A Thesis for the Degree of Master

The study of vomeronasal organ in Korean black goat, *Capra hircus coreanae*

Department of Veterinary Medicine

GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

Wonjun Yang

2020. 8.

The study of vomeronasal organ in Korean black goat, *Capra hircus coreanae*

Wonjun Yang

(Supervised by Professor Taekyun Shin)

A thesis submitted in partial fulfillment of the requirement for the degree of Master in Veterinary Medicine

2020. 6.

This thesis has been examined and approved.

.....

Thesis director, Seung-Dam Heo, Ph.D., Hyundai Animal Hospital

.....

Jeongtae Kim, Ph.D., Kosin University

.....

Taekyun Shin, Ph.D., Jeju National University

.....

Department of Veterinary Medicine

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

CONTENTS

List of Abbreviation ---------- 2

List of Figures ---------- 3

List of Tables ---------- 4

1. Abstract ---------- 6

2. Introduction ---------- 7

3. Materials and Methods ---------- 8

4. Results ---------- 12

5. Discussion ---------- 24

References ---------- 30

Abstract in Korean ---------- 34

Acknowledgements ---------- 36

List of Abbreviations

ABC	avidin-biotin complex
AOB	accessory olfactory bulb
BSL	*Bandeiraea (Griffonia) simplicifolia* lectin
DAB	3-3'-diaminobenzidine
DBA	*Dolichos biflorus* agglutinin
LCA	*Lens culinaris* agglutinin
OMP	olfactory marker protein
PAS	periodic acid Schiff
PBS	phosphate-buffered saline
PGP 9.5	protein gene product 9.5
PHA-L	*Phaseolus vulgaris* leucoagglutinin
PNA	*Arachis hypogaea* (peanut) agglutinin
SBA	*Glycine max* (soybean) agglutinin
UEA	*Ulex europaeus* agglutinin
VNO	vomeronasal organ
VNSE	vomeronasal non-sensory epithelium
VSE	vomeronasal sensory epithelium
WGA	*Triticum vulgaris* (wheat germ) agglutinin

List of Figures

Figure 1. Histological examination of vomeronasal organs (VNOs) from the domestic goat, *Capra hircus coreanae*---------- 13

Figure 2. Periodic acid-Schiff (PAS) reaction and Alcian blue staining (pH 1.0 and pH 2.5) in Bowman's glands of the VNSE---------- 14

Figure 3. Immunohistochemical staining of protein gene product 9.5 (PGP 9.5) in VNOs from Korean black goat-------- 16

Figure 4. Immunohistochemical staining of olfactory marker protein (OMP) in VNOs from Korean black goat------- 17

Figure 5. Lectin histochemistry for WGA, DBA, and BSL-I in the VSE of Korean black goat fetuses (A, D and G), one-day-old goats (B, E and H), and adult goats (C, F and I)---------- 20

Figure 6. Lectin histochemistry for WGA, DBA, and BSL-I in the VNSE of Korean black goat fetuses (A, D and G), one-day-old goats (B, E and H), and adult goats (C, F and I)---------- 21

Figure 7. Immunohistochemical staining of Giα2 and Goα in the VNO of Korean black goat---------- 27

List of Tables

Table 1 Binding specificities of lectins used in the present study---------- 11

Table 2 Expression patterns of OMP and PGP9.5 in the vomeronasal organ (VNO) of Korean black goat---------- 18

Table 3 Lectin binding in the vomeronasal sensory epithelium (VSE) of Korean black goat---------- 22

Table 4 Lectin binding in the vomeronasal non-sensory epithelium (VNSE) of Korean black goat---------- 23

Table 5 Lectin binding in the vomeronasal organ of various animals------ 28

The study of vomeronasal organ in Korean black goat, *Capra hircus coreanae*

1. Abstract

The appearance of olfactory signal transduction proteins, including olfactory marker protein (OMP), a marker for olfactory sensory neurons, is related to olfactory function in the vomeronasal organ (VNO) in animals. The present study examined the localization of OMP in the VNO of the Korean black goat (*Capra hircus coreanae*) in the prenatal and postnatal periods using immunohistochemistry. In fetal goats and 1-day-old goats, OMP was occasionally identified in receptor cells of the VNO sensory epithelium, while protein gene product 9.5 (PGP9.5), a marker for immature neurons, was localized in both the sensory and non-sensory epithelia. In VNOs from adult goats, OMP was abundant in the sensory epithelium and occasionally observed in single cells in the non-sensory epithelium. Mucus histochemistry revealed that both neutral and acidic polysaccharides were observed in VNO glands during the fetal period, with more intense reactivity in the VNO of adult goats. Furthermore, the free borders of sensory epithelia were positive for 7 lectins, and 6 lectins were moderately and/or highly abundant in receptor cells. Supporting cells, basal cells, and nerve bundles had similar expression patterns. In VNSE, 7 lectins were observed in the free border, and 6 lectins were observed in ciliated cells, goblet cells, basal cells, and gland acini. Collectively, these results show that OMP production was initiated in the VNO sensory epithelium at the fetal stage, and that its activity increased in adult VNO receptor cells and solitary cells in the non-sensory epithelium of the Korean black goat.

Keywords: Development; Korean black goat; Olfactory marker protein; Protein gene product 9.5; Vomeronasal organ.

2. Introduction

Olfactory marker protein (OMP) is expressed in mature chemoreceptor neurons of the olfactory epithelium (Oboti et al., 2011) and is known to be an important protein for olfactory signal transduction in the initial early stages of olfaction through the clearance of calcium ions (Ca^{++}) (Buiakova et al., 1996, Kwon et al., 2009). OMP is also a marker for receptor cells in the sensory epithelium of the vomeronasal organ (VNO), septal organ and Grüeneberg ganglions (Breer et al., 2006).

VNO, a sensory organ for the detection of pheromones, is situated at the base of the nasal septum. It consists of sensory and non-sensory epithelia encased by VNO cartilage, and the axons of VNO receptor cells terminate in accessory olfactory bulbs (AOB) in the caudal part of the olfactory bulbs (Halpern et al., 1998, Halpern and Martinez-Marcos, 2003). VNO receptor cells contain both the mature neuron marker OMP (Breer et al., 2006) and the immature neuron marker protein gene product 9.5 (PGP 9.5) (Park et al., 2014). The VNO of goat has been widely used for research on reproductive behavior (Gelez and Fabre-Nys, 2004). The morphological features of the VNO and its connection to AOB have been widely studied in goats at the fetal stage (Takigami et al., 2004) and the adult stage (Takigami et al., 2000). Previously, we reported that the VNO of Korean black goats consisted of both vomeronasal sensory and non-sensory epithelia, and that PGP 9.5 was present in VNO receptor cells and in some isolated cells in the VNO non-sensory epithelium (Park et al., 2013).

Although the morphological features of the VNO are well-characterized across developmental stages in goat, the appearance of OMP in the VNO of goat requires further investigation. The present study examined the expression of OMP and PGP 9.5 in the VNO of Korean black goat at different developmental stages.

3. Materials and Methods

3.1. Tissue preparation

Korean black goats (*Capra hircus coreanae*, 2-year-old females) were obtained from a local farm in Jeju city, Korea. Some samples analyzed in our previous paper (Park et al., 2013), were re-evaluated using a different immunohistochemical approach. Two samples of fetal VNO with unknown ages were obtained from pregnant goats. Two samples of VNO were obtained at day 1 after birth. For light microscopy, the VNOs were removed immediately after death and fixed in 10% buffered formalin for 48 h. All experimental procedures were conducted in accordance with Jeju National University Guidelines for the Care and Use of Laboratory Animals (Permission number: 2017-0019).

