A Thesis
Doctoral Thesis of Philosophy

# Morphological characterization of main and accessory olfactory systems of horses

Department of Veterinary Medicine

GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

Kwang-Hyup Lee

2017. 2.

# Morphological Characterization of main/accessory olfactory systems of horses

Kwang-Hyup Lee

(Supervised by professor Taekyun Shin)

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

2016. 12.

This thesis has been examined and approved.

Thesis director, Seungjoon Kim, Prof. of Dept. of Veterinary Medicine

Youngheun Jee, Prof. of Dept. of Veterinary Medicine

Changjong Moon, Prof. of Dept. of Veterinary Medicine

Meejung Ahn, Research Prof. of School of Medicine

Taekyun Shin, Prof. of Dept. of Veterinary Medicine

Department of Veterinary Medicine

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

# CONTENTS

# List of Abbreviation

| | |
|---|---|
| AB 2.5 | alcian blue, pH 2.5 |
| ABC | avidin-biotin-complex |
| AOB | Accessory olfactory bulb |
| BSL-I | *Bandeiraea simplicifolia* lectin-I |
| BSL-II | *Bandeiraea simplicifolia* lectin-II |
| Con A | Concanavalin A |
| DAB | diaminobenzidine |
| DBA | *Dlichos biflorus* |
| DSL | *Datura stramonium* lectin |
| ECL | *Erythrina cristagalli* lectin |
| ET | ethmoturbinates |
| FS | frontal sinus |
| GTP | guanine triphosphate |
| HE | Hematoxylin-eosin |
| HRP | Horseradish peroxidase |
| LCA | *Lens culinaris* agglutinin |
| LEL | *Lycopersicon esculentum* lectin |
| LP | Lamina propria |
| MT | Maxilloturbinate |
| NP | nasopharynx |
| NT | Nasoturbinate |
| OB | Olfactory bulb |
| OE | Olfactory epithelium |
| OMP | Olfactory marker protein |
| PAS | Periodic acid-Schiff |
| PBS | Phosphate-buffered saline |
| PGP 9.5 | Protein gene product 9.5 |
| PHA-E | *Phaseolus vulgaris* erythroagglutinin |
| PHA-L | *Phaseolus vulgaris* leucoagglutinin |

| | |
|---|---|
| PNA | Peanut agglutinin |
| PSA | *Pisum sativum* agglutinin |
| RCA 120 | *Ricinus communis* agglutinin-1 |
| s-WGA | succinylated-wheat germ agglutinin |
| SBA | Soybean agglutinin |
| SJA | *Sophora japonica* |
| STL | *Solanum tuberosum* lectin |
| UEA-I | *Ulex europaeus* agglutinin-I |
| VNC | vomeronasal cartilage |
| VNO | Vomeronasal organ |
| VNSE | Vomeronasal non-sensory epithelium |
| VSE | Vomeronasal sensory epithelium |
| VVA | *Vicia Villosa* agglutinin |
| WGA | Wheat germ agglutinin |

# List of Figures

# List of Tables

# General introduction

## Two olfactory systems in mammals

The mammals are highly dependent on volatile and non-volatile odorous information for social interaction, territory disputes, reproduction, and sexual behavior (Baum and Cherry, 2015; Breer et al., 2006; Keller and Lévy, 2012). The odorous information transmits through the two olfactory systems including main and accessory vomeronasal olfactory system. In the main olfactory system, olfactory receptor cells in the olfactory mucosa receive conventional odors and project olfactory nerves to the main olfactory bulb (OB). The OB transmits signals to specific areas of the brain, including the anterior olfactory nucleus, piriform cortex, olfactory tubercle and anterior cortical amygdala (Breer et al., 2006; Yokosuka, 2012). The vomeronasal organ (VNO) is the receptor organ of the vomeronasal system, which perceives various molecules related to social and reproductive communication and transmits signals to the accessory olfactory bulb (Brennan, 2001; Park et al., 2014; Takami, 2002; Yokosuka, 2012; Zufall et al., 2002). The lumen of the VNO is covered by the vomeronasal sensory epithelium (VSE) medially and the vomeronasal non-sensory epithelium (VNSE) laterally (Lee et al., 2003). The VNO glands are located in the lamina propria and secrete mucous and/or seromucous elements into the luminal surface of the VNO epithelium, playing a role in the detection of odorous molecules by the receptor cells of the VSE (Carmanchahi et al., 2000).

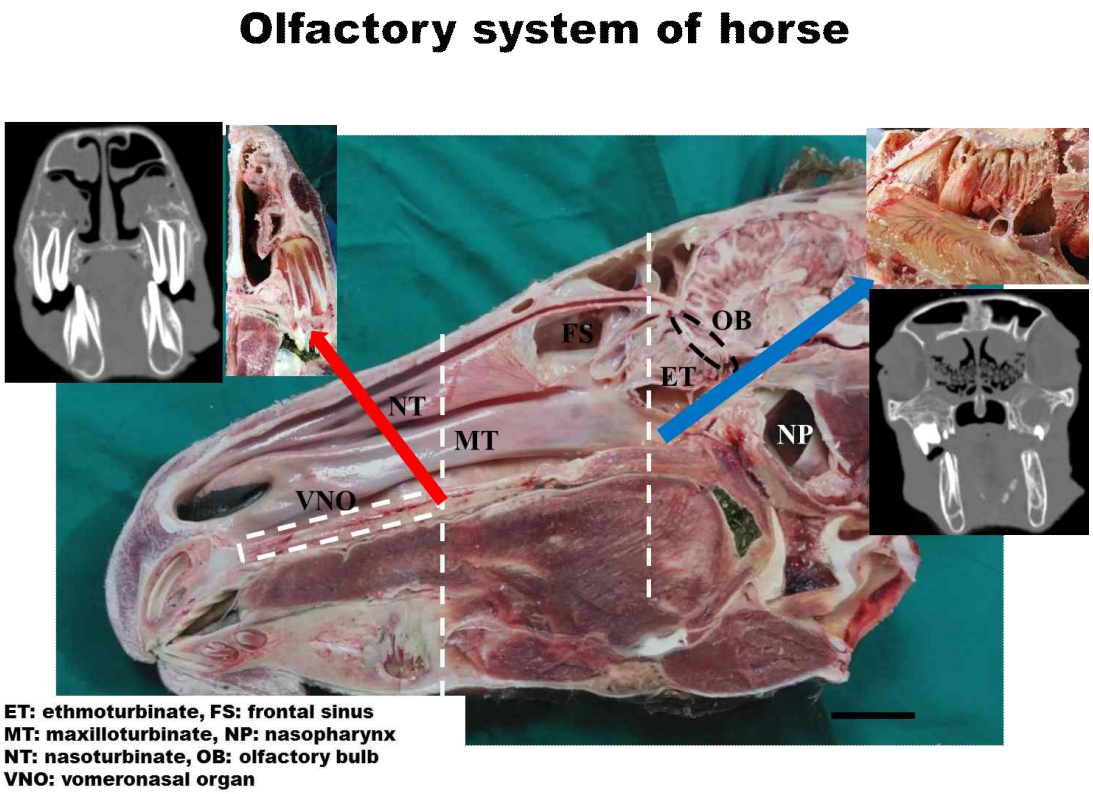


**Figure 1**. Gross and Computed tomography (CT) finding of the two olfactory systems including main and accessory vomeronasal olfactory system in horse.

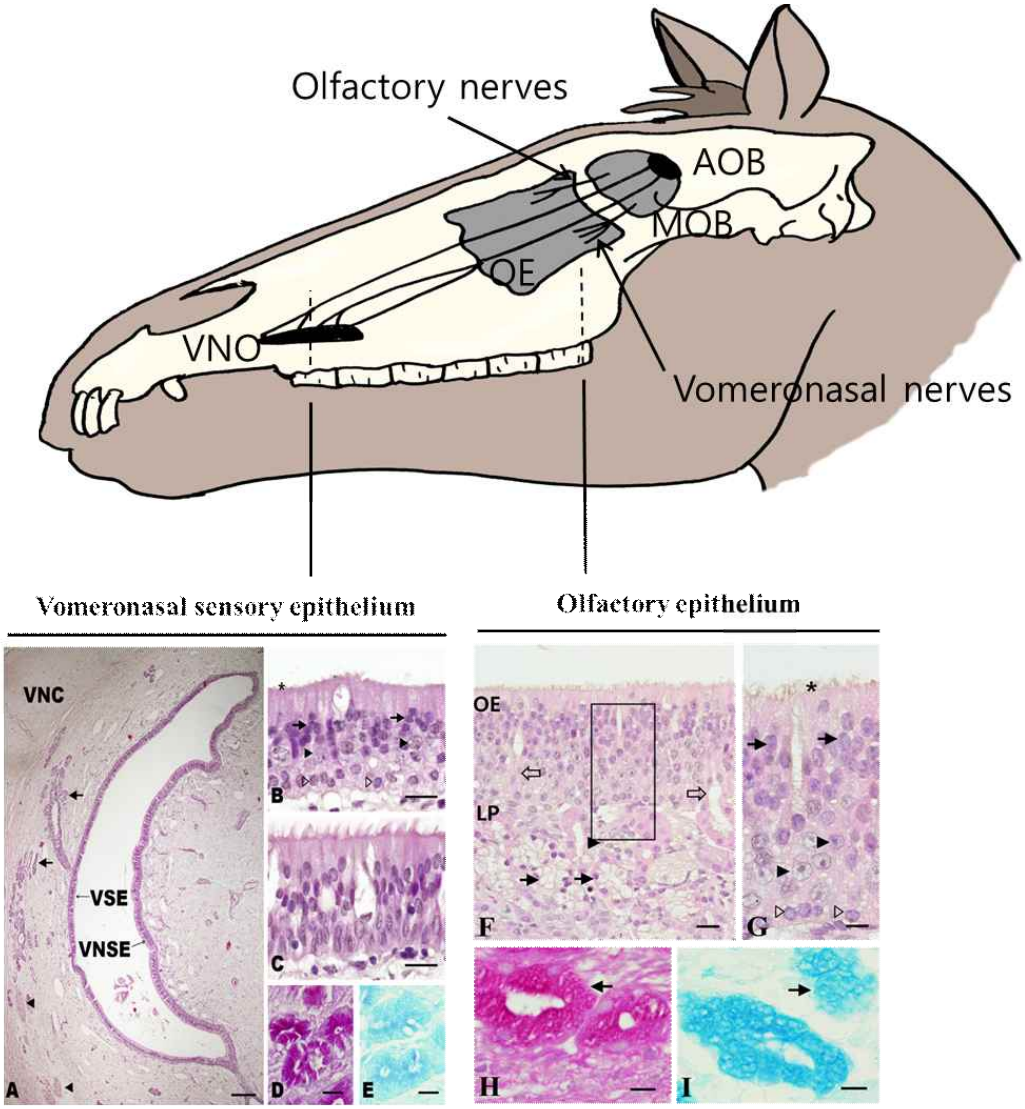


**Figure 2.** Schematic illustrations of sagittal section and histological findigns of the horse head showing the vomeronasal and main olfactory systems. The vomeronasal organ (VNO) and its nerve projection to the accessory olfactory bulb (AOB), and the olfactory epithelium (OE) and its nerve projection to the main olfactory bulb (MOB). (A-E) Histological findings of the horse vomeronasal organ. (A) Dotted line indicates the position of the coronal section of the VNO. (B) Higher magnification of the VSE. The VSE is

