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## The Developmental Changes of mRNAs Expression Levels of GHSR Gene in Sheep

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### Abstract

Different age group of Male Kazak sheep and Xinjiang fine wool sheep were used in the current study to investigate the developmental changes of GHSR mRNA expression. After measurement of body weight, hypothalamus and pituitary were sampled to extract total RNA for determining the abundance of GHSR mRNA by RT-PCR and real-time PCR. The results indicated that: (1) For both breeds, body weight among different ages was significantly different ( $P < 0.05$ ), and from day 30 to 90, the Kazak's was notable higher than the Xinjiang's ( $P < 0.01$ ); (2) Hypothalamus GHSR mRNAs expression level increased firstly and then declined with growing in both breeds. From day 60 to 90, the mRNAs expression level of the Kazak was extremely higher than that of the Xinjiang ( $P < 0.01$ ); (3) Pituitary GHSR mRNAs expression level increased with growing in both breeds, and was highly positively correlated with the cumulative growth curves. From day 2 to 90, the pituitary GHSR expression level of the Kazak was extremely higher than that of the Xinjiang ( $P < 0.01$ ). The results predicated that it may be pituitary GHSR but not hypothalamus GHSR through which ghrelin influenced sheep growing.

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## 1. Introduction

GHSR was a protein firstly identified from human and porcine hypothalamus and pituitary, which mainly acts as promotion factor of GH secretion. Howard et al. (1996) cloned GHSR cDNA and proved that GHSR was a G protein-coupled receptor, being a seven transmembrane  $\alpha$  helix protein receptor. There are two subtypes of GHSR, GHSR Ia and Ib.

The expression of GHSR mRNA is researched by in situ hybridization or an RNase protection assay mainly in ventromedial nucleus of the hypothalamus and in the pituitary (Guan, X.M. et al. 1997). The main function of GHSR is to promote GH secretion and release in conjunction with GHS, which is closely correlative with animal growth.

In this article, male Kazak sheep and Xinjiang fine wool sheep, with different growth rates during the early growth period, were selected to study the developmental changes of GHSR mRNA expression developmental changes and explored the effect of GHSR on growing, so as to provide reference for the study on sheep growth.

## 2. Materials and Methods

### 2.1. Animals

Twenty-four male Kazak sheep and thirty male Xinjiang fine wool sheep, six for each different age group (days 2, 30, 60, 90, and 120), were selected from the Ziniqan Sheep Pasture in Shihezi City of Xinjiang Autonomous Region for the present study; however, there was no 120-day-old Kazak sheep. After measurement of body weight, animals were slaughtered for sampling hypothalamus and pituitary. The removed samples were stored at  $-70^{\circ}\text{C}$  for total RNA analysis later.

### 2.2. Primer design

According to the gene sequences of ovine GHSR and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA, oligonucleotide primer sets for the three genes were designed using Primer premier 5.0 software and described in detail in table 1. GAPDH was used as an internal standard for the determination of targeted mRNA levels.

Table 1. Parameters of gene-specific primers for ghrelin, GHSR and GAPDH genes

Target genes	GenBank Accession number	Primer Sequence	Product Size (bp)	Annealing temperature ( $^{\circ}\text{C}$ )
<i>GHSR</i>	GenBank AF118636	P1: 5'-CGACCCTCGGGACACTAA-3'	174	58
		P2: 5'-ACCACTTCGCTGCGTCTT-3'		
<i>GAPDH</i>	GenBank AF030943	P1: 5'-ACTTTGGCATCGTGAGG-3'	379	56
		P2: 5'-GAAGAGTGAGTGTGCTGTTG-3'		

### 2.3. Total RNA extraction

Using the acid-guanidinium thiocyanate/phenol chloroform extraction method to extract the total RNA of hypothalamus and pituitary (GAO et al. 2003). RNA concentration and purity was detected by its optical density ratio at 260/280 nm ( $\text{OD}_{260}/\text{OD}_{280}=1.8-2.0$ ).