3.2. Histological examination

Formalin-fixed VNOs were trimmed and decalcified in sodium citrate-formic acid solution (Luna et al., 1968). The solution was replaced several times, until the bony pieces softened, as described previously (Park et al., 2014). Then, decalcified VNO was dehydrated in a graded ethanol series (70%, 80%, 90%, 95%, and 100%), cleared in xylene, embedded in paraffin, and sectioned at a thickness of 5 μm. After deparaffinization, the sections were stained with hematoxylin and eosin, periodic acid Schiff (PAS), and Alcian blue (pH 1.0 and pH 2.5).

3.3. Immunohistochemistry

Immunohistochemisty was performed using a Vector ABC Peroxidase Kit (Vector Laboratories, Burlingame, CA, USA) as described previously (Park et al., 2012a). Briefly, sections (5 μm) of paraffin-embedded tissue were deparaffinized and heated in a microwave (800 W) in citrate buffer (0.01 M, pH 6.0) for 3 min. After cooling

the slides, the sections were exposed to aqueous 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. Non-specific binding was then blocked with 10% normal goat and/or horse serum, washed in phosphate-buffered saline (PBS, pH 7.4) for 1 h, and allowed to react with the primary antibodies, including goat anti-OMP (1:500; sc-49070, Lot B27141104; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-PGP 9.5 (1:800; ab72911, Lot GR241185-1; Abcam, London, UK), for 1 h at room temperature. After washing in PBS, the sections were reacted for 45 min with matching biotinylated antibodies (Vector Kits; Vector Laboratories). After washing again in PBS, the sections were incubated for 45 min with the avidin-biotin peroxidase complex prepared according to the manufacturer's instructions. After washing in PBS, the peroxidase reaction was developed for 3 min using a diaminobenzidine substrate (DAB Kit; Vector Laboratories), prepared according to the manufacturer's instructions. Sequentially, the sections were counterstained with hematoxylin for 30 s, washed in running tap water for 20 min, dehydrated through a graded ethanol series, cleared with xylene, and mounted with Canada balsam (Sigma-Aldrich, St. Louis, MO, USA).

3.4. Lectin specificity

Lectin histochemistry was performed as described elsewhere (Lee et al., 2016, Park et al., 2013, Park et al., 2012b). Briefly, the sections were deparaffinized with serial alcohol solutions and rehydrated. Then, the sections were reacted with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase. After three washes with PBS, the slides were incubated with 1% bovine serum albumin in PBS to block non-specific activity. The sections were incubated with each lectin (Table 1) at 4℃ overnight in a humidified chamber. After incubation, the sections washed with PBS three times. Signals were developed using a DAB Kit (Vector Laboratories), and sections were then counterstained with hematoxylin for 10 s before mounting. Negative controls for lectin histochemistry were generated by preincubation

of the lectins with appropriate inhibitors in Tris buffer for 1 h at room temperature before use (Lee et al., 2016).

Table 1 Binding specificities of lectins used in the present study

Lectin abbreviations	Source	Concentration (μg/ml)	Sugar specificity	Inhibitor or eluting sugar*
WGA	Wheat germ agglutinin	1.0×10^{-2}	GlcNAc NeuAc, SA	0.2M GlcNAc
DBA	*Dolichos biflorus*	1.0×10^{-2}	αGalNAc	0.2M GalNAc
SBA	*Glycine max*	1.0×10^{-2}	α>βGalNAc	0.2M GalNAc
BSL-I	*Bandeiraea simplicifolia*	4.0×10^{-3}	αGal, αGalNAc	0.2M GalNAc
UEA-I	*Ulex europaeus*	2.0×10^{-2}	αFuc	0.1M L-fucose
PNA	*Arachis hypogaea*	4.0×10^{-3}	Galβ3GalNAc	0.2M βGal
LCA	*Lens culinaris*	4.0×10^{-3}	αMan, αGlc 4(Fucα1, 6)GlcNAc	0.2M MeαMan 0.2M MeαGlc
PHA-L	*Phaseolus vulgaris*	2.5×10^{-3}	Galβ4GlcNAcβ6 (GluNAcβ2Man α3) Man α3	0.1M acetic acid

Abbreviations: BSL, *Bandeiraea (Griffonia) simplicifolia* lectin; DBA, *Dolichos biflorus* agglutinin; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; LCA, *Lens culinaris* agglutinin; Man, mannose; MeαMan, α-Methylmannoside; MeαGlc, α-Methylglucoside; PHA-L, *Phaseolus vulgaris* leucoagglutinin; PNA, *Arachis hypogaea* (peanut) agglutinin; SA, sialic acid; SBA, *Glycine max* (soybean) agglutinin; UEA, *Ulex europaeus* agglutinin; WGA, *Triticum vulgaris* (wheat germ) agglutinin; NeuAc, N-acetyl-neuraminic acid.

* The acronyms and lectin specificities including sources, preferred sugar specificity and inhibitor were obtained from the data sheet (Lectin screening kit I-III, Vector laboratory) and a previous study (Lee et al., 2016).

4. Results

4.1 *Histological examination in VNOs of Korean black goat*

Cyto-architectural features of the VNO from Korean black goat at the fetal stage to adult stage are shown at low magnification in Fig. 1. The VNO samples were classified as vomeronasal sensory epithelium (VSE) or vomeronsal non-sensory epithelium (VNSE) and were capsulated by vomeronsal cartilage (Fig. 1A-C). In fetal samples, the VSE, located in the medial part of the VNO, was composed of receptor cells, supporting cells, and basal cells (Fig. 1A and 1D). On the other hand, the VNSE was located in the lateral part of the VNO and was composed of ciliated cells (arrowhead in Fig. 1E) and basal cells (hollow arrowhead in Fig. 1E). The vomeronasal nerves (VNn; asterisk in Fig. 1D) were distributed under the VSE. In the lamina propria of fetal VNSE, blood vessels and well-defined vomeronasal glands were observed (Fig. 1A and 1E). The VNOs exhibited similar cyto-architectural structures at postnatal day 1 (Fig. 1B, F, and 1G). Adult VNOs, including VSE and VNSE, were more highly developed (Fig. 1C, 1H and 1I) compared with fetal VNOs. The glandular cells in vomeronasal glands of the VNSE at all ages stained positive for PAS (Fig. 2A-C) and Alcian blue staining (pH 1.0 and pH 2.5; Fig. 2D-I).