composed of three discernible layers (supporting cells, arrows; receptor cells, arrowheads; basal cells, hollow arrowheads). The asterisk indicates the free border. (C) Higher magnification of the VNSE. The VNSE consists of non-sensory, ciliated, pseudostratified epithelium. H&E staining. (D) PAS staining of the acinar cells of the vomeronasal glands. (E) Alcian blue pH 2.5 staining of the acinar cells of the vomeronasal glands. (F) The olfactory mucosa comprises the olfactory epithelium (OE) and the lamina propria (LP). Pyramidal acinar cells (arrows) of Bowman's glands, the ducts of Bowman's glands intervening in the OE (hollow arrows) and the olfactory nerve bundles (arrowhead) in the LP are shown. (G) Higher magnification of the OE is shown in the marked rectangle in Figure F. The asterisk indicates the free border. The nuclei in the olfactory supporting cells are oval (arrows). The secretory duct is vertically positioned in the olfactory mucosa. The olfactory receptor cells (black arrowheads) between basal cells (hollow arrowheads) and supporting cells (black arrows) are shown. (H) Periodic acid-Schiff (PAS) reaction in the olfactory mucosa. PAS reactivity observed in Bowman's gland acini (arrow). (I) Alcian blue (pH 2.5) staining in the olfactory mucosa. Scale bar in (A) = 20 μm. Scale bars in (B)–(D) = 10 μm. (A) Scale bar = 200 μ m; (B) to (E) scale bars = 20 μm**;** (G) Scale bar = 20 μm;(H) to (I) scale bars= 10 μm.

**Two types of VNO receptor cells in mammals**

VNO receptor cells have been classified into two types expressing vomeronasal receptor type 1 or vomeronasal receptor type 2, which are known as positive $G_{\alpha i2}$ and $G_{\alpha o}$, respectively (Hagino-Yamagishi, 2008; Yokosuka,

2012). While rodent VNO receptor cells are positive for both $G_{\alpha i2}$ and $G_{\alpha o}$ (Hagino-Yamagishi, 2008; Yokosuka, 2012), those of other domestic animals, including goats, are only positive for $G_{\alpha i2}$ (Takigami et al., 2004). The identification of phenotypes of VNO receptor cells remains to be further studied in horse.

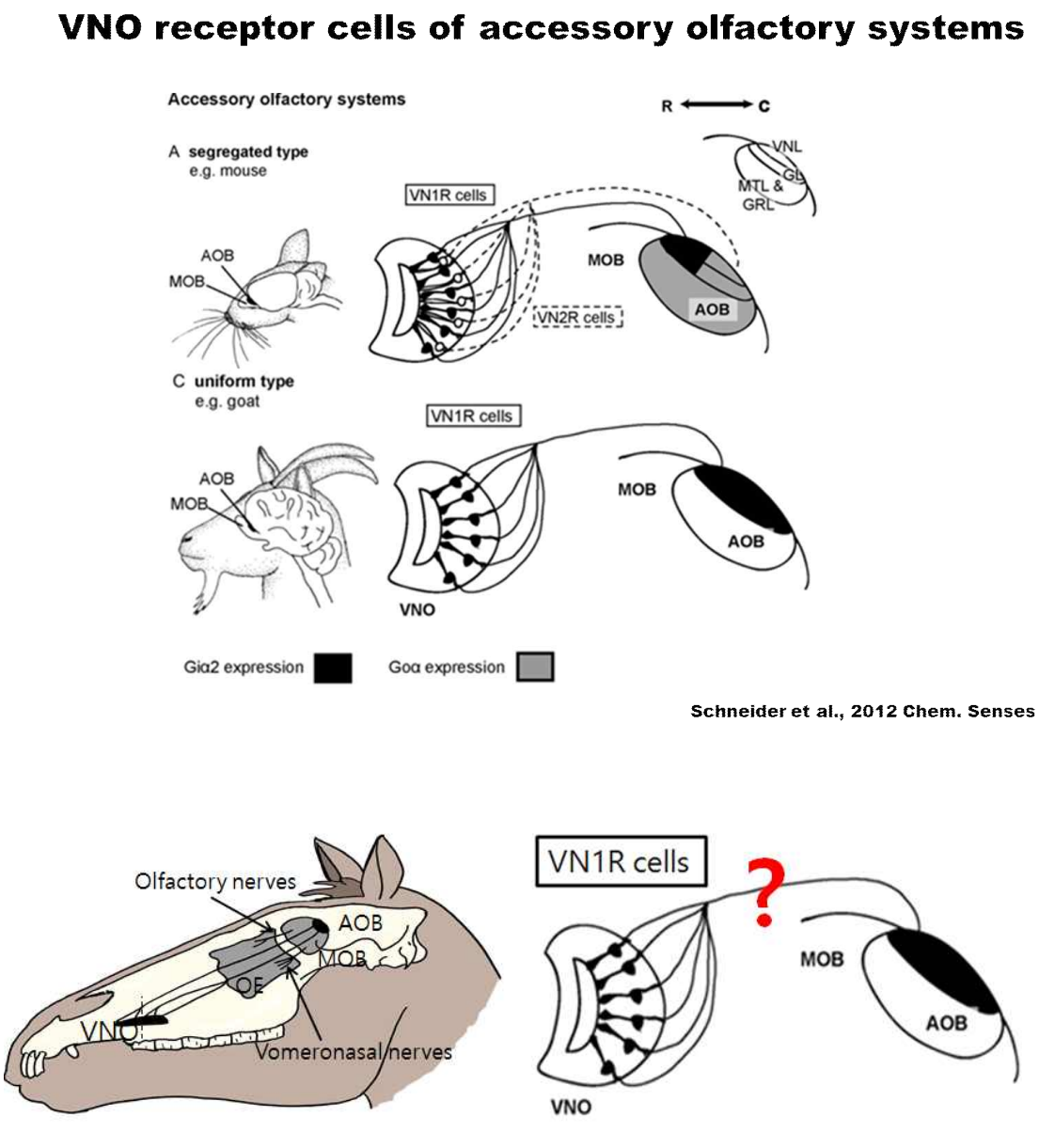


**Figure 3.** Two types of VNO receptor cells in mammals.

**Characterization of carbohydrate residues in olfactory mucosa**

Gycoconjugates in the mucosa has been known to play an important role in the cell-to-cell self-recognition, extracellular matrix interactions, embryonic development, cell growth, cell differentiation, cell signaling, cell adhesion, apoptosis, and inflammation (Nimrichter et al., 2004; Opdenakker et al., 1993). Lectin histochemistry is a choice for the discrimination of different sugar residues in the mucosa (Bies et al., 2004; Spicer and Schulte, 1992).

In olfactory systems, identification of glycoconjugates detected by lectin histochemistry have been used to discriminate the subpopulation of olfactory receptor cells (Plendl and Sinowatz, 1998). In the accessory olfactory system (Plendl and Sinowatz, 1998), both the sensory and non-sensory mucosa of the VNO contain exogenous and endogenous carbohydrate moieties that play crucial biological roles in mice (Salazar and Quinteiro, 2003), rats (Lee et al., 2012), sheep (Ibrahim et al., 2013), pigs (Park et al., 2012), and the common marmoset (Nakajima et al., 1998).

A morphological characteristic of the horse olfactory mucosa and VNO remain to be studied with special reference to the identification of sugar residues and of phenotyping of VNO receptor cells in horses.

# CHAPTER I

# Histochemical study of the olfactory mucosae of the horses

## 1. Abstract

The olfactory mucosae of the horse were examined by using histology and lectin histochemistry to characterize the carbohydrate sugar residues therein. Histological findings revealed that olfactory epithelium (OE) consisted of both olfactory marker protein (OMP)- and protein gene product (PGP) 9.5-positive receptor cells, supporting cells and basal cells with intervening secretory ducts from Bowman's glands. Mucus histochemistry showed that Bowman's gland acini contain periodic acid-Schiff (PAS) reagent-positive neutral mucins and alcian blue pH 2.5-positive mucosubstances. Lectin histochemistry revealedthat a variety of carbohydrate sugar residues, including N-acetylglucosamine, mannose, galactose, N-acetylgalactosamine, fucose and complex type N-glycan groups, are present in the various cell types inthe olfactory mucosa at varying levels. Collectively, this is the first descriptive study of horse olfactorymucosa to characterize carbohydrate sugar residues in the OE and Bowman's glands.

**Keywords**: Carbohydrate; horse; lectin; mucus; olfactory mucosa.

## 2. Introduction

Olfaction in mammals is a primary sense for social interaction and reproductive behavior and is processed by the main and accessory olfactory systems (Baum and Cherry, 2015; Breer et al., 2006; Keller and Levy, 2012). In the main olfactory system, olfactory receptor cells in the olfactory mucosa receive conventional odors and project olfactory nerves to the main olfactory bulb (OB). The OB transmits signals to specific areas of the brain, including the anterior olfactory nucleus, piriform cortex, olfactory tubercle and anterior cortical amygdala (Breer et al., 2006; Yokosuka, 2012). The vomeronasal system, a distinct pathway to OB (Yokosuka, 2012), senses pheromones through receptor cells in the vomeronasal organ (VNO), which sends signals to the accessory olfactory bulb (AOB), which in turn conveys signals to the vomeronasal amygdala in the brain (Yokosuka, 2012).

A recent study showed that OB is also involved in chemosignal transduction in mammalian reproduction (Baum and Cherry, 2015), suggesting that OB is also important for animal reproduction. Olfactory mucosa consists of olfactory receptor cells, supporting cells and basal cells, as well as Bowman's glands in the lamina propria (LP) (Kavoi et al., 2010). Even though the morphology of olfactory systems has been widely studied in mice (Barrios et al., 2014a), domestic animals including dogs and sheep (Barrios et al., 2014b; Kavoi et al., 2010) and wild life roe deer (Park et al., 2015), comparative morphological studies on the olfactory mucosa of thehorse remain to be performed.

