Expression of the NSE, SP, NFH and D β H in Normal and Cryptorchid Testes of Bactrian Camel

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Abstract

Background: Neuroendocrine substances play important roles in regulating the normal physiological functions of testicles. The purpose of this study is to explore the localization and effects of four neuroendocrine markers (NSE, SP, NFH and D β H) in normal and cryptorchid testes of Bactrian camels.

Methods: The cryptorchid testes were located in the abdominal cavity and were collected by orchiectomy. Fresh testes tissues were processed into small pieces and then divided into three samples. One sample was frozen in liquid nitrogen for western blotting hybridization reaction, while the other sample was fixed with 4% paraformaldehyde solution for histochemical analysis, and the third fixed in glutaraldehyde for transmission electron microscopy observation.

Results: The results showed that cryptorchidism caused a reduction in layers of spermatogenic epithelium and decreased glycogen positivity in the basement membrane. The ultrastructure revealed that macrophages were always found around the Leydig cells, which were crowded with swelling mitochondria in cryptorchidism. Expression of NSE in the Leydig cells of cryptorchidism was significantly weakened compared to that in the normal group ($p < 0.01$). We found that SP was always distributed along the nerve fibers in normal testes and was expressed in the Leydig cells of cryptorchidism. However, expression of NFH in the cryptorchidic tissue was strongly positive in spermatogenic epithelium, with limited expression in Leydig cells and no expression in peritubular myoid cells. Therefore, expression of D β H in the Sertoli cells was comparatively strong in both the normal and cryptorchidism groups. NFH and D β H expression was significantly increased in the cryptorchidism group compared with the normal group ($p < 0.01$).

Conclusions: These findings indicated that the underdeveloped seminiferous epithelium and pathological changes in cryptorchid tissue in Bactrian camels were potentially related to a disorder in glycoprotein metabolism. Our results suggest that NSE and SP could be helpful to judge the pathological changes of cryptorchidism. The present study provides the first evidence at the protein level for the existence of NFH and D β H in Sertoli and Leydig cells in Bactrian camel cryptorchidism and provides a more in-depth understanding of neuroendocrine regulation is crucial for animal cryptorchidism.

Background

Gametogenesis in mammals is a very complex process in which paracrine and autocrine mechanisms in addition to the hypothalamic-pituitary-testicular endocrine axis influence are intimately involved in complicated feedback loops [1, 2]. Leydig cells originate in the adult testis by differentiation from embryonic neuroectoderm precursor cells and mesenchymal cells and express a variety of neural and neuroendocrine markers [3, 4]. Studies have suggested that in addition to steroid hormones the Leydig cells of different rodents and humans produce and possess a number of neuronal markers such as 5-hydroxytryptamine (5-HT), neural cell adhesion molecule (N-CAM), and microtubule-associated protein (MAP-2) catecholamine and norepinephrine protease [3, 5, 6]. The distribution and localization of

PGP9.5, neuropeptide Y, neuron-specific enolase (NSE), substance P (SP), dopamine- β -hydroxylase (D β H), and tyrosine hydroxylase-related peptides in the testicles of mammals, such as cats [7], donkeys [8], ruminants [9], dromedaries [10] and humans [11] were studied by immunohistochemistry, and results showed that such neuroendocrine substances play important roles in regulating the normal physiological functions of the testicles. Previous researches have shown that the expression of NSE, SP and NFH (High Neurofilaments) in the testicular tissues of adult rats is regulated by serum sex hormone concentrations [12]. The occurrence of numerous marker substances, such as SP, NFP-200, NSE and D β H characterized human Leydig cells as new members of the "diffuse neuroendocrine system" or the family of paraneurons [1, 4]. Normal levels of neuroendocrine markers are indispensable for spermatogenesis, and malfunction of one or more of the neuroendocrine regulated processes within the testis is closely related to the occurrence of sterility [13, 14, 15]. However, the neuroendocrine nature of camel infertility is not well characterized. Here, we present histochemical results and the distribution characteristics of four neuroendocrine markers (NSE, SP, NFH and D β H) in cryptorchidism of camels. These results are necessary to clarify the capacity of Leydig cells to synthesize these substances, and to establish their functional significance in reproduction.

The Bactrian camel (*Camelus Bactrianus*) is an important domestic animal in some of the desert and semidesert areas of the world. Its ability to reproduce in very poor living conditions is related to its unique breeding mechanism. Cryptorchidism is a reproductive system disease that causes sterility in camels. A few studies have reported the pathological characteristics of testes in infertile male Bactrian camels, such as abnormalities in the seminiferous tubule and the decrease in the synthetic ability of Leydig cells was a meaningful index for the further research of the steroid biosynthesis [16, 17]. However, there is no detailed report about the neuroendocrine characteristics of cryptorchid testes in Bactrian camels. Additionally, we have compared the expression of the PGP9.5 and neuropeptide Y in Leydig cells between the normal and cryptorchid testes of Bactrian camels, and reported for the first time the role of neural markers on Bactrian camel infertility [15]. Based on these findings, the aim of this study was to compare the distribution of NSE, SP, NFH and D β H in Leydig cells from the normal and cryptorchid testicular tissues of Bactrian camels, we also investigated the microstructural characteristics and markers of Leydig cells to explore the changes of the Leydig neuroendocrine characteristics, suggest that the young adult cryptorchid camels is innervated by NFH and D β H, which may serve as major endocrine markers for testicular functions at this age. The expression of SP was significantly decreased in cryptorchidism provide a morphological basis for further research on the roles in testes descent. The innervation of Bactrian camels testis by cholinergic and adrenergic nerves present are summarized in this study and include evidence of indirect, as well as rare direct neuroendocrine character of cryptorchidism Bactrian camels.

Methods

All protocols involving the use of animals were performed in accordance with the approved Guidelines for Animal Experiments of Gansu Agricultural University, and were approved by the Animal Experimental Ethical Inspection Committee of Gansu Agricultural University.

Experimental animals and test reagent

A total of 11 male unmated about two-year-old Bactrian camels were collected in winter from breeding farmers in Laohe County, Wuzhong City, Ningxia Hui Autonomous Region. These home-bred animals were divided into two groups: the normal (five of samples) and cryptorchidism groups (six of samples) for comparative analyses. The cryptorchid testes were located in the abdominal cavity (Intra-abdominal cryptorchidism). Tissue specimens were collected by orchiectomy.

The samples were treated according to different test schemes. Fresh testes were processed into small pieces and then divided into three samples. One sample was frozen in liquid nitrogen for western blotting hybridization reaction, while the other sample was fixed with 4% paraformaldehyde solution for histochemical analysis, and the third fixed in glutaraldehyde for transmission electron microscopy observation. The study design was approved by the academic committee of the College of Veterinary Medicine of Gansu Agricultural University.

All antibodies were purchased from commercial suppliers. Rabbit polyclonal antibody NSE (bs-10445R-HRP, anti-NSE/HRP), SP (bs-0065R-HRP, anti-SP/HRP), NFH (bs-10680R-HRP, anti-NFH/HRP) and D β H (bs-0596R-HRP, anti-D β H/HRP), anti-rabbit IgG antibody (bs-0295G-AF488) and DAB chromogenic kit (ZLI-9018) were purchased from Beijing ZSGB-BIO Co., Ltd., Beijing, China. Immunohistochemical staining kit (SP-0023), produced by ZYMED Laboratories Co., Ltd., San Diego, USA, purchased from Jiamay Biotech Co., Ltd., Beijing, China. And other chemicals were obtained commercially and of reagent grade.

