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Histology and Immuno-reactivity of S-100 Protein in Cranial Cervical Ganglia of Camels (*Camelus dromedarius*)

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ABSTRACT

The cranial cervical ganglion (CCG) is a part of the cervical sympathetic chain located in the head and neck where it provides sympathetic input to their structures. In addition, CCG is associated with some neuropathies including Horner's syndrome. S-100 is a biologically active protein that is initially detected in peripheral nerves and glial cells. Its immunoreaction in mammalian peripheral nervous system has been thoroughly illustrated. Similar data, however, seem to be lacking regarding dromedary camels. This study aimed to describe the histological structure and S-100 immuno-reactivity of dromedary camel CCG using conventionally processed microscopic slides. The CCG was covered by a dense capsule of connective tissue which sent connective tissue septa to form ganglionic units; the units mainly contained principal ganglion neurons (PGNs), satellite glial cells (SGCs), and small intensely fluorescent cells (SIFCs). Myelinated and non-myelinated nerve fibers, Schwann cells, collagen fibers, fibroblasts, and blood capillaries were also present between the ganglionic units. Small-sized, medium-sized, and large-sized PGNs were detected with significantly different diameter lengths ($P<0.05$). S-100 immuno-staining was negative in the connective tissue septa, ganglionic connective tissue capsule, fibroblasts, blood vessels, and SIFCs. PGNs were S-100 positive with some neurons exhibiting negative to weak reactions. S-100 immunoreactivity was present in the glial capsules, SGCs, myelinated axons, and Schwann cells. In conclusion, as in other mammals, the ganglionic units of dromedary camel CCG are mainly formed of PGNs, glial cells, and SIFCs comprising the ganglion's morphological triads. Further, the study indicated the immuno-reactivity of S-100 protein both in neuronal and glial elements of camel CCG.

Key words: Cranial cervical ganglia, Dromedary camel, Histology, S-100 protein expression, Animal Tissue

INTRODUCTION

The cranial and caudal cervical ganglia that form part of the cervical sympathetic chain are found ventral to the transverse processes of the cervical vertebrae, dorsal to the vagus nerve in the neck region (Özgel et al. 2004; Kabak 2007). The postganglionic nerve fibers arising from the neurons of cranial cervical ganglion (CCG) transfer sympathetic nerve supply to the head (Ladizesky et al. 2000; Hayakawa et al. 2000). The morphology of the CCG in the different mammalian species including dromedary camels has been extensively investigated (Kiran 2002; Gagliardo et al. 2003; Fioretto et al. 2007; Eroschenko 2008; Nourinezhad et al. 2015; Abumandour and El-defrawy 2016; Bamohabat et al. 2018). These studies show that the CCG is formed of ganglionic units containing principal ganglion neurons (PGNs), satellite glial cells (SGCs), small intensely fluorescent cells (SIFCs) and nerve fibers.

S-100 proteins belong to the family of calcium-binding proteins which are soluble in a 100% saturated solutions with ammonium sulphates at neutral pH (Sedaghat and Notopoulos 2008). The calcium-binding protein family has comprised twenty-four proteins sub-divided into three main sub-groups: those exert intracellular regulatory effects, those that mainly exert extracellular regulatory

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functions and those exert intracellular and extracellular functions (Donato et al. 2013). Although S-100 proteins were initially discovered in peripheral nerves and glial cells and considered specific to the nervous system, they have been detected in other mammalian organs (Amselgruber et al. 1992; Alkafafy et al. 2011; Ibrahim 2015; Ibrahim et al. 2017; Ibrahim et al. 2020). S-100 proteins are biologically active proteins because they contribute to the regulation of cellular growth, cellular differentiation, calcium homeostasis, enzymatic activities, protein phosphorylation, transcription factors, the inflammatory response and the dynamics of cytoskeletal contents (Donato et al. 2013, Wolf et al. 2008; D'Ambrosi et al. 2021). In the nervous system, S100 protein levels in serum and cerebrospinal fluid are a useful marker of CNS damage and tumor (De Vries et al. 2001; Elting et al. 2000; Mrak and Griffin 2001; Pemberton and Brew 2001; Aghdam et al. 2019; Jaiswal et al. 2023; Tomalty et al. 2023). Contribution of S-100 members to gastric cancer progression with possible development of S-100 targeted reagents for treating gastric cancer has also been reported (Wang et al. 2019). S-100 protein was immune-localized in the different cell types of the central (Henry et al. 2012; Lunde et al. 2014), peripheral (Cocchia and Michetti 1981; Vega et al. 1991; Gonzalez-Martinez et al 2003) and enteric (Chen et al. 2009) nervous system. However, no similar data are available in the literature reviewed regarding the immuno-reactivity of S-100 proteins in the nervous system of camels. The current study, therefore, aimed to elucidate the immunoreactivity of this biologically active S-100 protein in the CCG of the dromedary camel. A complementary histological appearance has also been shown. Moreover, structural and immunohistochemical analysis of CCG in dromedary camels could be helpful as a source of basic information about the autonomic nervous system between animal species.

