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HISTOCHEMICAL AND IMMUNOHISTOCHEMICAL STUDIES ON THE TESTES OF THE BACTRIAN CAMEL (*Camelus bactrianus*)

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ABSTRACT

The characteristics of the testes in the bactrian camel was investigated with histochemical and immunohistochemical methods. The results showed that the testes, which were enveloped by thick tunica albuginea stretching into the testicular interstitium. The testes were filled with the interstitial tissue and seminiferous tubules. The interstitium bear abundant vessels, collagenous and reticular fibres and interstitial cells including Leydig cells stained with inhomogeneous fluorescence by Hoechst 33412. Nerve bundles revealed by the antibody to neurofilament (NF) 200. These were primarily distributed in the capsule near the vessels in the testes. It was suggested that abundant vessels and nerve bundles play an important role in controlling the blood supply and temperature of the testes to regulate the function of the organ. NF200 was also expressed in the cytoplasm of the Leydig and Sertoli cells. The pattern of NF200 expression in Sertoli cells was accompanied with the different stages of the spermatogenesis in the bactrian camel.

Key words: Bactrian camel, neurofilament, testes

The bactrian camel, one of the most important animals in the desert and semi-desert areas of the world, is getting less and less in their grazing districts (McCarthy, 2000; Niasari-Naslaji, 2008) because of the intrinsic factors, such as low reproductivity and extrinsic threatens such as slaughter and diminishing rangeland, especially to wild camels (Ibrahim and AR, 2008; Reading *et al,* 1999). Maintenance of a high level of reproduction is essential not only for profitable production, but also for maximum opportunity for selection of the species. Thus, the studies on the camel reproduction are extremely important for the conservation of this species.

The reproductive gonads in male is the testes in which the paternal germ cells generate. Various investigators have studied the testes of camels (Abdel-Raouf *et al,* 1975; Osman and el-Azab, 1974; Osman *et al,* 1979; Tingari *et al,* 1984). Testes of dromedary and bactrian camel resemble morphologically (Hafez and Hafez, 2001; Skidmore, 2004). However, the fine structures of the bactrian camel testes have not been studied completely. Therefore, the present study was conducted with histochemical and immuno-histochemical methods to investigate the construction of the testes in the bactrian camel and to acquire the information contributing to improve the reproduction and conservation of the species.

Materials and Methods

Eight testes were collected from 5-7 years old bactrian camels immediately after slaughter in winter in the slaughterhouse of the Right Alasan Banner Food Company in Inner Mongolia an autonomous region of China. The animals apparently did not show any disease. Testes were immersed in 10% neutral formalin for 2 weeks. Blocks about $1cm^3$ volumes were sliced from different regions of the testes and were fixed in neutral formalin. Blocks were later dehydrated and embedded in paraffin wax. Sections (6µm thick) were mounted on the 3-aminopropyltriethoxysilane (APES)-coated slides and kept in 37°C in oven for 2 days. All sections were dewaxed and rehydrated before histochemical or immunohistochemical processes (Hematoxylin and Eosin stain not depicted).

Mallory stain

Sections were prestained with 0.5% acid fuchsin for 5 min after the slides were handled in 5% potassium dichromate for 30 minutes. These were rinsed in distilled water; stained with aniline blue-orange G solution (0.5% aniline blue, 2% orange G, 1% phosphotungstic acid) for 1 hour and then douched with 80% ethanol. The collagenous (C) and reticular (R) fibres were blue, commonly with crocus elastic (E)

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Fig 1. **(A)** the mediastinum with vessels (asterisk and arrow) spread into the interstitium, HE stain; **(B)** the tubules with lumens (asterisk) occupied the majority of the testes, HE stain; **(C)** a binucleated Leydig cell (arrow), hematoxylin stain; **(D)** the connective tissues with multilaminar fibres (blue) surrounded vessels (V) with blood cells (orange) and enveloped tubules (T). Sertoli cells (arrow, orange red) located on the base of the tubules, Mallory stain. Bar=100μm.