Experiment 1: H&E staining, histochemical staining, and observation of tissue samples

Testicular tissue samples (1×1×0.6 cm) were fixed with paraformaldehyde and rinsed in running water for 24h before conventional gradient ethanol dehydration. Afterwards, they were made transparent with xylene, embedded in paraffin, and 5 μ m serial sections were cut every fifth section was mounted and stained either with standard haematoxylin and eosin (H&E) to examine the general morphology, followed by Alcian blue (AB pH=2.5, 30 min) and periodic acid Schiff reaction (PAS) and examined for the presence of positive staining cells, the acidic mucin and proteoglycan were blue, with the nuclei being reddish [18].

Experiment 2: transmission electron microscopic technique

The testes were cut into 0.1cm×0.1cm×0.1cm, fixed in the 2.5% glutaraldehyde in 0.1M phosphate-buffered saline (pH=7.4) or 48h, and then post fixed in 1% osmium tetroxide at 4°C for 1h. The pieces were dehydrated with a graded ethanol series, and then embedded in epoxy resin (Epon812, American). Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and then examined under a JEM-100CX electron microscope (NEC, Japan).

Experiment 3: immunofluorescence staining

Green immunofluorescence staining: Paraffin sections of testicular tissues were prepared following the same procedure as demonstrated in section 1.2.1. Slice thickness was 5 μ m, high-pressure antigen retrieval

was performed, 30g/L H_2O_2 aqueous solution was added to block peroxidase for 10min. Sections were incubated with goat serum albumin for 15min. A volume of 50 μ L of primary antibody (i.e., rabbit polyclonal anti-NSE, anti-SP, anti-NFH and anti-D β H) was added dropwise at a dilution ratio of 1:1,000. For the negative control group, 0.01mol·L⁻¹ phosphate buffer solution buffered saline (PBS) was used as the substitute for the primary antibody. Incubation was carried out for 2h at 37°C before the fluorescent secondary antibody was added dropwise. Alexa Fluor 488-labeled goat anti-rabbit IgG antibody (1:1,000, bs-0295G-AF488) was used as the secondary antibody. After incubation at 37°C for 2h, observations were made directly under the microscope. Positive cells displayed fluorescent green and the immunofluorescence images were collected by an ECHO fluorescence microscope (REVOLVE RVL-100-G).

Experiment 4: immunohistochemical staining

The streptavidin-peroxidase method was used for immunohistochemical staining. The paraffin sections were routinely dewaxed and differentiated by gradient ethanol; 3% H_2O_2 in methanol solution was used to block peroxidase for 10min. Normal goat serum albumin was incubated for 15min; 50 μ L of rabbit polyclonal anti-mouse NSE (bs-10445R), SP (bs-0065R), NFH (bs-10680R) or D β H (bs-0596R) antibodies were diluted at 1:400 and added dropwise to each section separately. After incubation at 37°C for 2h and washing by PBS shaking, 50 μ L of biotinylated goat anti-rabbit IgG working solution was added dropwise to each section, followed by the addition of 50 μ L of horseradish-labeled streptavidin working solution. The sections were then incubated at 37°C for 15min. Freshly prepared DAB substrate solution was then added for microscopic examination, followed by routine dehydration, making transparent, and mounting. Positive cells displayed as brown-yellow, with nuclei showing as blue after counterstaining. The negative control group was stained with 0.01mol·L⁻¹ of PBS instead of the primary antibody.

Experiment 5: western blot analysis

Total protein was extracted from testicular tissue was extracted. 100mg of liquid nitrogen-preserved testicular tissue was weighed and crushed. Radioimmunoprecipitation assay buffer containing phenylmethylsulfonyl fluoride was added proportionally. The mixture was then homogenized with a glass homogenizer until fully lysed, and then subjected to hypothermal centrifugation at 14,000 $\times g$ for 5min. The supernatant was transferred to another tube, and reagent with half of the volume of the solution was used to dissolve the precipitate. After standing still at 25°C for 30 min, the mixture was then centrifuged at 4°C and 19,575 $\times g$ for 20min. The supernatant was analyzed for protein concentration according to the instructions of the BCA protein assay kit (Beyotime Biotechnology Research Institute), dispensed, and stored at -70°C for use.

The protein was extracted and loaded following the routine procedure: the protein was separated by polyacrylamide gel electrophoresis (SDS-PAGE); 5% stacking gel and 12% separating gel were prepared. 30 μ g of protein was loaded to the gel sample well for electrophoresis, and the protein in the gel with the target band was subjected to wet transfer to the membrane. The membrane was incubated with primary antibody NSE, SP, NFH or D β H (1:1,000) at 4°C overnight, and then was washed by Tris-buffered

saline+Tween 20(TBST).The horseradish peroxidase-labeled cow anti-rabbit IgG was used as the secondary antibody for incubation for 2h at 25°C,and TBST was used to wash the membrane three times for 10min each.The polyvinylidene fluoride membrane was then subjected to color development.The chemiluminescent substrate solutions A and B were mixed at a ratio of 1:1,and were allowed to react at 25°C.The transfer membrane was photographed for analysis. β -actin was used as the internal reference.

Measurement and statistical analysis

Tissue sections were imaged using a Nikon Eclipse 80i microscope camera system.Leydig cell characteristic index:ten sections were randomly selected from each group,and six non-repeating fields were randomly selected from each section(bar=20 μ m,400 $\times$).The transverse and longitudinal diameters of Leydig nuclei,as well as the mean area of Leydig nuclei in the triangular and quadrangular mesenchymal tissues of each field were randomly counted(n=40).Statistical analyses were performed by Image Pro Plus 6.0 software.

The intensity of immunofluorescence results was scored as follows:-:no positive expression;+/-:occasional positive expression;+:positive expression;+:medium-intensity positive expression;+++:strong positive expression;++++:high-density strong positive expression[19].

The sections were imaged using a NIKON ECLIPSE 80i microscope camera system.Five sections were selected randomly from each group for immunohistochemical staining.Six non-repetitive fields(bar=20 μ m,400 $\times$)were randomly selected for each section.The mean positive signal intensity and positive area of each field were statistically analyzed by Image Pro Plus 6.0 software to evaluate the average light absorbance.A total of 30 statistical data were collected for each group,and the results were expressed as mean $\pm$ standard deviation(mean $\pm$ SD).SPSS15.0 software was used for statistical analysis,and the expression difference of NFH,SPNSE and D β H between normal and cryptorchid testes was analyzed by one-way analysis of variance.Paired t-tests were carried out,and the level of statistical significance was set at $p < 0.05$.

The western Blot expression band was first selected,the gray curve of which was then analyzed using Image J 1.48.The area under the peak was calculated as the band density value.The density of β -actin was taken as the base value,and the relative densities were obtained by comparing the densities of NSE,SPNFH or D β H expression bands in the normal testis group(Simply marked as N-NSE,N-SPN-NFH or N-D β H)and the cryptorchidism group(Simply marked as C-NSE,C-SPC-NFH or C-D β H).The relative density values were then statistically processed by SPSS 21.0 statistical software.All data were expressed as mean $\pm$ standard deviation.Difference between variables were analyzed by t-tests difference between variables were analyzed using paired t-tests,and the level of statistical significance was set at $p < 0.05$.