MATERIALS AND METHODS

The study experiments were approved by the Institutional Animal Ethics Committee, Sudan University of Science and Technology. All regulations of animal welfare were maintained during animal handling, transport, and slaughter.

Twenty-eight CCG were carefully dissected and separated from fourteen heads of adult dromedary camels; the camels which were slaughtered at Al-Salam slaughterhouse, Sudan, appeared healthy and were of both sexes (9 males and 5 females) and different ages (5-15 years). The separated ganglia were transversely cut into six equal pieces (1cm thick) and quickly fixed using 10% buffered formaldehyde. The fixed specimens were routinely processed (Culling 1975). Samples were dehydrated by ethyl alcohol using ascending grades protocol, cleared using xylene, and blocked in paraffin wax. After that, 5µm thick sections of the processed samples were cut using a microtome. The cut samples were stretched on gelatin-coated slides for further processing. For histological investigation, the slides were stained using Masson's trichrome, hematoxylin and eosin (H&E), toluidine blue, and Luxol fast blue with cresyl violet (Culling 1975).

For histometric measurements, neuron diameters from the left and right CCG of five camels were measured using H&E-stained sections. For each animal, three sections from different parts of each ganglion were selected and the diameter of ten neurons of different sizes were measured using ocular micrometer lens X6, Olympus microscope (CH20-Japan). The mean value for each measurement was calculated, and data were analyzed using Student's t-test and the difference was considered statistically significant at $P<0.05$.

For immunoreactivity of S-100 protein, dewaxed sections were hydrated using descending grades of ethyl alcohol and then rinsed in distilled water. Blocking of endogenous peroxidase was performed by rinsing sections in 3% hydrogen peroxide in absolute methyl alcohol for 15 minutes and then quickly rinsed 3 times in distilled water. Antigen retrieval was done by immersing sections in citrate buffer (pH 6) using a plastic container heated in a microwave for 14 minutes and then washed by distilled water. Using normal horse serum (dilution 1:10) for antibody non-specific protein binding was blocked by rinsing sections for 20 minutes at room temperature. After that sections were incubated for 40 minutes, at 1:2000 dilutions, with rabbit polyclonal anti-S-100 primary antibody (Z0311, Dako, Glostrup, Denmark). This antibody is known to cross-react with S-100 protein in different species such as rat, mouse, horse, swine, dromedary camel and cat (Alkafafy et al. 2011; Ibrahim 2015; Ibrahim et al. 2017). Then, sections were washed in diluted buffer for 10 minutes and incubated in Dako HRP/DAB Envision kit (DAKO, K5007, Glostrup, Denmark) as described by instructors. Sections were finally counterstained using haematoxylin, dehydrated in ascending ethyl alcohol up to 100% and cleared in xylene. Negative control immunostaining was performed by omitting the S-100 primary antibody, whereas, positive control immuno-reactivity was carried out according to the manufacturer's instruction. A light microscope (Olympus, Tokyo, Japan) was used to examine the glandular histological structure and S-100 immunoreaction. The intensity of the S-100 immunoreactivity was quantified using ImageJ software (can be downloaded from http://fiji.sc).

RESULTS

Histological Structure of Dromedary CCG

CCG was surrounded by a dense capsule of connective tissue from which originated connective tissue septa; the capsule and septa were mainly formed of dense collagenous fibers, nerve fibers, and blood vessels surrounding a number of ganglionic units that contained PGNs of different sizes (Fig. 1A, B). The analysis of histometric data revealed that the mean diameters of large-sized PGNs (251.41±29.64µm), medium-sized (126.27±6.06 m) and small-sized (78.01±4.49µm) neurons were significantly different as compared to each other. In addition to PGNs the ganglonic units mainly contained SGCs and SIFCs forming the structural triad of the CCG; scattered fibroblasts, blood vessels and myelinated and non-myelinated nerve fibers associated with Schwann cells were present in the ganglionic units (Fig. 1C, D). PGNs were oval to spindle-shaped and multipolar neurons with

cytoplasmic processes and granules of Nissl's substance; their nuclei were large and light-stained with eccentric nuclei which contained dispersed chromatin and prominent nucleoli (Fig. 1C). Around each PGN there were SGCs which were small and oval or cuboidal in shape and had light-stained cytoplasm; their capsule and processes together with the collagen fibers and blood capillaries surrounded the PGNs; each PGN and its glial capsule formed a distinct ganglionic sub-unit which was isolated from other sub-units (Fig. 1C). SIFCs were smaller than PGNs and were spherical or polyhedral in shape; their central nuclei were dark and large, and their small cytoplasm was clear and Nissl's bodies-free (Fig. 1D).

