

## Expression of Neuronal and Non-Neuronal Markers in the Vomeronasal Organs of One-Humped Camel (*Camelus dromedarius*) and Egyptian Water Buffalo (*Bubalus bubalis*)

Expresión de Marcadores Neuronales y No Neuronales en los Órganos Vomeronasales del Camello Jorobado y el Búfalo Egipcio de Agua

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**SUMMARY:** Fourteen vomeronasal organs (VNOs) of adult males one-humped camels (*Camelus dromedarius*) and Egyptian water buffaloes (*Bubalus bubalis*) (n=7/each) were examined immunohistochemically with neuronal markers; synaptophysin (SYP) and glial fibrillary acidic protein (GFAP) to clarify the distribution of the vomeronasal (VN) receptor cells and nerve fibers, in addition to elucidate the existence of non-neuronal elements via S-100 and endothelial nitric oxide synthase (eNOS). In both animals, the VNO was lined medially with VN sensory (olfactory) epithelium and non-sensory (respiratory) epithelium laterally. Immunohistochemically, both animals showed SYP immunolabeling only in the receptor cells of VN sensory epithelium while GFAP labeled the ensheathing cells of the nerve fibers of VNOs. Both S-100 and eNOS labeled non-neuronal elements of the VNO; the supporting cells of sensory epithelium and the VN glands. In view of these observations, we postulate that the VNOs of both animals contain various cells populations that express several neuronal and non-neuronal markers. As well as, SYP and GFAP are suggested as markers for receptor cells and ensheathing cells of nerves of the VNOs respectively. However, no clear differences can be detected in the expressions of neuronal and non-neuronal markers in VNOs of camel and buffalo since they are ruminant species.

**KEY WORDS:** Vomeronasal organ; Camel; Buffalo; Immunohistochemistry.

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### INTRODUCTION

Male individuals of most domestic animals commonly use the flehmen response as an olfactory mechanism for identifying the reproductive state of females of the same species based on transferring pheromones in the female's urine or genitalia into the vomeronasal organ (VNO). VNO is considered as an accessory olfactory system (Witt *et al.*, 2002; Halpern & Martínez-Marcos, 2003; Karimi *et al.*, 2007). It acts as a chemosensory organ where VN sensory neurons detect chemical signals such as pheromones that evoke sexual behavior (Zufall *et al.*, 2002; Keverne, 2004; Kimoto *et al.*, 2005). Both one-humped camel (*Camelus dromedarius*) and Egyptian water buffaloes (*Bubalus bubalis*), as large ruminants, are very important for the Egyptian food security. Males of both animals exhibit different sexual behavioral response during courtship of their females. Considering this, we aimed to describe the functional immunohistochemistry difference between their VNOs.

We examined their VNOs immunohistochemically with neuronal markers; SYP and GFAP to clarify the distribution of VN receptor cells and nerve fibers in the VNOs. In addition, to elucidate the existence of non-neuronal elements, we examined the VNOs with S-100 and eNOS.

SYP is a glycoprotein present in synaptic vesicles in almost all neurons and it is used as an immunohistochemical marker for neuronal tissues (Smith *et al.*, 1993) while, GFAP is a major intermediate filament found in glial cells (Eng *et al.*, 1985) and it has been expressed in the supporting cells of the olfactory epithelium (Kim *et al.*, 2005). S-100 protein is an acidic calcium-binding protein that has been detected in the olfactory mucosa and VNO (Wang *et al.*, 1994) but, eNOS is an enzyme that produces nitric oxide which is suggested to be involved in olfactory perception (Yamamoto *et al.*, 2003; Endo *et al.*, 2011).

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## MATERIAL AND METHOD

**Animals and tissue processing:** Heads from 14 apparently healthy, adult males' one-humped camels (*Camelus dromedarius*) and Egyptian water buffaloes (*Bubalus bubalis*) (7 heads/each) were obtained from a local slaughterhouse (Toukh, Kalubyia, Egypt) during winter of 2015. The animals were slaughtered according to guidelines of Benha University animal care committee. The heads were dissected to obtain the VNOs as a pair of tubes situated at the base of the nasal septum. Small pieces (1/2 cm) from the mid part of the VNOs were immersed in 10 % neutral buffered formalin, then routinely processed and embedded in paraffin, and cut transversally at 5 mm. Sections were stained with haematoxylin and eosin for general histological structure.