Results

Comparison of the histochemical characteristics between normal and cryptorchid testes of Bactrian camels

As shown in Fig. 1, H&E staining revealed that the seminiferous epithelium in normal testes of Bactrian camels was developed well with seminiferous cells located on both sides of Sertoli cells, and round, oval, or irregular-shaped Leydig cells of considerably large size (Fig. a). We performed a histochemical analysis to identify the cellular changes underpinning the changes in cryptorchidism. We detected glycogen by PAS staining and distinct purple-red glycogen-positive bands in the lamina propria and interstitial capillary walls were observed (Fig. b); Acid mucins were also clearly visible in the interstitial capillary wall and lamina propria as demonstrated by AB staining (Fig. c); Examination of the H&E images revealed that cryptorchidism causes a reduction in layers of spermatogenic epithelium, the Leydig cells lacked distinct structural characteristics and the nucleus was round, elliptical, or irregular, with relatively large cell bodies (Fig. d); We next examined whether the reduction in seminiferous tubule cross-section was caused by a decrease in glycoprotein metabolism. PAS staining of the cryptorchid testes showed positive expression in the basement membranes and the wall of interstitial blood vessels (Fig. e), while the AB blue positive band was almost invisible in the interstitial tissue of the cryptorchid group (Fig. f).

Statistical analyses indicated that the transverse and longitudinal diameters of the nuclei in Leydig cells of cryptorchid Bactrian camels were significantly increased compared to those in normal testicles ($p < 0.05$). The average areas of the Leydig cells were significantly larger in cryptorchid testes than in normal testes ($p < 0.01$, Table. 1).

Comparison of the ultrastructure of Leydig cells between normal and cryptorchid Bactrian camels

Electron micrographs showed that Leydig cells of the normal testis with the typical features of granular reticulum in the cisternae in direct continuity with this type of agranular reticulum. The granular reticulum were also sparse, the Golgi apparatus and mitochondria were dispersed, and lipid droplets were not obvious (Fig. 2a). Furthermore, collagen fibers were distributed around the interstitial capillaries, which revealed endothelial cells with flat nuclei and clear basement membranes in normal testicular tissue (Fig. 2b). The villi protrusions of the Leydig cell were unobvious and the swelling mitochondria and indistinct reticulum crowded in the cytoplasm which also consists of few lipid droplets. Moreover, the macrophages around Leydig cells exhibited obvious pseudopodia, but many lysosomes of different sizes and residual bodies were observed in cytoplasm (Fig. 2c). The capillary vessel was smaller with mostly clear epithelial basal lamina, and scattered particles were observed between adjacent microvessel endothelial cells, while perivascular collagen fibrils were richer in the cryptorchid group (Fig. 2d).

Comparisons of the distributions of NSE, SP, NFH and DβH in normal and cryptorchid testes of Bactrian camels

In normal tissues (Fig. 3A a-d), the expression of NSE was primarily observed in Leydig cells and vascular endothelial cells, not in seminiferous epithelial cells (Fig. 3A-a), while the obvious SP immunopositive nerve fibers in the interstitial tissue surrounding the Leydig cells (Fig. 3A-b); In addition, almost no NFH expression was observed in testicular tissues (Fig. 3A-c), whereas DβH was positively expressed in Sertoli cells, but not in seminiferous cells, Leydig cells or the surrounding tissues (Fig. 3A-d). No negative control had a positive reaction (Fig. 3A-e). Compared to normal testes, the expressions of NSE were obvious in

Sertoli cells, peritubular myoid cells, and Leydig cells in cryptorchid tissues (Fig. 3A-f), but SP expression in the seminiferous epithelium and Leydig cells was significantly decreased (Fig. 3A-g), and NFH was clearly expressed in Leydig and seminiferous cells (Fig. 3A-h). In cryptorchid tissues, D β H was highly expressed in cytoplasm rich primary spermatocytes, with occasional expression observed in atypical nuclear shaped Sertoli cells (Fig. 3A-i). No expression of endocrine markers was detected in the negative control group (Fig. 3A-j).

In addition, no significant difference in NSE was observed in testicular tissues between normal and cryptorchid testes in Bactrian camels, and both the SP and NFH average absorbance were increased in cryptorchid testicular tissues. A much higher abundance of D β H was observed in the cryptorchid group than in the normal group (Fig. 3B).

Expressions of neuroendocrine markers were further examined in normal and cryptorchid testes of Bactrian camels by western blotting. As shown in Fig. 3C and D, the protein levels of NFH and D β H were significantly increased in cryptorchid testicular tissues ($p < 0.01$).

Comparisons of the localization of NSE, SP, NFH and D β H between normal and cryptorchid testes of Bactrian camels.

Green immunofluorescence analysis indicated that NSE was mainly expressed in Leydig cells and weakly expressed in the seminiferous epithelium (Sertoli cells, peritubular myoid cells and spermatogenic cells) of the normal testicular tissues (Fig. 4a), and SP was prominently expressed in Leydig cells and nerve fibers in interstitial tissue (Fig. 4b). NFH was weakly stained in Leydig cells of normal testes (Fig. 4c). Similarly, D β H was weakly expressed in Leydig cells, whereas strong staining was detected in Sertoli cells, and no expression was detected in peritubular myoid cells of the normal group (Fig. 4d, Table 2).

As shown in Fig. 4e-f, in cryptorchid tissues, weak staining of NSE was observed in the whole testicular tissues (Fig. 4e), and SP was weakly expressed in the interstitial tissue (Fig. 4f). NFH protein was highly expressed in cytoplasm rich primary spermatocytes and Leydig cells, with occasional expression observed in atypical nuclear shaped Sertoli and peritubular myoid cells (Fig. 4g). D β H protein was strongly stained in the Sertoli and spermatogenic cells, while no expression was observed in peritubular myoid cells or Leydig cells (Fig. 4h, Table 2).

Discussion

Cryptorchidism is often associated with testicular heat stress, which leads to pathological changes, including decreased sperm motility, delayed development of adult stem cells, meiotic disorders, and reduced number of germ cells [20, 21]. Cryptorchidism of six-month-old Ziwuling Black goats caused a reduction in the number of spermatogenic epithelial cells, a significant decrease in the average diameter of the seminiferous tubules, abnormal differentiation of Sertoli cells, and retarded sperm development in the testes [22]. Male Bactrian camels reach sexual maturity at approximately four years

old and breeding starts at five years old. The age of Bactrian camels studied here was two-year-old haven't reached mating age yet, the seminiferous tubules were developed with three to five layers of seminiferous cells, but lack of obvious sperm in the seminiferous epithelia. and the cryptorchidism caused a reduction in layers of spermatogenic epithelium, which only comprised one to three layers. Therefore, the next experiments were designed to explore the underlying mechanisms of spermatogenic defects in cryptorchidism.

The interstitial connective tissue of mammalian testes is composed of different ingredients, such as blood capillaries, lymphoid vessels and Leydig cells. Leydig cells in testicular sections play crucial roles in male development and the maintenance of reproductive functions[23]. We have demonstrated that the swelling of mitochondria in Leydig cells was related to oxidative stress in cryptorchid yaks[24]. Novo et al. [25] revealed that oxidative stress mediates mitochondrial dysfunction and programmed cell death. In the present study, the villus protrusions of Leydig cells were not obvious and with swelling mitochondria and indistinct reticulum crowding in the cytoplasm of Leydig cells in cryptorchidism. A previous study focused on the similarities between aging-associated and cancer-associated oxidative stress and mitochondrial dysfunction as their common phenotype[26]. In our study, the pathological changes of cryptorchidism in camel should be closely related to the decrease in oxidative capacity of swollen mitochondria in Leydig cells.