Generally, the ganglionic connective tissue capsule and septa, fibroblasts, connective tissue septa and blood vessels showed negative reaction to S-100 (Fig. 2A). While some PGNs appeared with negative to weak S-100 immuno-staining (Table 1), others exhibited positive S-100 reaction especially the small and medium size neurons. The strongest S-100 reaction was detected in the myelinated nerve fibers and SGCs (Fig. 2B, C). The SIFCs cells, interstitial connective tissue and blood capillaries appeared with negative S-100 immunoreactivity (Fig. 2D).

DISCUSSION

The present histological results show that the camel CCG is encapsulated and is divided into ganglionic units. These units contain ganglion neurons, nerve fibers, Schwann cells, fibroblasts, glial cells and SIF cells. This arrangement is closely related to that described in different mammals (Kiran 2002; Eroschenko 2008; Fioretto et al. 2007; Gabella et al. 1988; Miolan and Niel 1996; Ribeiro et al. 2002). The present study found that each ganglionic unit mainly contains a cellular triad of PGN, SIFs, and glial cells constituting its cytological basis. Considering this ganglionic arrangement, camel CCG could be described as a ganglionic complex instead of a classical single sympathetic ganglion. It has also been reported that the large mammal's superior cervical ganglion (SCG) is in the form ganglionic complex rather than the classical arrangement as a single sympathetic ganglion found in laboratory animals (Fioretto et al. 2007; Miolan and Niel 1996; Ribeiro et al. 2002). This structural arrangement in large animals is supposed to be related to the existence of very specific SCG innervation areas and target organs as observed when injecting retrograde neuro-tracers that revealed the exact location of the labelled neurons in SCG (Kabak 2007; Gagliardo et al. 2003).

Table 1: S-100 immune-reactivity in the different parts of camel cranial cervical ganglia (CCG)

Tissue	Reaction Intensity
Capsule and septa	-
Blood vessels	-
PGNs	±
SGCs	++
SIFCs cells	-
Myelinated fibers	++
Non-yelinated fibers	-

Negative (-), weak (±), medium (+) and strong **(++)** reactions.

