VIA MEDICA

ORIGINAL ARTICLE

Folia Morphol.
Vol. 80, No. 4, pp. 857–869
DOI: 10.5603/FM.a2020.0126

ISSN 0015–5659
eISSN 1644–3284
journals.viamedica.pl

Heat shock protein 60 expression and localisation in different tissues and testis development of male cattle (cattle-yak and yak)

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[Received: 5 September 2019; Accepted: 11 November 2019; Early publication date: 15 October 2020]

Background: *Heat shock protein 60 (Hsp60) play important roles in protecting testicular development and production of sperms. This study was conducted to investigate Hsp60 gene expression and localisation in testicular development to ascertain its influence on infertility and in different tissues of the male cattle-yak and yak. A total of 54 cattle (24 cattle-yak and 30 yak) were examined.*

Materials and methods: *Heat shock protein 60 mRNA of cattle-yak was cloned first and amino acid variations were found leading to differences at protein spatial structure compare with the yak. Real-time quantitative polymerase chain reaction analysis revealed that Hsp60 mRNAs expression were different in cattle-yak and yak.*

Results: *The results showed disparity in Hsp60 expression among different tissues and in different developmental stages of the testis. High Hsp60 expression was observed in juvenile and adult testicles. Moreover, Hsp60 expression in cattle-yak was significantly higher than yak ($p < 0.01$). The location of Hsp60 in tissue and testis was detected by immunohistochemistry and immunofluorescence. The results demonstrated that Hsp60 proteins located in epithelial cells, spermatocytes, sperm cells and mesenchymal cells.*

Conclusions: *The Hsp60 proteins are expressed in different tissues, and the highest expression level was observed in the testis of the cattle-yak, which suggests that infertility of cattle-yak have some correlation with up-regulation of Hsp60.* (Folia Morphol 2021; 80, 4: 857–869)

Key words: yak, tissue, Hsp60, expression, localisation

INTRODUCTION

Cattle-yak (*Bos* cattle-yak) and yak (*Bos grunniens*) are important animals for local herdsmen of the Qinghai-Tibet Plateau in China. Cattle-yak as a hybrid, exhibits obvious heterosis of yak and cattle, such as tallness, robustness, fast growth speed, drought tolerance, and disease resistance [39]. Different organs play different roles in the metabolism of the body [3]. The testis is one most important male reproductive organs, and the heat shock protein 60 (Hsp60) location of its cell proliferation and development have significant importance [24]. Although infertility is a worldwide problem in male yak-cattle, the studies on causal mechanism are lacking.

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Recent studies have shown that Hsp60 could protect spleen lymphocytes cells from damage, organ failure, spleen inflammatory diseases, and body shock [42]. Hsp60 not only participates directly or indirectly in endothelial dysfunction, atherosclerosis, cardiovascular diseases, and growth and development but also protects reproductive cell activity and regulates protein metabolism of organisms [2]. Previous studies have demonstrated that Hsp60 up-regulates in monocytic cells of the immune system, and tumour cells in stomach cancer, colon cancer, liver cancer, breast cancer, and lung cancer [17, 19]. Moreover, co-culturing of human myoblasts revealed that molecular patterns of Hsp60 induced interleukin-10 to protect THP-1 cells [38]. The level of expression of HSPs throughout the period of heat stress was highest in buffaloes, followed by bos indicus and taurus [18]. In addition, the HSPA2 expression levels significantly differed in the various tissues of yak, especially in the testes and breast, followed by the brain, kidney, heart, lung, and liver, and its weakest expression was found in the spleen [32]. In relation to these backdrops, this study was performed to investigate whether Hsp60 was differentially expressed in the various tissues of cattle-yak and yak.

The testes are most important male reproductive organs. When testicular tissue was exasperated by metals such as arsenic, copper and cadmium or a chemical (DEHP), mRNA and protein levels of Hsp60, Hsp70, and Hsp90 were up-regulated [8, 34]. Meanwhile, Hsp40, Hsp60, and Hsp70 expression increased respectively, when mice testis was buoyed up by high temperature [44]. Many factors are responsible for the infertility of male cattle-yak. These factors include testicular dysplasia, hindered spermatogenesis, metamorphosis phase, and sustentacular cells assisting the germ cell differentiation in the seminiferous tubule [23]. Thus, studies on the fertility of male cattle-yak and male yak have focused on distinguishing histologic structural development, the effects of genes expression, and the intensity of protein involved in the production and control of sperms [32].

Previous studies have shown that the cattle-yak infertility not only reflects histologically, but also manifestations of differential expression of related genes and proteins generated by sperms [33]. As creatures have low content of eight differential proteins containing triosephosphate isomerase, glycerol kinase, and HSP [36]. The Hsp60 high expression is used as a marker for semen quality, sperm concentration and functional capacity [7, 25]. In the sperm of human beings, the expression of HSPs in sperm cells are significantly higher than that of the primary spermatocyte, and diverse HSP chaperones are accessible for surface labelling on human sperm [27]. HSPs (Hsp60, Hsp70, Hsp90) are highly expressed in different physical, mechanical, and chemical stresses, an indication that these proteins might function in various processes such as mitochondrial function, gamete interaction, and receptor activity regulation [14, 35]. Research has shown that Hsp10 and Hsp60 were inducible and functional in testis development and migration process of Coilia nasus, suggesting their essential roles in these processes. The results also indicated that Hsp60 may be one indicator of properly working mitochondrial import and refolding in the fish testis [9]. However, the correlation between male sterility and Hsp60 of yak and cattle-yak is still unclear. Therefore, we determined the relationship between Hsp60, tissue specificities of cattle, and the regulation of testicular development and sperm production.

MATERIALS AND METHODS

Experimental animals

Cattle were sampled from a pasture in the Tibetan plateau in Qinghai, China [11]. A total of 54 healthy cattle (24 cattle-yak and 30 yaks) were used in this study (Table 1), All cattle were kept under the same natural conditions (altitude, approximately 2,300 m a.s.l.; temperature, 2∼5°C; and oxygen content, 14.97%) [39]. Male cattle-yak without feeding value from herders were excluded, and no samples were taken from the senile group.

The cattle with conventional disease were considered healthy individuals. All experiments were performed in accordance with relevant guidelines and regulations. The euthanasia procedure was carried by atropine plus diazepam given intramuscularly as premedication for narcosis, and overdose intravenous injection of sodium pentobarbital sodium with IV administration when study was finished (Nembutal, l50 mg/kg; Abbot Labs, USA). The study was approved by the Animal Ethics Committee of Gansu Agricultural University, China (SYXK [Gan] 2015-0001).