There are two distinct populations of Leydig cells, fetal and adult, that arise at different times during the development of the testis[27]. The adult population, appearing at or just before puberty, is responsible for spermatogenesis and the maintenance of male secondary sex characteristics[27]. Leydig cells are first recognized morphologically 2 days after birth with the appearance of lipid droplets in the cytoplasm of certain interstitial cells. The lipid content closely matches the steroid content of the developing testis and marks the maturation of the steroid synthesis pathway in tammar testis[28]. Research has shown that the minimal concentration of testosterone in testicular tissues occurs in 2 year-old camels during the nonbreeding season, which was lower than that in 1.5 year-old and 3 year-old young camels and then increased with advancing age[29]. Furthermore, the onset of puberty coincides with a dramatic increase in the average Leydig cell size, which accompanied by a peak in the steroid-producing capacity per Leydig cell[30]. The present results show that the lipid droplets in Leydig cells were non-significant in cryptorchidism tissue compared to the normal camel, which was still at the early stage of pre-pubertal and sexual activity was not obvious and Leydig cells were in the process of development. Much evidences suggested that there is a significant correlation between the testosterone concentration and the size and number of Leydig cells[31, 32]. Our results showed that the average area of Leydig nuclei in cryptorchid tissues was significantly lower than that in normal testes, it was consistent with that of cryptorchid goats[22]. Ezeasor[33] demonstrated that the increase of the accumulation of hormone precursors in Leydig cells in cryptorchid goats stimulates immune stress due to decreases in the synthetic activity of Leydig cells. Therefore, our findings regarding the ultrastructural alterations in the Leydig cell indicate defects in the steroid synthesis pathway in cryptorchidism, and also we observed that cryptorchidism caused obvious phagocytic characteristics of the macrophages near Leydig cells, it may be an clinical cue for degraded testes immune microenvironmental system.

Glycoproteins are important biological macromolecules that present at the cell membrane, intercellular substance, plasma, and mucus. Glycogen and glycoprotein are abundantly expressed in neurons, endocrine, and neuroendocrine cells [34, 35]. Prior studies showed that the positive bands of PAS were primarily distributed in the vascular wall and the basement membrane of seminiferous tubules in the testes of dromedary camels. It was clearly indicated that the camel testis contains a wide range of glycoconjugates and specific carbohydrate structures are required for spermatogenesis during periods of rutting season and particularly apparent in the breeding period [36, 37]. Methods for the detection of mucin are considered with the AB staining, acidic mucus containing hydroxyl and sulfate groups forms an insoluble complex which stains blue [18, 38]. The levels of acidic mucus increase with establishment of the Leydig cell groups and the maturity of spermatogenic cells [39]. Our previous study observed PAS-positive bands distributed in the vascular wall and lamina propria of seminiferous tubules in the normal goat testes, while cryptorchidic goat caused a decrease in glycoproteins, which may indicate that saccharides function as essential components of basic nutrition metabolism, and play crucial roles in spermatogenesis [22]. Here, we observed the same results in cryptorchidic Bactrian camel. The sulfated glycoproteins protect the normal activity of sperm [40]. Our studies revealed that the cryptorchidism decreased the diameter of the Leydig cell ($p < 0.05$) and induced glucose metabolism disorders in the surrounding connective tissue, which may contribute to the abnormal distribution of local neurohormones in testis.

Soluble brain protein 14-3-2 first described by Moore and McGregor in 1965 is now known to be a cell specific isoenzyme of the glycolytic enzyme enolase, designated neuron specific enolase (NSE) [41]. It is not only a marker for all types of neurons, but also for all neuroendocrine or paraneuronal cells [42]. It has been proven that Leydig cells of human testis possess neuroendocrine properties and are therefore a member of the diffuse neuroendocrine (paraneuron) system, and NSE is immunopositive in the cytoplasm of human Leydig cells [43]. Comparative immunocytochemical studies have demonstrated that NSE was detected in Leydig cells of the golden hamsters, guinea pigs, and rats at all stages studied: fetal, neonatal and adult. NSE may be synthesized by Leydig cells and was involved in the synthesis of steroids [43, 44]. Furthermore, the expression intensity of NSE in the testes of young rats and hamsters was higher than that of adults, and NSE is positively expressed not only in Leydig cells, but also in sperm cells and Sertoli cells in adult guinea pig testes [44]. The demonstration that tumors of the nervous system and of neuroendocrine origin contain NSE was promoted the NSE as a possible tumor marker. Immunohistochemical staining revealed positive expression of NSE in human testicular tumors [45]. NSE was predominantly detected in Sertoli cells caused by Leydig cell tumors in canine cryptorchidism [46]. Here, the expression of NSE was only observed in Leydig cells and vascular endothelial cells of normal testes. More importantly, NSE was expressed not only in Leydig cells but also in Sertoli cells in cryptorchidism. Although there was no significant difference between the two groups, this difference in NSE expression is helpful to judge the pathological changes of testicular tumors. Our results suggested that cryptorchidism of the Bactrian camel has a tendency toward malignant transformation.

SP was accidentally isolated from the intestinal plain muscle and brain of horses in the 1930s by Euler and Gaddum, which lowers arterial blood-pressure and stimulates the tone and rhythm of the rabbit's

isolated intestine[47].Euler demonstrated that human semen and extracts of the prostate glands of various animals have pharmacological actions due to SP[48].SP immunoreactivity can be detected in Leydig cells,particularly human testes,and to a lesser degree in mouse Leydig cells,it could be potentially paracrine substance regulating intratesticular function[49].SP is present in Leydig and Sertoli cells of humans,mice,hamsters,marmosets,and rats,and evidence suggests that it has potential regulatory roles regarding in spermatogenesis,spermatozoa function and motility,and testicular steroidogenesis[50].The SP immunopositive fibers in camel testes were mainly distributed in the caput and cauda of testes[10].Expression of SP in cremaster muscle was decreased in cryptorchid tissues,resulting in a decrease in testicular autonomic nerve regulation,and testicular retention in the groin[51].Our study showed that immunostaining of SP in Leydig cells and peripheral nerve fibers in normal testes was strongly positive,however,its distribution in cryptorchid tissues was significantly decreased.Therefore,our results suggest the distribution differences of SP in cryptorchidism may reducing the local autonomic innervation and related to steroidogenesis of Bactrian camel.

Neurofilaments(NFs)are composed of light(NF-L),medium(NF-M),and heavy(NF-H)subunits of approximately 68,145,and 200 kDa,respectively.All three subunits are phosphorylated and most of the phosphorylation sites are located in the tail domain of NFH[52]New evidence that NFH exists within axons and influences neurotransmission suggests that NFH might contribute to normal synaptic function and neuropsychiatric disease states[53].A correlation was found between the expression of NFH immunoreactivity in Sertoli cells and the stage of spermatogenesis;in human cryptorchidism,Sertoli cells exhibited strong NFH immunoreactivity whereas Sertoli and Leydig cells showed no or only weak reactivity in normal testes[54].NFH immunoreactivity in perivascular locations,intermingled with interstitial cells and close to the wall of seminiferous tubules was found only in the testes from immature rhesus monkeys.Furthermore,it show a marked degree of plasticity during development,especially around the time of puberty,and in cells of the tubular wall and vascular cells directly and/or indirectly via intermediation of mast cells[55].Immunohistochemical analyses showed that expression of NFH was significantly increased in Sertoli cell only syndrome and germ cell arrest biopsies in males[56].The results showed that during the recovery of rat testicular Leydig cells following ablation with ethane dimethanesulfonate,expression of NFH in Leydig cells increased,suggesting that NFH contributes to the proliferation of Leydig cells[57].We also discovered that immature of Leydig cells,the abnormal proliferation of the Sertoli cells and arrested spermatogenesis were the main characteristics in cryptorchid testes of Bactrian camels[5, 16].The present study provides the first evidence at the protein level for the existence of NFH in Sertoli and Leydig cells of the Bactrian camel cryptorchidism,and also western blot studies demonstrated that NFH was significantly increased in cryptorchid testicular tissues.Our study demonstrates that NFH correlates with developmental abnormalities of Sertoli and Leydig cells in Bactrian camel cryptorchidism.