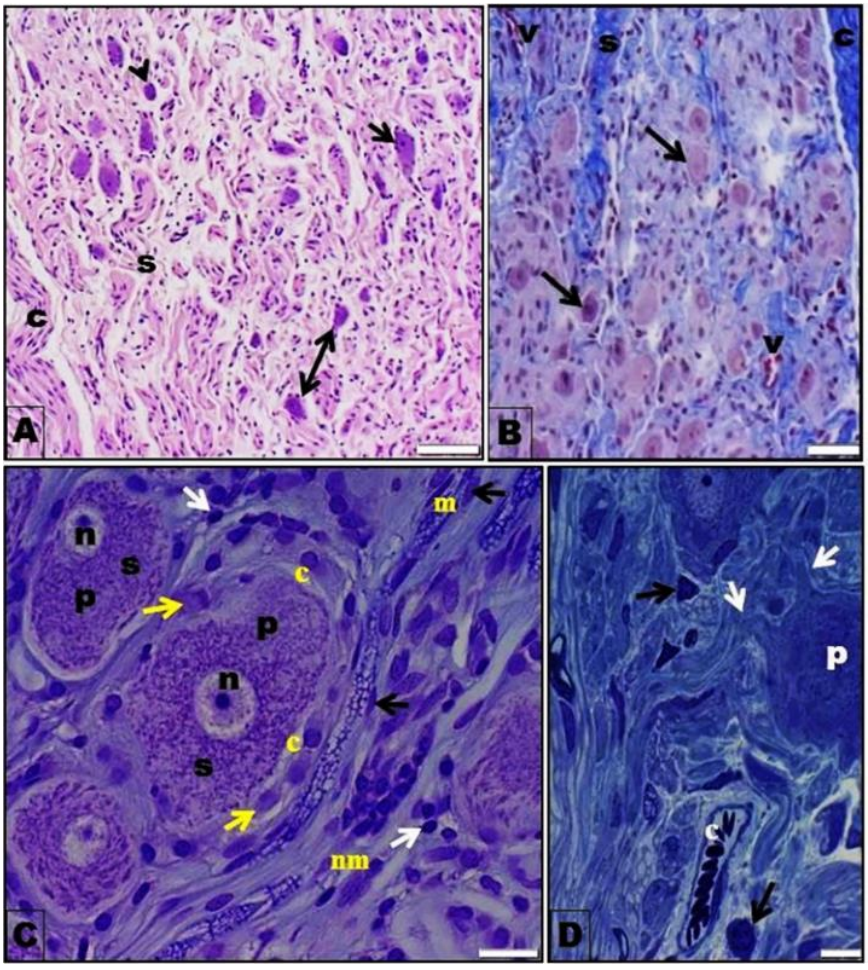

Fig. 1: Photomicrographs of camel CCG. A) Shows the dense connective tissue capsule (c) and septa (s). Note the small PGN (arrowhead), medium PGN (tow-caped arrow) and large PGN (arrow) .H&E stain. Image bar=100µm; B) Shows the collagenous fibers in the CCG capsule (c) and septa (s) forming the ganglionic units with PGNs (arrows) and blood vessels (v). Masson's trichrome stain. Image bar=50µm; C) Shows the principal ganglionic neurons (p) with cytoplasmic Nissls substance (s) and large nuclei (n), surrounded by SGCs (yellow arrows) and blood capillaries (c). Note the non-myelinated nerve fibers (nm), myelinated nerve fibers (m), Schwann cell nuclei (black arrows) and fibroblasts (white arrows. Luxol fast blue with cresyl violet. Image bar=20µm; and D) Shows SIFCs (black arrows), principal ganglion neuron (p) with processes (white arrows) and blood capillary (c). Semi-thin section stained by toluidine blue. Image bar=10µm.

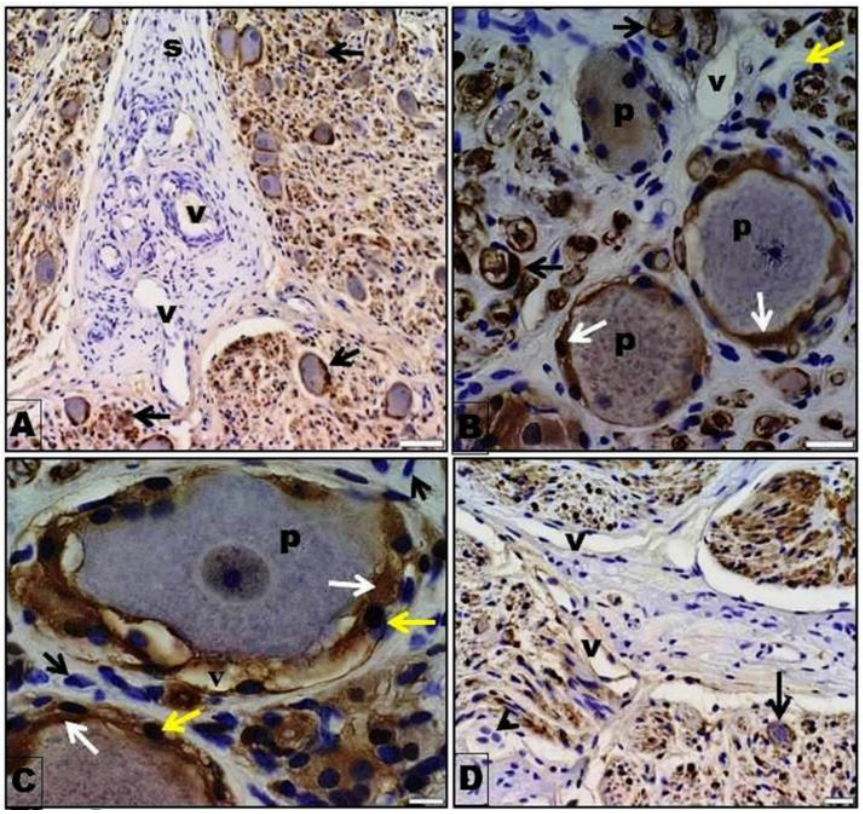

Fig. 2: Photomicrographs of camel CCG immune staining for S-100. A) Shows the ganglionic connective tissue septa (s) and blood vessels (v) with S-100 negative reaction. Some small and medium size PGNs show S-100 positive (arrows). Image bar=50µm; B) Shows a strong S-100 immunostaining in, glial capsule (white arrows) and myelinated nerves (black arrow). Variable reaction is shown in PGNs (p). Non-myelinated nerve fibers (yellow arrows) and blood capillaries (v) show negative staining. Image bar=20µm; C) Shows a strong S-100 labelling in SGCs cytoplasm (white arrows) and nuclei (yellow arrows). Large PGNs (p) show negative to weak reaction. Blood vessels (v) and fibroblasts (black arrows) are S-100 negative. Image bar=10µm; and D) Shows negative S-100 immunoreactivity in the SIF (arrowhead), blood vessels (v) and PGN (arrow). Image bar=20µm.

