/−} male mice became progressively sub/infertile because of germ cell loss resulting from increasing apoptosis (Friedman and Kaestner 2006). In the present study, *FOXA3* was significantly downregulated after acute heat stress. Consistent with earlier reports, then, acute heat stress regulated sheep reproduction (Nasiri et al. 2018; Sejian et al. 2014). The circadian rhythm in animals is an adaptation to predictable variations in environmental conditions. Wan et al. (Wan et al. 2013) found that heat stress and dramatic differences between nighttime and daytime temperatures are the main selection pressures for diel activity in mammals. NOS (nitric oxide synthase) has been implicated in mammalian biological rhythms (Spessert and Rapp 2001; Golombek et al. 2004). Several reports suggested that NO transmits photic information from the retina to the suprachiasmatic nucleus (SCN), which is the main circadian oscillator (Watanabe et al. 1994; Melo et al. 1997). In the present study, we found that *NOS1* was upregulated after acute heat stress. We confirmed that heat stress affected animal circadian rhythms, and our findings were consistent with those of Wan (Wan et al. 2013).

Conclusions

We predicted that acute heat stress disrupted calcium homeostasis, inhibited biogenesis, caused ROS accumulation, damaged innate defense, and induced apoptosis via the P53 signaling pathway. Moreover, it initiated a resistant apoptosis mechanism, influenced circadian entrainment, induced organ damage, and decreased growth, development, and reproduction in Hu sheep (Fig. 6). These data provide a theoretical basis for understanding the molecular mechanism of heat stress and possibly providing corresponding protective measures to protect sheep from heat stress such as keeping calcium homeostasis, protecting biogenesis, and increasing antioxidant defense and the ability to innate defense and so on.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval The use of the animals was in agreement with the Committee on Ethics of Animal Experimentation from the Jiangsu Academy of Agricultural Sciences. The experiment was performed according to regulations and guidelines established by this committee.

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