Dopamine β -hydroxylase(D β H) is an adrenergic monoamine enzyme.D β H immuneoreactivity in the cytoplasm of interstitial Leydig cells in human testes provides strong evidence for the neuroendocrine nature of the Leydig cells[1].Immunohistochemical studies in pigs suggest that D β H-positive neurons should be considered elements of highly testosterone-dependent autonomic circuits involved in the

regulation of urogenital function[58].A previous study identified Leydig cells as the presumed sites of catecholamine production in both fetal and mature human testes and further supported the recognized neuroendocrine characteristics of this cell type[59].Romeo et al[60]found that D β H is a neurotransmitter involved in the regulation of testicular function and activates Leydig cell receptors,especially,during psychogenic or somatic stress environments,which could contribute to the high concentration of catecholamines and to suppression of testicular functions.Considering the controversial data presented in previous reports,compared to the control cases,Leydig cell-specific expression of D β H was significantly weaker in human cryptorchidism and suggested that Leydig cells are not essentially involved in the pathogenesis of the disturbances[61].These results are in accordance with our immunohistochemical and western blotting data for D β H,which was weakly expressed in Leydig and peritubular myoid cells in Bactrian camel cryptorchidic tissue.We suggest that D β H expression in Leydig cells has little to do with whether they are normal.However,more importantly,the results presented here provide evidence that D β H is strongly expressed in the Sertoli and spermatogenic cells,which is very different from other reports.

Sertoli cells are involved in the regulation of testis-specific differentiation processes,such as prevention of germ cell entry into meiosis,Leydig and peritubular myoid cell differentiation,regression of the Müllerian duct,and the upper part of the vagina,also hide from the host immune system and secrete trophic factors[62].Sertoli cells can be divided into three stages: embryonic Sertoli cells (eSCs),immature Sertoli cells,and mature Sertoli cells,eSCs possess multiple supporting functions and research value in gonadal development and sex determination[63, 64].In addition,the effect of GDNF secreted by Sertoli cells on the differentiation potential of dopaminergic neurons has been reported[62,63.64].Negative expression of D β H by immunostaining and RT-PCR in human embryonic stem cells supported that the differentiated neurons induced by Sertoli cells are dopaminergic rather than noradrenergic[64];Our study suggests that expression of D β H from Sertoli cells of Bactrian camels is very important for normal development of both testicular somatic cells and germ cells,and it may provide a promising method for therapeutic application and basic neuroscience research in the Sertoli-derived D β H neuron differentiation.The production of sperm and androgens depends on the normal development of both testicular somatic cells and germ cells, and spermatogenic cell development is entirely dependent on somatic cells,especially Sertoli cells[65].Cryptorchidism can occur due to malfunctions in both somatic and germ cells[65] In cryptorchid patients,the diminished number and functional defects of Sertoli cells prove its pivotal role in germ cell development[66, 67].In the current study,D β H expression in the cryptorchidism group was significantly higher than that in the normal group,D β H was strongly stained in the Sertoli cells,indicating a close relationship between D β H and Sertoli cell.Dysplasia of Sertoli cells and abnormal proliferation of spermatogenic epithelial cells may be one of the reasons for the increased expression of D β H. Furthermore, additional neuroendocrine characteristic studies specifically in Sertoli cells are needed to amplify our findings.

Conclusions

The current study demonstrated that the underdeveloped seminiferous epithelium and pathological changes in cryptorchid tissue in Bactrian camels are potentially related to disorders in glycoprotein

metabolism. Therefore, our results suggest that distribution differences of NSE, NFH, SP and D β H in normal and cryptorchid testes of Bactrian camels are very important to analyze the neuroendocrine mechanism of cryptorchidism. NSE and SP were correlate with developmental abnormalities of Leydig cells in Bactrian camel cryptorchidism. The present study provides the first evidence at the protein level for the existence of NFH and D β H in Sertoli and Leydig cells in Bactrian camel cryptorchidism and indicates a close relationship between D β H and Sertoli cells. These results provide a more in-depth understanding of neuroendocrine regulation that is crucial in animal cryptorchidism. Nevertheless, the mechanisms of neuroendocrine activity in Sertoli cells need to be further investigated.

List Of Abbreviations

Abbreviation	Complete name
AB	Alician blue
Am	Acidic mucoprotein
C- D β H	Cryptorchidism- D β H
C- NFH	Cryptorchidism - NFH
C- NSE	Cryptorchidism - NSE
C- SP	Cryptorchidism - SP
D β H	Dopamine- β -hydroxylase
Gly	Glycogen
Ley	Leydig cell
NFH	Neurofilament-200
NPY	Neuropeptide Y
NSE	Neuron specific enolase
N- D β H	Normal- D β H
N- NFH	Normal- NFH
N- NSE	Normal- NSE
N- SP	Normal- SP
PAS	Periodic acid Schiff reaction
PGP9.5	Protein gene product 9.5
ST	Seminiferous tubule
Sc	Sertoli cell
SP	Substance P

Declarations

Ethical Approval and Consent to participate

All protocols involving the use of animals were performed in accordance with the approved Guidelines for Animal Experiments of Gansu Agricultural University, and were approved by the Animal Experimental Ethical Inspection Committee of Gansu Agricultural University. We have provided an attachment for this statement.

Consent for publication

Not applicable

Availability of supporting data

All data and models generated during the study are available in a repository or online in accordance with funder data retention policies. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

No potential conflicts of interest related to this article were reported. The author acknowledged all the laboratory assistants and technicians who contributed to this study. The authors declare that they have no competing interests.

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Authors' contributions

Ligang Yuan put forward the concept and design research, data analysis and drafting article, edited the manuscript to make it finally approved for submission. Hua Wang, Hongzao Yang, Shaoyu Chen carried out the experiment, provided part of the test results for the manuscript. Dapeng Yang collected samples by surgical operation. Yong Zhang assisted the experimental work. All the authors have approved the final version of the manuscript.

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References

1. Tony P. 60 years of Neuroendocrinology: The hypothalamo-pituitary-gonadal axis. *J Endocrinol* 2015;226:41-54. <https://doi.org/10.1530/JOE-15-0113>
2. Khanezhad M, Abbaszadeh R, Holakuyee M, Modarressi M H, Nourashrafeddin M S. FSH regulates RA signaling to commit spermatogonia into differentiation pathway and meiosis. *Reprod Biol Endocrinol* 2021 19. <https://doi.org/10.1186/s12958-020-00686-w>
3. Davidoff M S, Middendorff R, Müller D, Holstein AF. The Neuroendocrine Leydig Cells and their Stem Cell Progenitors, the Pericytes. *Advances in anatomy, embryology, and cell biology* 2009;1-107.
4. Davidoff MS, Middendorff R, Köföncü E, Müller D, Ježek D, Holstein AF. Leydig cells of the human testis possess astrocyte and oligodendrocyte marker molecules. *Acta Histochem* 2002;104:39-49.

<https://doi.org/10.1078/0065-1281-00630>.