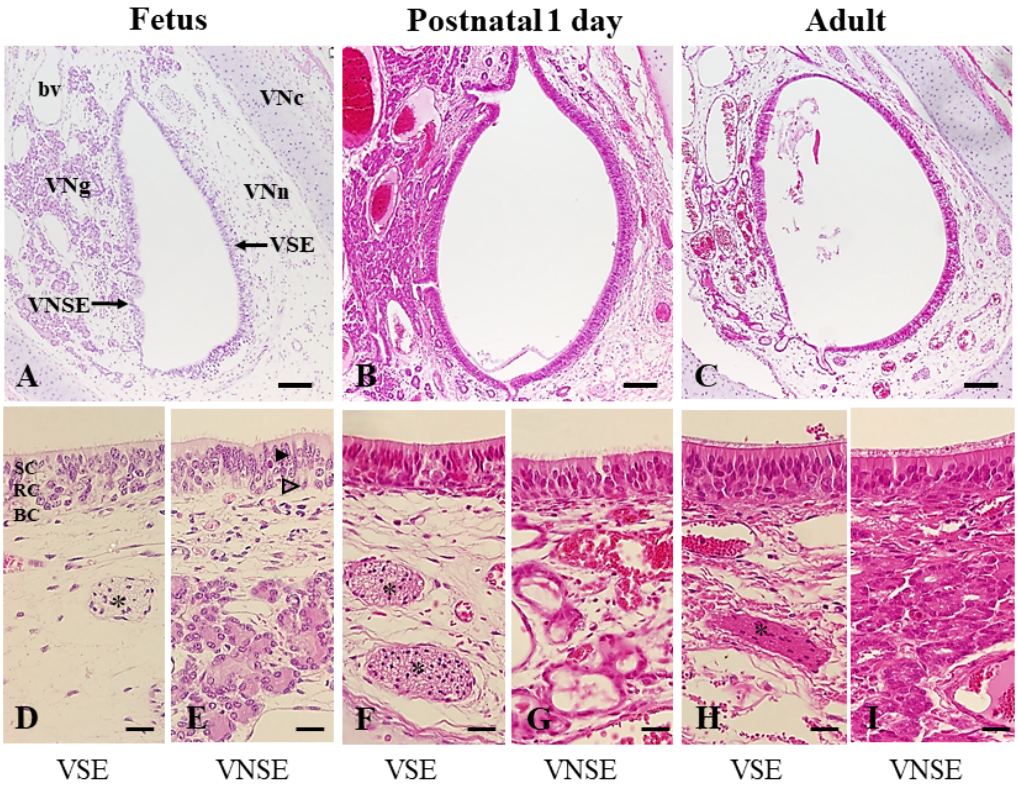

Figure 1. Histological examination of vomeronasal organs (VNOs) from the domestic goat, *Capra hircus coreanae.* (A, D and E) Fetal stage. (B, F, and G) One-day-old goats. (C, H and I) Adult goats. VNOs, including the VSE, VNSE, VNg, and bv, were encapsulated by VNc. BC, basal cell; bv, blood vessels; RC, receptor cell; SC, supporting cell; VNc, vomeronsal cartilage; VNg, vomeronasal glands; VNn, vomeronasal nerves; VNSE, vomeronasal non-sensory epithelium; VSE, vomeronasal epithelium. Asterisk, VNn; arrowhead, ciliated cells in the VNSE; hollow arrowhead, basal cells in the VNSE. Hematoxylin and eosin staining. Scale bars = 100 μm (A-C), 20 μm (D-I).

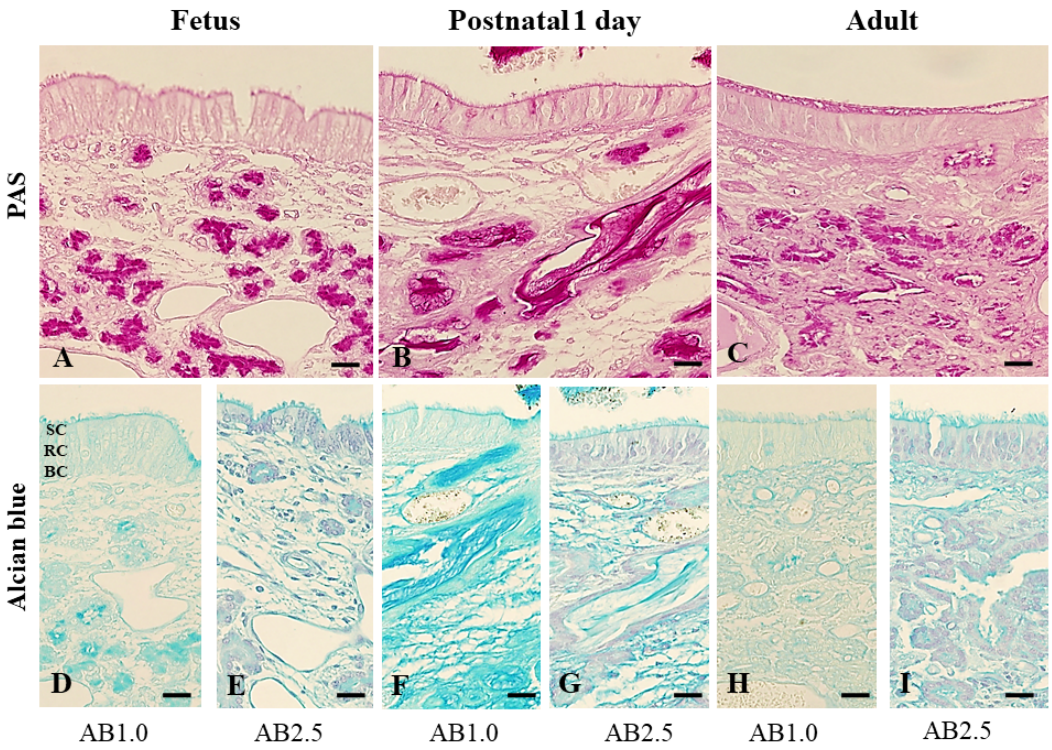

Figure 2. Periodic acid-Schiff (PAS) reaction and Alcian blue staining (pH 1.0 and pH 2.5) in Bowman's glands of the VNSE. (A, D and E) Fetal stage. (B, F and G) One-day-old goats. (C, H and I) Adult goats. Both PAS and Alcian blue reactions were detected in the acinar cells of Bowman's glands from the fetal to adult stage. Scale bars = 100 μm (A-C), 20 μm (D-I).

4.2. *Immunostaining of PGP9.5 and OMP in VNOs of Korean black goat*

First, we carried out immunohistochemical staining for PGP9.5 and OMP, markers for immature and mature receptor cells, respectively (Park et al., 2014). PGP 9.5-positive immunoreactivity was strongly detected in the receptor cells, free borders, and nerve bundles of VSE from fetal to adult goats (Fig. 3A-C). PGP 9.5-positive receptor cells and their dendrites extended to the lumen of VNOs (Fig. 3D-F). PGP 9.5-positive immunoreactivity was occasionally detected in some ciliated cells of the VNSE (Fig. 3G-I).

OMP-positive immunoreactivity was weakly detected in the receptor cells of the middle VSE layer in samples from fetal goats (Fig. 4A and 4D) and 1-day-old goats (Fig. 4B and 4E). However, the receptor cells of adult VNOs exhibited strong OMP-positive immunoreactivity (Fig. 4C and 4F). Neither PGP 9.5 nor OMP was detected in supporting cells in the VSE or VNSE. These results are summarized in Table 2.