**Immunohistochemistry:** Sections of 5 mm thickness were stained by immunohistochemical avidin-biotin method as follows. After rehydration, the sections were incubated for 30 min in 3 % hydrogen peroxide in methanol then incubated for 30 min in 1 % normal goat and rabbit serum diluted in PBS. Sections were incubated with the primary antibodies (Table I) for 1 h at room temperature (RT). Sections were incubated for 30 min at RT with anti-rabbit and anti-goat IgG then with Vectastain<sup>®</sup>Elite (Vector Laboratories, Burlingame, CA, USA) for the same time. A diaminobenzidine solution was used for the final staining. The specificity of the immunoreaction was detected substituting the primary antibodies with non-immune goat and rabbit IgG (Santa Cruz Biotechnology, CA, USA) to obtain negative control sections. The stained sections were finally captured by light microscope Leica DM3000 LED.

## RESULTS

**Histological observation:** The VNOs of both one-humped camels and Egyptian water buffaloes had VN lumen and are encapsulated by hyaline cartilage (Figs. 1A, B). The VN lumen was lined with respiratory epithelium (non-sensory epithelium) at the lateral convex surface of the lumen while the medial concave surface of the lumen was lined with VN

sensory (olfactory) epithelium. The respiratory epithelium consisted of pseudostratified ciliated columnar epithelium with goblet cells (Figs. 1C, D). In the VN sensory epithelium, nuclei of the basal cells were situated at the basal region, while those of the receptor cells were situated at the middle region. In addition, nuclei of the supporting cells were located at the apical region of the epithelium (Fig. 1E, F). Propria-submucosa consisted of a vascular loose connective tissue which gave cavernous appearance (Fig. 1). Numerous serous and mucous VN glands were found in propria-submucosa, with small secretory ducts ending in the lumen of the organ (Figs. 1C, D).

**Immunohistochemical observation:** Depending on blind examination by two independent observers, the intensities of immunostaining were scored as negative (-), weak (+), moderate (++) or strong (+++). The expressions of neuronal and non-neuronal markers in the VNOs of both animals were summarized in Table II. The findings showed unclear differences in the expression of one-humped camel and Egyptian water buffalo for neuronal and non-neuronal protein markers.

SYP was restricted only to the VN sensory epithelium. SYP labeled only the receptor cells in the VN sensory epithelium, but did not label the supporting or basal cells. The SYP labeled cells appeared as pear shaped cells (Figs. 2A, B). In both of camel and buffalo, GFAP immunostaining could not be seen in the VN sensory, non-sensory epithelium or glands. GFAP labeled only ensheathing cells of the scattered nerve fibers in lamina propria of VNO (Fig. 2C, D).

S-100 intensely labeled the apical part of supporting cells of the VN sensory epithelium in addition to scattered nerve fibers and VN glands in lamina propria-submucosa of the VNO (Fig. 3).

Moderate eNOS immunostaining was observed mainly in the free border of the VN sensory epithelium (Figs. 4A, B) while weak eNOS was seen in the VN glands (Figs. 4C, D). Immunostainings for SYP, GFAP, S-100, and eNOS were considered positive in comparison to negative control sections as shown in Fig. 5.

Table I. Data of the used antibodies in the present study.

Antibody	Catalogue number	Source	Cross reactivity	Dilution
Goat polyclonal anti-SYP	sc-7569	Santa Cruz Biotech., CA, USA	Equine, bovine, porcine	1:200
Goat polyclonal anti-GFAP	sc-6170	Santa Cruz Biotech., CA, USA	Equine, canine, bovine	1:150
Rabbit polyclonal anti-eNOS	sc-7849-R	Santa Cruz Biotech., CA, USA	Equine, canine, bovine	1:200
Rabbit polyclonal anti-S100	ab5589	Abcam, Cambridge, MA, USA	Cow, pig, dog	1:100

