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Differential expression of luteinizing hormone receptor, androgen receptor and heat-shock protein 70 in the testis of long-distance transported mice

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ABSTRACT. Spermatogenesis, is a complex process of precisely regulated intracellular events, where it is affected by many factors. Long-distance transport of animals is one of the stressors that may influence spermatogenesis and sperm quality. The present study chose luteinizing hormone receptor (LHR), androgen receptor (AR), and heat-shock protein 70 (HSP70) as our target genes to investigate their mRNA and protein expression in the testes of long-distance transported (about 1000 km) mice. Histological analysis showed that there was a reduction in the thickness of the seminiferous epithelium in the transported mice, and a significant decrease in body weight and sperm count in the epididymis was also observed. mRNA expression was determined by QPCR in the testis of transported and control mice. The levels for AR

decreased significantly in transported mice. LHR and HSP70 expression in the testes of the transported mice was slightly higher than that of control mice but did not reach a significant level. A similar tendency of protein expression was also observed by Western blot analysis. The levels of LHR and HSP70 increased slightly after transportation. However, none of the changes were statistically significant compared with the control mice. In conclusion, long-distance transport has an adverse effect on reproductive organs and spermatozoa in adult mice.

Key words: Transport stress; Male reproduction; Androgen receptor; Luteinizing hormone receptor; Heat-shock protein 70

INTRODUCTION

With the rapid development of animal husbandry and the emergence of many new dairy farms, transportation has become an unavoidable thing. However, road transportation especially long-distance transportation as a stressor could do much harm to the dairy industry. The economic losses due to transport stress include decreased body weight (Greer et al., 2011; Gonzalez et al., 2012), decreased milk production and changes in meat quality (Honkavaara et al., 2003; Hoffman and Fisher, 2010; Smiecinska et al., 2011) and higher incidence of diseases. Significantly higher egg production of *Dicrocoelium dendriticum* was observed in the feces of transported ewes (Sotiraki et al., 1999). Transportation stress alters immune function (Riondato et al., 2008; Lv et al., 2011), which further increases the incidence of respiratory diseases (Sporer et al., 2008). Significantly decreased mean total serum antioxidant capacity and significantly increased serum malondialdehyde concentrations have been investigated, showing that they are related to episodes of bovine respiratory disease and mortality in calves (Chirase et al., 2004).

Hormonal regulation is essential to spermatogenesis, which is regulated by the hypothalamus-pituitary-testis axis, especially by follicle stimulating hormone (FSH) and luteinizing hormone (LH), which functionally connect the brain with the testis (Alves et al., 2013). Hormones exert their biological effects through binding with their receptors. The male carriers of homozygous mutation of the LHR gene display delayed puberty, micropenis, and oligospermia, so LHR mutations may be responsible for male hypogonadism with reduced spermatogenesis (Bruysters et al., 2008). Gonadotropins and androgen receptors (AR) play a role in gonadal development (Omran Nel, 2012), and FSH and testosterone are the main hormonal regulators of spermatogenesis (Sofikitis et al., 2008). Heat shock proteins (HSP) also play pivotal roles in spermatogenesis. HSP70 has been found to be present in the spermatozoa of mature bulls, and redistribution of the protein occurs during capacitation and the acrosome reaction (Kamaruddin et al., 2004). Significantly increased expression of HSP70 in infertile male may indicate that HSP70 is a protective protein against apoptosis in spermatozoa (Erata et al., 2008).

Transport stress first activates the hypothalamic-adrenal cortex and then sympathetic-adrenal-medulla (Mitchell et al., 1988) and alters the concentrations of a series of hormones, such as CRH, GnRH, TH, and ACTH. Numerous studies have shown that stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis plays an important role in inhibiting the hypothalamic-pituitary-gonadal (HPG) axis (Cevik et al., 2004; Chand and Lovejoy, 2011). Studies have suggested plasma cortisol levels and HSP70 as indicators of

the transport stress. An elevation of LHR secretion has been observed during transport stress (Bieglmayer et al., 1980). The effect of transport stress on disease incidence, immune response, meat quality and also indicators of the extent of stress has been studied by many researchers. However, to date, there is little scientific literature with regard to the effect of transport stress on male reproduction performance. To elucidate the mechanisms of transport stress on male reproduction performance and further provide evidence for reducing or eliminating the harm it does to dairy industry, LHR, AR and HSP70 were selected as candidate genes to study their expression profiles through QPCR and Western blot after long-distance transport using mice as the animal model.