#### 2.4. Reverse transcription (RT)

Two micrograms of total RNA was used for reverse transcription in a final volume of 25  $\mu$ L containing 200 U MMLV reverse transcriptase (Promega, Inc. Madison, USA), 20 U RNase inhibitor (Promega, Inc. Madison, USA), 1  $\mu$ g of random primer, 5  $\mu$ L of 5 $\times$ RT buffer (250 mmol/L Tris-HCl pH8.3, 50 mmol/L  $MgCl_2$ , 250 mmol/L KCl, 50 mmol/L DTT, 2.5 mmol/L Spermidine), and 0.4 mmol/L each of dNTP. RNA sample, random primer, dNTP, and sterile  $H_2O$  (final volume 10  $\mu$ L) were first mixed in a 0.5-mL microcentrifuge tube and incubated at 70°C for 5 min, and cooled on ice for 2 min. The rest of the reagents were then added into the reaction tube to a final volume of 25  $\mu$ L and incubated at 37°C for 1 h. The reaction was terminated by heating at 95°C for 5 min and quickly cooled on ice. RT products were stored at –20°C.

#### 2.5. Polymerase chain reaction (PCR)

RT products (0.5  $\mu$ L) were amplified in a 10  $\mu$ L PCR reaction containing 1 U Taq DNA polymerase (TaKaRa, Inc. Dalian, China), 1  $\mu$ L of 10 $\times$ PCR buffer (100 mmol/L Tris-HCl pH 8.3, 500 mmol/L KCl), 0.25 mmol/L each of dNTP, 1.25 mmol/L  $MgCl_2$ , and 0.5  $\mu$ mol/L each of gene-specific primers.

The following amplification conditions were used: one cycle of 1 min at 94°C followed by 40 PCR cycles of 30 s at 94°C, 30 s at the annealing temperature of the primers, 30 s at 72°C, and a final extension for 5 min at 72°C.

The product of PCR was isolated by 8% polyacrylamide gel electrophoresis, after silver staining, which was subsequently analyzed with a computer flatbed scanner.

#### 2.6. Cloning and sequence analysis of the amplified fragments

PCR products were excised after being confirmed by electrophoresis on a 1% agarose gel and purified by V-gene DNA Purification Kit (V-gene Biotechnology, Ltd. Hangzhou, China) according to the manufacturer's manual and then cloned into pMD18-T simple vector. Subsequently, the ligation products were transformed into JM109 cells. Positive clones based on blue-white selection were picked out for plasmid extraction by V-gene Kit (V-gene Biotechnology, Ltd. Hangzhou, China) according to the manufacturer's recommendation and then identified by PCR using gene-specific primers. Plasmids containing inserts of the right size were sequenced by Invitrogen Biotechnology Co., Ltd. (Shanghai, China).

#### 2.7. Real-time polymerase chain reaction (real-time PCR)

The abundance of GHSR mRNA was estimated by real-time reverse transcription polymerase chain reaction with a fluorescence temperature cycler (DNA Engine Opticon Real-time PCR Systems, MJ Research, Inc., Waltham, Massachusetts, USA). The final reaction volume was 20  $\mu$ L containing 1  $\mu$ L of the RT products, 1 U EX Taq HS DNA polymerase (TaKaRa, Inc. Dalian, China), 4  $\mu$ L of 5 $\times$ PCR buffer, 0.3 mmol/L each of dNTP, 3.75 mmol/L  $MgCl_2$ , 0.5  $\mu$ mol/L each of primers, and 1  $\mu$ L of 20 $\times$ SYBR green. PCR conditions were as follows: one cycle of 1 min at 95°C; 45 PCR cycles of 10 s at 95°C, 10 s at the annealing temperature of the primers, 15 s at 72°C, plate-reading; this was followed by an extension of 10 min at 72°C; plate-reading every other 0.2°C from 65°C to 94°C for drawing melting curves; and then the reaction was stopped with an extension of 5 min at 72°C.