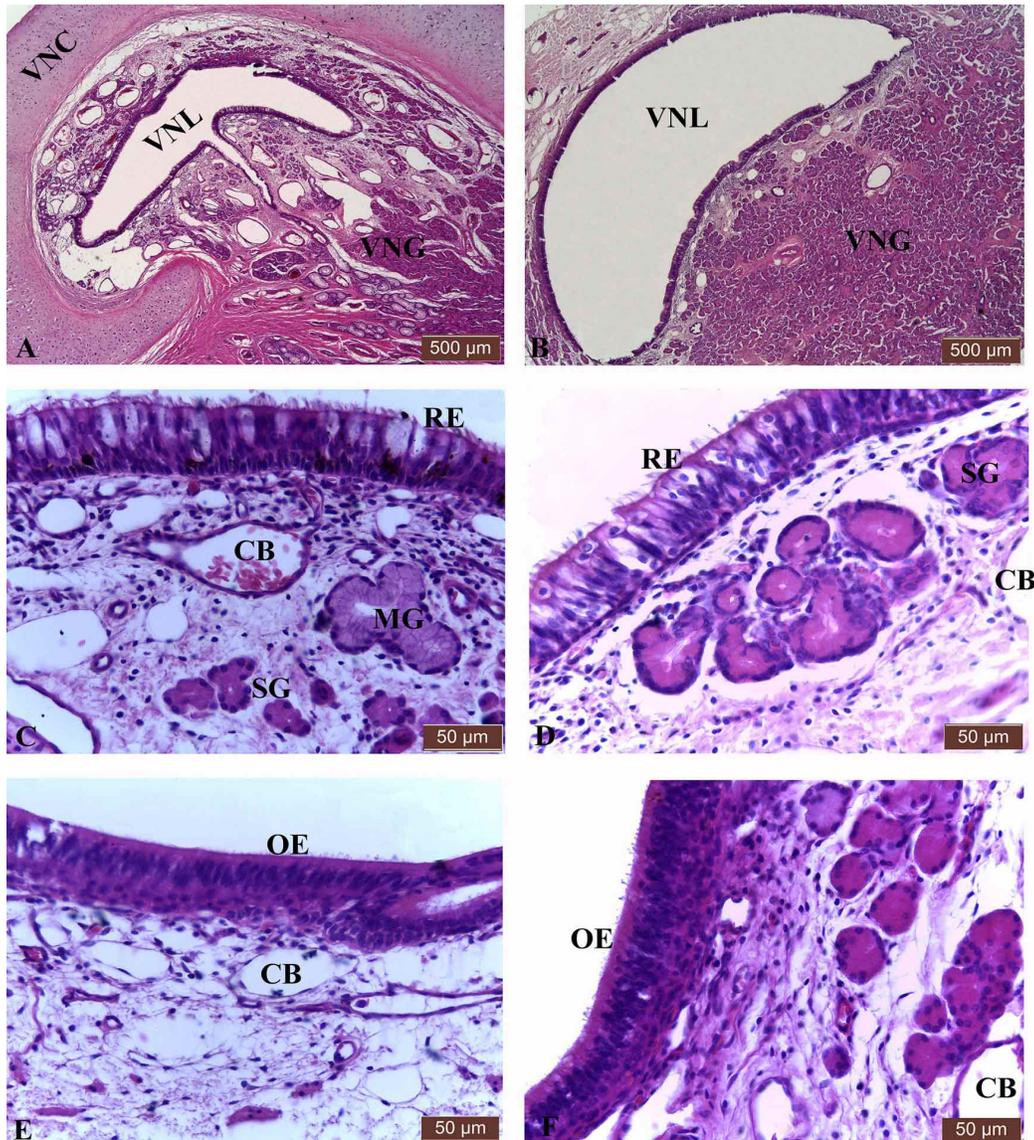


Fig. 1. Transverse sections of the VNO in the camel (Figs. A, C, E) and buffalo (Figs. B, D, F) stained with H&E. Figs. A and B. General view of VNO of camel and buffalo. VNL, vomeronasal lumen; VNG, vomeronasal gland; VNC, vomeronasal cartilage. Scale bar = 500 μm. Figs. C and D. Respiratory epithelial lining of the VNO of camel and buffalo. RE, respiratory epithelium; MG, mucous gland; SG, serous gland; CB, caves of blood. Scale bar = 50 μm. Figs. E and F. Olfactory epithelial lining of the VNO of both camel and buffalo. OE, olfactory epithelium; CB, caves of blood. Scale bar = 50 μm.