Lectins are carbohydrate-binding proteins (Bies et al., 2004). Lectin histochemistry has been used to discriminate carbohydrate terminals in

chemosensory olfactory mucosae in sheep (Ibrahimet al., 2014) and armadillo (Ferrari et al., 1999). In the chemoreception of main olfactory systems, carbohydrate terminals are known to play important roles in the perception of odorous substances (Plendl and Sinowatz, 1998). Recently, a lectin histochemical study showed that cell components in the olfactory mucosae contain a variety of different carbohydrate sugar residues in sheep (Ibrahimet al., 2014) and roe deer (Park et al., 2015). Although the light and scanning electron microscopic features of the nasal turbinates of the horse have been studied (Kumar et al., 2000), characterization of carbohydrates in the olfactory mucosa is poorly understood in this species. In the present study, we examined the morphological features of olfactory mucosa in the horse and characterized carbohydrate sugar residues using lectin histochemistry.

## 3. Materials and Methods

### 3.1. Tissue preparation

Three adult horses (males, 2.3 years of age) were obtained from the Korea Horse Race Association and local abattoir. The three horses used in this study were sexually mature. All experimental procedures were conducted in accordance with the Jeju National University Guidelines for the Care and Use of Laboratory Animals.

### 3.2. Histological examination

For light microscopic evaluation, the endoturbinate part of ethmoturbinates revealed by parasagittal sections were immediately removed after death and fixed in 10% buffered formalin for 48 h. Formalin-fixed ethmoturbinates were trimmed and decalcified in a sodium citrate-formic acid solution with several changes of thesolution, until the bony pieces softened, as described in our previous study (Park et al., 2012). The decalcified ethmoturbinates were dehydrated in a graded ethanol series (70%, 80%, 90%, 95%, and 100%), cleared in xylene, embedded in paraffin and sectioned into 5-μm slices. After deparaffinization, the serial sections were stained with hematoxylin-eosin (HE), and examined to determine whether the olfactory mucosa contained pathological lesions. The three samples showed no inflammation in the olfactory mucosa and were therefore further studied.

### 3.3. Mucus histochemistry

To observe the mucosal characteristics, periodic acid-Schiff (PAS) reaction was used for neutral or weakly acidic glycoconjugates, while alcian

blue at pH 2.5 (AB 2.5) was used for the confirmation of sulfate esters and carboxyl groups in glycoconjugates (Luna, 1968).

### 3.4. Immunohistochemistry

Routine immunohistochemical staining was done as shown in our previous paper (Park et al., 2015). 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 5 min. After cooling, the sections were exposed to aqueous 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. Then, non-specific binding was blocked with 10% normal goat serum (ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA), washed in phosphate-buffered saline (PBS, pH 7.4) for 1 h, and allowed to react with either the goat anti-olfactory marker protein (OMP) antibody (1:400) (catalog No. sc-49070, Santa Cruz Biotechnology, CA, USA) or rabbit anti- protein gene product 9.5 (PGP 9.5) antibody (1:800) (catalog No. RP 087.05, Bio trend, Koln, Germany) for 1 h at room temperature. After washing in PBS, the sections were reacted for 45 min with biotinylated rabbit anti-goat IgG (1:100; Vector Laboratories) or biotinylated goat anti-rabbit IgG (1:100; Vector Laboratories), and followed by the avidin-biotin peroxidase-complex (ABC Elite Kit; Vector Laboratories) prepared according tothe manufacturer's instructions. After washing in PBS, the peroxidase reaction was developed for 3 min using a diaminobenzidine substrate kit (DAB Kit; Vector Laboratories), prepared according to the manufacturer's instructions. 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).

### 3.5. Lectin histochemistry

Lectin histochemistry was performed as described in previous studies (Lee et al., 2012; Park et al., 2015, 2014, 2012). Three lectin screening kits (Biotinylated lectin kit I; catalog No. BK-1000, Biotinylated lectin kit II; catalog No. BK-2000, Biotinylated lectinkit III; catalog No. BK-3000) were purchased from Vector Laboratories (Burlingame, CA, USA). Based on previous papers, lectins were classified into six groups according to the binding specificity and inhibitory sugars, including N-acetylglucosamine, mannose, galactose, N-acetylgalactosamine, fucose and complex type N-glycangroups (Table 1) (Ibrahim et al., 2014; Kaltner et al., 2007).

In the present study, we focused on the characterization of carbohydrate sugar residues in the olfactory mucosa. In brief, the paraffin-embedded ethmoturbinates were sectioned into 5-μm slices using a microtome. The sections were mounted on glass microscope slides, the paraffin was removed and the sections were rehydrated. Endogenous peroxidase activity was blocked using0.3% hydrogen peroxide in methanol for 30 min. After three washes with phosphate buffered saline (PBS), the sections were incubated with 1% bovine serum albumin to block non-specific reactivity. The sections were rinsed with PBS and incubated with the avidin-biotin-complex (ABC) method using 21 biotinylated lectins (Table 1) from the lectin screening kits I-III (Vector Laboratories) at 4℃ overnight. Horseradish peroxidase (HRP) were developed using a diaminobenzidine substrate kit (DAB Kit; Vector Laboratories). The sections were counterstained

with hematoxylin before mounting. For negative controls, a primary reagent was omitted during the staining procedure.

**Table 1. Binding specificities of lectins used in the present study ***

| Lectin abbreviations | Source | Concentration (mg/mL) | Sugar specificity |
|---|---|---|---|
| N-acetylglucosamine binding lectins | | | |
| s-WGA | *Triticum vulgaris* | $1.0 \times 10^{-2}$ | GlcNAc |
| WGA | *Triticum vulgaris* | $1.0 \times 10^{-2}$ | GlcNAc |
| BSL-II | *Bandeiraea simplicifolia* | $4.0 \times 10^{-3}$ | α or βGlcNAc |
| DSL | *Datura stramonium* | $4.0 \times 10^{-3}$ | $(GlcNAc)_{2-4}$ |
| LEL | *Lycopersicon esculentum* | $2.0 \times 10^{-2}$ | $(GlcNAc)_{2-4}$ |
| STL | *Solanum tuberosum* | $1.0 \times 10^{-2}$ | $(GlcNAc)_{2-4}$ |
| Mannose binding lectins | | | |
| ConA | *Canavalia ensiformis* | $3.3 \times 10^{-3}$ | αMan, αGlc |
| LCA | *Lens culinaris* | $4.0 \times 10^{-3}$ | αMan, αGlc |
| PSA | *Pisum sativum* | $4.0 \times 10^{-3}$ | αMan, αGlc |
| Galactose binding lectins | | | |
| $RCA_{120}$ | *Ricinus communis* | $2.0 \times 10^{-3}$ | Gal |
| BSL-I | *Bandeiraea simplicifolia* | $4.0 \times 10^{-3}$ | αGal, αGalNAc |
| Jacalin | *Artocarpus integrifolia* | $5.0 \times 10^{-4}$ | Galβ3GalNAc |
| PNA | *Arachis hypogaea* | $4.0 \times 10^{-3}$ | Galβ3GalNAc |
| ECL | *Erythrina cristagalli* | $2.0 \times 10^{-2}$ | Galβ4GlcNAc |
| N-acetylgalactosamine binding lectins | | | |
| VVA | *Vicia villosa* | $4.0 \times 10^{-3}$ | GalNAc |
| DBA | *Dolichos biflorus* | $1.0 \times 10^{-2}$ | αGalNAc |
| SBA | *Glycine max* | $1.0 \times 10^{-2}$ | α>βGalNAc |
| SJA | *Sophora japonica* | $2.0 \times 10^{-2}$ | βGalNAc |
| Fucose binding lectin | | | |
| UEA-I | *Ulex europaeus* | $2.0 \times 10^{-2}$ | αFuc |
| Complex type N-glycan binding lectins | | | |
| PHA-E | *Phaseolus vulgaris* | $5.0 \times 10^{-3}$ | Complex oligosaccharides |
| PHA-L | *Phaseolus vulgaris* | $2.5 \times 10^{-3}$ | Complex oligosaccharides |

Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; Man, mannose

*, Twenty one lectins were grouped into as shown in a previous paper (Kaltner et al., 2007).

# 4. Results

## 4.1. Histological features of the olfactory and respiratory mucosae

The olfactory mucosa consisted of the olfactory epithelium (Fig. 4A) and the underlying lamina propria (LP) (Fig. 4A). The olfactory epithelium consisted of the supporting cells, receptor cells and basal cells (Fig. 4B), while secretory ducts of Bowman's glands were occasionally found between olfactory epithelium (Fig. 4A, hollow arrows). The free border of the olfactory epithelium was observed at the lumen side (Fig. 4B, asterisk). In the LP, nerve bundle (Fig. 4A, arrowhead) and Bowman's glands were positively stained for PAS (Fig. 4C) and/or AB 2.5 (Fig. 4D).

In the respiratory mucosa of the adult horse, respiratory epithelium (RE; Fig. 5A) consisted of goblet cells (Fig. 5A, arrows), ciliated pseudostratified epithelium (Fig. 5A, arrowheads) and basal cells(Fig. 5A, hollow arrows), distinct from olfactory mucosa (Fig. 4A); in the LP of blood vessels, respiratory epithelium consisted of connective tissues and gland acini (Fig. 5A), which were positive for PAS (Fig. 5B) and/or AB 2.5 (Fig. 5C).

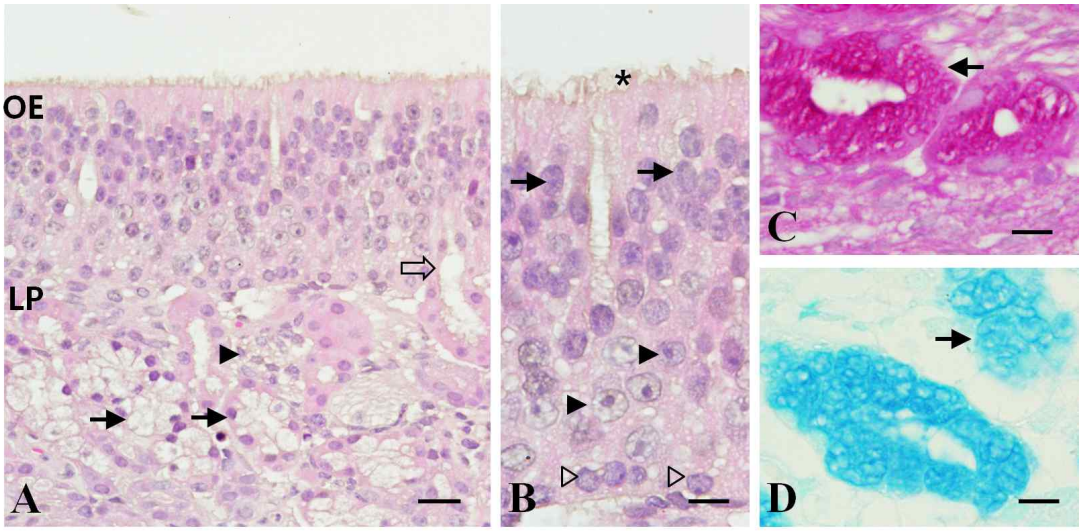


**Figure 4.** Histological results of the olfactory mucosa of adult horse. (A) Coronal section of the olfactory mucosa stained with hematoxylin-eosin (HE). The olfactory mucosa comprises the olfactory epithelium (OE) and the lamina propria (LP). Pyramidal acinar cells (arrows) of Bowman's glands, the ducts of Bowman's glands intervening in the OE (hollow arrows) and the olfactory nerve bundles (arrowhead) in the LP are shown. (B) Higher magnification of the OE is shown in the marked rectangle in Figure A. The asterisk indicates the free border. The nuclei in the olfactory supporting cells are oval (arrows). The secretory duct is vertically positioned in the olfactory mucosa. The olfactory receptor cells (black arrowheads) between basal cells (hollow arrowheads) and supporting cells (black arrows) are shown. (C) Periodic acid-Schiff (PAS) reaction in the olfactory mucosa. PAS reactivity observed in Bowman's gland acini (arrow). (D) Alcian blue (pH 2.5) staining in the olfactory mucosa. Alcian blue reaction in the acinar cells (arrow) of Bowman's glands. Scale bar in (A) = 20 μm. Scale bars in (B)-(D) = 10 μm.