MATERIAL AND METHODS

Studied animals

All procedures involving animals were approved by the Animal Care and Use Committee of Huazhong Agricultural University. A total of 30 mature male Kunming mice between 9 and 10 weeks old were obtained from the Animal Experiment Centre of Wuhan University. All mice were conventionally fed 2 weeks prior to the experiment to facilitate their adaptation to the new environment. At the beginning of the experiment, the mice were randomly divided into control group and experimental group, with 15 mice in each group. The experimental group was transported approximately 1000 km (time, 15 h) on a commercial road in November.

Samples, body, and organ weight

All animals including control group were sacrificed by cervical dislocation after one day's rest. Testis and epididymis samples were collected and weighed and organ indices were calculated according to the formula below:

$$\text{Organ index (mg/100 gwt)} = \text{organ weight (mg)/body weight (g)} \times 100$$

The testes for routine histology studies were fixed in 4% paraformaldehyde, while those for biochemical analysis were frozen in liquid nitrogen.

Sperm count

A small piece of the cauda epididymis of each animal was dissected and placed in 1 mL of 0.9% saline, and then cut it softly to make spermatozoa swim out into the medium. Sperm count was evaluated with a colorimeter (Ibersan, Portuguesa) according to manufacturer instructions.

Histopathology

Formalin-fixed, paraffin-embedded testes were serially cut into 5-µm sections. Each section was routinely stained with hematoxylin and eosin and examined by light microscopy.

RNA extraction, reverse transcription, and quantitative real-time PCR

Total RNA isolated from testes was prepared using Trizol reagent (Invitrogen, USA), and 2 μg total RNA were then used for cDNA synthesis by SuperScript reverse transcriptase (Invitrogen, USA). cDNA (1 μL) was added to 20 μL reaction mixture containing 10 μL DyNAmo SYBR Green qPCR kit (FINNZYMES, USA) and 1 μL 12.5 μM primer (forward and reverse). Quantitative real-time PCR was carried out using the LightCycler® 480 System (Roche, Basel, Switzerland) for cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min. This was continued through 40 cycles. Primer pairs for quantitative RT-PCR are shown in Table 1.

Table 1. Primer sequences and amplicon information for qRT-PCR analysis.

Genes	Primer sequence (5' to 3')	Anneal temperature (°C)	Product size (bp)
LHR	F: 5'-TAGAGAAGCGAATAACGAG-3' R: 5'-AGGAGAACAAAGAGGACTG-3'	60	231
AR	F: 5'-GGAGGTTACGCCAAAGGATT-3' R: 5'-GCCAGCGGAAAGTTGTAGTA-3'	60	170
HSP70	F: 5'-GAAGGTGCTGGACAAGTGC-3' R: 5'-GCCAGCAGAGGCCTCTAATC-3'	60	237
β-actin	F: 5'-GTATGCCTCGGTCGTACCA-3' R: 5'-CTTCTGCATCCTGTCAGCAA-3'	60	499

Western blotting

Testis tissue explants were homogenized in 10 mL lysis buffer (10% SDS in PBS containing protease inhibitors) and placed on ice for 2 h with vortexing every 10 min. Samples were centrifuged at 12000 *g* for 30 min. Total protein concentration was determined by the BCA assay (Pierce, Rockford, USA), and 50 μg total protein were subjected to gel electrophoresis. Proteins were separated on a 12% polyacrylamide gel and transferred to PVDF membranes (Millipore, Bedford, MA). Membranes were first blocked with Tris-buffered saline (TBS) containing 5% skimmed milk for 1 h and washed with TBS three times (10 min each), and the membranes were then incubated overnight at 4°C with primary antibody. Anti-HSP70, anti-LHR and anti-androgen receptor (Boster, Beijing) were used for primary antibody. After incubation with the primary antibody, membranes were washed three times with PBS containing 0.1% Tween 20, incubated for 1 h at room temperature with 3000-fold diluted HRP labeled goat anti-rabbit secondary antibodies, and washed three times with PBS containing 0.1% Tween 20 (10 min each). Signal was detected using an ECL kit (CWBIO, Beijing, China). The band intensities were measured with the AlphaEaseFC software (Alpha Innotech, USA).

Statistical analysis

The significance of differences between each group was conducted by one-way analysis of variance (ANOVA) using the SAS 9.0 software. Comparison of the mean value of the transport group with that of control group was carried out using the Duncan test for multiple comparisons.