Preparation of mRNA and proteins

Tissue samples were selected from 21 healthy cattle. Tissues were dissected, collected in frozen

Table 1. Specimens examined

Number of cattle-yaks and yak	Group	Age	Source	Application
6 + 6	Newborn	1~7 days	Xi'ning City Qinghai Province	Molecular biology Measurement
6 + 6	Calf	6~8 months		
6 + 6	Juvenile	1~1.5 years		
6 + 6	Adult	3~4 years		
0 + 6	Senile	8~10 years		

Table 2. Primer sequences of target and house-keeping genes

Primer name	Sequence (5′→3′)	Tm (°C)	Note
Hsp60-1R	CGGAGTCGGGCGATTGT	59.5	RT-PCR
Hsp60-1F	CTCCAAATACTGCACCCCCA	60.6	1016bp
Hsp60-2R	TCTCATCTGGTGATAGCAAG	61.7	RT-PCR
Hsp60-2F	CCTTGTTTTGAATAGGTTGA	60.2	1293bp
β-actin-R	GCTCGGCTGTGGTGGTAAA	59.0	RT-qPCR
β-actin-F	AGGCTGTGCTGTCCCTGTATG	59.0	207bp
Hsp60-R	GCCAAAGGGAAGGACAGT	54.3	RT-qPCR
Hsp60-F	ATGACCGTGCGAGATAACG	54.6	204bp

liquid nitrogen, and then stored at −80°C before use. The RNA from the cattle tissues were isolated using a TRIzol kit (R1100, USA), and reverse-transcription polymerase chain reaction (RT-PCR) was performed to clone the cDNA.

Frozen samples were ground in liquid nitrogen and transferred to centrifuge tubes with RIPA/PMSF (Solarbio, China). After thoroughly blending and mixing, a pink colour was obtained. The sample tubes were incubated on a spiral oscillator for 2 h (200 r/h) on ice and centrifuged at 4°C for 10 min (12,000 r/h) to obtain the complete divided protein. The total protein concentration for each sample was measured. The total protein concentration was then adjusted to the same level, and 4× sample buffer at 100°C was added for 12 min to completely denature the proteins.

Molecular cloning of Hsp60

The degenerate primers used to amplify the Hsp60 sequence were based on the published partial sequence of Hsp60 mRNA in the National Centre for Biotechnology Information (NCBI) database (Accession No. NM-001012670.2). Primers for cloning the initial fragment of Hsp60 mRNA were designed according to the predicted conserved sequences in other *Bos* cattle-yaks (Table 2). The amplified segments were inserted into the cloning vector pMD-18T and were transfected into *Escherichia coli* JM109 competent cells. The primers for 5′ Hsp60 and 3′ Hsp60 were designed using the sequencing data. Then, the segments from 5′ to 3′ Hsp60 from the first-strand cDNA were used for cloning and sequencing.

Analysis of Hsp60 protein

The open reading frames (ORF) in the complete mRNA sequence of HSP90 were identified using an ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/orfig.cgi), and the nucleotide sequences were translated into amino acids using the Vector NTI 11 software [12]. The Hsp60 sequence was analysed using the ODC Hsp60 finder software, and the codon region and frameshifting site were identified [10].

Homology searches were performed using BLASTn and BLASTp in NCBI. The conserved domain. Search service was used to identify the conserved domains in the predicted protein sequences (http://www.ncbi.nlm.nih, https://web.expasy.org/protparam). The three-dimensional structure of the predicted protein was envisaged according to the methods described in the website (http://bioinf.cs.ucl.ac.uk/psipred/). The deduced amino acid sequence of HSP90 was aligned using the course line computer (CLC) main workbench software (http://www.clcbio.com) with known homologous proteins of the Hsp60 class obtained from the GenBank. A phylogenetic tree was constructed using the CLC main workbench software through the neighbour-joining method for the amino acid sequences of Hsp60 from the SwissProt databank/GenBank.

Expression of Hsp60 gene in different tissues

The distribution of Hsp60 in the tissues of cattle-yak and yaks were detected during the development of testicles and in the non-reproductive system (kidney, heart, cerebellum, liver, lung, and spleen). The expression levels of Hsp60 in the different tissues were detected using quantitative real-time PCR (Invitrogen, USA) with Hsp60 specific primers. Actin was used as a reference gene to normalise the amount and quality of each cDNA, because this gene is expressed constitutively in the different tissues [13].

Immunofluorescence and immunohistochemical assays

The different tissues of the cattle-yak and yak were fixed in 4% paraformaldehyde solution at room

temperature for hebdomad. Tissues were clipped and paraffin-embedded, and the sections were sliced (4 μm), dried, and stored.

Samples were dewaxed using dimethyl benzene and then dehydrated with an increasing alcohol gradient for immunohistochemical staining to investigate the Hsp60 expression levels. The endogenous peroxidase was eliminated with 3% deionised H_2O_2 (18––22 min), and the sections were rehydrated and sealed with goat serum (18–22 min). After overnight incubation at 4°C with primary rabbit anti-Hsp60 monoclonal antibodies (1:2500, Abcam, Hong Kong), the sections were then incubated with the secondary antibody. Then, the labelled samples were counterstained with 3,3′-diaminobenzidine, and the nuclei were observed [11]. Two antibodies showed different colours in immunofluorescence (BX53MTRF-S, OLYMPUS, Japan), and re-dyeing the nuclei was not necessary.

Measurement and statistical analyses

The intensity of immunofluorescence and immunohistochemical assays were measured using integrated optical density and Image-Pro plus 6.0. Data were analysed using SPSS 21.0. Spearman correlation of coefficients was analysed between β-actin and sample protein levels. Other data were analysed by one-way ANOVA and Duncan's post hoc test. P-values less than 0.05 between groups were considered statistically significant [12].

RESULTS

Analysis of physical and chemical properties of Hsp60

The cDNA sequence of Hsp60 was cloned and submitted to GenBank with accession number KF690729.

The nucleotide sequence and predicted amino acid sequence of Hsp60 were presented. Hsp60 nucleic acids were 2572 bp long. Results from the analysis of the contig showed that the predicted Hsp60 cDNA of 2572 bp contained an ORF of 1604 bp from 479 bp to 2083 bp. Analysis of the Hsp60 cDNA sequence in our study confirmed this conclusion. RT-PCR results show successful isolation of a cDNA fragment of 1604 bp from the cattle-yak total RNA of the heart. Finally, this confirmed cDNA sequence was deposited in the GenBank under accession number KF690729. To obtain the genomic DNA of Hsp60, the publicly available cow genome database at the NCBI Bovine Genome Resources (http://www.ncbi.nlm.nih.gov/projects/genome/guide/cow/) was screened using the Hsp60 cDNA sequence as a query. A cow (*Bos taurus*) contig (GenBank Accession No. NM_001166608.1) which encompasses the entire Hsp60 gene was identified by BLASTGen analysis.

The basic physical and chemical properties of Hsp60 were analysed. The atomic number of the protein coding region was 26828. Hsp60 has a molecular formula of $C_{7704}H_{12838}N_{2572}O_{3228}S_{486}$, molecular weight of 56633.35 Da, and theoretical isoelectric point of 5.091. Hsp60 has a half-life of approximately 30 h, instability index of 34.82, fat soluble index of 29.70, and average hydrophobicity index of 0.725.