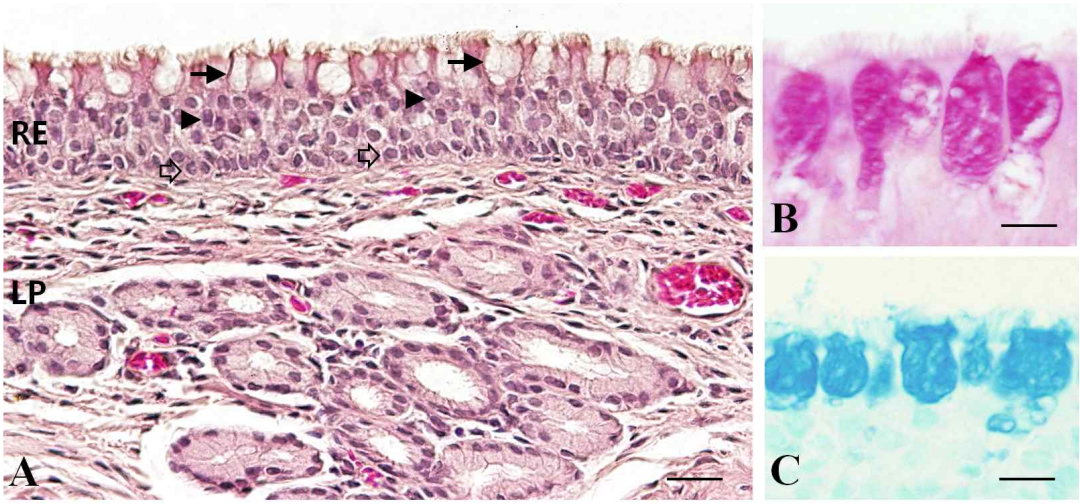


**Figure 5.** Histological results of the respiratory mucosa of adult horse. (A) Coronal section of the respiratory mucosa stained with HE. The OE consists of the goblet cells (arrows), ciliated cells (arrowheads) and basal cells (hollow arrows). The nasal glands are located in the LP. (B) PAS reaction of the goblet cells. (C) Alcian blue (pH 2.5) staining of the goblet cells. Scale bar in (A) = 20 μm. Scale bars in (B) and (C) = 10 μm.

### 4.2. Immunohistochemical labeling of olfactory receptor cells

To observe the receptor cells in the olfactory mucosa using immunohistochemistry with anti-OMP and anti-PGP 9.5 antibodies, olfactory receptor cells (Fig. 6A, arrows) with dendrites (Fig. 6A, arrowheads) were positive for OMP antibody, suggesting that olfactory receptor cells are mature-type. Some receptor cells near the basal cell layer were not positive for OMP (Fig. 6A, hollow arrow). In PGP 9.5 immunostaining (Fig. 6B), the reactivity of PGP 9.5 in receptor cells (Fig. 6B, arrows) was similar to OMP, while several receptor cells in the basal side also showed PGP 9.5 immunoreactivity (Fig. 6B, hollow arrow), suggesting that some cells are undergoing development. Additionally, some PGP 9.5-positive spindle-shaped cells (Fig. 6B, arrowheads) were observed at the luminal side of the olfactory epithelium.

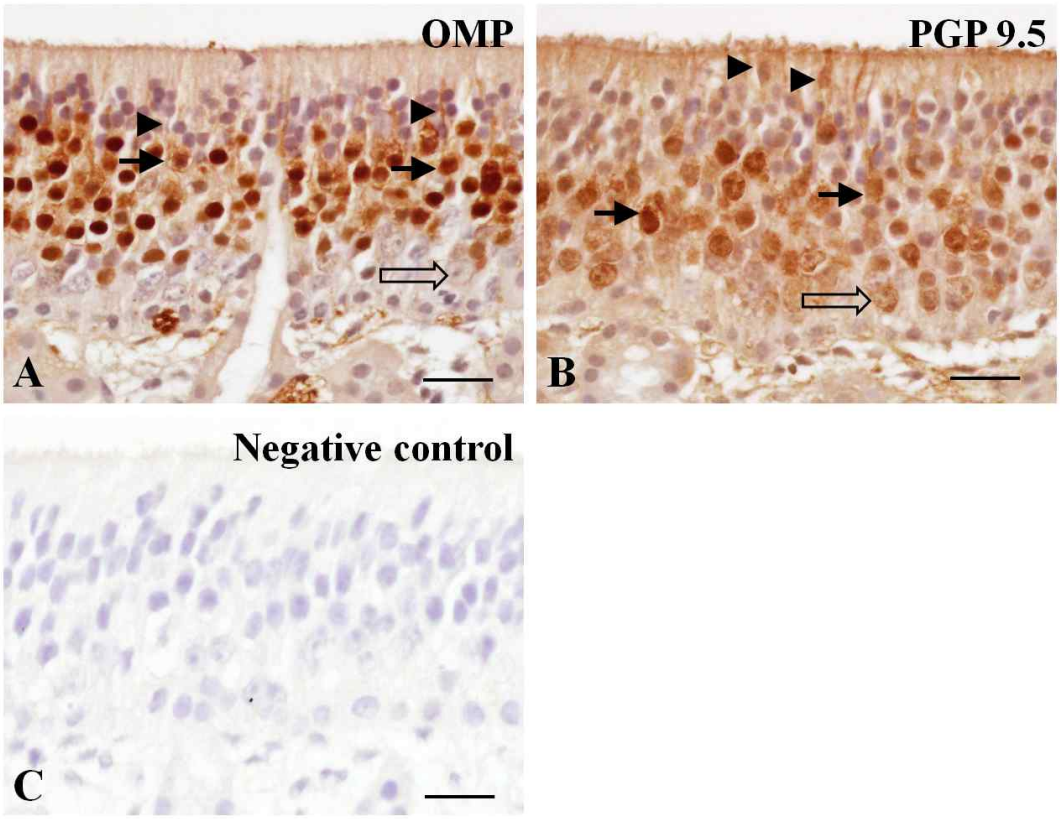


**Figure 6.** Immunohistochemical localization of OMP (A) and PGP 9.5 (B) in the OE. (A) The receptor cells are strongly positive for OMP in the OE (arrows). The dendritic processes of OMP-positive receptor cells extend to the free border in the OE (arrowheads). Hollow arrow indicates OMP-negative receptor cells. (B) PGP 9.5-positive cells (arrows) are located in the middle layer of the OE with their dendrites extending to the free border. PGP 9.5 was also stained in spindle-shaped cells near the nasal cavity and upper level of supporting cells (arrowheads). Hollow arrow indicates PGP 9.5-positive receptor cells probably undergoing differentiation. (C) Negative control. Scale bars in (A)-(C) = 20 μm.

### 4.3. Lectin histochemistry in the olfactory mucosa

#### 4.3.1. *N*-acetylglucosamine binding lectins

All N-acetylglucosamine binding lectins including s-WGA (Fig. 7A), WGA, DSL (Fig. 8A), LEL and STL, but not BSL-II (Fig. 7B), were diffusely labeled in the free border, receptor cells, supporting cells and basal cells, as well as in the nerve bundle and the aciniin Bowman's glands (Table 2). Both DSL and LEL, but not s-WGA, WGA, BSL-II or STL, were occasionally labeled in the secretory ductof Bowman's gland.

#### 4.3.2. Mannose binding lectins

Mannose binding lectins - Con A, LCA (Fig. 7C) and PSA - were intensely labeled in the free border, receptor cells, supporting cells, basal cells and nerve bundle in the LP, while their reactivity in Bowman's glands was less intense than in the olfactory epithelium (Table 2). Both LCA (Fig. 7C, arrow) and PSA were also intensely localized in the perinuclear lesion in the receptor cells. Con A was also labeled in the ducts of Bowman's glands, but both LCA and PSA were not.

#### 4.3.3. Galactose binding lectins

As shown in Table 2, three lectins including $RCA_{120}$, Jacalin and ECL were intensely labeled in the olfactory epithelium and Bowman's gland acini, while BSL-I (Fig. 7D) showed reactivity in the olfactory epithelium but not in Bowman's glands. PNA (Fig. 7E, arrow) was specifically labeled in receptor cells, free border and Bowman's glands, but not in supporting cells or basal

cells, suggesting that PNA plays a role in odor perception. Both Jacalin and PNA clearly reacted with the perinuclear lesion of receptor cells.

#### 4.3.4. *N*-actylgalactosamine binding lectins

The reactivity of N-acetylgalactosamine binding lectins including VVA, DBA, SBA and SJA varied by cell type (Table 2). Both VVA and SBA (Fig. 7F) were positive for receptor cells with perinuclear staining (Fig. 7F, arrow) and Bowman's gland acini (VVA; Fig. 8B), but not in supporting cells. DBA was labeled in the ductal epithelium but not in any type of cell in the olfactory mucosa. Additionally, VVA, SBA and SJA showed no reactivity in the ductal epithelium. SJA was weakly positive for Bowman's gland acini, but was intensely labeled in the free border, but not in olfactory mucosal epithelial cells (Fig. 7G; Table 2). All four lectins - VVA, DBA, SBA and SJA - showed no reactivity in the nerve bundle, while two lectins (VVA and SBA) were clearly positive in the receptor cells (Table 2). The discrepancy of lectin reactivity between receptor cells and nerve bundle in the LP was observed in VVA (Fig. 8B) and SBA.