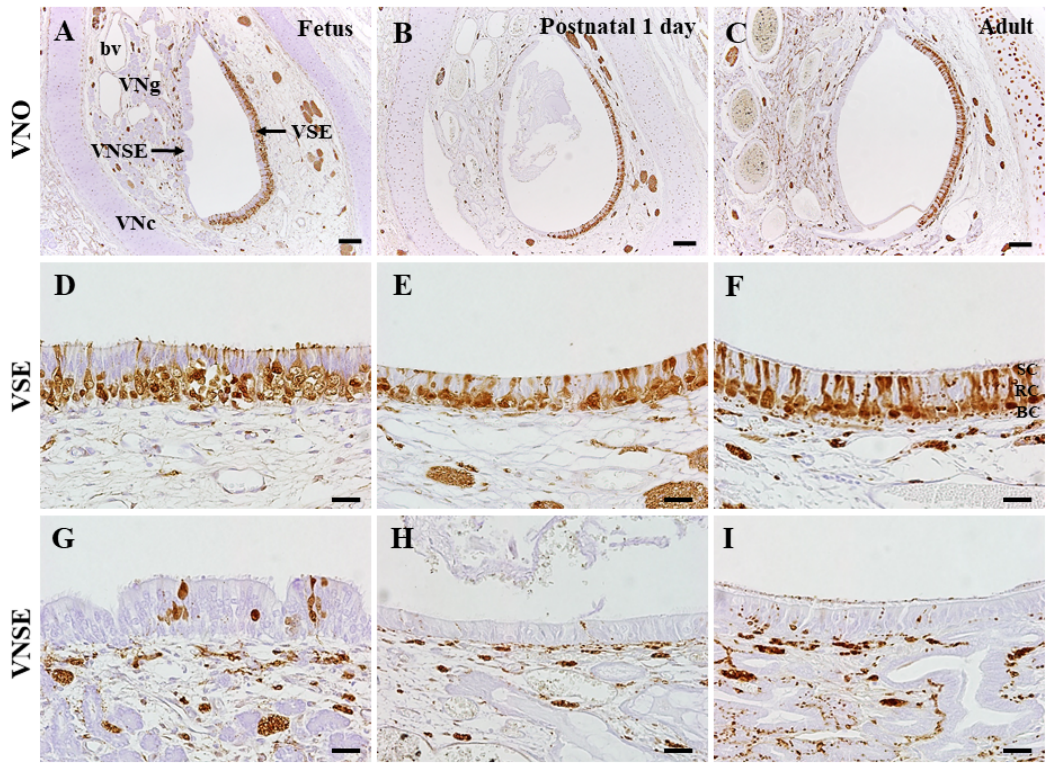

Figure 3. Immunohistochemical staining of protein gene product 9.5 (PGP 9.5) in VNOs from Korean black goat. (A, D and G) Fetal stage. (B, E and H) One-day-old goats. (C, F and I) Adult goats. PGP 9.5 was localized in receptor cells, basal cells, and nerve bundles (A-C). Under high magnification, strong PGP 9.5-positive immunoreactivity was detected in the apical dendrites of receptor cells (D-F). Some ciliated cells exhibited PGP 9.5-positive immunoreactivity (G-I). BC, basal cell; bv, blood vessels; RC, receptor cell; SC, supporting cell; VNc, vomeronsal cartilage; VNg, vomeronasal glands; VNn, vomeronasal nerves; VNSE, vomeronasal non-sensory epithelium; VSE, vomeronasal epithelium. Scale bars = 100 μm (A-C), 20 μm (D-I).

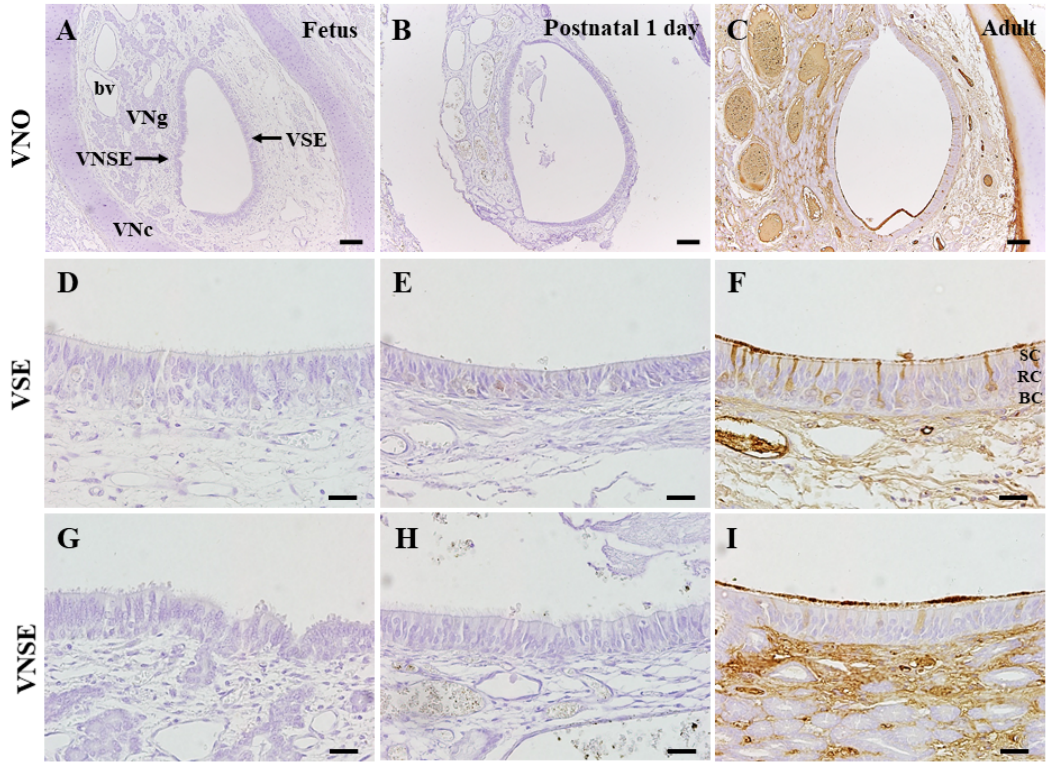

Figure 4. Immunohistochemical staining of olfactory marker protein (OMP) in VNOs from Korean black goat. (A, D and G) Fetal stage. (B, E and H) One-day-old goats. (C, F and I) Adult goats. OMP was not detected in the VSE or VNSE in fetal goats (A, D and G). In VNOs from one-day-old goats, OMP-positive immunoreactivity was weakly detected in the basal lamina of the VSE, but not in the VNSE (B, E and H). OMP was localized in receptor cells of the VSE and some ciliated cells of the VNSE at the adult stage (C, F and I). BC, basal cell; bv, blood vessels; RC, receptor cell; SC, supporting cell; VNc, vomeronsal cartilage; VNg, vomeronasal glands; VNn, vomeronasal nerves; VNSE, vomeronasal non-sensory epithelium; VSE, vomeronasal epithelium. Scale bars = 100 μm (A-C), 20 μm (D-I).

Table 2 Expression patterns of OMP and PGP 9.5 in the vomeronasal organ (VNO) of Korean black goat

Antibody	Stage	VSE			VNSE	
		Receptor cells	Supporting cells	Basal cells	Ciliated cells	Basal cells
PGP 9.5	Fetus	+++	-	+++	+[a]	-
	Day 1	+++	-	+++	+[a]	-
	Adult	+++	-	+++	+[a]	-
OMP	Fetus	±	-	-	+[a]	-
	Day 1	±	-	-	+[a]	-
	Adult	++	-	-	+[a]	-

Abbreviation: OMP, olfactory marker protein; PGP 9.5, protein gene product 9.5; VNSE, vomeronasal non-sensory epithelium; VSE, vomeronasal sensory epithelium.

Stained sections were scored as follows: -, negative; +/-, weak positive; +, positive; ++, moderatively positive; +++, intensively positive.

[a] Some cells are positive.

4.3. *Lectin histochemistry in VNOs of Korean black goat*

Strong lectin staining was observed in the free border of the VSE for WGA (Fig. 5A-C), LCA, BSL-I (Fig. 5G-I), PHA-L, and PNA (Table 3). Conversely, SBA and UEA-I and BLS-I exhibited moderate and/or faint staining (Table 3). DBA-positive staining was not detected in goats of any age in the present study (Fig. 5D-F, Table 3). WGA was strongly detected in the receptor cells and nerve bundles in 1-day-old (Fig. 5B) and adult goats (Fig. 5C). BSL-I was strongly detected in receptor cells from fetal goats (Fig. 5G); however, the intensity was lower in 1-day-old (Fig. 5H) and adult goats (Fig. 5I). Table 3 summarizes the intensities of varying lectins, including WGA, DBA, SBA, BSL-I, LCA, PNA, PHA-L, and UEA-I, in the VSE of Korean black goat.

In the VNSE of Korean black goat, WGA was strongly detected in the free border, goblet cells, and gland acini from fetal to adult goats (Fig. 6A-C), whereas ciliated cells and basal cells were weakly and/or not stained (Table 4). DBA was not detected in the VNSE in any age group (Fig. 6D-F). BSL-I was detected in the free border, ciliated cells, and goblet cells, but not in basal cells or gland acini from fetal to adult goats (Fig. 6G-I). Table 4 summarizes the intensities of various lectins, including WGA, DBA, SBA, BSL-I, LCA, PNA, PHA-L, and UEA-I, in the VNSE of Korean black goat.