A molecular phylogenetic tree was constructed to analyse the evolutionary relationship of Hsp60 nucleotide sequences (Fig. 1). Analysis of the gene family tree showed that the cattle-yak HSP90 evolutionarily shared a higher sequence identity with *B. grunniens*, *Ovis aries*, *Bos taurus*, *Orcinus orca*, *Sus scrofa*, *Canis familiaris*, *Ceratotherium simum*, *Homo sapiens*, *Pan troglodytes.*

Analysis of structure specificity with proteins of Hsp60

The predicted amino acid sequence of Hsp60 was aligned with known *B. grunniens* sequences through BLASTp. The cattle-yak Hsp60 protein sequence shared a low percentage of similarity to other known Hsp60 protein sequences. This result indicated that the Hsp60 proteins were different from other members in the HSP family. The amino acid sequence of Hsp60 was approximately 99.87%, 98.54%, 97.39%, 95.62%, and 83.75% identical to those of HSPs from *B. grunniens*, *Ovis aries*, *B. taurus*, *Orcinus orca*, *Sus scrofa*, *Canis lupus.* The highest level of similarity appeared near the C-terminal, while the very low similarity was found in the N-terminal and in the middle of the amino acid sequence.

The cattle-yak Hsp60 protein sequence showed that mutational nucleotide caused the change of amino acids, such as the G into V, T into N, A into E, G into P and so on (Fig. 2). The most important result was the change in the protein spatial structure and differences in protein function, such as the H-key number (390), spiral number (25), link number (22) and corner number (54). In addition, compared with yak, the initiating terminal and terminal of the cattle-yak are longer.

Expression and distribution of Hsp60 in different tissue

The Hsp60 mRNA in tissues of cattle-yak and yak were investigated through RT-qPCR (Fig. 3-I), with the

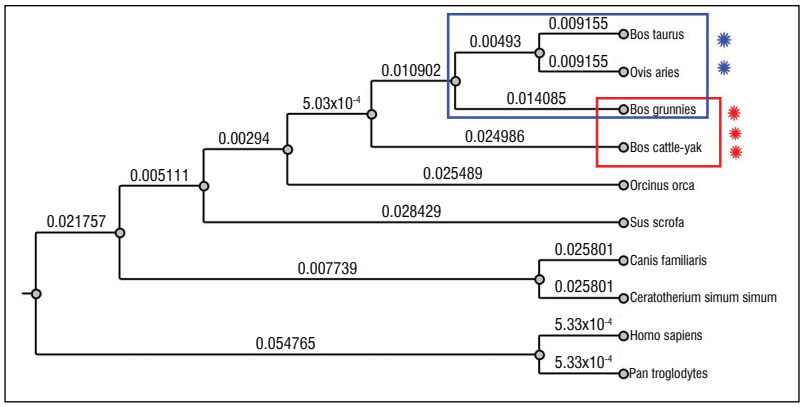

Figure 1. The heat shock protein 60 (Hsp60) phylogenetic tree of cattle-yak. The phylogenetic tree was constructed with course line computer main workbench software using the neighbour-joining method for the amino acid sequences of Hsp60 from the Swiss-Prot databank/GenBank.