#### 4.3.5. Fucose binding lectins

UEA-I was weakly labeled in the free border and basal cells, but not in the receptor cells and supporting cells. However, UEA-I was strongly labeled in Bowman's gland acini and nerve bundles (Fig. 8C). Discrepant UEA-I reactivity was found between receptor cells and nerve bundle. In addition, UEA-I was localized in slender spindle-shaped cells in the olfactory epithelium (Fig. 7H, arrows).

#### 4.3.6. Complex type *N*-glycan binding lectins

PHA-E was intensely labeled in all epithelial cells and Bowman's gland acini, while PHA-L only showed reactivity in basal cells (Fig. 7I) and Bowman's gland acini (Fig. 8D), but not in receptor cells (Fig. 8I) or nerve bundle (Fig. 8D). The lectin-binding patterns in the olfactory mucosa are summarized in Table 2.

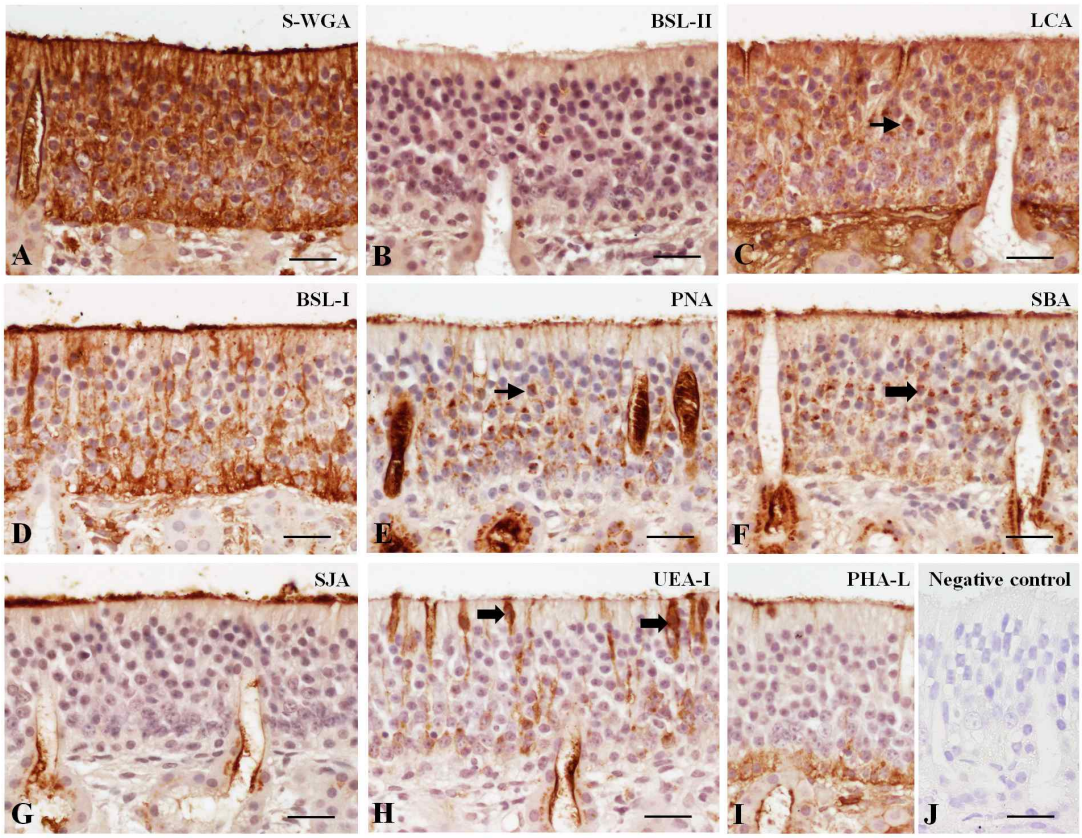


**Figure 7.** Lectin histochemistry in the horse OE. (A) All layers of the OE exhibit intensive s-WGA positivity. (B) No positive reaction for BSL-II was observed in the OE. (C) The free border, the supporting cells and the basal cells are moderately labeled by LCA. Arrow indicates LCA reactivity at the perinuclear lesion on the receptor cells. (D) The horizontal basal cells are strongly stained by BSL-I. (E) Intense staining for PNA was observed in the free border and the receptor cells. Arrow indicates PNA reactivity at the perinuclear lesion on the receptor cells. (F) The free border, the receptor cells and basal cells show a positive reaction for SBA. Arrow indicates SBA reactivity at the perinuclear lesion in the receptor cells. (G) Only the free border shows SJA reactivity in the OE. (H) UEA-I was weakly stained in the free border and basal cells. UEA-I occasionally stained some slender

spindle-shaped cells (arrows) and the upper level of supporting cell nuclei. (I) PHA-L was detected in the free border and basal cells. (J) Negative control. All sections in (A)-(J) were counterstained with hematoxylin. Scale bars in (A)-(J) = 20 μm.

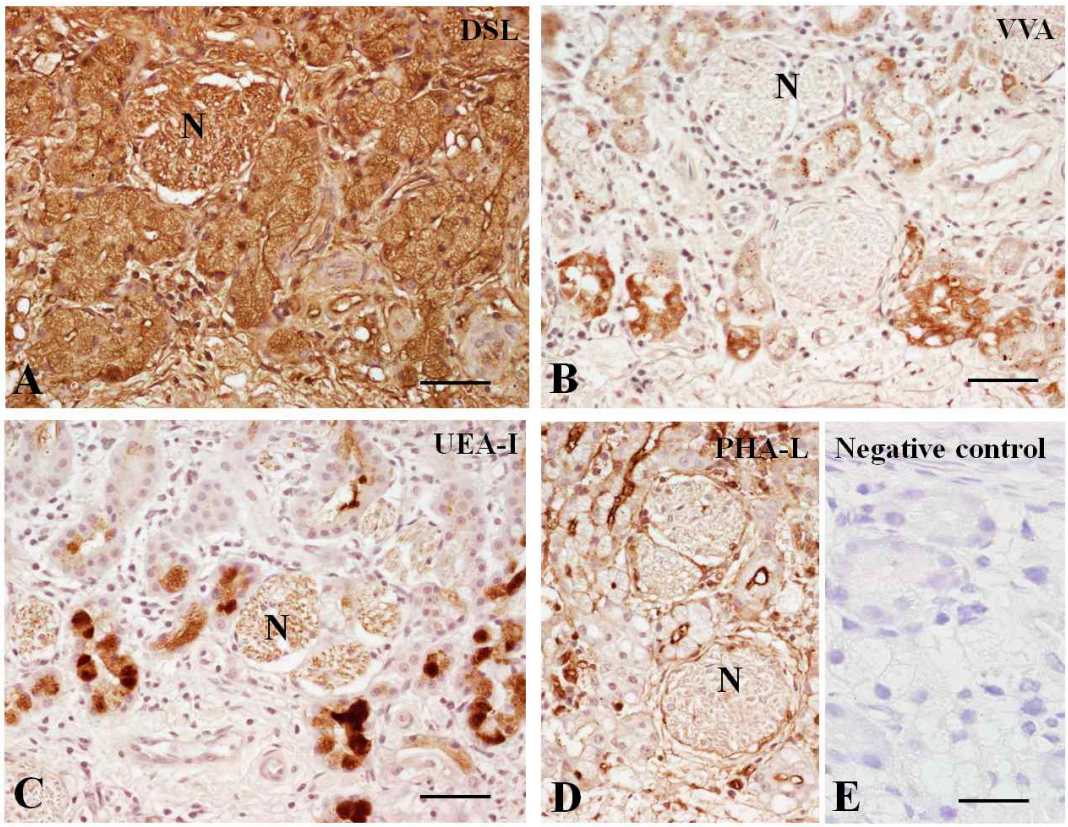


**Figure 8.** Lectin histochemistry in Bowman's glands. (A) DSL homogenously labels Bowman's gland acini and nerve bundles (N). (B) The glandular acini cells are moderately stained by VVA but not in the nerve bundles (N). (C) Glandular acini cells and nerve bundles (N) in the LP were stained by UEA-I. (D) PHA-L was labeled in the apical portion of Bowman's gland acini but not in nerve bundles (N). (E) Negative control. All sections in (A)-(E) were counterstained with hematoxylin. Scale bars in (A)-(E) = 20 μm.

**Table 2. Lectin binding patterns in the olfactory epithelium, nerve bundles and Bowman's glands in the horse**

| Lectin | Free border | Receptor cell | Supporting cell | Basal cell | Nerve bundle in lamina | Bowman's gland duct | Bowman's gland acini |
|---|---|---|---|---|---|---|---|
| N-acetylglucosamine binding lectins | | | | | | | |
| s-WGA | +++ | +++ | +++ | +++ | +++ | - | +++ |
| WGA | +++ | ++ | ++ | ++ | ++ | - | ++ |
| BSL-II | - | - | - | - | - | - | - |
| DSL | +++ | +++ | +++ | +++ | ++ | + | +++ |
| LEL | +++ | +++ | +++ | +++ | ++ | + | +++ |
| STL | +++ | +++ | +++ | +++ | ++ | - | +++ |
| Mannose binding lectins | | | | | | | |
| ConA | +++ | ++ | +++ | +++ | + | + | + |
| LCA | ++ | +++* | ++ | ++ | + | - | + |
| PSA | ++ | +++* | ++ | ++ | + | - | + |
| Galactose binding lectins | | | | | | | |
| RCA120 | +++ | +++ | +++ | +++ | ++ | - | +++ |
| BSL-I | +++ | + | + | ++ | + | - | - |
| Jacalin | +++ | +++* | +++ | +++ | ++ | - | +++ |
| PNA | ++ | ++* | - | - | + | - | +++ |
| ECL | +++ | ++ | ++ | ++ | + | + | +++ |
| N-acetylgalactosamine binding lectins | | | | | | | |
| VVA | + | ++* | - | - | - | - | ++ |
| DBA | - | - | - | - | - | + | - |
| SBA | +++ | ++* | - | ++ | - | - | ++ |
| SJA | +++ | - | - | - | - | - | + |
| Fucose binding lectin | | | | | | | |
| UEA-I | + | - | - | + | + | - | +++ |
| Complex type N-glycan binding lectins | | | | | | | |
| PHA-E | +++ | +++ | +++ | +++ | ++ | - | +++ |
| PHA-L | + | - | - | ++ | - | - | + |

–, Negative staining; +, Faint staining; ++, Moderate staining; +++, Intense staining