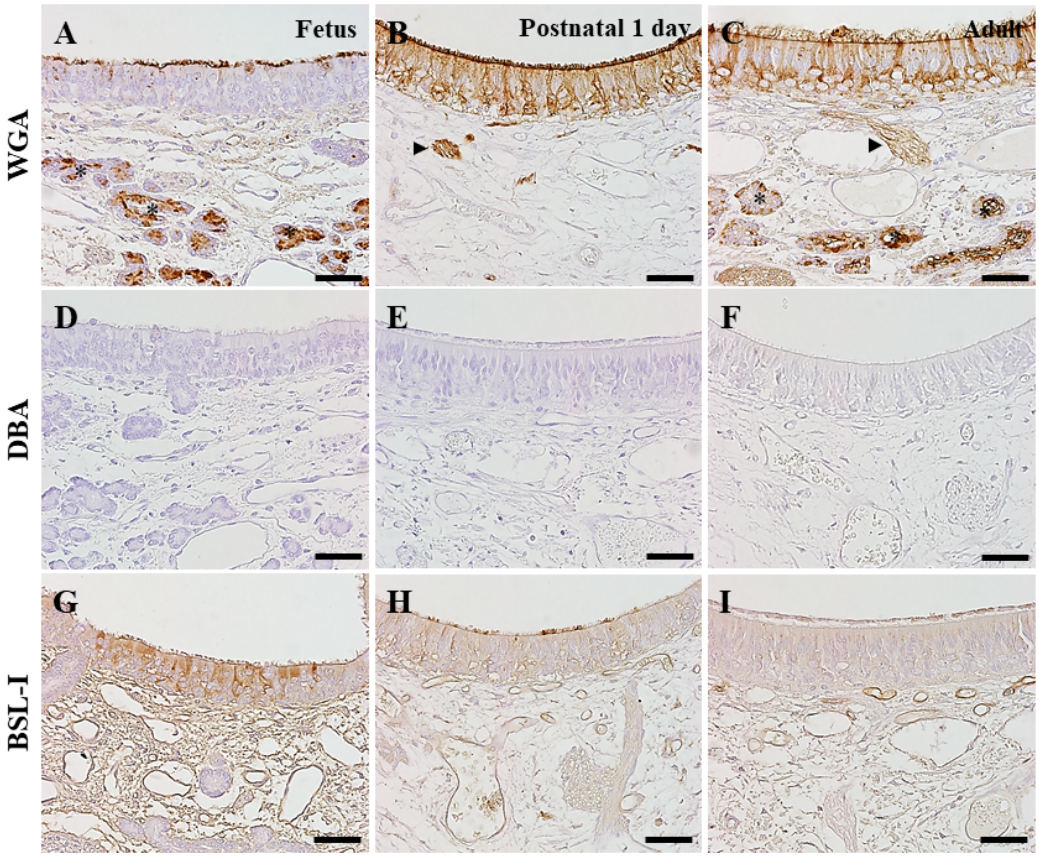

Figure 5. Lectin histochemistry for WGA, DBA, and BSL-I in the VSE of Korean black goat fetuses (A, D and G), one-day-old goats (B, E and H), and adult goats (C, F and I). (A-C) WGA. (D-F) DBA. (G-I) BSL-I. In the VSE, WGA was labelled in the free border, receptor cells, gland acini (asterisks), and nerve bundles (arrowhead) from the fetal to adult stage (A-C). However, DBA was not labelled in the VSE at any stage (D-F). BSL-I was labelled in the free border and receptor cells, but the intensity decreased during development (G-I). Arrowhead, nerve bundles; asterisks, gland acini. Hematoxylin counterstaining. Scale bars = 40 μm (A-I).

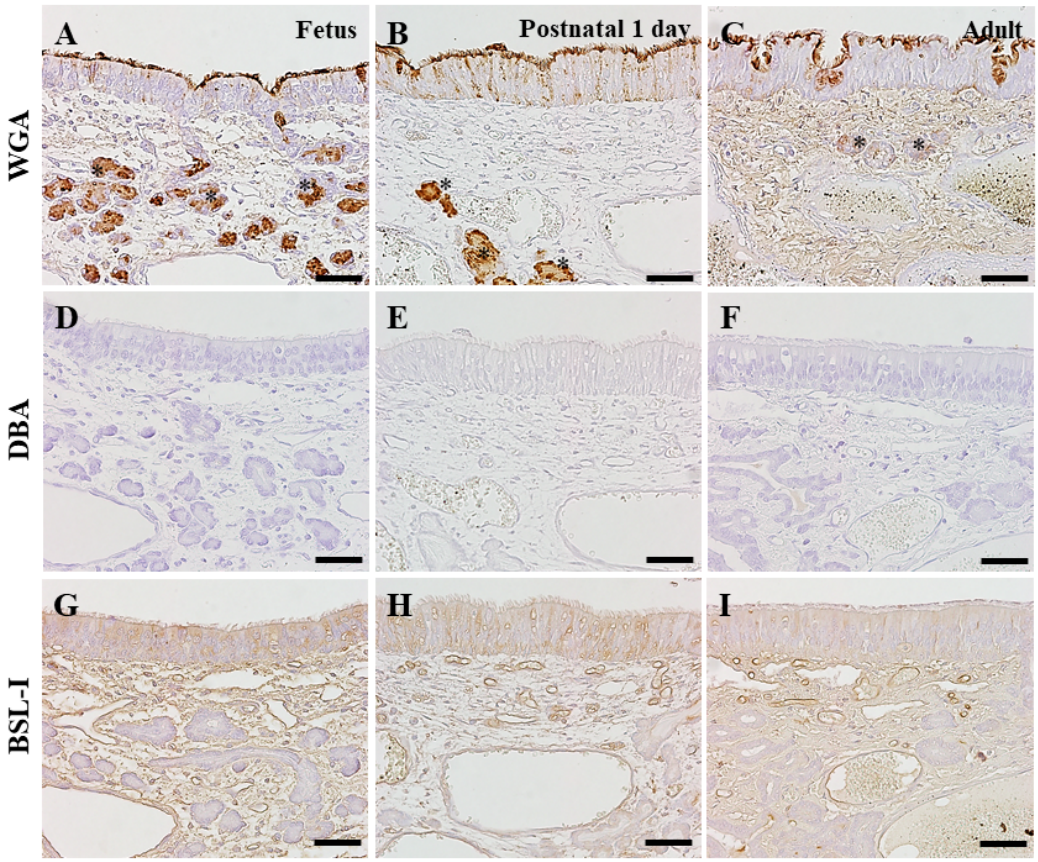

Figure 6. Lectin histochemistry for WGA, DBA, and BSL-I in the VNSE of Korean black goat fetuses (A, D and G), one-day-old goats (B, E and H), and adult goats (C, F and I). (A-C) WGA. (D-F) DBA. (G-I) BSL-I. In the VNSE, WGA was labelled in free border, ciliated cells, goblet cells, and gland acini (asterisks) from the fetal to adult stage (A-C). DBA was not labelled in the VNSE at any stage (D-F). BSL-I was labelled in the free border and ciliated cells (G-I). Asterisks, gland acini. Hematoxylin counterstaining. Scale bars = 40 μm (A-I).

Table 3 Lectin binding in the vomeronasal sensory epithelium (VSE) of Korean black goat

Lectins	Stage	Structure				
		Free border	Receptor cells	Supporting cells	Basal cells	Nerve bundle
WGA	Fetus	++	±[a]	-	-	±
	Day 1	++	++	+	-	++
	Adult	++	++	+	-	++
DBA	Fetus	-	-	-	-	-
	Day 1	-	-	-	-	-
	Adult	-	-	-	-	-
SBA	Fetus	+	-	-	-	-
	Day 1	+	-	-	-	-
	Adult	+	-	-	-	-
BSL-I	Fetus	++	±	++	-	-
	Day 1	+	±	+	-	-
	Adult	±	±	±	-	-
LCA	Fetus	+	+	+	++	+
	Day 1	++	++	+	++	+
	Adult	++	++	+	+	+
PNA	Fetus	++	+	-	-	-
	Day 1	++	++	-	±	+
	Adult	++	++	-	±	+
UEA-I	Fetus	+	++	++	++	++
	Day 1	+	++	++	++	++
	Adult	+	++	++	++	++
PHA-L	Fetus	++	++	+	++	+
	Day 1	++	++	+	++	+
	Adult	++	++	+	++	++

Abbreviation: BSL, *Bandeiraea simplicifolia* lectin; DBA, *Dolichos biflorus agglutinin*; LCA, *Lens culinaris* agglutinin; PHA-L, *Phaseolus vulgaris* leucoagglutinin; PNA, *Arachis hypogaea* (peanut) agglutinin; SBA, *Glycine max* (soybean) agglutinin; UEA, *Ulex europaeus* agglutinin; VNO, vomeronasal organ; WGA, *Triticum vulgaris* (wheat germ) agglutinin.