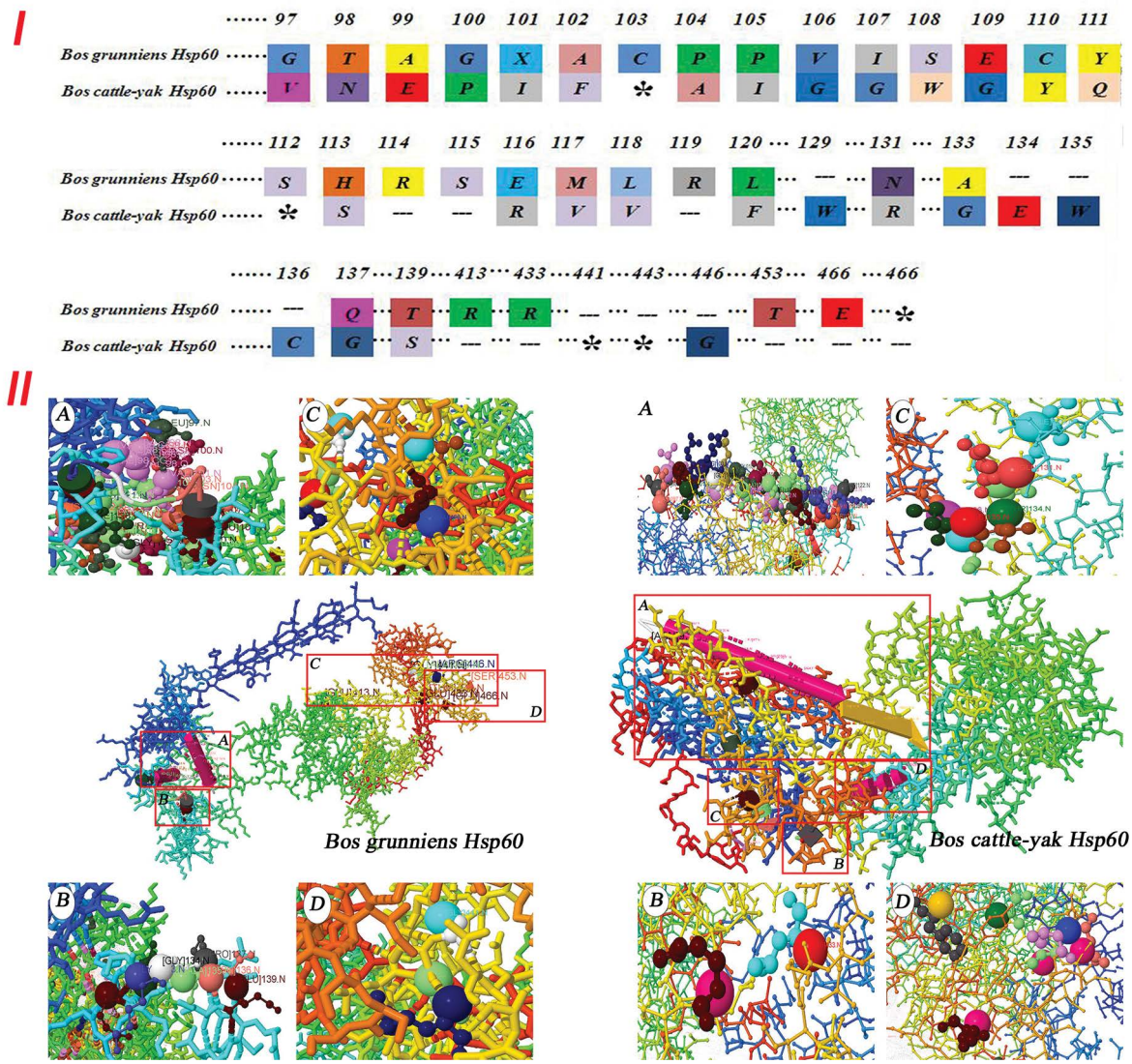

Figure 2. Analysis of heat shock protein 60 (Hsp60) protein structure of cattle-yak and yak; **I.** The Hsp60 amino acid sequence analysis that the sites and types of mutation in cattle-yak and yak; **II.** The Hsp60 protein structure of yak; **A.** The initiating terminal of the yak is mutation. as follows: 97~120 (G, T, A, G, X, A, C, P, P, V, I, S, E, C, Y, S, H, R, S, E, M, L, R, L); **B, C, D.** Mutation site and amino acids are as follows: 131(N), 133(A), 137(Q), 139(T), 413(R), 433(R), 453(T), 466(E); **III.** The Hsp60 protein structure of cattle-yak. 97~120 (V, N, E, P, I, F, *, A, I, G, G, W, G, Y, Q, *, S, R, V, V, F); **B, C, D.** Mutation site and amino acids are as follows: 129(W), 131(R), 133(G), 134(E), 135(W), 136(C), 137(G), 139(S), 441(*), 433(*), 446(G); Asterisk (*) is the unknown amino acids.

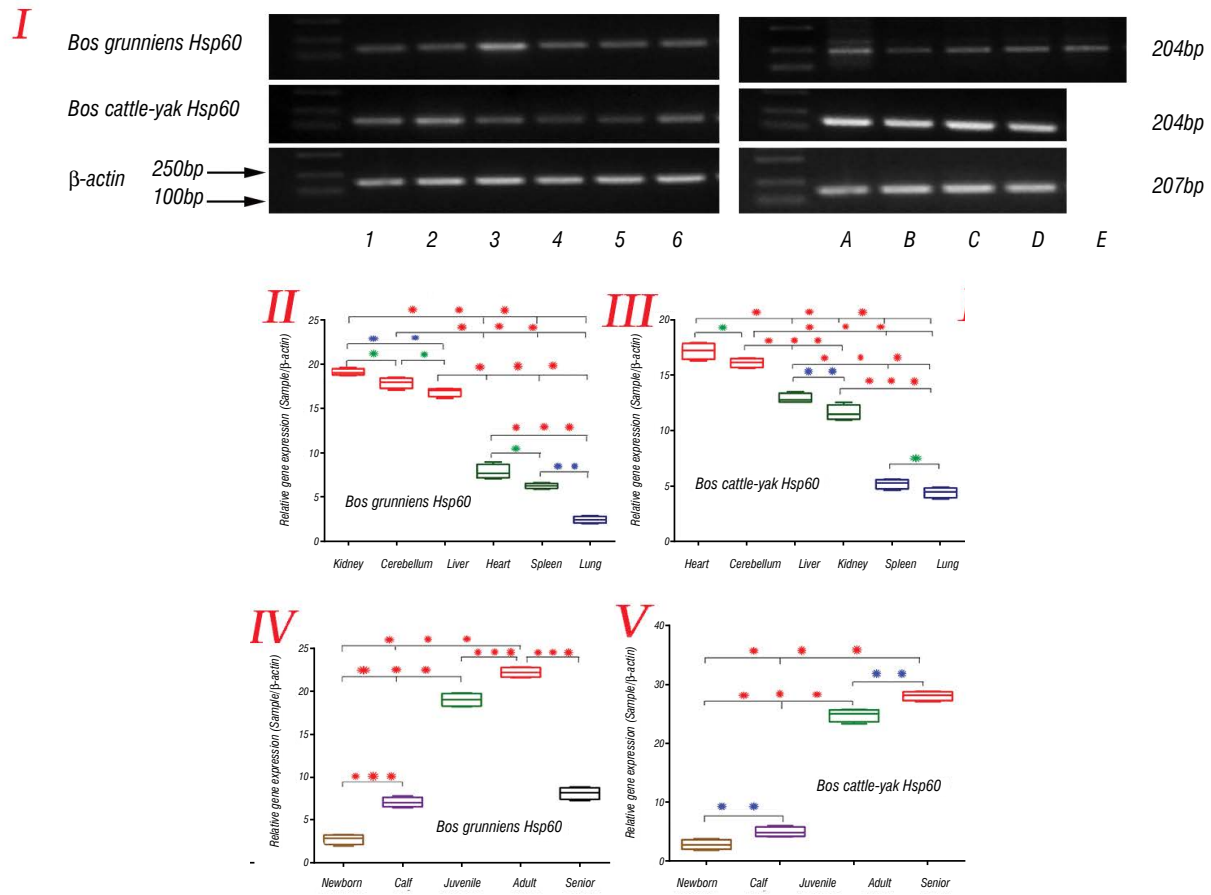

Figure 3. The heat shock protein 60 (Hsp60) gene expression in different tissues of cattle-yak and yak; **I.** The results of real time-polymerase chain reaction; 1 — kidney; 2 — cerebellum; 3 — liver; 4 — heart; 5 — spleen; 6 — lung; A — newborn; B — calf; C — juvenile; D — adult; E — senile; **II, III.** The gene expression levels of Hsp60 in different tissue of male yak and cattle-yak; **IV, V.** The gene expression levels of Hsp60 in testicular tissue of yak and cattle-yak. The columnar represent the expression trend of Hsp60 protein in different organizations and testis development stages. Different colours represent different expressions of Hsp60 in the organizations and testicular tissue of cattle-yak and yak. The asterisk represents the difference in positive expression. The more asterisks, the more significant the difference.

total RNA isolated from cattle-yak and yak tissues as templates. As shown in Fig. 3-II and 3-III, 6 tissues in cattle-yak and yak were examined. The expression of Hsp60 was widely distributed in all tissues. According to our data, Hsp60 expression was reduced in yak, with a trend from kidney, cerebellum, liver, heart, and spleen to the lungs. But Hsp60 expression increased in the cattle-yak, with a trend from heart, cerebellum, liver, kidney, and spleen to the lungs. There was significant difference about Hsp60 in these tissues, but interestingly the Hsp60 expression levels in yak were consistently higher than cattle-yak in almost all the tested tissues ($p < 0.01$). Although the expression of Hsp60 was higher in cerebellum and liver in yak and cattle-yak, the expression pattern in different tissues and levels were irregular. Taken together our data suggest that Hsp60 expression in yak was consistently higher than cattle-yak in all the tested tissues ($p < 0.01$).

The Hsp60 was mainly observed in the kidney tubules, cardiac muscle cells, hepatocytes, Purkinje cells, red pulp, and cerebellar medulla (Fig. 4-I, 4-II and Fig. 5-I, 5-II). Position analysis indicated that Hsp60 protein was mainly concentrated in the connective tissue and epithelia. Thus, the proteins were mainly concentrated in the cell membrane and in the cytoplasm but not in the nucleus. Furthermore, Hsp60 protein expression was reduced in the yak, with a trend from kidney, cerebellum, liver, heart, and spleen to the lungs ($p < 0.01$), except in the kidney. But Hsp60 protein expression was same as gene expression of cattle-yak (Fig. 4-III and Fig. 5-III).