*: apical perinucelar labeling

## 5. Discussion

This is the first histochemical study of horse olfactory mucosa to include visualization of olfactory receptor cells and each carbohydrate residue on the olfactory mucosa. Regarding the histological features of olfactory mucosa in horse, the general structure is similar to those of domestic animals, including dogs (Kavoi et al., 2010) and sheep (Ibrahim et al., 2014). In the present study, we found that OMP is specifically localized in the receptor cells in the olfactory epithelium, while PGP 9.5 is occasionally localized in immature-type receptor cells located between OMP-positive receptor cells and basal cells. This finding implies that some neurons continuously differentiate in the olfactory epithelium, suggesting that cells in the olfactory epithelium, from which specific stem cells originate, are dynamically regenerating (Duan and Lu, 2015; Murrell et al., 2005). Regarding the mucus characteristics in the olfactory Bowman's glands, some acini in the glands were both positive either for PAS or alcian blue (pH 2.5) - or for both - suggesting that Bowman's glands secrete neutral or weakly acidic glycoconjugates as well as sulfate esters in glycoconjugates. This finding is similar to those in other domestic animals including sheep (Ibrahim et al., 2014).Regarding lectin histochemistry, we found that a variety of carbohydrate residues detected by lectins (19 lectins; see Table 2)used in this study were identified on the free border of olfactory epithelium, but not BSL-II or DBA. This finding is partly in contrast to those reported in sheep, in which the free borders of both the olfactory and respiratory epithelia were stained with 12 lectins including WGA, s-WGA, LEL, STL, DSL, SBA, BSL-I, RCA-120, ECL, ConA, PHA-E, and PHA-L (Ibrahim et al., 2014). Regarding the source of

carbohydrate sugar residues in the free border, we postulate thatsome originated from secretions from Bowman's glands because acini in Bowman's glands contained a variety of carbohydrate sugar residues, except BSL-I-, BSL-II- and DBA-binding carbohydrates, inthe present study. Additionally, we do not exclude the possibil-ity that some carbohydrate sugar residues in the free border areoriginated from the olfactory epithelial cells.In the olfactory epithelial cells, seventeen lectins, but not BSL-II, DBA, SJA and PHA-L, bound to the receptor cells, suggestingthe many different sugar residues are partly involved in the cell biology of receptor cells. Regarding the location of lectin-binding sugar residues on the receptor cells, lectins including LCA, PSA,Jacalin, PNA, VVA and SBA were clearly labeled at the apical perinuclear lesions, suggesting that the lectin-binding carbohydratesugar residues are associated with rough endoplasmic reticulum-associated protein synthesis. A similar finding was also reported inthe receptor cells of sheep VNO, in the form of supranuclear granules of VVA reactivity (Ibrahim et al., 2013). Thus, we postulate that receptor cells in the present study were actively involved in protein synthesis. The lectin reactivity between receptor cells and nerve bundleswas basically the same for each lectin used in this study except VVA and SJA. Both VVA and SJA were intensely labeled in the receptor cells with apical perinuclear staining but not in the nerve bundles in the lamina propria. We postulate that this discrepancy is due to the modification of sugar residues in the locations. Regarding the lectin reactivity in supporting cells and basal cells,N-acetylgalactosamine binding lectins including VVA, DBA and SJA were largely negative or faint, suggesting N-acetylgalactosamineis less involved in the maintenance of these cells. Considering that cell glycoconjugates are involved in cell-cell

communicationand adhesion (Spicer and Schulte, 1992), other groups including N-acetylglucosamine, mannose, galactose and complex-type N-glycans are more actively involved in cell–cell communicationsthan N-acetylgalactosamine groups. Because of the broad lectin reactivity in olfactory mucosa, we postulate that carbohydrate sugar residues in the cells of olfactory mucosa play a role in cell-cell communication. Regarding the lectin reactivity in the ductal epithelium of Bowman's glands, we found that four lectins - DSL, LEL, ECL and DBA - were occasionally labeled, while all examined lectins, except BSL-II, BSL-I and DBA, were positive for Bowman's gland acini. This finding suggests that acini contain more diverse sugar residues thanthe secretory ductal epithelium of Bowman's glands (Table 2). Regarding the involvement of spindle-shaped cells in the olfactory mucosa, we found that spindle-shaped cells reacted by UEA-I were detected at the apical side of the epithelium, beyond the layer of supporting cell nuclei. This type of cell was positive for phospholipase C (PLC)-beta I antibody in mouse olfactory mucosa (Elsaesser et al., 2005; Montani et al., 2006), mouse VNO (Oguraet al., 2010) and the sensory and non-sensory epithelium of camel VNO (Ibrahim et al., 2015), suggesting that this cell type is a solitary chemosensory cell in VNO (Ogura et al., 2010). The precise characteristics of this type of cell in olfactory epithelium remain to beelucidated in the horse. Collectively, the present study results contribute to the comparative study of chemosensory epithelium in the horse. Furthermore, this is the first descriptive study of carbohydrate sugar residues inthe horse olfactory mucosa.

# CHAPTER II

# Histological and lectin histochemical studies of the vomeronasal organ of horses

## 1. Abstract

The morphological characteristics and glycoconjugate composition of the vomeronasal organ (VNO) of the horse was investigated using histological, immunohistochemical, and lectin histochemical methods. The VNO is bilaterally located at the base of the nasal septum, has a tubular structure surrounded by cartilage, and consists of sensory and non-sensory epithelia. Immunohistochemical examination showed that the vomeronasal sensory epithelium (VSE) consisted of receptor cells positive for both olfactory marker protein (OMP) and protein gene product 9.5 (PGP 9.5), supporting cells, and basal cells. VNO receptor cells were positive for G protein $G_{\alpha i2}$ (vomeronasal receptor type 1 marker), but not $G_{\alpha o}$ (vomeronasal receptor type 2 marker). Lectin histochemical studies using 21 biotinylated lectins showed that the free border of the VSE was positive for 20 lectins. The receptor and supporting cells reacted with 16 lectins while the basal cells reacted with 15 lectins, with varying intensities. In the vomeronasal non-sensory epithelium, the free border was positive for 19 lectins. The cilated cells were positive for 17 lectins and the basal cells were positive for 15 lectins. The vomeronasal glands, positioned in the lamina propria, were stained with both periodic acid Schiff (PAS) and alcian blue (pH 2.5). Eighteen lectins stained the acinar cells of the vomeronasal glands with various binding patterns. These findings suggest that horse VNO receptor cells express vomeronasal receptor type 1, and the VNO glands have mucous to seromucous characteristics. Moreover, each lectin differentially binds each cell type in both the VNO sensory and non-sensory epithelia.

**Keywords**: Horse; Lectin; Vomeronasal organ; Carbohydrate.

## 2. Introduction

The vomeronasal organ (VNO) is the receptor organ of the vomeronasal system, which perceives various molecules related to social and reproductive communication and transmits signals to the accessory olfactory bulb (Brennan, 2001, Park et al., 2014, Takami, 2002, Yokosuka, 2012, Zufall et al., 2002). The lumen of the VNO is covered by the vomeronasal sensory epithelium (VSE) medially and the vomeronasal non-sensory epithelium (VNSE) laterally (Lee et al., 2003). The VNO glands are located in the lamina propria and secrete mucous and/or seromucous elements into the luminal surface of the VNO epithelium, playing a role in the detection of odorous molecules by the receptor cells of the VSE (Carmanchahi et al., 2000). VNO receptor cells have been classified into two types expressing vomeronasal receptor type 1 or vomeronasal receptor type 2, which are known as positive $G_{\alpha i2}$ and $G_{\alpha o}$, respectively (Hagino-Yamagishi, 2008, Yokosuka, 2012). While rodent VNO receptor cells are positive for both $G_{\alpha i2}$ and $G_{\alpha o}$, those of other domestic animals, including goats, are only positive for $G_{\alpha i2}$ (Takigami et al., 2004).

Lectins are carbohydrate binding proteins that protrude from glycolipids and glycoproteins (Bies et al., 2004) and can differentiate macromolecules in mammalian tissues (Spicer and Schulte, 1992). They are involved in biological activities through the process of glycoconjugation, including cell-to-cell self-recognition, extracellular matrix interactions, embryonic development, cell growth, cell differentiation, cell signaling, cell adhesion, apoptosis, and inflammation (Nimrichter et al., 2004, Opdenakker et al., 1993).

In olfactory systems, glycoconjugates detected by lectin histochemistry are considered to discriminate the subpopulation of olfactory receptor cells

(Plendl and Sinowatz, 1998). In the accessory olfactory system (Plendl and Sinowatz, 1998), both the sensory and non-sensory mucosa of the VNO contain exogenous and endogenous carbohydrate moieties that play crucial biological roles in mice (Salazar and Sanchez Quinteiro, 2003), rats (Lee et al., 2012), sheep (Ibrahim et al., 2013), pigs (Park et al., 2012b), and the common marmoset (Nakajima et al., 1998).

With regards to the horse VNO, Taniguchi et al. investigated the fine structure of the horse VSE (Taniguchi and Mikami, 1985), while Salazar et al. studied the vomeronasal cartilage (Salazar et al., 1995). In terms of analyzing the cell phenotypes of the VNO, Garcia-Suarez et al. studied the expression of the Trk A-like and epidermal growth factor receptors (Garcia-Suarez et al., 1997). In line with previous reports, we examined the histological characteristics and cellular localization of signals including protein kinase C and nitric oxide synthase (Lee et al., 2001), and also examined the lectin binding patterns of the VSE using 6 lectins (Lee et al., 2003). However, our previous study was insufficient to fully understand the characteristics of carbohydrate moieties in the horse VNO, including the VSE and the VNSE.

The aim of the present study is to evaluate the morphological characteristics of the horse VNO, with special reference to lectin histochemistry, to clarify the characteristics of carbohydrate specificity, as well as to confirm the VNO receptor type in horses.

## 3. Materials and Methods

### 3.1. Tissue preparation

Three horse samples (mature, Table 3) were obtained from the Korea Horse Racing Association and local abattoir. Animals were sacrificed through the administration of over-dosed of succinylcholine. For light microscope evaluation, the VNOs were removed immediately after death and fixed in 10% buffered formalin for 48 h. All experimental procedures were conducted in accordance with the Jeju National University Guidelines for the Care and Use of Laboratory Animals.

### 3.2. Histological examination

Formalin-fixed VNOs were trimmed and decalcified in a sodium citrate–formic acid solution. The solution was changed several times, until the bony pieces softened, as described in our previous study (Park et al., 2012). The decalcified VNOs were 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 serial sections were stained with hematoxylin-eosin (HE), periodic acid Schiff (PAS), and alcian blue (pH 2.5). The morphological evaluation of the VNO focused on male horses, as only one VNO sample from a female horse was obtained and was histologically similar to that of the males.