Table II. Summary of immunostaining intensities of neuronal and non-neuronal markers in the VNO of both one-humped camel and Egyptian buffalo.

	VN sensory epithelium			VN glands	VN nerve fibers
	Supporting cells	Receptor cells	Basal cells		
SYP	-	++	-	-	-
GFAP	-	-	-	-	+++
S-100	+++	+	-	+++	+
eNOS	++	+	-	+	-

-, negative; +, weak immunostaining; ++, moderate immunostaining; +++, strong immunostaining.

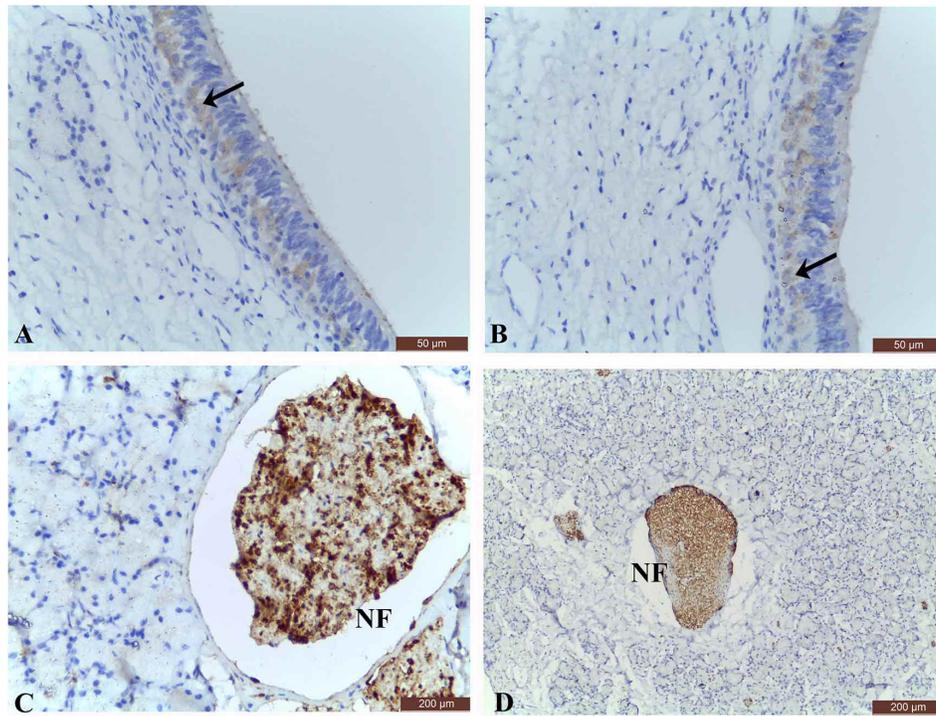


Fig. 2. Immunohistochemical stainings for SYP and GFAP in the VNO of camel (Figs. A, C) and buffalo (Figs. B, D). Figs. A and B. SYP immunostaining in the receptor cells of the VN sensory epithelium (arrow). Scale bar = 50 μm. Figs. C and D. GFAP immunostaining in the ensheathing cells of the nerve fibers (NF) in the lamina propria of VNO. Scale bar = 200 μm.