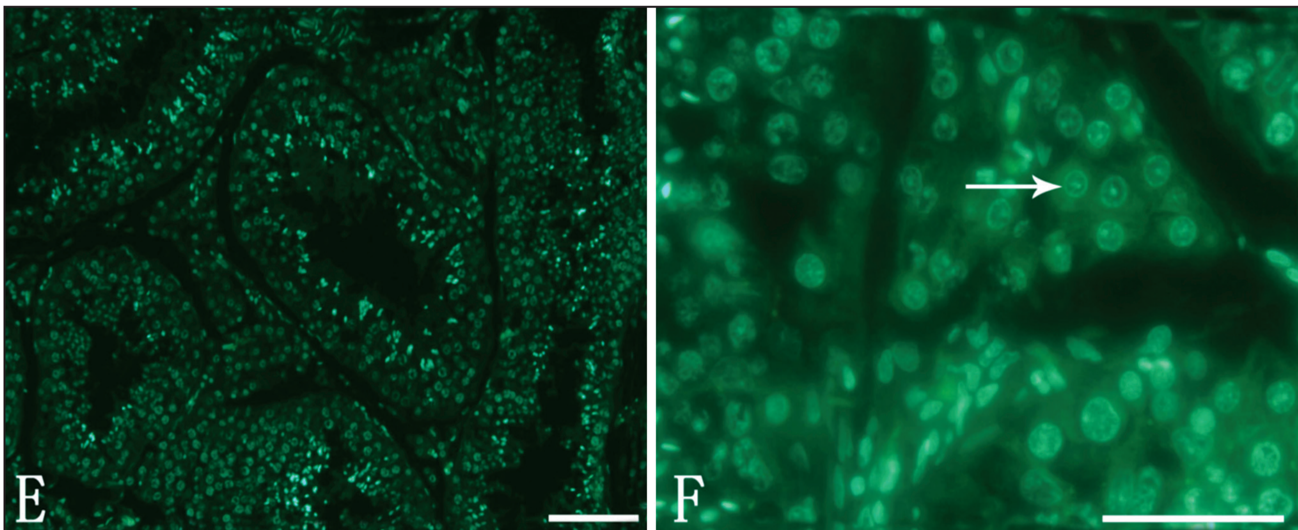

Fig 2. **(E)** sperms were illuminated by bright fluorescence of Hoechst 33342; **(F)** Leydig cells (arrow) showed inhomogenous stain by Hoechst 33342 with a circle and a condensated spot near the centre of the nucleus. Bar=100μm.