**Table 3. Description of the horses used in this study**

| Age | Weight (kg) | Sex |
|---|---|---|
| 3 years | 510 | Male |
| 3 years | 500 | Male |
| 4 years | 530 | Male |

### 3.3. Antibodies

To confirm the presence of receptor cells in the VSE, immunohistochemistry was performed using a goat polyclonal antibody against the olfactory marker protein (OMP, Santa Cruz Biotechnology, CA, USA) and a rabbit polyclonal antibody against protein gene product 9.5 (PGP 9.5, Bio trend, Köln, Germany). To identify vomeronasal receptors types, a rabbit polyclonal anti-$G_{\alpha i2}$ antibody and a rabbit polyclonal anti-$G_{\alpha o}$ antibody (Santa Cruz Biotechnology) were used. The primary antibodies are listed in Table 4.

**Table 4. Antibodies used in this study**

| Antibody | MAB/PAB | Host | Dilution | Company |
|---|---|---|---|---|
| Anti-olfactory marker protein (OMP) | PAB | Goat | 1:400 | Santa Cruz, Texas, USA |
| Anti-protein gene product 9.5 (PGP 9.5) | PAB | Rabbit | 1:800 | Bio trend, Köln, Germany |
| Anti-$G_{\alpha i2}$ | PAB | Rabbit | 1:200 | Santa Cruz |
| Anti-$G_{\alpha o}$ | PAB | Rabbit | 1:200 | Santa Cruz |

### 3.4. Immunohistochemistry

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 5 min. After cooling, the sections were exposed to aqueous 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. Then, non-specific binding was blocked with 10% normal goat serum (ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA), washed in phosphate-buffered saline (PBS, pH 7.4) for 1 h, and allowed to react with the goat anti-OMP antibody (1:400), rabbit anti-PGP 9.5 antibody (1:800), rabbit anti-$G_{\alpha i2}$ antibody (1:200), or rabbit anti-$G_{\alpha o}$ antibody (1:200) for 1 h at room temperature. After washing in PBS, the sections were reacted for 45 min with biotinylated rabbit anti-goat IgG (1:100; Vector Laboratories) or biotinylated goat anti-rabbit IgG (1:100; Vector Laboratories). The sections were washed in PBS and incubated for 45 min with the avidin–biotin peroxidase complex (ABC Elite Kit; Vector Laboratories) prepared according to the manufacturer's instructions. After washing in PBS, the peroxidase reaction was developed for 3 min using a diaminobenzidine substrate kit (DAB Kit; Vector Laboratories), prepared according to the manufacturer's instructions. 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).

### 3.5. Lectin histochemistry

Three lectin screening kits (I-III) were purchased from Vector Laboratories (Burlingame, CA, USA). Lectins were classified according to the binding specificity and inhibitory sugars, including N-acetylglucosamine,

mannose, galactose/N-acetylgalactosamine, complex type N-glycan (PHA-E and PHA-L), and fucose groups (Table 5) (Kaltner et al., 2007) . The following sugars were obtained from Sigma-Aldrich and used for competitive inhibition (See Table 3): α-methyl mannoside/α-methyl glucoside (Cat#, M6882, Sigma-Aldrich), ß-D-galactose (Cat#, G6637, Sigma-Aldrich), lactose (Galß1, 4Glc) (Cat#, L0100000, Sigma-Aldrich), melibiose (Galα1, 6Glc) (Cat#, M5500, Sigma-Aldrich), N-acetyl-D-galactosamine (α-D-GalNAc) (Cat#, A2795, Sigma-Aldrich), N-acetyl-D-glucosamine (ß-D-GlcNAc) (Cat#, A8625, Sigma-Aldrich). Chitin hydrolysate (Cat#, SP-0090, Vector) was purchased from Vector Laboratories.

Lectin histochemistry was performed as described in previous studies (Lee et al., 2012; Park et al., 2014; Park et al., 2012). Briefly, the paraffin-embedded VNOs were sectioned at a thickness of 5 μm using a microtome. The sections were mounted on glass microscope slides, the paraffin was removed, and the sections were rehydrated. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide in methanol for 30 min. After three washes with PBS, the sections were incubated with 1% bovine serum albumin to block non-specific activity. The sections were rinsed with PBS and incubated using the ABC method with 21 biotinylated lectins (Table 3) at 4°C overnight. Signals were developed using a diaminobenzidine substrate kit (DAB Kit; Vector Laboratories). The sections were counterstained with hematoxylin before mounting. Negative controls for the lectin histochemistry included (1) omission of primary reagent (biotinylated lectins) and (2) preincubation of the lectins with the appropriate inhibitors (0.2 M- 0.5 M in Tris buffer) for 1 hour at room temperature as shown in a previous paper (Kaltner et al., 2007).

**Table 5. Binding specificities of lectins used in this study**

| Lectin abbreviations | Source | Concentration (μg/ml) | Sugar specificity | Inhibitor or eluting sugar* |
|---|---|---|---|---|
| N-acetylglucosamine binding lectins | | | | |
| s-WGA | Succinylated-Wheat germ agglutinin | $1.0 \times 10^{-2}$ | GlcNAc | 0.2M GlcNAc |
| WGA | Wheat germ agglutinin | $1.0 \times 10^{-2}$ | GlcNAc NeuAc, SA | 0.2M GlcNAc |
| BSL-II | *Bandeiraea simplicifolia* | $4.0 \times 10^{-3}$ | α or β GlcNAc | 0.2M GlcNAc |
| DSL | *Datura stramonium* | $4.0 \times 10^{-3}$ | $(GlcNAc)_{2-4}$ | 0.5M chitin hydrolysate |
| LEL | *Lycopersicon esculentum* | $2.0 \times 10^{-2}$ | $(GlcNAc)_{2-4}$ | 0.5M chitin hydrolysate |
| STL | *Solanum tuberosum* | $1.0 \times 10^{-2}$ | $(GlcNAc)_{2-4}$ | 0.5M chitin hydrolysate |
| Mannose binding lectins | | | | |
| ConA | *Canavalia ensiformis* | $3.3 \times 10^{-3}$ | αMan, αGlc, 4GlcNAc | 0.2M MeαMan/0.2M MeαGlc |
| LCA | *Lens culinaris* | $4.0 \times 10^{-3}$ | αMan, αGlc 4(Fucα1,6)GlcNAc | 0.2M MeαMan /0.2M MeαGlc |
| PSA | *Pisum sativum* | $4.0 \times 10^{-3}$ | αMan, αGlc 4(Fucα1,6)GlcNAc | 0.2M MeαMan/0.2M MeαGlc |
| Galactose/ N-acetylgalactosamine binding lectins | | | | |
| $RCA_{120}$ | *Ricinus communis* | $2.0 \times 10^{-3}$ | Gal | 0.2M lactose |
| BSL-I | *Bandeiraea simplicifolia* | $4.0 \times 10^{-3}$ | αGal, αGalNAc | 0.2M GalNAc |
| VVA | *Vicia villosa* | $4.0 \times 10^{-3}$ | GalNAc | 0.2M GalNAc |
| DBA | *Dolichos biflorus* | $1.0 \times 10^{-2}$ | αGalNAc | 0.2M GalNAc |
| SBA | *Glycine max* | $1.0 \times 10^{-2}$ | α>βGalNAc | 0.2M GalNAc |
| SJA | *Sophora japonica* | $2.0 \times 10^{-2}$ | βGalNAc | 0.2M GalNAc |
| Jacalin | *Artocarpus integrifolia* | $5.0 \times 10^{-4}$ | Galβ3GalNAc | 0.2M melibiose |
| PNA | *Arachis hypogaea* | $4.0 \times 10^{-3}$ | Galβ3GalNAc | 0.2M βGal |
| ECL | *Erythrina cristagalli* | $2.0 \times 10^{-2}$ | Galβ4GlcNAc | 0.2M lactose |
| Complex type N-glycan ( Complex oligosaccharide) binding lectins | | | | |
| PHA-E | *Phaseolus vulgaris* | $5.0 \times 10^{-3}$ | Galβ4GlcNAcβ 2 Man α6 (GlcNAcβ4)(GlcNAcβ4Manα3) Manβ4 | 0.1M acetic acid |
| PHA-L | *Phaseolus vulgaris* | $2.5 \times 10^{-3}$ | Galβ4GlcNAcβ6 (GluNAcβ2Man α3) Man α3 | 0.1M acetic acid |
| Fucose binding lectin | | | | |
| UEA-I | *Ulex europaeus* | $2.0 \times 10^{-2}$ | αFuc | 0.1M L-fucose |

Fuc, fucose; gal, galactose; GalNAc, N-acetylgalactosamine; glc, glucose; GlcNAc, N-acetylglucosamine; man, mannose. MeαMan, α-Methylmannoside; MeαGlc, α-Methylglucoside; NeuAc, N-acetylneuraminic acid; SA, sialic acid.

* The acronyms and lectin sepcificities including sources, preferred sugar specificity and inhibitor were obtained from the data sheet (Vector laboratory).

## 4. Results

### 4.1. Gross anatomy of the nasal cavity

The nasal cavity, defined as the first air passage cavity surrounded by bones, extended from the nostril to the nasopharynx. In the rostral three-quarters of the nasal cavity, the air passage was divided by the nasoturbinate (NT) and the maxilloturbinate (MT), while the VNO was located below the MT of nasal cavity (Fig. 9). The VNO had a prominent tubular-shaped structure, supported by a thin cartilage, adjacent to the vomer. The ethmoturbinates were positioned at the caudal one-fourth of the nasal cavity and had a more complex lamellar folding and branching pattern than the NT and the MT (Fig. 9).

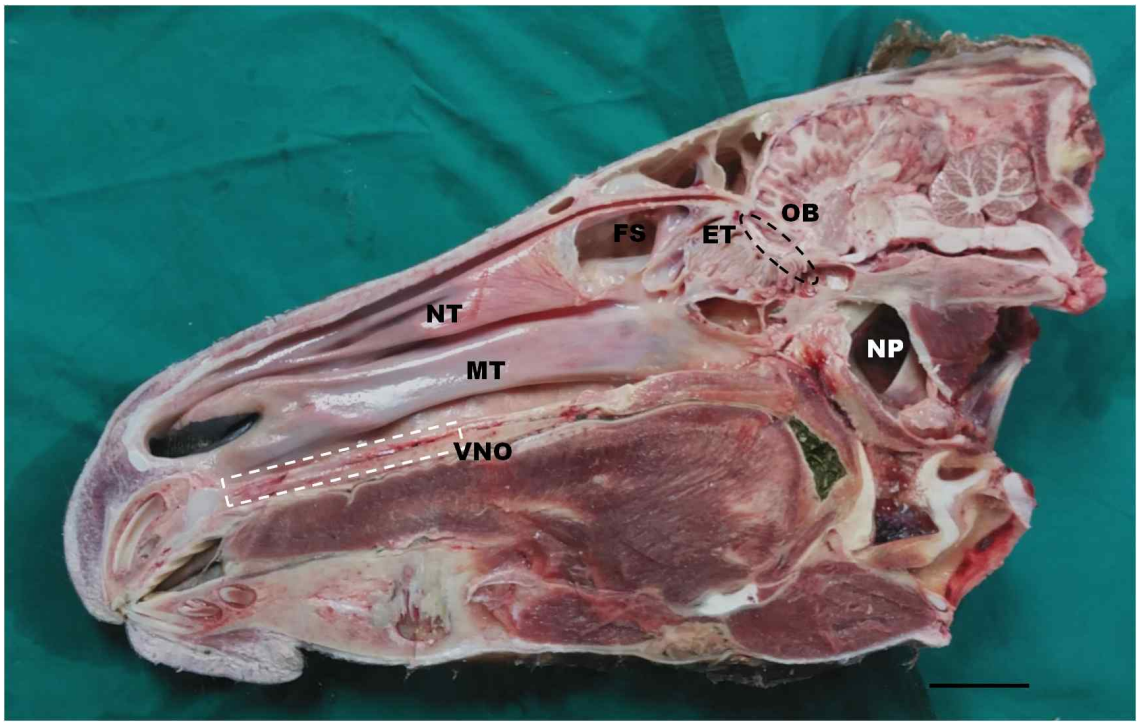


**Figure 9.** Gross anatomy of the rostral part of the head of the adult horse on the right lateral side. The ethmoturbinates (ET), located in the caudal portion of the nasal cavity, are lined by the olfactory mucosa. FS, frontal sinus; MT, maxilloturbinate; NP, nasopharynx; NT, nasoturbinate; OB, olfactory bulb; VNO, vomeronasal organ. Scale bar represents 5 cm.