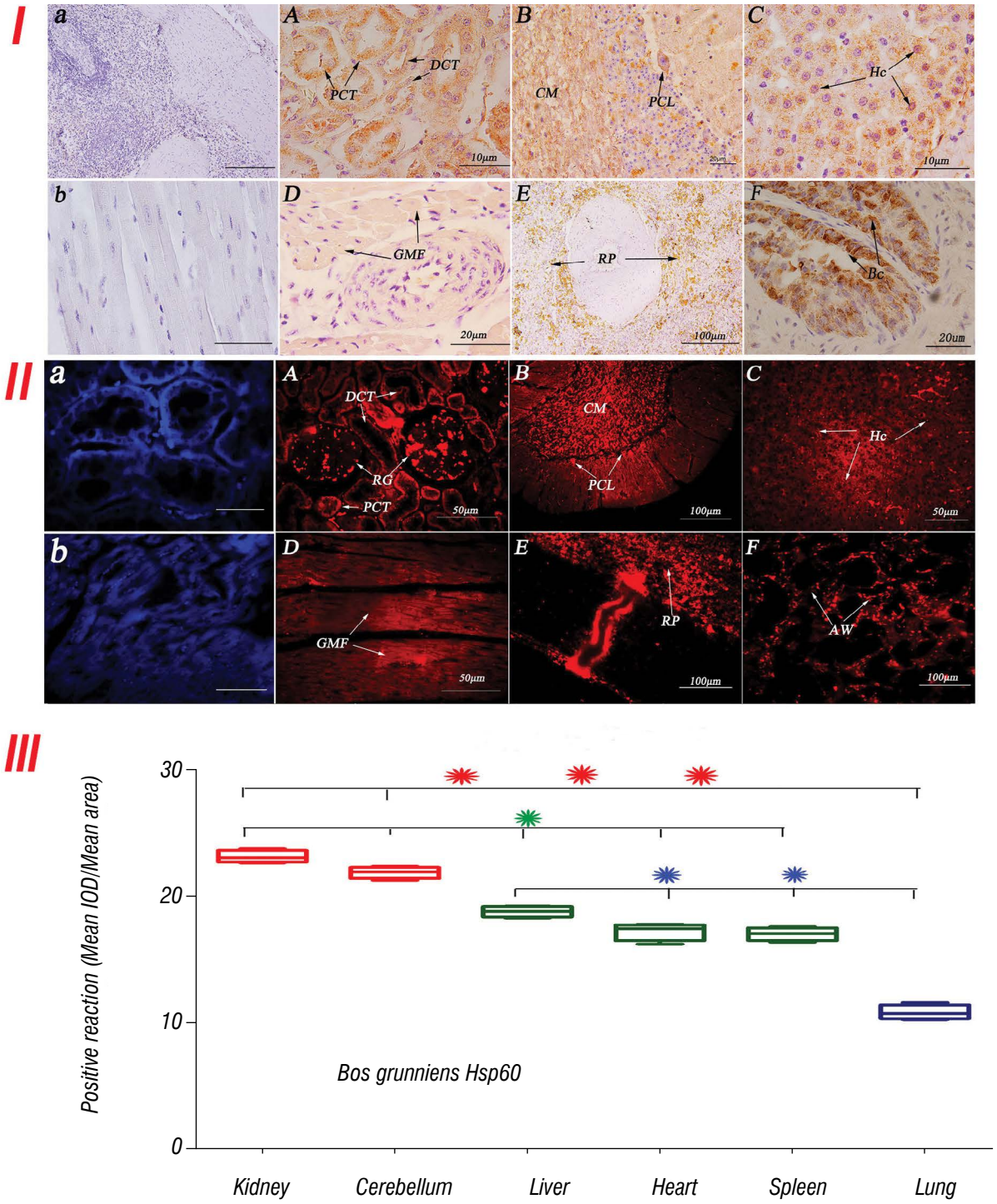

Figure 4. Immunohistochemistry and immunofluorescence localisation of heat shock protein 60 (Hsp60) in the different tissue of yak; **I, II: a, b.** The control sections collected from the different tissue of yak, without immunoreactions (negative control); **A.** Positive staining for Hsp60 was observed in the distal convoluted tubule and proximal convoluted tubule of kidney; **B.** Positive staining for Hsp60 was observed in the cerebellar medulla, granular layer and Purkinje cell layer of cerebellum; **C.** Positive staining for Hsp60 was observed in the hepatocytes of liver; **D.** Positive staining for Hsp60 was observed in the cardiac muscle fibres of heart; **E.** Positive staining for Hsp60 was observed in the red pulp of spleen; **F.** Positive staining for Hsp60 was observed in the terminal bronchioles of the lungs; **III.** The result of optical density analysis value. The columnar represent the expression trend of Hsp60 protein in different organizations; TB — terminal bronchiole; Hc — hepatocyte; CV — central vein; CMF — cardiac muscle fibres; DCT — distal convoluted tubule; PCL — Purkinje cell layer; RG — renal glomerulus; Bf — biofilm;Tc — trabecula; RP — red pulp; WP — white pulp; CM — cerebellar medulla; ML — molecular layer; GL — granular layer; PCT — proximal convoluted tubule; IOD — integral optical density. The main mode of positive expression was measured by image software. The asterisk represents the difference in positive expression. The more asterisks, the more significant the difference.