5. Müller D, Davidoff MS, Bargheer O, Paust HJ, Pusch W, Koeva Y, Jezek D, Holstein AF, Middendorff R. The expression of neurotrophins and their receptors in the prenatal and adult human testis: Evidence for functions in Leydig cells. *Histochem Cell Biol* 2006;126:199–211. <https://doi.org/10.1007/s00418-006-0155-8>.
6. Krobert KA, Bach T, Syversveen T, Kvingedal AM, Levy FO. The cloned human 5-HT₇ receptor splice variants: A comparative characterization of their pharmacology, function and distribution. *Naunyn Schmiedeberg's Arch Pharmacol* 2001;363:620–32. <https://doi.org/10.1007/s002100000369>.
7. Suburo AM, Chiocchio SR, Soler MVCC, Nieponice A, Tramezzani JH. Peptidergic Innervation of Blood Vessels and Interstitial Cells in the Testis of the Cat. *J Androl* 2002;23:121–34. <https://doi.org/10.1002/j.1939-4640.2002.tb02605.x>.
8. Wrobel KH, Moustafa MNK. On the innervation of the donkey testis. *Ann Anat* 2000;182:13–22. [https://doi.org/10.1016/S0940-9602\(00\)80116-8](https://doi.org/10.1016/S0940-9602(00)80116-8).
9. Maddocks S, Kern S, Setchell B. Investigating local regulation of the testes of ruminants. *J Reprod Fertil Suppl* 1995;49:309–19. <https://doi.org/10.2307/27800477>.
10. Saleh AMM, Alameldin MA, Abdelmoniem ME, Hassouna EM, Wrobel KH. Immunohistochemical investigations of the autonomous nerve distribution in the testis of the camel (*Camelus dromedarius*). *Ann Anat* 2002;184:209–20. [https://doi.org/10.1016/S0940-9602\(02\)80106-6](https://doi.org/10.1016/S0940-9602(02)80106-6).
11. Gong YG, Feng MM, Hu XN, Wang YQ, Gu M, Zhang W, Ge RS. Peptidergic not monoaminergic fibers profusely innervate the young adult human testis. *J Anat* 2009;214:330–8. <https://doi.org/10.1111/j.1469-7580.2008.01038.x>.
12. Ortega HH, Lorente JA, Salvetti NR. Immunohistochemical study of intermediate filaments and neuroendocrine marker expression in Leydig cells of laboratory rodents. *Anat Histol Embryol* 2004;33:309–15. <https://doi.org/10.1111/j.1439-0264.2004.00559.x>.
13. Huo SY, Xu ZH, Zhang XX, Zhang JF, Cui S. Testicular denervation in prepuberty rat inhibits seminiferous tubule development and spermatogenesis. *J Reprod Develop* 2010;56:370–8. <https://doi.org/10.1262/jrd.10-009N>.
14. Manfredi-Lozano M, Roa J, Tena-Sempere M. Connecting metabolism and gonadal function: Novel central neuropeptide pathways involved in the metabolic control of puberty and fertility. *Front Neuroendocrinol* 2018;48:37–49. <https://doi.org/10.1016/j.yfrne.2017.07.008>.
15. Yuan L, Yan Z, Tao J, Liu Y, Chen G. The distribution of PGP9.5 and NPY in the normal testis and cryptorchidism of Bactrian camel. *Acta Vet Zootech Sin* 2015;46:303–8. <https://doi.org/10.11843/j.issn.0366-6964.2015.02.018>.
16. Yuan L, Lu Y, Tao J, Zhang Y. Comparison of histochemical and ultrastructural characteristics of extracellular matrix components in the scrotal and cryptorchid testes of the Bactrian camel. *Acta Theriol Sin* 2017;37:189–99. <https://doi.org/10.16829/j.slxb.201702010>.
17. Yuan L, Qu Y, Li C, Lu Y, Yan Z. The histologic and ultrastructural characteristics of the Bactrian camel testis in cryptorchidism. *Acta Vet Zootech Sin* 2016;5:993–1000.

<https://doi.org/10.11843/j.issn.0366-6964.2016.05.017>.

18. Culling CFA. Handbook of histopathological and histochemical techniques. 1974.
19. Amadio S, Montilli C, Picconi B, Calabresi P, Volonté C. Mapping P2X and P2Y receptor proteins in striatum and substantia nigra: An immunohistological study. *Purinergic Signal* 2007;3:389–98. <https://doi.org/10.1007/s11302-007-9069-8>.
20. Ali A, Derar R, Al-Sobayil F, Mehana S, Al-Hawas A. Impotentia generandi in male dromedary camels: Clinical findings, semen characteristics, and testicular histopathology. *Theriogenology* 2014;82:890–6. <https://doi.org/10.1016/j.theriogenology.2014.07.001>.
21. Pinart E, Sancho S, Briz MD, Bonet S, Garcia N, Badia E. Ultrastructural study of the boar seminiferous epithelium: Changes in cryptorchidism. *J Morphol* 2000;244:190–202. [https://doi.org/10.1002/\(SICI\)1097-4687\(200006\)244:3<190::AID-JMOR4>3.0.CO;2-B](https://doi.org/10.1002/(SICI)1097-4687(200006)244:3<190::AID-JMOR4>3.0.CO;2-B).
22. Yuan L, Wang H, Wang Q, Li C, Yang D. INSL-3 protein expression in normal and cryptorchid testes of Ziwuling black goats. *Reprod Domest Anim* 2021;56:725–35. <https://doi.org/10.1111/rda.13911>.
23. Zhuang S, Dang Y, Schnellmann RG. Requirement of the epidermal growth factor receptor in renal epithelial cell proliferation and migration. *Am J Physiol Physiol* 2004;287:365–72. <https://doi.org/10.1152/ajprenal.00035.2004>.
24. Chen G, Yuan L, Li C, Yan Z. The histologic characteristics of Yak cryptorchidism. *Acta Vet Zootech Sin* 2015;46:2282–90. <https://doi.org/10.11843/j.issn.0366-6964.2015.12.021>.
25. Novo N, Ferreira P, Medina M. The apoptosis-inducing factor family: Moonlighting proteins in the crosstalk between mitochondria and nuclei. *IUBMB Life* 2020;73:568–81. <https://doi.org/10.1002/iub.2390>.
26. Kudryavtseva A V, Krasnov GS, Dmitriev AA, Alekseev BY, Kardymon OL, Sadritdinova AF, Fedorova M S, Pokrovsky A V, Melnikova N V, Kaprin A D, Moskalev A A, Snezhkina A V. Mitochondrial dysfunction and oxidative stress in aging and cancer. *Oncotarget* 2016;7:44879–905. <https://doi.org/10.18632/oncotarget.9821>.
27. Martin LJ. Cell interactions and genetic regulation that contribute to testicular Leydig cell development and differentiation. *Mol Reprod Dev* 2016;83:470–87. <https://doi.org/10.1002/mrd.22648>.
28. Butler CM, Shaw G, Clark J, Renfree MB. The functional development of Leydig cells in a marsupial. *J Anat* 2007;212:55–66. <https://doi.org/10.1111/j.1469-7580.2007.00837.x>.
29. El-Harairy MA, Attia KA. Effect of age, pubertal stage and season on testosterone concentration in male dromedary camel. *Saudi J Biol Sci* 2010;17:227–30. <https://doi.org/10.1016/j.sjbs.2010.04.006>.
30. Pasha R, Qureshi AS, Lodhi L, Jamil H, Masood A, Hamid S, Iqbal J, Kamran Z, Khamas W. Seasonal changes in the anatomy of testis of one-humped camel (*Camelus dromedarius*). *J Camel Pract Res* 2011;18:145–53. <https://www.researchgate.net/publication/215599135>
31. Curley M, Milne L, Smith S, Jørgensen A, Frederiksen H, Hadoke P, Potter P, Smith LB, Smith L. A young testicular microenvironment protects Leydig cells against age-related dysfunction in a mouse model