RESULTS

Body and organ weight, sperm count

The body and organ weights of the sacrificed mice were examined (Table 2). There was a statistically significant reduction in average body weight in the transport group. Considering organ weights, there were no significant differences between the two groups, but the epididymis index increased significantly in the transport group. Considering the concentration of sperm in the epididymis, a significantly decreased sperm concentration was observed with transport.

Table 2. Effect of transportation stress on body weight, organ weight organ index, and sperm count.

	N	Body weight	Testis weight	Epididymis weight	Testis index	Epididymis index	Sperm count
Control	9	34.34 ± 3.14	141.01 ± 316.80	40.978 ± 4.86	411.02 ± 88.27	119.46 ± 10.57	17.75 ± 3.96
Stress	9	31.50 ± 1.63*	140.11 ± 19.78	40.93 ± 4.60	445.04 ± 61.04	130.01 ± 13.26*	10.58 ± 4.27*

Data reported are means ± SD.

Histology of testes

Histological analysis showed that a reduction in seminiferous epithelium thickness in the transported mice (Figure 1).

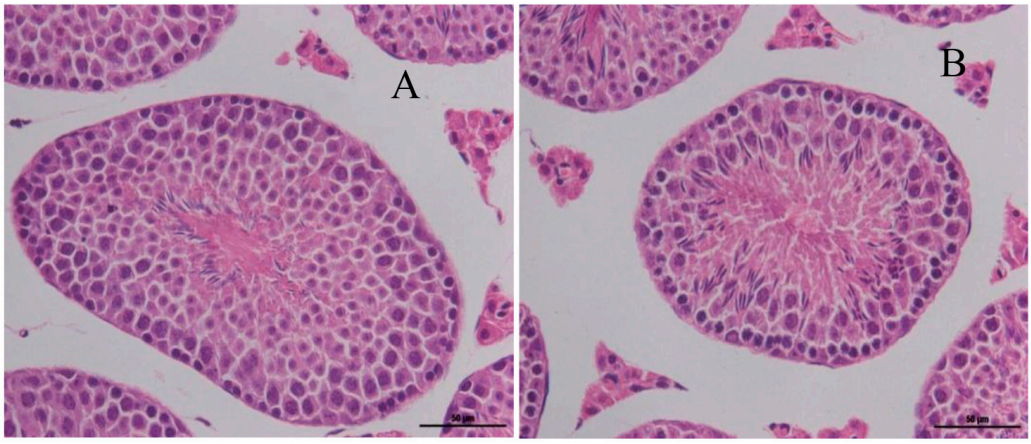

Figure 1. Representative image of histological sections of the seminiferous tubules in adult male mice. **A.** control group with no treatment; **B.** transportation group.

Expression of LHR, AR, and HSP70 in the testes of mice

mRNA and protein expression levels of LHR, AR, and HSP70, normalized to β-actin, in the testes of transported mice and control mice are shown in Figures 2 and 3.

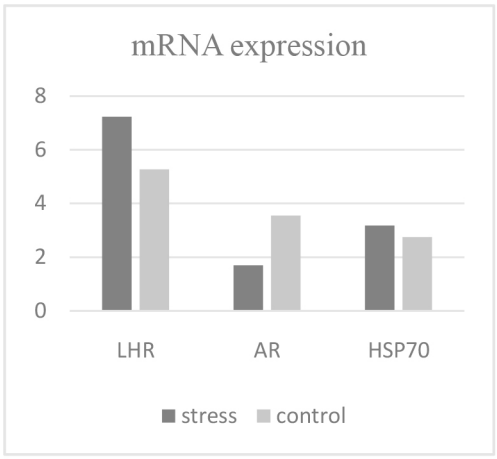

Figure 2. Expression profiles of LHR, AR, and HSP70 in mice subjected to transportation stress. Gene expression changes were measured by qRT-PCR.