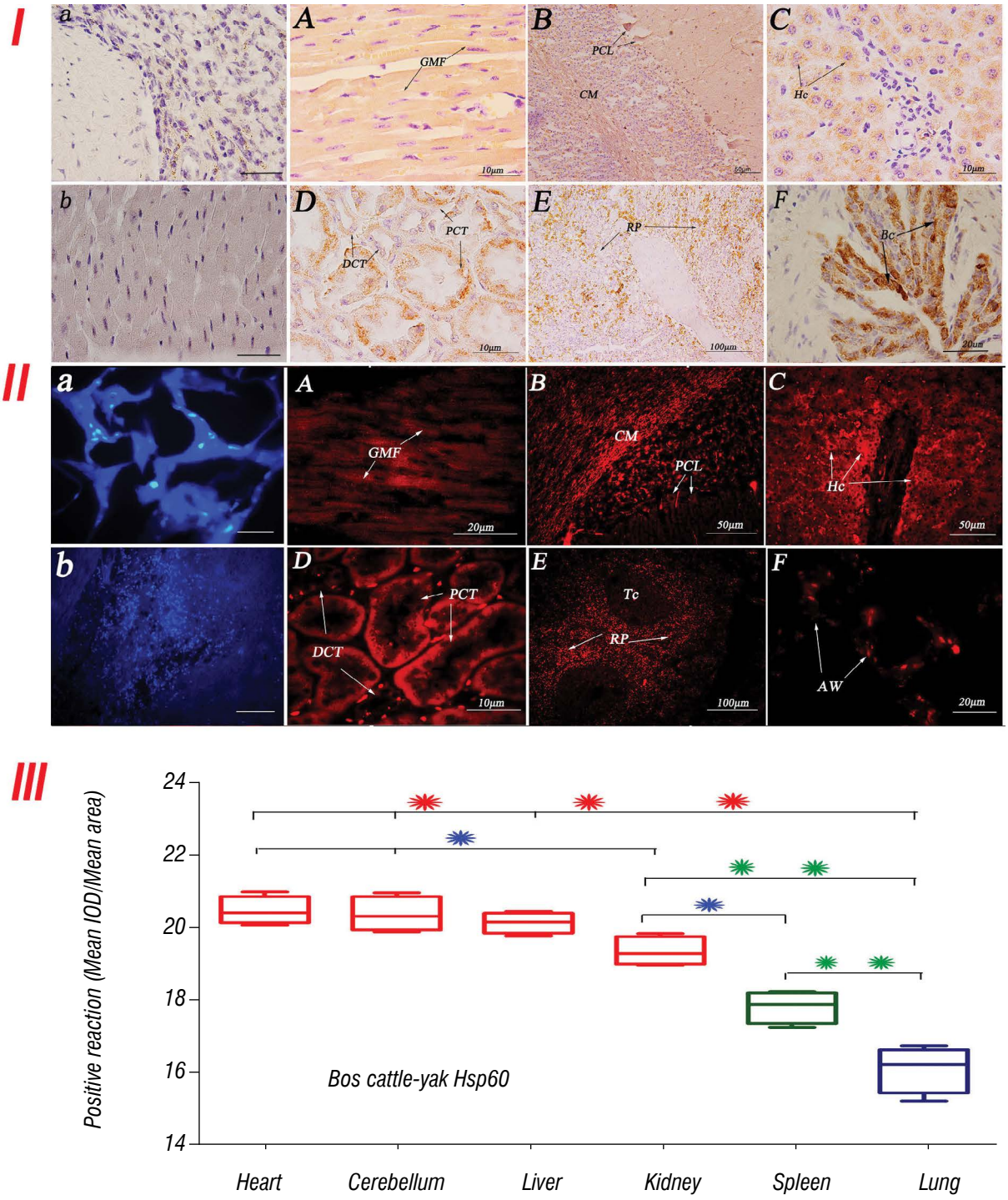

Figure 5. Immunohistochemistry and immunofluorescence localisation of heat shock protein 60 (Hsp60) in the different tissue of cattle-yak; **I, II: a, b.** The control sections collected from the different tissue of cattle-yak, without immunoreactions (negative control); **A.** Positive staining for Hsp60 was observed in the cardiac muscle fibres of heart; **B.** Positive staining for Hsp60 was observed in the cerebellar medulla, granular layer and Purkinje cell layer of cerebellum; **C.** Positive staining for Hsp60 was observed in the hepatocytes of liver; **D.** Positive staining for Hsp60 was observed in the distal convoluted tubule and proximal convoluted tubule of kidney; **E.** Positive staining for Hsp60 was observed in the red pulp of spleen; **F.** Positive staining for Hsp60 was observed in the terminal bronchioles of the lungs; **III.** The result of optical density analysis value. The columnar represent the expression trend of Hsp60 protein in different organizations; TB — terminal bronchiole; Hc — hepatocyte; CV — central vein; CMF — cardiac muscle fibres; DCT — distal convoluted tubule; PCL — Purkinje cell layer; RG — renal glomerulus; Bf — biofilm;Tc — trabecula; RP — red pulp; WP — white pulp; CM — cerebellar medulla; ML — molecular layer; GL — granular layer; PCT — proximal convoluted tubule; IOD — integral optical density. The main mode of positive expression was measured by image software. The asterisk represents the difference in positive expression. The more asterisks, the more significant the difference.