- of premature aging. *FASEB J* 2019;33:978–95. <https://doi.org/10.1096/fj.201800612R>.
32. Mularoni V, Esposito V, Di Persio S, Vicini E, Spadetta G, Berloco P, Fanelli F, Mezzullo M, Pagotto U, Pelusi C, Nielsen JE, Rajpert-De Meyts E, Jorgensen N, Jorgensen A, Boitani C. Age-related changes in human Leydig cell status. *Hum Reprod* 2020;35:2663–76. <https://doi.org/10.1093/humrep/deaa271>.
 33. Ezeasor D. Light and electron microscopical observations on the Leydig cells of the scrotal and abdominal testes of naturally unilateral cryptorchid West African dwarf goats. *J Anat*;1985; 141:27–40. PMID: 2867081; PMCID: PMC1166386.
 34. K Buckley; R B Kelly. Identification of a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells. *J Cell Biol*;1985;100:1284–94. <https://doi.org/10.1083/jcb.100.4.1284>
 35. Terzano G.M, Barilen V L, Borghese A. Overview on reproductive endocrine aspects in buffalo. *J Buffalo Science*;2012;1:126–38. <https://doi.org/10.6000/1927-520X.2012.01.02.01>
 36. Abd-Elmaksoud A, Sayed-Ahmed A, Kassab M, Aly K. Histochemical mapping of glycoconjugates in the testis of the one humped camel (*Camelus dromedarius*) during rutting and non-rutting seasons. *Acta Histochem*;2008;110:124–33. <https://doi.org/10.1016/j.acthis.2007.10.004>.
 37. Osman DI, Moniem KA, Tingari MD. Studies on the testis of the camel (*Camelus dromedarius*). III. Histochemical observations. *Histochem J* 1976;8:579–90. <https://doi.org/10.1007/BF01003959>.
 38. Walsh MD, Jass JR. Histologically Based Methods for Detection of Mucin. *Glycoprotein Methods Protoc.*, Totowa, NJ: Humana Press;2000.29–44. <https://doi.org/10.1385/1-59259-048-9:029>.
 39. Moore BC, Hamlin HJ, Botteri NL, Lawler AN, Mathavan KK, Guillette LJ. Posthatching development of alligator mississippiensis ovary and testis. *J Morphol*;2010;271:580–95. <https://doi.org/10.1002/jmor.10818>.
 40. Liu M. Capacitation-Associated Glycocomponents of Mammalian Sperm. *Reprod Sci* 2016;23:572–94. <https://doi.org/10.1177/1933719115602760>.
 41. Moore BW, McGregor D. Chromatographic and electrophoretic fractionation of soluble proteins of brain and liver. *J Biol Chem* 1965;240:1647–53. [https://doi.org/10.1016/S0021-9258\(18\)97483-1](https://doi.org/10.1016/S0021-9258(18)97483-1).
 42. Schmechel D, Marangos PJ, Brightman M. Neurone-specific enolase is a molecular marker for peripheral and central neuroendocrine cells. *Nature*;978;276:834–6. <https://doi.org/10.1038/276834a0>.
 43. Schulze W, Davidoff MS, Ivell R, Holstein AF. Neuron-specific enolase-like immunoreactivity in human Leydig cells. *Andrologia* ;2009;23:279–83. <https://doi.org/10.1111/j.1439-0272.1991.tb02560.x>.
 44. Angelova P, Davidoff M, Baleva K, Staykova M. Substance P- and neuron-specific enolase-like immunoreactivity of rodent Leydig cells in tissue section and cell culture. *Acta Histochem* 1991;91:131–9. [https://doi.org/10.1016/S0065-1281\(11\)80266-7](https://doi.org/10.1016/S0065-1281(11)80266-7).
 45. Lu C, Zhang Z, Jiang Y, Yang Z, Yang Q, Liao D, Bu, H. Primary pure carcinoid tumors of the testis: Clinicopathological and immunophenotypical characteristics of 11 cases. *Oncol Lett* 2015;9:2017–22. <https://doi.org/10.3892/ol.2015.3046>.

46. Owston MA, Ramos-vara JA. Histologic and Immunohistochemical Characterization of a Testicular Mixed Germ Cell Sex Cord-Stromal Tumor and a Leydig Cell Tumor in a Dog. *Vet Pathol* 2007;44:936–43. <https://doi.org/10.1354/vp.44-6-936>.
47. Euler US., Gaddum JH. An unidentified depressor substance in certain tissue extracts. *J Physiol* 1931;72:74–87. <https://doi.org/10.1113/jphysiol.1931.sp002763>.
48. Euler USV. An adrenaline-like action in extracts from the prostatic and related glands. *J Physiol* 1934;81:102–12. <https://doi.org/10.1113/jphysiol.1934.sp003119>.
49. Chiwakata C, Brackmann B, Hunt N, Davidoff M, Schulze W, Ivell R. Tachykinin (Substance-P) Gene Expression in Leydig Cells of the Human and Mouse Testis. *Endocrinology* 1991;128:2441–8. <https://doi.org/10.1210/endo-128-5-2441>.
50. Blasco V, Pinto FM, González-Ravina C, Santamaría-López E, Cadenas L, Fernández-Sánchez M. Tachykinins and Kisspeptins in the Regulation of Human Male Fertility. *J Clin Med* 2019;9:113. <https://doi.org/10.3390/jcm9010113>.
51. Tanyel FC, Ertunç M, Büyükpamukçu N, Onur R. Mechanisms involved in contractile differences among cremaster muscles according to localization of testis. *J Pediatr Surg* 2001;36:1551–60. <https://doi.org/10.1053/jpsu.2001.27042>.
52. Laser-Azogui A, Kornreich M, Malka-Gibor E, Beck R. Neurofilament assembly and function during neuronal development. *Curr Opin Cell Biol* 2015;32:92–101. <https://doi.org/10.1016/j.ceb.2015.01.003>.
53. Yuan A, Rao M V., Veeranna, Nixon RA. Neurofilaments and Neurofilament Proteins in Health and Disease. *Cold Spring Harb Perspect Biol* 2017;9:a018309. <https://doi.org/10.1101/cshperspect.a018309>.
54. Davidoff MS, Middendorff R, Pusch W, Müller D, Wichers S, Holstein AF. Sertoli and Leydig cells of the human testis express neurofilament triplet proteins. *Histochem Cell Biol* 1999;111:173–87. <https://doi.org/10.1007/s004180050347>.
55. Frungieri MB, Urbanski HF, Höhne-Zell B, Mayerhofer A. Neuronal Elements in the Testis of the Rhesus Monkey: Ontogeny, Characterization and Relationship to Testicular Cells. *Neuroendocrinology* 2000;71:43–50. <https://doi.org/10.1159/000054519>.
56. Mayerhofer A, Frungieri M, Fritz S, Bulling A, Jessberger B, Vogt H. Evidence for catecholaminergic, neuronlike cells in the adult human testis: changes associated with testicular pathologies. *J Androl* 1999;20:341–7. <https://doi.org/10.1002/j.1939-4640.1999.tb02527.x>.
57. O'Shaughnessy PJ, Morris ID, Baker PJ. Leydig cell re-generation and expression of cell signaling molecules in the germ cell-free testis. *Reproduction* 2008;135:851–8. <https://doi.org/10.1530/REP-07-0529>.
58. Kaleczyc J, Kasica-Jarosz N, Pidsudko Z, Przyborowska A, Sienkiewicz W. The expression of androgen receptor in neurons of the anterior pelvic ganglion and celiac-superior mesenteric ganglion in the male pig. *Pol J Vet Sci* 2019;22:151–9. <https://doi.org/10.24425/pjvs.2018.125609>.