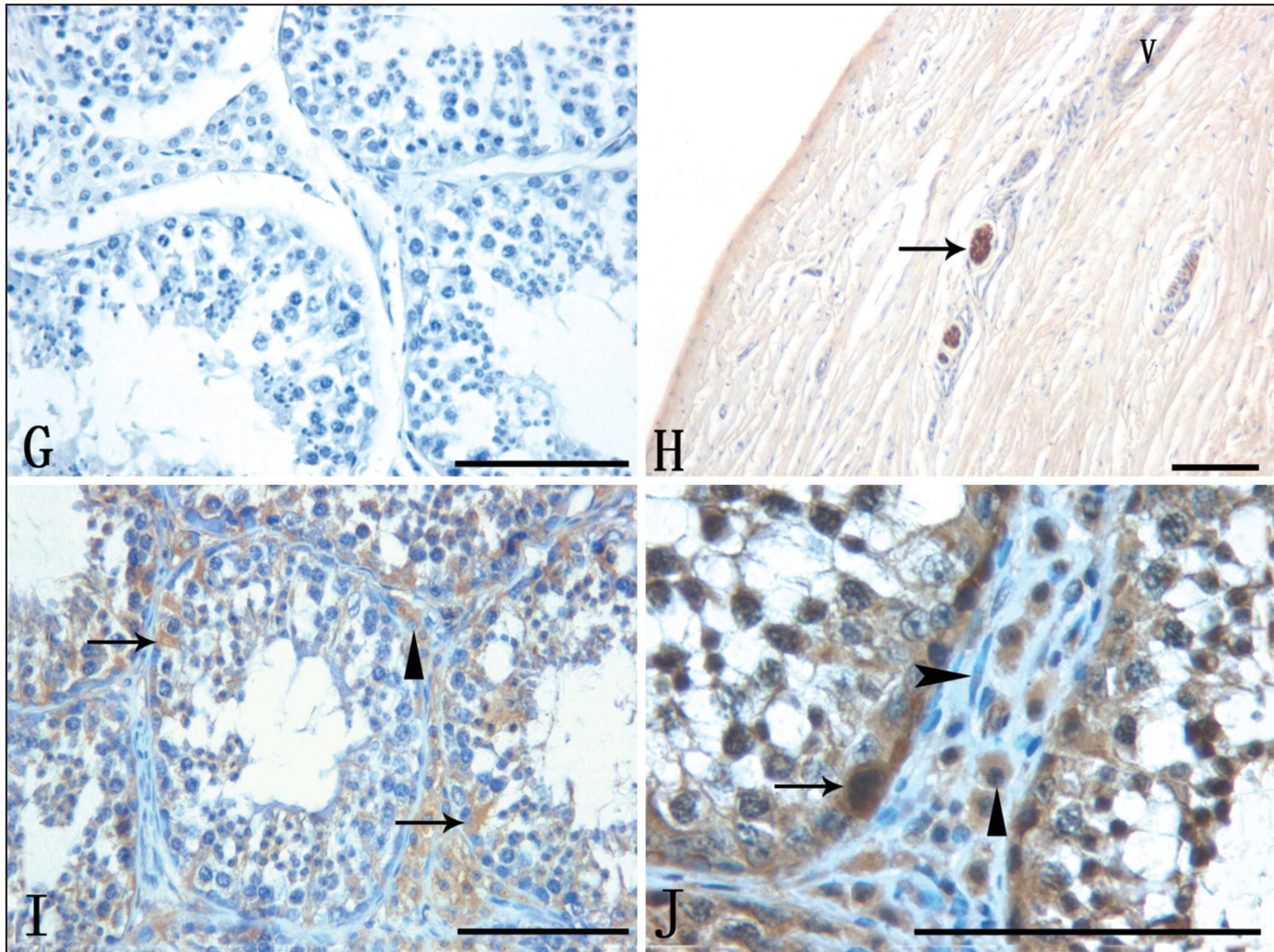

Fig 3. **(G)** the negative control for NF200 didn't show any positive stain; **(H)** nerve bundles (arrow) showed by the antibody to NF200 in the capsule near the vessels (V); **(I, J)** the immunoreactivity of NF200 was detected in the cytoplasm of Leydig (upward triangle) and Sertoli cells (arrow) but not fusiform myoid cells (arrowhead). The positive stain was mainly around the nucleus of the Sertoli cells in **J**, while it flamed towards the lumen and adhered the spermatocytes in **I**. Bar=100µm.

fibres, pink muscle fibres and orange blood cells (Rui *et al*, 1982) after Mallory stain.

Hoechst stain

Sections were covered with Hoechst 33342 (Sigma, 10µg/µl) in phosphate buffered saline (PBS, 0.01M, pH 7.4) in dark for 30min at room temperature. After rinsed in PBS, sections were coverslipped with 75% glycerol in PBS and visualised under Leica fluorescence microscopy. Apoptosis of the cell was identified by the condensation and fragment of the nucleus with bright fluorescence (Ilic *et al*, 1998; Susin *et al*, 2000).

Immunohistochemistry

The expression of neurofilament 200 (NF200) was detected with polyclonal antibody (Sigma, N4142). Antigen retrieval was performed by microwaving. Sections were dipped in 3% H_2O_2 in PBS for 30 minutes to remove the activity of endogenous peroxidase followed by washing with PBS. Non-specific binding was blocked with 10% normal goat serum in PBS for 1 hour at room temperature. The sections were incubated with the primary antibody anti-NF200 (1:200 diluted in PBS) overnight at 4°C followed by washing, and then incubated with biotinylated goat anti-rabbit IgG (Zhongshan, Beijing, China, 1:150) for 3 hours at RT. Subsequently, the sections were rinsed in PBS and incubated with streptavidin-labeled peroxidase complex (Zhongshan, 1:150) for 3 hours at room temperature. The peroxidase was visualised with fresh diaminobenzidine (DAB, Sigma, 0.5 mg/ml in PB, containing 0.03% hydrogen peroxide). Sections were counterstained with haematoxylin. The primary antibody was omitted or replaced by PBS in negative control. The mouse brain was used for the positive control.