Stained sections were scored as follows: -, negative; ±, weak positive; +, moderate positive; ++, strong positive.

a Not detection in the present study.

Table 4 Lectin binding in the vomeronasal non-sensory epithelium (VNSE) of Korean black goat

Lectins	Stage	Structure				
		Free border	Ciliated cell	Goblet cell	Basal cells	Gland acini
WGA	Fetus	++	+	++	-	++
	Day 1	++	+	++	-	++
	Adult	++	+	++	-	++
DBA	Fetus	-	-	-	-	-
	Day 1	-	-	-	-	-
	Adult	-	-	-	-	-
SBA	Fetus	+	-	++	-	±
	Day 1	+	-	-	-	++
	Adult	+	-	-	-	++
BSL-I	Fetus	+	+	+	-	±
	Day 1	±	+	+	-	±
	Adult	±	+	+	-	±
LCA	Fetus	±	±	+	+	-
	Day 1	±	+	+	+	+
	Adult	++	++	+	+	+
PNA	Fetus	++	±	-	-	++
	Day 1	+	+[b]	-	-	++
	Adult	+	+[b]	-	-	++
UEA-I	Fetus	+	++	-	++	++
	Day 1	+	++	-[a]	++	++
	Adult	+	++	-[a]	++	++
PHA-L	Fetus	++	++	-	++	++
	Day 1	++	++	-	++	++
	Adult	++	++	-	++	++

Abbreviation: BSL, *Bandeiraea simplicifolia* lectin; DBA, *Dolichos biflorus agglutinin*; LCA, *Lens culinaris* agglutinin; PHA-L, *Phaseolus vulgaris* leucoagglutinin; PNA, *Arachis hypogaea* (peanut) agglutinin; SBA, *Glycine max* (soybean) agglutinin; UEA, *Ulex europaeus* agglutinin; VNO, vomeronasal organ; WGA, *Triticum vulgaris* (wheat germ) agglutinin.

Stained sections were scored as follows: -, negative; ±, weak positive; +, moderate positive; ++, strong positive.

a Not detection in the present study; b Some ciliated cells are positive.

5. Discussion

This is the first description of developmental changes in Korean black goat VNO, the initial site for pheromone detection. PGP 9.5-positive immature receptor cells were detected in VSE and VNSE from the fetal stage to adult stage. Conversely, OMP-positive mature receptor cells were localized in the VSE of adult goats. Lectins, including WGA, SBA, DBA, BSL-I, PNA, and UEA-I, were detected at varying intensities, depending on the cell type. These results suggest that the VSE, including receptor cells, is well-developed at the prenatal stage in the Korean black goat.

Ungulate mammals, including deer and goats, rely on odor cues for foraging, sexual behavior, maternal behavior, and territory disputes (Asher et al., 1990, Gelez and Fabre-Nys, 2004, Wood, 2003). The VNO is the first site for the perception of volatile odorants such as pheromones. It includes sensory and non-sensory epithelia encased by VNC, and the axons of VNO receptor cells, which are V1 type receptors (Giα2-positive), terminate in the nerve layer of AOB (Halpern et al., 1998, Halpern and Martinez-Marcos, 2003). We previously reported the morphological structure of adult VNOs from Korean black goat (Park et al., 2013) based on immunohistochemistry and lectin histochemistry, but we did not investigate developmental changes in VNOs, including the sensory and non-sensory epithelia. In the present study, we focused on receptor cells using OMP and PGP 9.5 antibodies, markers for matured and immature receptor cells, respectively. We also analyzed the diversity of glycans by WGA, SBA, DBA, BSL-I, LCA, PNA, PHA-L and UEA-I staining.

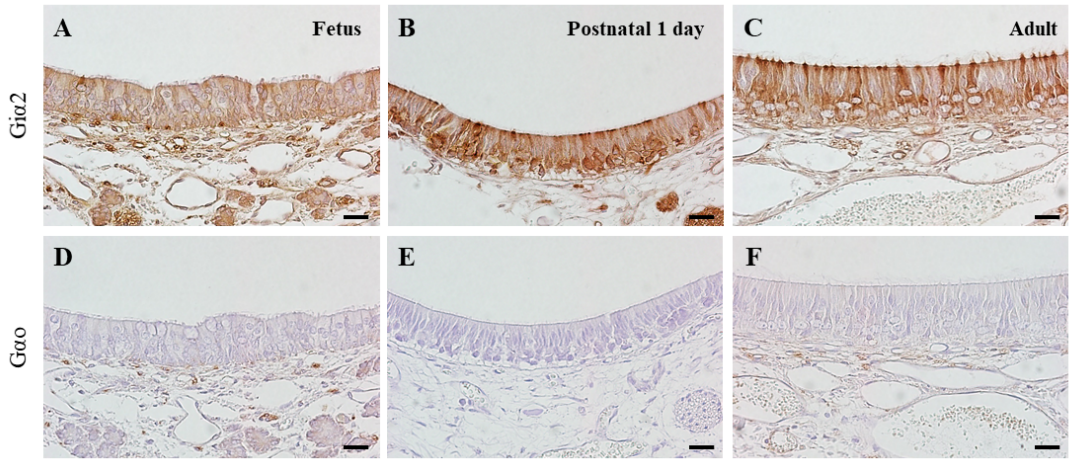

Figure 7. Immunohistochemical staining of Giα2 and Goα in the VNO of Korean black goat. (A and D) Fetal stage. (B and E) Postnatal 1 day. (C and F) Adult. Giα 2 was localized in vomeronasal receptor cells and nerve bundles (A-C). On the other hands, Gαo was not detected in the vomeronasal receptor cells. These results indicated that Korean black goat has the uniform vomeronasal receptor 1. Scale bars = 20 μm (A-F).

In VNOs from Korean black goat, numerous PGP 9.5-positive immature receptor cells were observed from the fetal to adult stage, and some slender cells in the VNSE exhibited PGP 9.5-positive immunoreactivity. In the rodent olfactory system, PGP 9.5 is detected in olfactory sensory neurons from the olfactory epithelium and mitral/tufted cells of the main olfactory bulb (Weiler and Benali, 2005). Conversely, we previously detected PGP 9.5-positive immunoreactivity in the receptor cells, basal cells, gland acini, and nerve bundles of adult Korean black goat VNOs (Park et al., 2013). Our results indicated that PGP 9.5-positive immunoreactivity remained consistent in the VSE and VNSE of Korean black goat from the late fetal stage onwards.

OMP-positive receptor cells were not detected in the VSE from fetal goats, but increased in density and intensity from 1-day-old to adult goats. OMP-positive immunoreactivity was also detected in some cells in the VSE of adult goat VNOs. Vomeronasal receptor 1 (V1R) cells, which are Giα2-positive, were detected in the VSE from the fetal stage to the adult stage (Figure 7). In the mammalian vomeronasal system, vomeronasal receptor cells are divided into V1R and vomeronasal receptor 2 (V2R) types, and only V1R is detected in the goat VNO (Takigami et al., 2000, Takigami et al., 2004). Furthermore, Giα2-positive receptor cells were detected in the VSE from postnatal day 1 onwards (Takigami et al., 2004). Therefore, our results suggest that OMP-positive V1R cells in the VNOs of Korean black goats develop after birth. Recently, OMP was also found in non-olfactory systems, including the heart, thymus, thyroid, and testis. In these organs, olfactory receptor-mediated chemoreception occurs through OMP, which is involved in repolarization through the efflux of intracellular Ca^{++} (Ferrer et al., 2016, Kang et al., 2015). Some ciliated cells of the VNSE exhibited OMP-positive immunoreactivity, which may be associated with chemoreception in Korean black goats. However, the precise role of OMP-positive cells in the VNSE requires further

investigation.