59. Davidoff MS, Ungefroren H, Middendorff R, Koeva Y, Bakalska M, Atanassova N, Holstein A F, Jezek D, Pusch W, Müller D. Catecholamine-synthesizing enzymes in the adult and prenatal human testis. *Histochem Cell Biol* 2005;124:313–23. <https://doi.org/10.1007/s00418-005-0024-x>.
60. Romeo R, Pellitteri R, Russo A, Marcello M F. Catecholaminergic phenotype of human Leydig cells. *Archivio italiano di anatomia ed embriologia* 2004;109:45-54. PMID:15141475.
61. Middendorff R, Davidoff M, Holstein AF. Neuroendocrine marker substances in human Leydig cells - changes by disturbances of testicular function. *Andrologia* 2009;25:257–62. <https://doi.org/10.1111/j.1439-0272.1993.tb02722.x>.
62. Barrionuevo F, Burgos M, Jiménez R. Origin and function of embryonic Sertoli cells. *Biomol Concepts* 2011;2:537–47. <https://doi.org/10.1515/BMC.2011.044>.
63. Yue F, Cui L, Johkura K, Ogiwara N, Sasaki K. Induction of midbrain dopaminergic neurons from primate embryonic stem cells by coculture with sertoli cells. *Stem Cells* 2006;24:1695–706. <https://doi.org/10.1634/stemcells.2005-0409>.
64. Procópio MS, de Avelar GF, Costa GMJ, Lacerda SMSN, Resende RR, de França LR. MicroRNAs in Sertoli cells: implications for spermatogenesis and fertility. *Cell Tissue Res* 2017;370:335–46. <https://doi.org/10.1007/s00441-017-2667-z>.
65. Mäkelä J-A, Koskenniemi JJ, Virtanen HE, Toppari J. Testis Development. *Endocr Rev* 2019;40:857–905. <https://doi.org/10.1210/er.2018-00140>.
66. Zivkovic D, Hadziselimovic F. Development of Sertoli Cells during Mini-Puberty in Normal and Cryptorchid Testes. *Urol Int* 2009;82:89–91. <https://doi.org/10.1159/000176032>.
67. Hamdi SM, Almont T, Galinier P, Mieusset R, Thonneau P. Altered secretion of Sertoli cells hormones in 2-year-old prepubertal cryptorchid boys: a cross-sectional study. *Andrology* 2017;5:783–9. <https://doi.org/10.1111/andr.12373>.

Tables

Table 1. The characteristics index of the leydig cells in Bactrian camel normal testis and cryptorchidism
 mean ± SD n=40

Group	The transverse diameter of the leydig cell nucleusµm	The Longitudinal diameter of the leydig cell nucleusµm	Average area of leydig cells nucleus(µm ²)
Normal group	12.217±2.745	12.864±2.282	124.428±18.539
Cryptorchid group	9.612±2.263*	10.156±2.689*	96.703±14.533**

Significant differences with normal group, **p*<0.05, ** *p*<0.01.

Table 2. Expression intensity of NFH, SP, NSE and D β H in different cells of the normal and cryptorchidism of Bactrian camel testis

Group	Sertoli cells	Spermatocytes	Leydig cells	Peritubular myoid cells	Endothelial cells
NSE-Normal group	++	+	++++	++++	++++
NSE-Cryptorchid group	+	+	++	++	+++
SP-Normal group	+	□	+++	++	++
SP-Cryptorchid group	+	+ -	++	□	□
NFH-Normal group	□	□	□	□	++++
NFH-Cryptorchid group	+	++	+	+	+++
D β H-Normal group	++++	+	□	+++	+++
D β H-Cryptorchid group	+++	++	□	□	+++

The intensity of immunofluorescence results was scored as follow: -: no positive expression; +/-: occasional positive expression; +: positive expression; ++: medium-intensity positive expression; +++: strong positive expression; ++++: high-density strong positive expression[19].

Figures

Figure 1

Comparison of the histochemical characters in normal and cryptorchid testes of Bactrian camels a-c: Normal Bactrian camels; d-f: Cryptorchid Bactrian camels; (a,d): stained with H&E; (b,e): stained with PAS; (c,d): stained with AB. a: Revealed that the seminiferous epithelium of Bactrian camels was developing well in the normal group; b: The glycogen was detected by PAS staining and distinct purple-red glycogen-positive band in the lamina propria and interstitial capillary walls; (c) The positive reaction of AB staining

were present clearly throughout the basement membranes and the wall of interstitial blood vessels in the normal testes; (d)The cryptorchidism causes a reduction in layers of spermatogenic epithelium; (e)Decreased PAS positive in the basement membrane of the seminiferous tubule; (f)The positive reaction of AB was almost invisible in the interstitial tissue of the cryptorchid group. Acidic mucoprotein(Am); Blood capillary(BC); Glycogen(Gly); Leydig cell(Ley); Small artery(SA); Sertoli cells(Sc); Seminiferous tubule(ST); scale bar=20μm

Figure 2

The ultrastructure of Leydig cell in the normal and cryptorchid testes of Bactrian camels a-b: Normal Bactrian camels; c-d: Cryptorchid Bactrian camels; (a) Elaborately developed organelles of the Leydig cells in the normal testis; (b) The Leydig cells and blood capillaries in the interstitial tissue of the normal group; (c) The macrophages were always found around the Leydig cells in cryptorchidism; (d) The epithelial basal lamina of the capillary vessel was clear and scattered with microfine-cytotic vesicles in cytoplasm. Collagen fiber(CF); Erythrocyte(E); Endothelial cells(Ec); Golgi apparatus(G); Leydig cell (Ley); lysosomes(Ly); Macrophages(M); Mitochondrion(Mi); Lipid droplet(Li); Microparticles(MP); Pericyte(P); Pseudopod(Ps); Vacuole(V). scale bar=2 μ m

Figure 3

Distribution(A,B) and protein (C,D) levels of NSE, SP, NFH and DβH levels in normal and cryptorchid testes of Bactrian camels. (A) Tissue sections were stained with specific antibody and counterstained with hematoxylin. Positive signals appeared brown in color, and counterstaining background appeared blue in color. a-e: normal testicular tissues; f-j: cryptorchid testicular tissues; a and f: stained with NSE polyclonal antibody; b and g: stained with SP polyclonal antibody; c and h: stained with NFH polyclonal antibody; d

and i: stained with D β H polyclonal antibody; e and j: negative control (no significant immunoreactivity was observed when normal rabbit serum instead of the primary antibody); Leydig cells(Ley); Sertoli cells(Sc); Seminiferous tubule(ST). scale bar=20 μ m. (B)Average absorbance of neuroendocrine markers (NSE, SP, NFH and D β H) in normal and cryptorchid testicular tissues of Bactrian camels. Data are expressed as fold change. The data are presented as mean $\pm$ SD. value from 40 statistical figures. Different letters indicate significant differences of the expressions in the testes of the Bactrian camel cryptorchidism compared with the normal groups (*p<0.05,** p<0.01). (C and D)Relative protein levels of NSE, SP, NFH and D β H in normal and cryptorchid testes of Bactrian camels.The density of β -actin was taken as the base value,and the relative densities were obtained by comparing the densities of NSE, SP, NFH or D β H expression bands in the normal testis group(Simply marked as N-NSE, N-SP, N-NFH or N-D β H) and the cryptorchidism group(Simply marked as C-NSE, C-SP, C-NFH or C-D β H). Data are expressed as fold change. The data are presented as mean $\pm$ S.E.M. value from 12 statistical figures. Different letters indicate significant differences in the expressions of same neuroendocrine marker between the groups (** p<0.01).

Figure 4

Localization of NSE, SP, NFH and DβH in the normal and cryptorchid testes of Bactrian camels. Green color: immunofluorescence representing the reaction of antibodies with antigens; a-d: Normal Bactrian camels; e-h: Cryptorchid Bactrian camels; (a,e): stained with NSE polyclonal antibody; (b,f): stained with SP polyclonal antibody; (c,g): stained with NFH; (d,h)stained with DβH polyclonal antibody; Blood

capillary(BC); Leydig cells(Ley); Myoid cells(Mc); Sertoli cells(Sc); Spermatocytes(Sp); Seminiferous tubule(ST). scale bar=20µm

Supplementary Files

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