The reactions were repeated twice for every sample. Plasmid DNA with the targeted DNA fragment was diluted to gradient concentrations, which were used to draw quantitative standard curves.

## 2.8. Statistical analyses

Data were described as  $\bar{x} \pm Sd$  and statistics was analyzed using SPSS11.5 For Windows Software. Differences of body weight and gene expression level between ages in the same breed, and those at the same age between the two breeds were analyzed by one-way ANOVA and independent-samples t-test, respectively.

## 3. Results

### 3.1. Cumulative growth curves

Results showed that for both Sheep breeds, body weight among different ages was significantly different ( $P < 0.05$ ). And from day 30 to 90, the body weight of the Kazak was extremely higher than that of the Xinjiang ( $P < 0.01$ ) (Fig. 1).

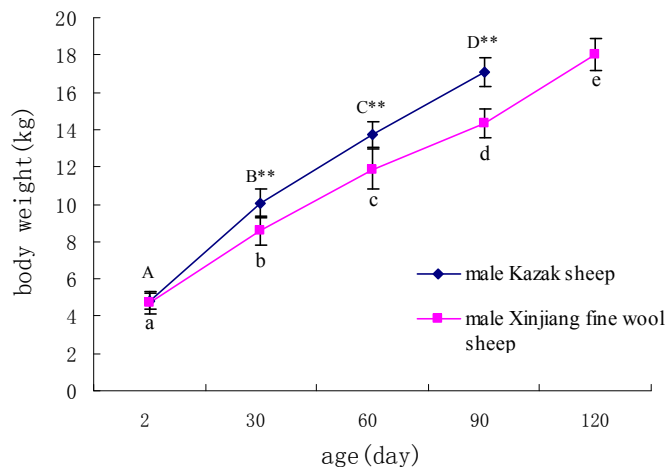


Fig. 1. Cumulative growth curves of Kazak sheep and Xinjiang fine wool sheep. Significant difference is denoted with letters (the capital for the Xinjiang and the small for the Kazak) and means without a common superscript indicate significant differences ( $P < 0.05$ ) between ages in the same breed. Double stars \*\* indicate extreme differences ( $P < 0.05$ ) between breeds at the same age.

### 3.2. RT-PCR of GHSR and GAPDH genes and sequence analysis of the amplified fragments

Total RNA from the abomasum and hypothalamus of Kazak sheep was used as an initial sample to amplify GHSR and GAPDH genes by RT-PCR, which produced a 174bp and a 379 bp cDNA fragment, respectively (Fig. 2).

The amplified GHSR and GAPDH cDNA fragments were then cloned into pMD18-T simple vector. After ligation, the plasmids containing inserts of the right size being sequenced were used to identify the products by PCR using gene-specific primers (Fig. 3). The sequences of the amplified fragments were aligned by DNASTar software with the corresponding reported sequences according to which the gene-specific primers were designed. The results showed that (1) There was 98.85% sequence identity for the GHSR gene; (2) There was 100% sequence identity for the GAPDH gene. These results indicated that the amplified cDNA fragments of the two genes were gene-specific products.



Fig. 2. RT-PCR of GHSR and GAPDH mRNA.

2-3: GHSR, 5-6: GAPDH, 1 and 4: DNA marker PUC18 DNA/Msp I.

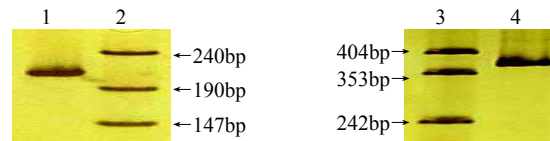


Fig. 3. PCR of GHSR-pMD18-T and GAPDH-pMD18-T.

1: GHSR, 4: GAPDH, 2 and 3 : DNA marker PUC18 DNA/Msp I.