Regarding the developmental changes of glycan epitopes in VNOs from Korean black goats, the limited sample size of the present study made it difficult to analyze the density and intensity of glycoconjugates in the VSE and VNSE from the fetal to adult stage. The intensities of WGA, LCA, and PNA increased in the receptor cells of the VSE, and the intensity of PNA increased in ciliated cells of the VNSE during development. The intensity of each lectin follows different patterns among animals, including sheep (Salazar et al., 2003), pig (Park et al., 2012b, Salazar et al., 2004), horse (Lee et al., 2016), rabbit (Villamayor et al., 2018) and roe deer (Shin et al., 2017) (Summarized in Table 5). However, considering the developmental changes in both the VSE and VSNE of Korean black goat, our results suggest that certain glycan epitopes increase with age in the VNO. These glycan epitopes may be involved with pheromone perception, given their functions in membrane interactions, cell-to-cell adhesion, and ion transport (Spicer and Schulte, 1992).

Collectively, our results suggest that VNOs from Korean black goat develop by the late fetal stage and undergo further differentiation after birth.

Table 5 Lectin binding in the vomeronasal organ of various animals

Species	Stage	Lectins	VSE	VNSE	Gland	Reference
Sheep	Fetus	LEA	++	+	++	(Salazar et al., 2003)
		BSL-I	-	-	-	
		UEA-I	-	-	-	
		DBA	-	-	-	
Pig	Fetus	LEA	++	++	-	(Salazar et al., 2004)
		UEA-I	-	-	-	
Pig	Fetus	WGA	±	NI	±	(Park et al., 2012)
		UEA-I	±	NI	±	
		BSL-I	-	NI	-	
		DBA	-	NI	-	
		SBA	-	NI	-	
	Day2	WGA	++	NI	+	
		UEA-I	++	NI	+	
		BSL-I	-	NI	-	
		DBA	-	NI	-	
		SBA	±	NI	±	
	Adult	WGA	±	NI	+	
		UEA-I	+	NI	+	
		BSL-I	±	NI	±	
		DBA	±	NI	±	
		SBA	++	NI	+	
Horse	Adult	WGA	++	+	++	(Lee et al., 2016)
		UEA-I	-	++	++[a]	
		BSL-I	+	+	-	
		DBA	++	-	++[a]	
		SBA	±	±	++[a]	
		LCA	+	+	±	
		PNA	++	±	++[a]	
Rabbit	67-70 days old	UEA	++	+	+/-	(Villamayor et al., 2018)
Roe deer	Adult	WGA	±	±	±	(Shin et al., 2017)
		UEA-I	±	±	±[a]	
		BSL-I	±	+[b]	±[a]	
		DBA	-	±	±	
		SBA	±	+[b]	±	
		LCA	+	+[b]	±	
		PNA	+	-	±[a]	

Stained sections were scored as follows: -, negative; ±, weak positive; +, moderate positive; ++, strong positive.

NI, Not investigated.

[a] Some acinar cells are positive.

[b] Some receptor cells are positive.

References

Asher, G.W., Fisher, M.W., Smith, J.F., Jabbour, H.N., Morrow, C.J., 1990. Temporal relationship between the onset of oestrus, the preovulatory LH surge and ovulation in farmed fallow deer, Dama dama. J Reprod Fertil 89, 761-7.

Breer, H., Fleischer, J., Strotmann, J., 2006. The sense of smell: multiple olfactory subsystems. Cell Mol Life Sci 63, 1465-75.

Buiakova, O.I., Baker, H., Scott, J.W., Farbman, A., Kream, R., Grillo, M., et al., 1996. Olfactory marker protein (OMP) gene deletion causes altered physiological activity of olfactory sensory neurons. Proc Natl Acad Sci U S A 93, 9858-63.

Ferrer, I., Garcia-Esparcia, P., Carmona, M., Carro, E., Aronica, E., Kovacs, G.G., et al., 2016. Olfactory Receptors in Non-Chemosensory Organs: The Nervous System in Health and Disease. Front Aging Neurosci 8, 163.

Gelez, H., Fabre-Nys, C., 2004. The "male effect" in sheep and goats: a review of the respective roles of the two olfactory systems. Horm Behav 46, 257-71.

Halpern, M., Jia, C., Shapiro, L.S., 1998. Segregated pathways in the vomeronasal system. Microsc Res Tech 41, 519-29.

Halpern, M., Martinez-Marcos, A., 2003. Structure and function of the vomeronasal system: an update. Prog Neurobiol 70, 245-318.

Kang, N., Kim, H., Jae, Y., Lee, N., Ku, C.R., Margolis, F., et al., 2015. Olfactory marker protein expression is an indicator of olfactory receptor-associated events in non-olfactory tissues. PLoS One 10, e0116097.

Kwon, H.J., Koo, J.H., Zufall, F., Leinders-Zufall, T., Margolis, F.L., 2009. Ca extrusion by NCX is compromised in olfactory sensory neurons of OMP mice. PLoS One 4, e4260.

Lee, K.H., Park, C., Kim, J., Moon, C., Ahn, M., Shin, T., 2016. Histological and lectin histochemical studies of the vomeronasal organ of horses. Tissue Cell 48, 361-9.

Luna, L.G., Armed Forces Institute of Pathology (U.S.), Armed Forces Institute of Pathology (U.S.). 1968. Manual of histologic staining methods of the Armed Forces Institute of Pathology. 3d ed. New York,: Blakiston Division.

Oboti, L., Peretto, P., Marchis, S.D., Fasolo, A., 2011. From chemical neuroanatomy to an understanding of the olfactory system. Eur J Histochem 55, e35.

Park, C., Ahn, M., Lee, J.Y., Lee, S., Yun, Y., Lim, Y.K., et al., 2014. A morphological study of the vomeronasal organ and the accessory olfactory bulb in the Korean roe deer, Capreolus pygargus. Acta Histochem 116, 258-64.

Park, C., Choi, S., Joo, H.G., Ahn, M., Taniguchi, K., Shin, T., 2012a. Galectin-3 immunohistochemistry in the vomeronasal organ of the domestic pig, Sus scrofa. Acta Histochem.

Park, C., Yang, W., Bae, Y., Lee, Y., Kang, W., Ahn, M., et al., 2013. A morphological study of vomeronasal organ of Korean black goat (Capra aegagrus hircus). Korean J Vet Res 53, 55-60.

Park, J., Lee, W., Jeong, C., Kim, H., Taniguchi, K., Shin, T., 2012b. Developmental changes affecting lectin binding in the vomeronasal organ of domestic pigs,

Sus scrofa. Acta Histochem 114, 24-30.

Salazar, I., Lombardero, M., Aleman, N., Sanchez Quinteiro, P., 2003. Development of the vomeronasal receptor epithelium and the accessory olfactory bulb in sheep. Microsc Res Tech 61, 438-47.

Salazar, I., Sanchez Quinteiro, P., Lombardero, M., Aleman, N., Fernandez de Troconiz, P., 2004. The prenatal maturity of the accessory olfactory bulb in pigs. Chem Senses 29, 3-11.

Shin, T., Kim, J., Choi, Y., Ahn, M., 2017. Glycan diversity in the vomeronasal organ of the Korean roe deer, Capreolus pygargus: A lectin histochemical study. Acta Histochem 119, 778-85.

Spicer, S.S., Schulte, B.A., 1992. Diversity of cell glycoconjugates shown histochemically: a perspective. J Histochem Cytochem 40, 1-38.