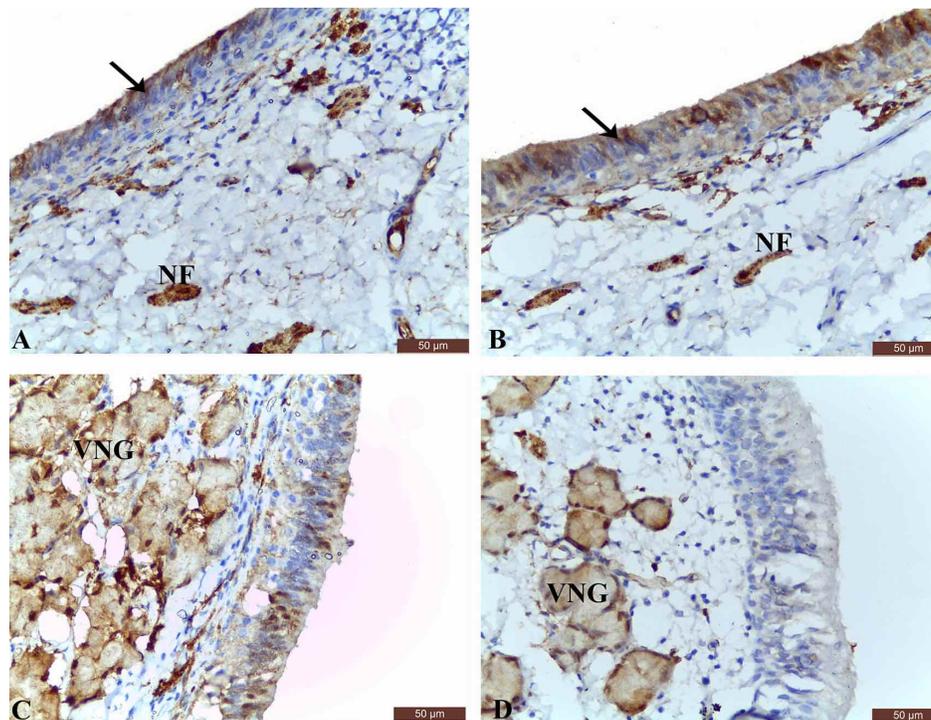


Fig. 3. Immunohistochemical staining for S-100 in the VNO of camel (Figs. A, C) and buffalo (Figs. B, D). Intense immunostaining for S-100 in the apical part of the supporting cells of the VN sensory epithelium (arrow), scattered nerve fibers (NF) and VN glands (VNG) in the lamina propria of VNO. Scale bar = 50 μm.

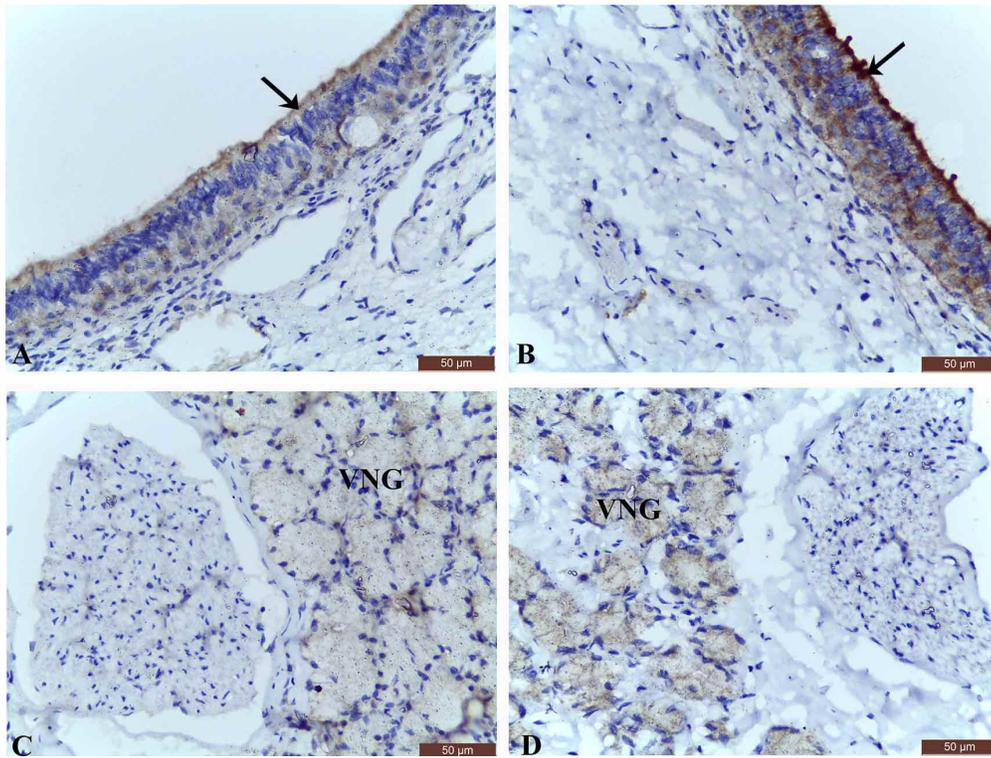


Fig. 4. Immunohistochemical staining for eNOS in the VNO of camel (Figs. A, C) and buffalo (Figs. B, D). Moderate eNOS immunostaining observed in the free border of the VN sensory epithelium (arrow). Weak eNOS immunostaining observed in the vomeronasal glands (VNG). Scale bar = 50 µm.