### 3.3. Developmental changes of GHSR mRNA expression in hypothalamus

Real-time PCR was used to quantify GHSR mRNA levels in hypothalamus of Kazak and Xinjiang fine wool sheep at different ages. Results showed that the hypothalamus GHSR mRNAs expression level increased firstly and then declined with growing in both breeds. From day 2 to 60, mRNAs expression level increased continuously in both breeds and showed significantly different ( $P < 0.05$ ) between ages except between day 30 and day 60 in Kazak sheep ( $P > 0.05$ ). After day 60 mRNAs expression level descended rapidly and also showed significantly different ( $P < 0.05$ ) between ages in both breeds. From day 60 to 90, the hypothalamus GHSR expression level of Kazak sheep was extremely lower than that of Xinjiang fine wool sheep ( $P < 0.01$ ) (Fig. 4).

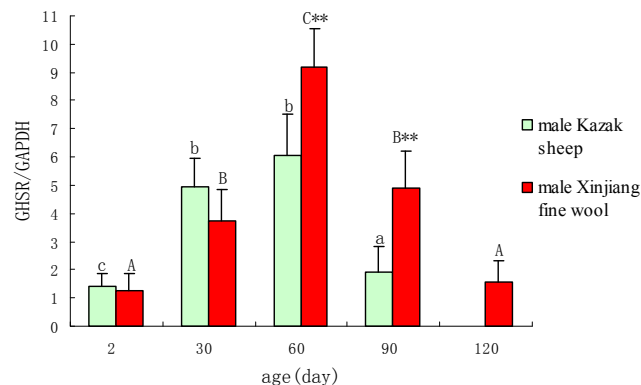


Fig. 4. The developmental changes of hypothalamus GHSR mRNA expression.

The difference is denoted with letters and means without a common superscript (the capital for the Xinjiang and the small for the HSK) indicate significant differences ( $P < 0.05$ ) between ages; Double-star \*\* indicates extreme differences ( $P < 0.01$ ) between breeds at the same age.

### 3.4. Developmental changes of GHSR mRNA expression in pituitary

Real-time PCR was used to quantify GHSR mRNA levels in pituitary of Kazak and Xinjiang fine wool sheep at different ages. Results showed that the GHSR mRNA level developmental change patterns of the two breeds were very similar. For the two breeds, it increased continuously from day 2 and 90 and showed significantly different ( $P < 0.05$ ) between ages. The mRNA expression level of Xinjiang fine wool sheep maintain steady. From day 2 to 90, the pituitary GHSR expression level of Kazak sheep was extremely higher than that of Xinjiang fine wool sheep ( $P < 0.01$ ) (Fig 5). In both breeds, the mRNAs expression level of pituitary GHSR was highly positively correlated with that of abomasum ghrelin ( $r = 0.8972$ ) ( $P < 0.01$ ), with  $r$  being of 0.8972 ( $P < 0.01$ ) in Kazak sheep and 0.9364 ( $P < 0.01$ ) in Xinjiang fine wool sheep. The patterns of pituitary GHSR mRNAs expression level changes were also highly positively correlated with those of the cumulative growth curves ( $r = 0.9319$ ) ( $P < 0.01$ ) in the two breeds, with  $r$  being of 0.9740 ( $P < 0.01$ ) in Kazak sheep and 0.9416 ( $P < 0.01$ ) in Xinjiang fine wool sheep.

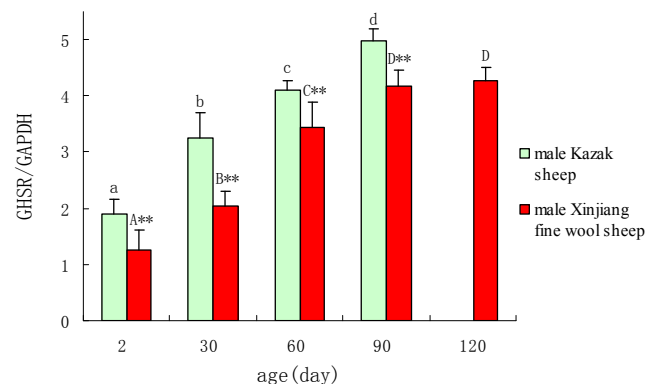


Fig. 5. The developmental changes of pituitary GHSR mRNA expression.