After staining, all sections except for Hoechst stain were dehydrated in gradient ethanol, hyalinised in xylene, coverslipped with neutral gummi and then visualised under Leica microscopy.

Seminiferous tubule diameter (STD) Analysis

After visualisation, STD was measured in 20 cross sections of tubules of each camel with Leica Qwin Image Processing and Analysis Software. The data was processed in Microsoft Excel and the value were recorded as mean ± standard deviation (SD).

Results

Morphology and STD

Haematoxylin and Eosin (or only H) stains showed the morphology of the testes in bactrian camel, which were enveloped by thick tunica albuginea and filled with the interstitial tissue and seminiferous tubules. The tunica with vessels stretched into the interstitium and formed the mediastinum spreading around the septulum (Fig 1A). In the superficial layer of the testes, the interstitial tissue had tight contact with seminiferous tubules, while the deep tissue was rather loose. Seminiferous tubules were composed of Sertoli cells and germ cells and fistular lumens and took up the majority of the space in the testes (Fig 1B). The nuclei of spermatogenic cells were dispersed and the lumen of tubule was large with few mature sperms. The tubules were wrapped by long fusiform peritubular myoid cells. Among the tubules were trigonal or polygonal districts dwelled by Leydig and other interstitial cells accompanied with microvessels and connective fibres. Small number of interstitial cells were diffused in the mediastinum. The occasional binucleated Leydig cells were observed in interstitial zone (Fig 1C). The data showed that STD in bactrian camel was 172.75±18.38μm.

Mallory stain

The testicular interstitium was filled with blue thin multilaminar fibres while crocus fibres were scarcely distributed. The vessels with orange blood cells were surrounded by blue fibres. All of the tubules were enveloped by a thin layer of the blue fibres. Most cells were stained blue-purple. However, Sertoli cells surprisingly exhibited orange red colour (Fig 1D).

Hoechst stain

The morphology of the testes was illuminated by Hoechst 33342. The fluorescence of sperms was rather bright than other cells (Fig 2E). The lumens of some but not all tubules were filled with sperms. It is strange that all of the Leydig cells seemed to be with fragment nucleotide adhering to the circular nuclear membrane and a condensed point near the centre of the nucleus (Fig 2F).

Immunohistochemistry

Nerve bundles showed by the antibody to NF200 existed in the capsule near the vessels (Fig 3H) while no positive bundles or fibres were found in the interstitium and seminiferous tubules. The immunoreactivity of NF200 was also detected in interstitial cells and seminiferous tubules in the testes. Moreover, the positive stain was exhibited in the cytoplasm of the big round Leydig cells, but not the fusiform myoid cells. The pattern of NF200 expression in Sertoli cells was different in tubules. The immunoreactivity of NF200 was mainly around the nucleus of the Sertoli cells in tubules which bore more spermatids and less spermatocytes, while the positive stain flamed towards the lumen and adhered the spermatocytes in tubules with less spermatids and more spermatocytes (Fig 3I,J). The negative control did not show any immunoreactivity (Fig 3H).

Discussion

The testicular morphology of the bactrian camel resembled that of other vertebrates (Bronson *et al,* 1994). The intratesticular construction showed rare differences between the one- and two-humped camel (Osman *et al,* 1976) except that STD of the latter in this study was slightly larger than that about 170.0μm than the former in the same season (Tingari *et al,* 1984). The camels collected in present study were in non-rutting season since the animals did not show any breeding behaviour (Hafez and Hafez, 2001) before slaughter and seminiferous tubules were with few mature sperms in the lumen. Tingari *et al* (1984) have reported the binucleated Leydig cell in testes of dromedary camel (Tingari *et al,* 1984), which had been demonstrated in present study. Since adult Leydig cells rarely divide (Moore *et al,* 1992), these cells may possess high activity with 2 copies of the template for transcription.