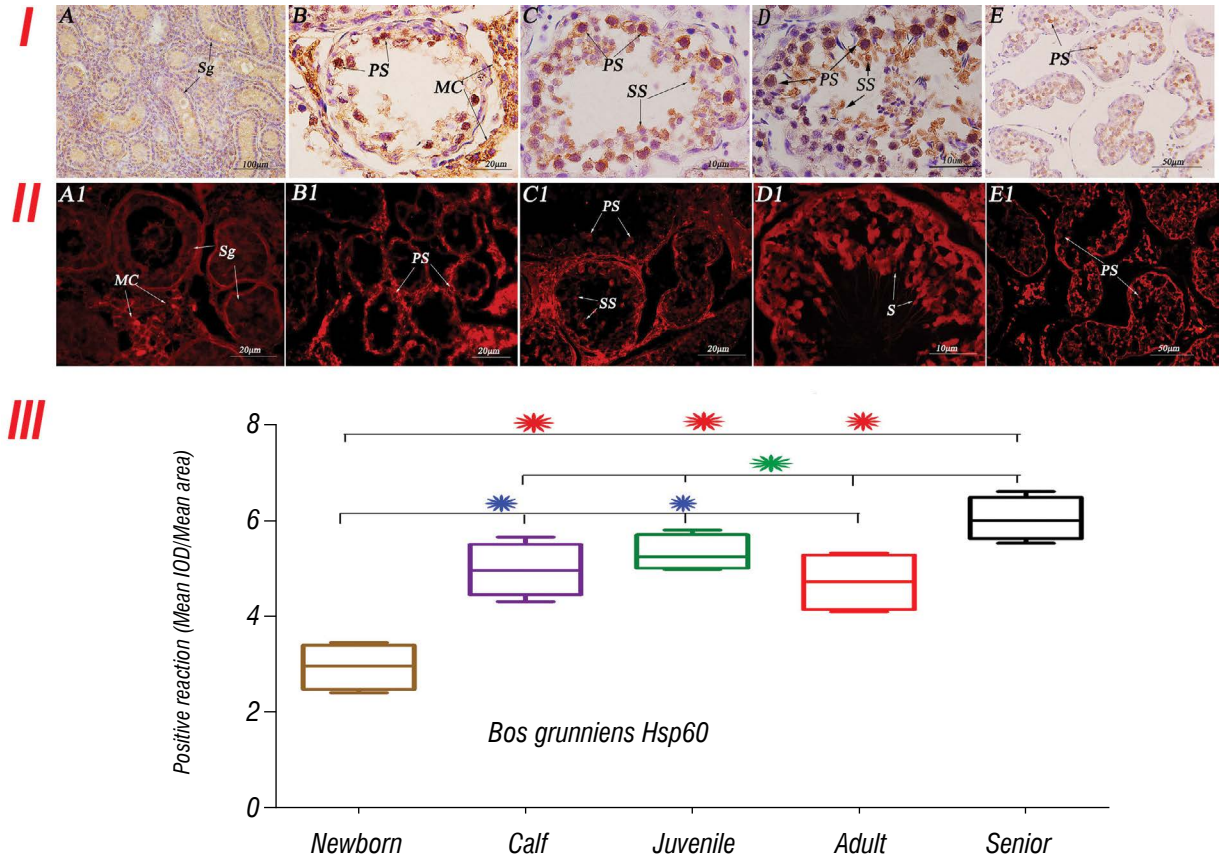

Figure 6. Immunohistochemistry and immunofluorescence localisation of heat shock protein 60 (Hsp60) in the testicular tissue of yak; **I, II: A, A1.** Positive staining for Hsp60 was observed in the spermatogonium of the newborn testis; **B, B1.** Positive staining for Hsp60 was observed in mesenchymal, primary spermatocyte of the calf testis; **C, C1.** Positive staining for Hsp60 was observed in the primary spermatocyte and secondary spermatocyte of the juvenile testis; **D, D1.** Positive staining for Hsp60 was observed in the primary spermatocyte and spermoblast of adult testis; **E, E1.** Positive staining for Hsp60 was observed in the primary spermatocyte of senior testis; **III.** The result of optical density analysis value. The columnar represent the expression trend of Hsp60 protein in different age of testis tissues; PS — primary spermatocyte; SS — secondary spermatocyte; Sg — spermatogonium; Sb — spermoblast; S — sperm; SC — Sertoli cells; MC — mesenchymal cells; MC — myoid cells; ST — seminiferous tubule; IOD — integral optical density. The main mode of positive expression was measured by image software. The asterisk represents the difference in positive expression. The more asterisks, the more significant the difference.

Expression and distribution of Hsp60 at the different developmental stages of the testis

In addition to founding the differences in Hsp60 expression between cattle-yak and yak in the 6 tissues, the testicles were further examined. As shown in Figure 3-IV, Hsp60 gene expression levels showed significant differences in the cattle-yak and yak testicular tissues at different developmental stages ($p < 0.01$). In the yak, the expression of Hsp60 showed an obvious increasing trend from newborn to senior, with the highest expression in the adult and this tends to decrease. Whereas in cattle-yak, Hsp60 expression was highest in the adult than other. In short, Hsp60 expression in the cattle-yak remained higher during every development stage of the testis (Fig. 3-V). It is worth noticing that Hsp60 expression in cattle-yak was significant higher than yak in every development stage of the testis.

The testicular seminiferous tubule of the newborn cattle-yak and yak was thin and sparse (Fig. 6-I, 6-II and Fig. 7-I, 7-II). The Hsp60 expression was strongest in the yak testis in primary spermatocyte, followed by that in the secondary spermatocyte. Sperm cells also had expression, contrary to the spermatogonium cells. We were interested in what was showed, as the organism developed, seminiferous tubules became closely packed, lumens became enlarged, and different amounts of sperm cells appeared during the development of sperms, with the largest count occurring during the adult stage. For the elderly yak,

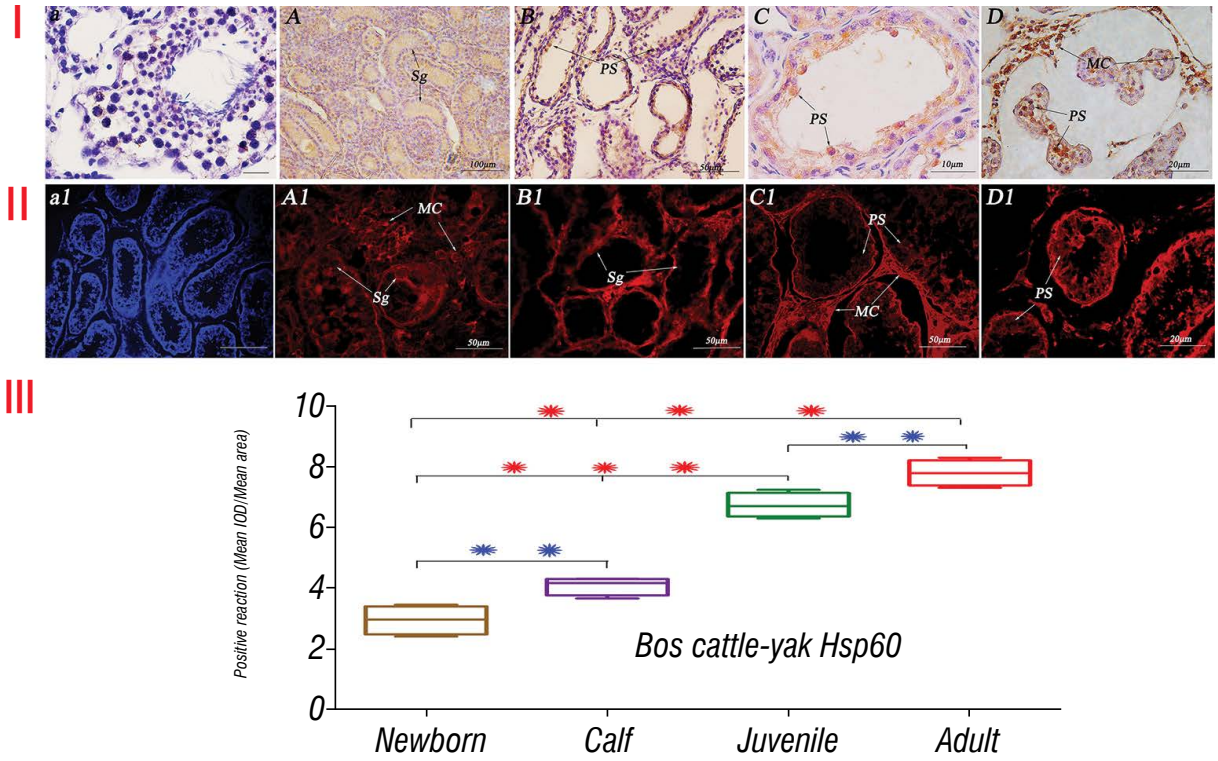

Figure 7. Immunohistochemistry and immunofluorescence localisation of heat shock protein 60 (Hsp60) in the testicular tissue of cattle-yak; **I, II: a, a1.** The control sections collected from the testicular tissue of adult yak, without immunoreactions (negative control); **A, A1.** Positive staining for Hsp60 was observed in the spermatogonium and mesenchymal of the newborn testis; **B, B1.** Positive staining for Hsp60 was observed in spermatogonium and primary spermatocyte of the calf testis; **C, C1.** Positive staining for Hsp60 was observed in the primary spermatocyte and mesenchymal of the juvenile testis; **D, D1.** Positive staining for Hsp60 was observed in the primary spermatocyte and mesenchymal of adult testis; **III.** The result of optical density analysis value. The columnar represent the expression trend of Hsp60 protein in different age of testis tissues. Abbreviated as shown in Figure 6. The asterisk represents the difference in positive expression. The more asterisks, the more significant the difference.

the amount of spermatogenic cells in the seminiferous tubule decreased significantly. As the cattle-yak aged, the number of spermatogenic cell at the different development stages decreased, and few sperm and sperm cells appeared. Hsp60 was highly expressed in all levels of spermatocyte in the cattle-yak testicular tissue, whereas the expression levels in the basement-membrane and myogenic cells were the lowest.