The organization of ganglionic neurons and glial capsules has been suggested to help in close mutual neuron–SGC interaction (Hanani 2005; Jasmin et al. 2010). Moreover, the close contact between ganglionic neurons and SGCs has been considered as a major key for predicting their function which is still not clear; for example, this contact could enable them to control homeostasis of neurons (Hanani 2005).

The current study reported that the PGN cell body diameter in camel CCG ranges from 78 to 251µm which indicates that its size is larger than those of horse, dog and cat (Fioretto et al. 2007). It has also been reported to be larger than dorsal root ganglia when compared with the findings in the horse (Adalbert et al. 2022), bovine (Fadda et al. 2016) and humans (Valtcheva et al. 2016). The latter two authors claimed that large neuronal size could reflect very long axons they have to support. Further, a correlation between neuronal size and animal body size has also been reported (Herculano-Houzel et al. 2014; Ho et al. 1992).

The presence of SIFCs in the ganglionic units of camel CCG has also been reported in other mammals including horses, cats and dogs (Fioretto et al. 2007). SIFCs are considered as one of the main types of cells in sympathetic ganglia (Hanani 2010).

To our knowledge, this is the first report on the immuno-histochemical distribution of S-100 protein in the CCG of the dromedary camels. In the study, the satellite glial cells, glial capsule and myelinated fibers with Schwann cells showed a strong S-100 immune-reactivity. Similar observations have been noted in mammalian dorsal root, sympathetic and enteric ganglia (Gonzalez-Martinez et al. 2003). Positive S-100 immuno-reaction in the glial cells is suggested to be involved in growth regulation and differentiation of these cells (Isobe et al. 1989). S-100 protein positive immunoreactivity has also been observed in the neuronal perikarya of some ganglionic principal neurons especially small and medium ones. Similar findings have also been shown in the ganglion principal neurons in the of rat dorsal root ganglion (Vega et al. 1991) and sympathetic and enteric ganglia of sheep, pig and buffalo were S-100 positive (Albuerne et al. 1998). It has been stated that the main role of S-100 protein is to regulate biosynthesis of catecholamine and serotonin in neurons and other synthesizing cells of monoamine (Isobe et al. 1989). Also in astrocytes, there are proteins from S-100 family expressed in Schwann cells and oligodendrocytes which aid in regulation of intracellular Ca2+ as well as having neurotrophic activities (Donato et al. 2013, Gonzalez-Martinez et al. 2003). However, S-100 proteins are not considered as ideal SGCs markers because a sub-population of Schwann cells and sensory ganglia neurons show positive reaction to them (Vega et al. 1991, Ichikawa et al. 1997). Immunoreaction of S-100 protein was reported in sensory ganglia and its possible roles need to be determined (Valtcheva et al. 2016). One of the S-100 proteins is S-100A4 that was up-regulated in Schwann cells of rat DRG following sciatic nerve injury (Sandelin et al. 2004). According to the latter authors the change in S-100A4 immuno-reactivity is only found in SGCs surrounding neurons with injured axons, suggesting a contribution of this protein to neuronal survival. Astrocytes and SGCs have also been found to express S-100 (Ho et al. 1992). Furthermore, Yang et al. (2020) indicate the neuroprotective role of S100A4 in retinal ganglia where they inhibit the apoptosis of ganglionic cells which might be a novel therapeutic protocol for glaucoma. On the other hand, Li et al. (2020) state that due to S100A4 degenerative or regenerative effects, its expression could be a result or cause of fibrotic disease progression.

Conclusion

We showed that CCG of dromedary camels consists of several ganglionic units; these units which contain PGNs of different sizes, glial cells and SIF cells have been designated as the basic structural constituents. This arrangement is similar to that found in other large mammals. The mean diameters of large sized PGNs, medium-sized and small-sized neurons are significantly different. The immuno-reaction for S-100 protein is present in the cytoplasm of some large and intermediate sized PGNs, SGCs, Schwann cells, myelinated and non-myelinated nerve fibers. However, connective tissue capsule, septa and blood vessels are S-100 negative.

Conflict of Interest: None declared.

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