The difference is denoted with letters and means without a common superscript (the capital for the Xinjiang and the small for the HSK) indicate significant differences ( $P < 0.05$ ) between ages; Double-star \*\* indicates extreme differences ( $P < 0.01$ ) between breeds at the same age.

## 4. Discussion

### 4.1. Sheep growth and development during the early growth period

Jia (2003) found that from day 30 to 255, the body weight of Kazak hair sheep was extremely higher than that of the first filial generation (F1) of Romilly Hillys  $\times$  Merino of China (Xinjiang Agricultural Reclamation line) wool sheep. The present research showed that from day 30 to 90, the body weight of Kazak sheep was extremely higher than that of Xinjiang fine wool sheep, which predicated that the former grew more rapidly than the latter, and the results were similar to that of Jia's.

### 4.2. Developmental changes of GHSR mRNA expression in hypothalamus and pituitary

GHSR expression was markedly increased in hypothalamus arcuat (ARC) and ventromedial (VMN) nuclei of GH-deficient dw/dw dwarf rats, which was restrained by bovine GH treatment. There were Similarly change in GHRH expression, whereas NPY expression was decreased and increased by bovine GH treatment. The

sensitivity of ARC GHSR expression to modulation by GH suggests that GHS-Rs may be involved in feedback regulation of GH (Bennett et al., 1997). According to research of Shuto et al (2002) ,they creat transgenic (Tg) rats expressing an antisense GHSR mRNA. Tg rats had lower body weight and less adipose tissue than did control rats. The stimulatory effect of GHS treatment on feeding was abolished and daily food intake was reduced in Tg rats. The GHSR gene lies on human chromosome 3q26 within multiple phenotypes linked to a quantitative trait locus strongly related to obesity and the metabolic syndrome. Baessler et al. (2005) found that common SNPs and haplotypes within the GHSR gene region were involved in the pathogenesis of human obesity . The above results suggested that GHSR was involved in the monitorship of GH secretion, adiposity, and food intake, so studying the developmental changes of sheep GHSR mRNA expression was useful to explore sheep growth and development and the regulatory mechanisms.

The present research showed that the hypothalamus GHSR mRNAs expression level in both breeds increased firstly and then declined with growing, and that in Kazak sheep was extremely lower than that of Xinjiang fine wool sheep from day 60 to 90. From day 2 to 90, the pituitary GHSR expression level of Kazak sheep was extremely higher than that of Xinjiang fine wool sheep, which was similar to the difference of body weight between the two sheep breeds in the early growth period. The mRNAs expression level of pituitary GHSR increased with growing in both breeds, and was highly positively correlated with the cumulative growth curves, which predicated that GHSR played some role in sheep growing.

A endogenous ligand of GHSR, ghrelin, was get in rat stomach tissue (Kojima et al., 1999). Ghrelin was able to stimulate the release of growth hormone (GH) after binding to GHSR. Moreover, it was soon established that ghrelin had function in weight regulation as its administration increased food intake and caused fat and weight gain in rodents. HUANG Zhi Guo et al. (2006) studied the hypothalamus and pituitary distribution and developmental changes of ghrelin mRNA expression in sheep and found ghrelin mRNA mostly existed in abomasum and was increased steadily in the early growth period. The present research found sheep pituitary GHSR mRNAs expression level was was highly positively correlated with abomasum ghrelin mRNAs expression level (the data about ghrelin mRNAs expression level see in HUANG Zhi Guo et al., 2006).The results predicated that it may be pituitary GHSR but not hypothalamus GHSR through which ghrelin influenced sheep growing.

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