As shown in Figures 6-III and 7-III, protein expression levels of Hsp60 were detected in the testicular tissues at different developmental stages of yak and cattle-yak. In yak, the expression of Hsp60 showed obvious trend from newborn to senior, that expression increased to a higher level in the juveniles and then it tends to decrease, and at the same time, its expression was highest in the senior yaks. In the cattle-yak, the Hsp60 expression increased to highest in the adult. The results showed that the Hsp60 protein expression levels of cattle-yak were significantly higher than those of yak in almost all the developmental stages ($p < 0.01$).

DISCUSSION

For the first time, cDNA clones that encoded Hsp60 from cattle-yak were isolated, sequenced, and characterised. Previous studies have found that protein space structural differences led to functional differences [1]. Using reference sequence, our analytical results showed that three ORFs in Hsp60 illustrated frame-shifting property of the sequence. We also present evidence that the phylogenetic tree to cattle-yak Hsp60 associated with *Bos grunniens*, *Ovis aries* and *Bos taurus* were very high, and that cattle-yak evolution with the above animals had very close genetic relationship. In eukaryotic cells different HSP gene promoters upstream of TATA, heat shock element with size of approximately 20 bp was also necessary in the transcription of specific nucleotide sequence (C-GAA-TTC-G) [15, 29].

The amino acid sequences of the various protein spatial structures differed. Among the HSPs in cattle, Hsp70 was relatively more expressed followed by Hsp60 indicating the action of molecular chaperones to stabilise the native conformation of proteins [18]. Results (Fig. 2) showed cattle-yak amino acids multipoint mutation relative to yak. So, the molecular data show that Hsp60 gene could be used as a single nucleotide polymorphism marker to evaluate the loss of genetic diversity caused by hatchery selection or internal control gene in cattle-yak. Thus, we believed that the amino acid mutation is an important factor for functional differences of Hsp60.

The gene and protein expression of Hsp60 was significant not only in the different species but also in the different tissues of the same species. Interestingly, HSP60 and HSP10 were found to be the second most abundantly expressed HSPs. The relative mRNA abundance of HSF1 significantly increased ($p < 0.001$) in Murrah buffalo compared to Tharparkar and Sahiwal cattle during summer and winter [20]. In addition, the Hsp60 expression was high in under-cold stress and selenium deficient induced oxidative stress in the spleen and it also influenced immune function in chicks [16]. It was hard to believe these data also verified the controversies regarding the appropriate physiological function of different tissues. We found that gene and protein expression of Hsp60 was higher in the kidney, cerebellum, and liver of yak. However, Hsp60 had higher expression in the heart, cerebellum, and liver of cattle-yak. Studies have shown that under low-salinity (17 ppt) stress, RpHSP60 mRNA levels were significantly increased at 3, 72, and 96 h ($p < 0.05$). These results suggest that HSP60 of *R. philippinarum* may play important roles in responding to high-temperature and low salinity stresses [6]. In addition, when hepatic cells were exposed by enrofloxacin, Hsp60 mRNA levels significantly increased in hepatocyte of *Ctenopharyngodon idellus* [5]. So, considering the high expression in the kidney, cerebellum, and heart of yak and cattle-yak, we speculated that Hsp60 could protect cells and tissue from the environmental stimulus.

Previous studies demonstrated that Hsp60 can protect lung tissue from damage caused by bronchitis and cold air, and local response occurring at the level of the alveoli, bronchioles and respiratory bronchiole epithelial cells and smooth muscle cells [40]. Gene expression levels of Hsp27/40/60/70/90 were significantly up-regulated when the treatment cytotoxicity in vitro spleen lymphocytes of chicken [45]. In addition, studies have shown that adenocarcinoma was characterised by the highest levels of Hsp10 and Hsp60 in epithelium and lamina propria [30]. Hsp60/70/90 possess the ability to modulate cellular anti-stress responses and play key roles in protecting organisms from high temperature and low salinity [4, 22]. This study demonstrated the localization of Hsp60 in the cell membrane and cytoplasm, not in the nuclei of organs. It is undeniable that, Hsp60 plays an active function in the tissue physiological adaptation in cattle-yak and yak.

Recent data have indicated that the expression level of Hsp60 has no apparent change during the developmental progress in rabbit testis, but high expression in cynomolgus monkeys The Hsp60 mRNA transcripts detected were highest during the developmental phase (in April), while the lowest levels were found in the resting phase (after spawning in late July) of fish testis [41, 43]. Interestingly, this result is largely consistent with our experimental result that Hsp60 expression level in the testicular tissue of cattle-yak increased gradually with age. Meanwhile, research shows that in situ and northern hybridizations significantly reduced levels of the Hsp60C transcripts in Hsp60C1 homozygous adult males (*Drosophila melanogaster*) [31]. In the human testis, Hsp60 expression in vitro was high than in vivo [21]. Encouragingly, Hsp60 expression in testicular tissue of cattle-yak was generally higher than yak, especially in the juvenile and adult. Thus, as in Figure 3, we speculated that the high expression of Hsp60 in the testicular tissue of male cattle that was activated offspring restore normal fertility of male cattle-yak.

Previous studies indicated that Hsp60 was detected in spermatogonia, premeiotic spermatocytes and germ cells of humans at different ages [28]. In the testis of immature rhesus, Hsp60 immunoreactivity was visible in spermatogonia and in Sertoli cells, whereas interstitial cells were negative [26]. In addition, Hsp60 immunoreactivity was detected in sperm midpiece of dog, cat, stallion and boar [37]. This study found that the Hsp60 detected in primary spermatocyte, secondary spermatocyte and sperm cell of yak and cattle-yak. Similarly, positive reactions have recently been described in Sertoli cells and spermatogonia, where it promotes testis development but may also underlie spermatogenesis [44]. Furthermore, the Hsp60 expression was low in early stages

of spermatogenesis and increased with the increase in age. Thus, high expression of Hsp60 caused the blockage of spermatocyte development. This study showed an important breakthrough in the study of male infertility.

CONCLUSIONS

The following conclusions can be drawn from this study. Changes in amino acids sequences were the main cause for protein functional differences of Hsp60. Hsp60 has an obvious differential expression in different organs and different ages of cattle-yak and yak. In addition, Hsp60 was mainly detected in epithelial of tissue, spermatocyte, sperm cells and mesenchymal cells in the testicles. Hsp60 showed the specific expression in yak and cattle-yak testicular tissue, and it has important correlation with cattle-yak infertility.

Funding

This study was supported by Nature Science Foundation of China (grant no. 31572478, 31360594).

Conflict of interest: None declared

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