Takigami, S., Mori, Y., Ichikawa, M., 2000. Projection pattern of vomeronasal neurons to the accessory olfactory bulb in goats. Chem Senses 25, 387-93.

Takigami, S., Wakabayashi, Y., Ohsako, S., Ohkura, S., Okamura, H., Ikai, A., et al., 2004. Fetal development of vomeronasal system in the goat. Brain Res Dev Brain Res 149, 113-20.

Villamayor, P.R., Cifuentes, J.M., Fdz-de-Troconiz, P., Sanchez-Quinteiro, P., 2018. Morphological and immunohistochemical study of the rabbit vomeronasal organ. J Anat 233, 814-27.

Weiler, E., Benali, A., 2005. Olfactory epithelia differentially express neuronal markers. J Neurocytol 34, 217-40.

Wood, W.F., 2003. Volatile components in metatarsal glands of sika deer, Cervus nippon. J Chem Ecol 29, 2729-33.

Abstract in Korean

한국 흑염소 보습코기관에 대한 연구

(지도교수: 신 태 균)

양 원 준

제주대학교 일반대학원 수의학과

보습코기관은 부후각신경계의 말초 감각 기관으로 동물의 성행동과 관련이 높은 페로몬을 감지한다. 보습코기관은 내측의 신경상피와 외측의 비신경상피인 호흡상피로 나뉘어진다. 신경상피의 감각기 세포에서 감지된 페로몬이 부후각망울로 신호를 전달하게 되고 이후 발정관련 호르몬이 분비되게 된다. 현재, 성체 한국 흑염소의 보습코기관의 조직학적 연구가 진행되었으나 출생 전부터 성체까지 보습코기관의 조직학적 변화는 아직 연구된 바가 없다.

본 연구에서는 출생 전 개체, 생후 1일령, 2년령의 성숙 개체 각 2마리씩을 이용하여, 육안해부학 및 조직학적 구조를 확인하였다. 각 개체에서 채취한 보습코기관은 탈회과정을 거치고, 파라핀에 포매한 후 조직 절편을 제작하였다. 조직 절편을 이용하여 보습코기관의 상피 및 점막 구조에 대하여 평가하였다. 보습코기관은 연골에 둘러싸인 반달모양의 구조물로 후각을 감지하는 수용체세포가 있는 신경상피와 수용체 세포가 존재하지 않는 호흡상피로 이루어져 있다. 호흡상피는 후각수용체세포, 지지세포, 바닥세포로 구성되어 있으며, 호흡상피는 거짓중층원주상피의 구성을 나타내었다. 중성 및 산성 다당류의 점액 염색에서는 두 종류 점액 성분이 태아기 때부터 성체기까지 강하게 염색되었다.

동물의 후각점막과 보습코기관의 기능과 관련이 있으며, 후각감각신경의 대표적인 표지 단백질인 olfactory marker protein (OMP)와 미성숙 후각신경세포의 표지 단백질인 protein gene product 9.5 (PGP 9.5)을 이용하여 면역염색을 실시하였다. 출생 전 개체와 생후 1일령의 흑염소 보습코기관 감각상피의 감각기세포에서 OMP가 발현이 약하게 확인되었고, 호흡상피에서도 일부 양성 반응이 관찰되었다. 성체 한국 흑염소 보습코기관에서 OMP는 감각상피의 감각기세포에서 강하게 발현되었으며, 호흡상피의 일부 세포에서도 확인되었다. 이에 반해, 미성숙 신경세포의 표지 단백질인 PGP 9.5는 출생 전 개체와 생후 1일령에서 강하게 발현되었고, 성체에서는 반응성이 감소하였다. 또한, 모든 연령의 호흡상피에서 양성 반응이 확인되었다.

렉틴조직화학 염색결과, 감각상피의 자유면에서는 맥아응집소(wheat germ agglutinin; WGA)를 포함한 7종의 렉틴이 모두 결합되었으며, 감각기 세포, 기저세포와 신경다발에서는 말콩응집소(*Dolichos biflorus* agglutinin; DBA)를 제외한 6종의 렉틴이 결합되었다. 호흡상피에서도 맥아응집소를 포함한 7종의 렉틴이 자유면에서 결합되었으며, 말콩응집소를 제외한 6종의 렉틴이 섬모세포, 술잔세포, 기저세포 그리고 샘포에서 양성으로 확인되었다.

이러한 결과는 태아기 흑염소 보습코기관의 감각상피에서 후각기능과 관련이 깊은 OMP가 생산되기 시작한다는 것을 시사하며, 성체가 될수록 보습코기관 감각상피의 후각감지세포에서 활성이 증가된다는 것을 보여준다.

주요어: 발달단계, 한국 흑염소, Olfactory marker protein, Protein gene product 9.5, 보습코기관.

감사의 글

2008년 겨울 수의해부학실험실 오픈랩에 참여한 계기로 존경하는 신태균 교수님께 큰 가르침을 받고 소중한 인연을 맺게 되었습니다. 다사다난했던 수년의 세월이 지나고 이제야 비로소 석사학위를 마치고 교수님을 비롯한 주변에 많은 소중한 분들에게 짧은 지면을 통해 감사한 말씀을 전할 수 있게 되었습니다.

먼저 저의 주례선생님이시자 스승이신 신태균 교수님께 감사드립니다. 예과 시절 처음 뵌 교수님께 “섬에서 세계를 보라”라는 말씀을 듣고 크게 감동했습니다. 교수님과 함께라면 더 많은 것을 보고 배울 수 있을 거란 믿음이 생겼고 그 이후 정말 감사하게도 저의 삶에 큰 가르침을 주셨습니다. 그리고 학위 논문이 완성되기까지 많은 가르침을 주신 허승담 원장님, 김정태 교수님께 진심으로 감사의 말씀 올립니다.

수의학과에 와서 학부 시절부터 많은 가르침을 주시고 학문의 정수를 알려주신 존경하는 박전홍 교수님, 배종희 교수님, 이두식 교수님, 이경갑 교수님, 임윤규 교수님, 우호춘 교수님, 이영재 교수님, 정종태 교수님, 손원근 교수님, 김재훈 교수님, 황규계 교수님, 주홍구 교수님, 윤영민 교수님, 이주명 교수님, 강태영 교수님, 지영흔 교수님, 박현정 교수님, 한창훈 교수님께 감사의 말씀 올립니다.

실험실 생활 동안 정신적으로 큰 버팀목이 되어주신 이용덕 박사님, 김승준 교수님, 강재윤 선배님, 김황룡 선배님, 김철 선배님, 진재광 박사님, 문창종 교수님, 강종철 원장님, 안미정 교수님, 이기현 선배님, 이광협 원장님, 김희철 박사님, 정경숙 선배님, 정찬우 선배님, 정진우 선배님께 진심으로 감사드립니다. 그리고 실험실 동기 오한슬, 배연지, 이철호, 강소희에게 고마운 마음을 전합니다. 논문작성에 큰 도움을 준 박창남, 최유나, 전지윤 선생님께도 감사드립니다.

바쁜 와중에 함께 고생해준 ㈜명성 이상호 원장님, 이상숙 대표님, 이강산 이사님, 김재윤 부장님 그리고 권호민 차장님께 깊은 감사의 말씀 올립니다. 덕분에 석사과정을 잘 마무리할 수 있었습니다. 한별팜텍 김동욱 원장님, 정필수 원장님, 도드람 동물병원 신준재 수의사님께도 감사드립니다.

지금의 저를 만들어 주신 부모님, 항상 응원해 주시는 장인어른, 장모님, 그리고 동생 원혁에게 감사드립니다. 특별히 물심양면으로 지원해 주시고 저에게 큰 힘이 되어주신 장영자 여사님께 감사드립니다.

마지막으로 사랑하는 아내 신영이와 저의 분신 서진이에게 감사의 말을 남깁니다.