The connective fibres are primarily composed of collagenous, reticular and elastic fibres. Collagenous fibres, principal structural elements of extracellular matrix, played a dominant role in maintaining the biological and structural integrity of various tissues and organs (Kolacna *et al,* 2007). They are tough and endure the tension, while elastic fibres are resilient and endows the organ with the variability. The reticular fibres usually form the basal lamina between tissues (Zou, 2004). Thus, the connective fibres in testicular

interstitium of bactrian camel are mainly blue C fibres while those around the tubules contain R fibres. Testes of the bactrian camel with mallory stain showed gross interstitial tissue with abundant vessels and connective fibres, which may contribute to the large compacted construction of this animal's "big" testes. Incidentally, Sertoli cells are easily distinguished in Mallory-stained sections since they clearly exhibited orange red colour different with the cells nearby.

Various researchers have studied the apoptosis of germ cells in mammalian testes, which occurs in either normal or diseased individuals, changes in the different stages of the ontogeny and is controlled by hormones (Billig *et al,* 1995; Mori *et al,* 1997; Sinha and Swerdloff, 1999). The bright fluorescence of the nucleus stained by Hoechst commonly represents wither of the cell (Ilic *et al,* 1998; Susin *et al,* 2000). However, the Hoechst phenotype of camel sperms should result from the condensation of the chromatin but not the sign of nuclear pyknosis. Likewise, the inhomogenous fluorescence of almost all the Leydig cells in camel testes should just be their intrinsic nature because the animal did not show any testicular disease before the slaughter. Anyway, apoptosis in camel testes and the morphology of the Leydig nucleus should be investigated with other approaches.

NF200 has been used as a marker of neuron and nerve fibres for long (Anesetti *et al,* 2001; Dees *et al,* 1995; Kimaro and Madekurozwa, 2006). Nerve fibres revealed by this polypeptide in the testes of the bactrian camel were commonly distributed in the capsule near the vessels like that of other vertebrates including dromedary camel, in which nerve fibres have been manifested by various pan-neuronal markers (Rauchenwald *et al,* 1995; Saleh *et al,* 2002; Wrobel and Abu-Ghali, 1997). Saleh *et al* (2002) have reported that an inverse relation between nervous and endocrine activities with a high 3b-hydroxysteroid dehydrogenase (3b-HStDH) activity in Leydig cells and a regression of intertubular innervation during winter while a weak or absent 3b-HStDH activity and an increase in intertubular nerve fibres in summer in the testes of dromedary camel (Saleh *et al,* 2002). Nerve fibres penetrating into the Leydig cells in bactrian camel were not seen, which might result from the regression of the intratesticular innervation in winter.

The testicular innervation of the vertebrates played an important part in the development and function of the testes (Bronson *et al,* 1994), which controls blood flow of the vessels and the temperature of the testes to influence the secretion of testosterone (Chiocchio *et al,* 1999) and spermatogenesis (Thonneau *et al,* 1998). Thus, it was suggested that the reproduction of the bactrian camel would be modulated directly or indirectly by the innervation of the testes.

Recently, NF200 and other 2 neurofilament proteins have been reported to express in human and rodent Leydig and Sertoli cells (Davidoff *et al,* 1999; Davidoff *et al,* 1993). Saleh *et al* (2002) didn't mention the expression of NF200 in testicular somatic cells in dromedary camel. However, the present study showed that this polypeptide exists in Leydig and Sertoli cells in the bactrian camel testes. Leydig cells have been speculated to originate from the neuroectoderm because of the expression of several neuroendocrine molecules (Davidoff *et al,* 2004). However, the role of NF in Leydig cells should further be confirmed.

It was reported that the variation of NF expression in Sertoli cells correlates to the on-arrest-shift of spermatogenesis and impaired spermatogenesis (Davidoff *et al,* 1999). In this study, we found that the pattern of NF200 expression in sertoli cells from bactrian camel testes were associated with the spermatic development either. It was suggested that this pattern conserved in the vertebrates and the disturbance of the variation of NF200 expression in sertoli cells may result in or from the dysfunction of the male gonads.

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