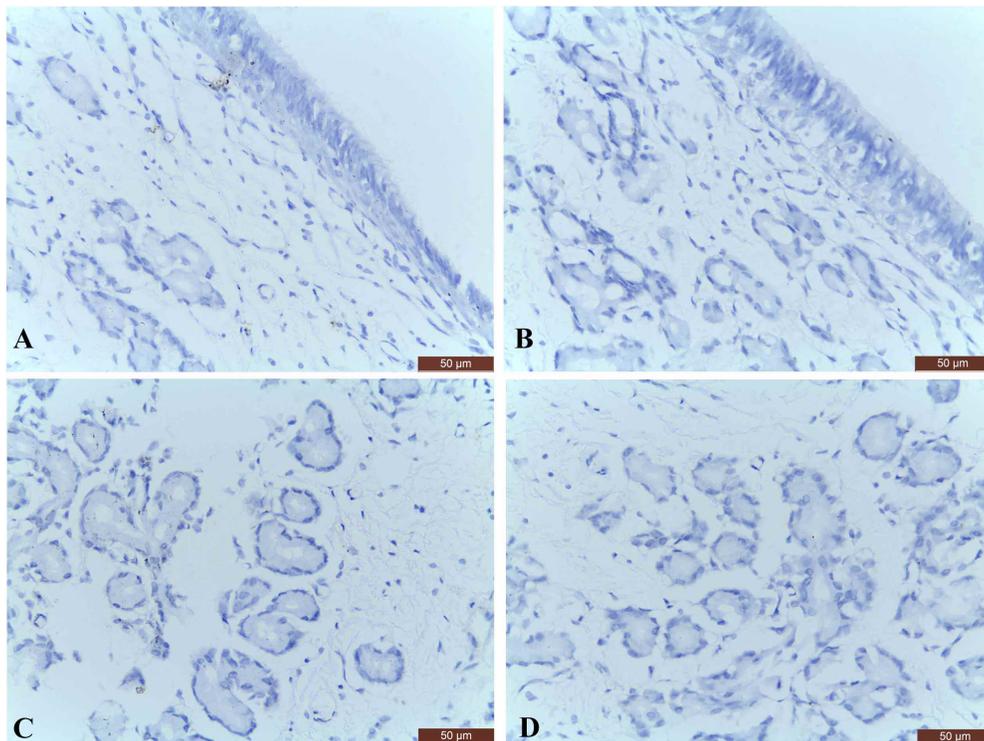


Fig.5. Negative control sections for SYP, GFAP, S-100 and eNOS. Immunohistochemical staining of VNO of camel (Figs. A, C) and buffalo (Figs. B, D) with non-immune IgG as negative control

## DISCUSSION

Like histological findings of Ibrahim *et al.* (2015) and Karimi *et al.* (2014) in camel, Ardalani *et al.* (2000) and Abbasi (2007) in buffalo, the VNOs of both one-humped camel and Egyptian buffalo in the present study are encapsulated by hyaline cartilage and lined medially with sensory epithelium and laterally with non-sensory epithelium. Propria-submucosa contains numerous VN glands whose secretion is essential for pheromones contacting VNO chemoreceptors (Zufall *et al.*; Nunez Chichet *et al.*, 2007). Moreover, the vascularity of propria-submucosa plays important role through the outflow and inflow of blood stream during breeding of the animals aiding in flehmen response (Abass *et al.*, 2012).

Immunohistochemically, it was interesting that the VNOs of both one-humped camel and Egyptian water buffalo showed unclear differences in the expressions of neuronal and non-neuronal markers. Such finding indicates the similarity of functional histology of the VNOs in both animals since they are ruminant species. In both animals, the localization of SYP in receptor cells of the VN sensory epithelium indicates the functional nature of VN receptors as signal transduction (Eisthen, 1992; Menco *et al.*, 2001). In accordance with the view that GFAP is a classical glial marker such as astrocytes, nerve surrounding cells and satellite cells in ganglia (Kim *et al.*), our results confirmed the expression of GFAP in the ensheathing cells of VN nerve axons in the propria-submucosa of VNOs of both animals. Contrary to Kim *et al.*, our results did not detect GFAP in the VN epithelium that was in consistence with Witt *et al.* Such finding does not suggest GFAP as a biomarker for supporting cells in VNOs of camel and buffalo that contradict Rio *et al.* (2002) and Smithson & Kawaja (2009) in mice. Therefore, we suggest a species variation in GFAP expression in the VNO of different animals but its expression in camel and buffalo is still similar. Furthermore, we suggest GFAP as markers for the ensheathing cells of nerve fibers in VNOs of both camel and buffalo.