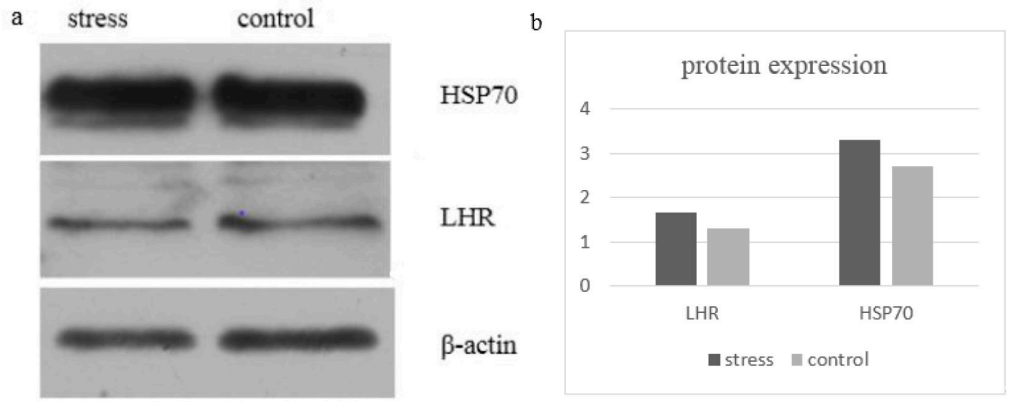

Figure 3. Western blot analysis (**a**) and protein expression levels of HSP70 and LHR in the testes of transported mice and control mice. (**b**) Comparison of HSP70 and LHR relative expression between the transported and the control group (means ± SD). The relative protein content was the ratio of the gray scale of the target protein band *vs* the gray scale of the β-actin band.

The mRNA expression levels of LHR and HSP70 in the testes of the transported mice were slightly higher than that of control mice ($P > 0.05$); however, they did not reach significant levels (Figure 2). Expression levels of AR decreased significantly after transport (Figure 2).

The levels of protein expression of LHR and HSP70 in the testes of the transported mice displayed a similar tendency as those of mRNA expression (Figure 3). The levels of LHR and HSP70 in the testes of the transported mice increased slightly after transportation. However, none of the changes were statistically significant compared with the control mice.

DISCUSSION

Spermatogenesis, as a complex of precisely regulated intracellular events, is affected by many factors, which include genetics and environment. A significant lower total sperm count was found in motorway tollgate workers compared with normal control (Calogero et al., 2011). Transport, especially long road transport, will cause serious stress to animals. The result of our present study also showed a significant reduction in sperm count in transported mice, the conditions of which are similar to those of motorway tollgate workers. Genetics is also an important factor that influences spermatogenesis and sperm quality. In another of our studies, we found that different genotypes of LHR had a significant effect on sperm density and fresh sperm motility, where the SNP site was located in an intron (Sun et al., 2012).

Plasma cortisol concentrations had a negative correlation with plasma dehydroepiandrosterone ($r = -0.55$, $P = 0.001$) and testosterone ($r = -0.43$, $P = 0.02$), while positively correlating with progesterone ($r = 0.59$, $P = 0.0007$) during transportation stress in beef bulls (Sporer et al., 2008). Many researchers have considered plasma cortisol as an indicator of stress, and have found a significant increase in plasma cortisol concentration (Tischner and Niezgoda, 2000; Leche et al., 2013). In our study, a significant reduction of AR expression was found in transported mice, and this may indicate that Sertoli cells function changed greatly in the testis, but the complex mechanism is still unknown. One reason for this maybe the reduction in androgens controlling the expression of AR at the transcriptional level, posttranscriptional level and protein level (Lee and Chang, 2003). Abnormalities in the androgen-AR signaling pathway further affect the proper function of male reproductive system, as in male infertility. A dramatic decrease in AR expression was also found after heat treatment in another study (Chen et al., 2008), the result of which is consistent with our research. The lack of AR in Sertoli cells or Leydig cells would result in the arrest of spermatogenesis; however, the deletion of AR gene in mouse germ cells does not affect spermatogenesis and male fertility (Wang et al., 2009).

LHR expression in our present study was slightly higher in the testes of transported mice compared with control mice. The results of our present study did not conflict with our previous research, because many researchers have confirmed that the SNPs in introns can affect gene expression, phenotype and consequently function (Spotter et al., 2010; Stinckens et al., 2010). HSP70 may play an important role in spermatogenesis and sperm maturation (Cao et al., 2009). In our present study, a slight increase in HSP70 in transported mice was observed. The increased HSP70 expression may be a protective mechanism against apoptosis in spermatozoa (Erata et al., 2008).

In summary, the results of our present study indicated that long road transport had an adverse effect on reproductive organs and spermatozoa in adult males.

Conflicts of interest

The authors declare no conflict of interest.

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