S-100 was intensely expressed in the supporting cells of VN epithelium in both animals that was similar to findings of Witt *et al.* Such finding supports Smithson & Kawaja who considered S-100 as biomarker for olfactory supporting cells. Also, S-100 was intensely expressed in the VN gland that supports Kahn *et al.* (1983) who detected S-100 in various exocrine glands in the body. Since S-100 is a calcium binding protein, such picture indicates an increase in calcium transport through, or demands within the VN epithelium and glands so that, S-100 may be involved in the exocytosis of VN glandular secretion.

eNOS was expressed in the free luminal surface of the VN sensory epithelium in both animals. Such finding was in accordance with Endo *et al.* as they detected eNOS in the free border of the olfactory epithelium. The expression of eNOS in the VNOs of both camel and buffalo confirms the suggestion of eNOS as a candidate enzyme to produce nitric oxide in the VNO epithelium which is involved in olfactory perception (Yamamoto *et al.*, 2003; Endo *et al.*).

In conclusion, the VNOs of both one-humped camels and Egyptian water buffaloes contain various cells populations that express several neuronal and non-neuronal markers. Expression of SYP and GFAP in the VNOs indicate the sensory and nervous function of VNOs, while expression of S-100 indicates calcium transportation or demand via VN epithelium and glands, as well as expression of eNOS indicates production of nitric oxide in the VNO epithelium that is involved in olfactory perception. Overall, since camel and buffalo are ruminant species, there are unclear differences in expressions of neuronal and non-neuronal markers in both VNOs. Furthermore, we suggest SYP and GFAP as markers for receptor cells and ensheathing cells of nerves of the VNO respectively.

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**EMAM, M. A.; EL-SHAFFEY, A. A. & KASSAB, A. A.** Expresión de marcadores neuronales y no neuronales en los órganos vomeronasales del camello jorobado *Camelus dromedarius*) y el búfalo egipcio de agua (*Bubalus bubalis*). *Int. J. Morphol.*, 34(4):1207-1210, 2016.

**RESUMEN:** En este estudio fueron examinados 14 órganos vomeronasales (OVN) de machos adultos de camellos de una joroba (*Camelus dromedarius*) y búfalos egipcios de agua (*Bubalus bubalis*) (n = 7 / cada uno) por inmunohistoquímica con marcadores neuronales, sinaptosina (SIP) y proteína ácida fibrilar glial (PAFG), para identificar la distribución vomeronasal (VN) del receptor de células y fibras nerviosas, además de dilucidar la existencia de elementos no neuronales a través de S-100 y óxido nítrico sintasa endotelial (ONSe). En ambos animales, el OVN se encuentra alineado en sentido medial con el epitelio sensorial (olfato) y lateralmente con el epitelio no sensorial (respiratorio). En el estudio inmunohistoquímico, ambos animales mostraron marcadores inmunológicos solamente en las células receptoras del epitelio sensorial VN, mientras que la proteína ácida fibrilar glial marcaba las fibras nerviosas de OVN. Tanto el S-100 como la óxido nítrico sintasa endotelial, marcaron elementos no neuronales del OVN, las células de revestimiento del epitelio sensorial y las glándulas VN. En relación a estas observaciones, se postula que los OVN de ambos animales contienen células que expresan varios marcadores neuronales y no neuronales. SIP y la PAFG se sugieren como marcadores para células receptoras y las células gliales de nervios del OVN, respectivamente. Sin embargo, debido a que son especies de rumiantes, no existen diferencias claras que se puedan detectar en las expresiones de los marcadores neuronales y no neuronales en el OVN de camello y búfalo.

**PALABRAS CLAVE:** Órgano vomeronasal; Camello; Búfalo; Inmunohistoquímica.

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