### 4.2. Histological features of the VNO

The morphological features of the VNO are shown at low magnification in Figure 10A. A lamina of cartilage wrapped around its entire structure, and the connective tissue, glands, vessels, and nerves making up the soft tissue of the VNO were organized around the vomeronasal duct. The VSE was located in the medial portion of the VNO and consisted of supporting cells, receptor cells, and basal cells (Fig. 10B). The free border of the VSE faced the lumen of the vomeronasal duct (Fig. 10B, asterisk). The VNSE was located in the lateral portion of the VNO and consisted of ciliated cells and basal cells (Fig. 10C). In the lamina propria, there were enlarged blood vessels and numerous vomeronasal glands (Fig. 10A). The glandular cells in the vomeronasal glands were positive for both PAS (Fig. 10D) and alcian blue staining (pH 2.5) (Fig. 10E).

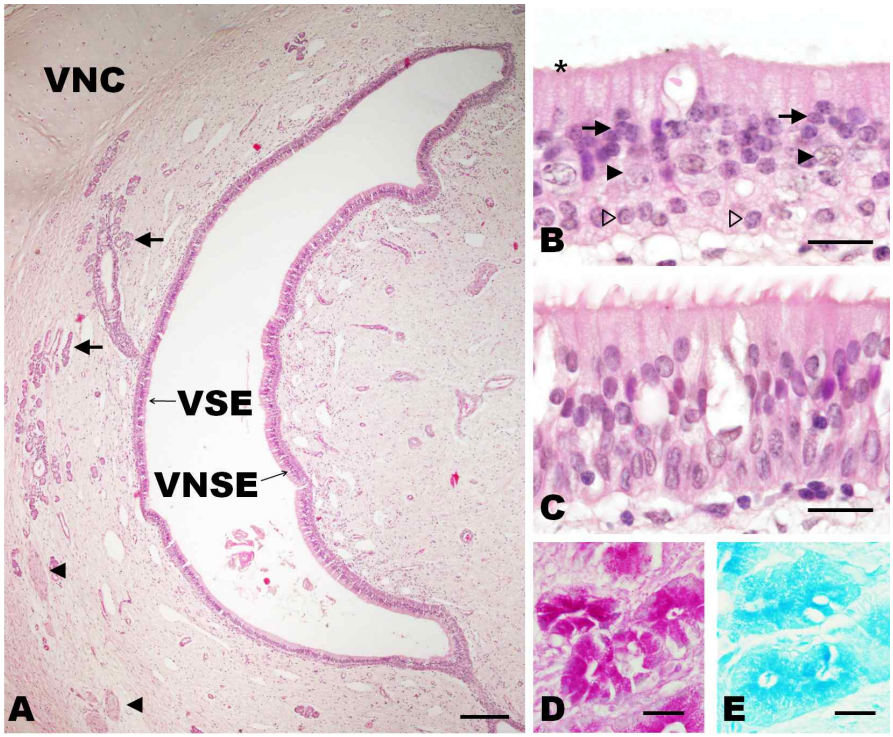


**Figure 10.** Histological findings of the horse vomeronasal organ. (A) A coronal section through the ventral part of the nasal septum. The vomeronasal cartilage (VNC) encapsulates the lamina propria. The vomeronasal ducts are lined by the vomeronasal sensory epithelium (VSE) and the vomeronasal non-sensory epithelium (VNSE). The vomeronasal nerve bundles (arrowheads) and the vomeronasal glands (arrows) are positioned in the lamina propria. (B) Higher magnification of the VSE. The VSE is composed of three discernible layers (supporting cells, arrows; receptor cells, arrowheads; basal cells, hollow arrowheads). The asterisk indicates the free border. (C) Higher magnification of the VNSE. The VNSE consists of non-sensory, ciliated, pseudostratified epithelium. H&E staining. (D) PAS staining of the acinar cells of the vomeronasal glands. (E) Alcian blue pH 2.5 staining of the acinar cells of the vomeronasal glands. (A) Scale bar = 200 μm; (B) to (E) scale bars = 20 μm.

### 4.3. Immunohistochemical analysis of the VSE

The receptor cells were well visualized by OMP immunoreaction in the middle layer of the VSE (Fig. 11A). OMP- and PGP 9.5-positive cells extended their dendrites to the free border (Fig. 11A and B, arrowheads). Both OMP and PGP 9.5 labelled mature olfactory receptor cells in the middle layer of the VSE (Fig. 11A and B, arrows). Both OMP and PGP 9.5 were not immunoreactive on supporting and basal cells in the present study.

With regard to the cellular expression of guanine-triphosphate (GTP)-binding proteins (G proteins), $G_{\alpha i2}$ was intensely immunostained in the receptor cells (Fig. 11C, arrows) in which their dendrites extended to the free border forming dendritic knobs (Fig. 11C, arrowheads). However, neither supporting cells nor receptor cells (Fig. 11D) were immunoreactive for $G_{\alpha o}$. Negative control shows no immunoreactivity (Fig. 11E).

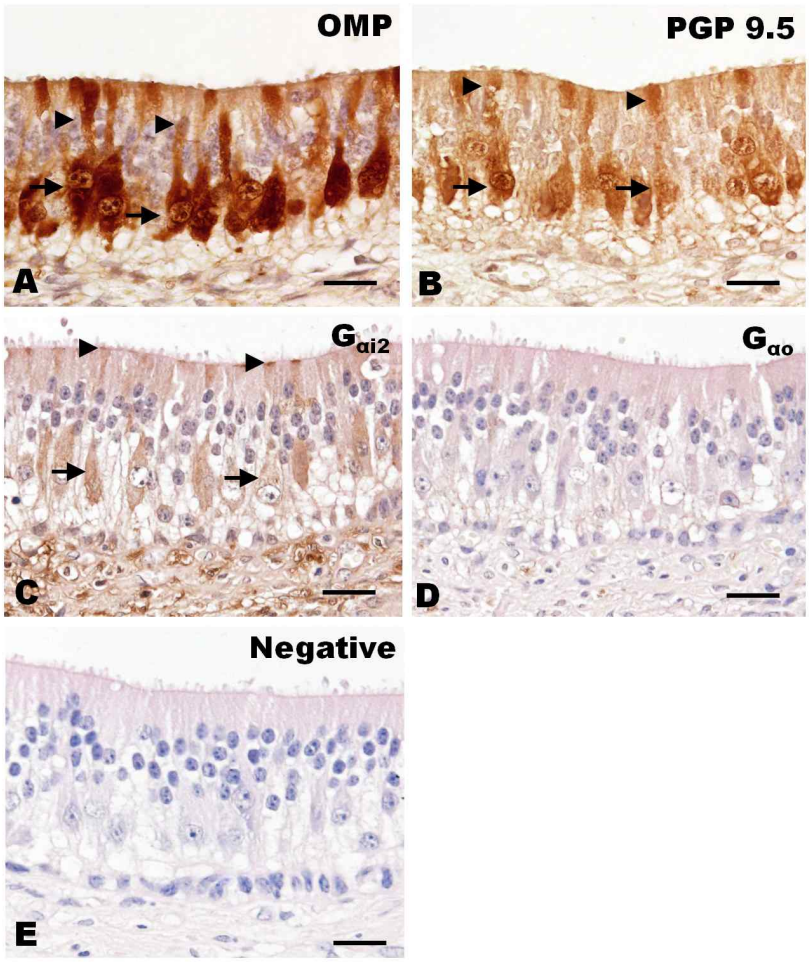


**Figure 11.** Immunohistochemical localization of OMP, PGP 9.5, and $G_{\alpha i2}$ and $G_{\alpha o}$ proteins in the VSE. (A) The receptor cells are strongly positive for OMP in the VSE (arrows). The dendritic processes of OMP-positive receptor cells extend to the free border of the VSE (arrowheads). (B) PGP 9.5-positive cells (arrows) are located in the middle layer of the VSE with their dendrites extending to the free border (arrowheads). (C) The receptor cells are intensely stained by protein $G_{\alpha i2}$ (arrows) and their dendrites elongate and form dendritic knots on the free border (arrowheads). (D) $G_{\alpha o}$ protein was not labeled on VNO receptor cells nor supporting cells. Scale bars = 20 μm.

### 4.4. Lectin histochemistry in the vomeronasal sensory epithelium

#### 4.4.1. N-acetylglucosamine binding lectins

The free border of the VSE was intensely labeled with s-WGA (Fig. 12A) and LEL (Fig. 12B). WGA, DSL, and STL also labeled the free border (Table 6). The supranuclear cytoplasm of the receptor cells and basal cells were strongly positive for s-WGA lectin, while the supporting cells were moderately stained (Fig. 12A). LEL (Fig. 12B) labeled the all layers of the VSE. WGA, DSL, and STL labeling patterns are similar to that of LEL (Table 6). There was no lectin binding observed for BSL-II in the VSE (Fig. 12C). Negative controls using lectins pretreated with appropriate inhibitors (Table 5) showed no reactivity (Fig. 12F).

#### 4.4.2. Mannose binding lectins

Con A strongly labeled the free border and all layers of the VSE (Fig. 12D). Intense labeling for LCA was observed in the free border and the receptor cells—(Fig. 12E), while the supporting cells were moderately stained with LCA and the basal cells were weakly positive for LCA (Fig. 12E). PSA showed a similar labeling pattern to LCA (Table 6).

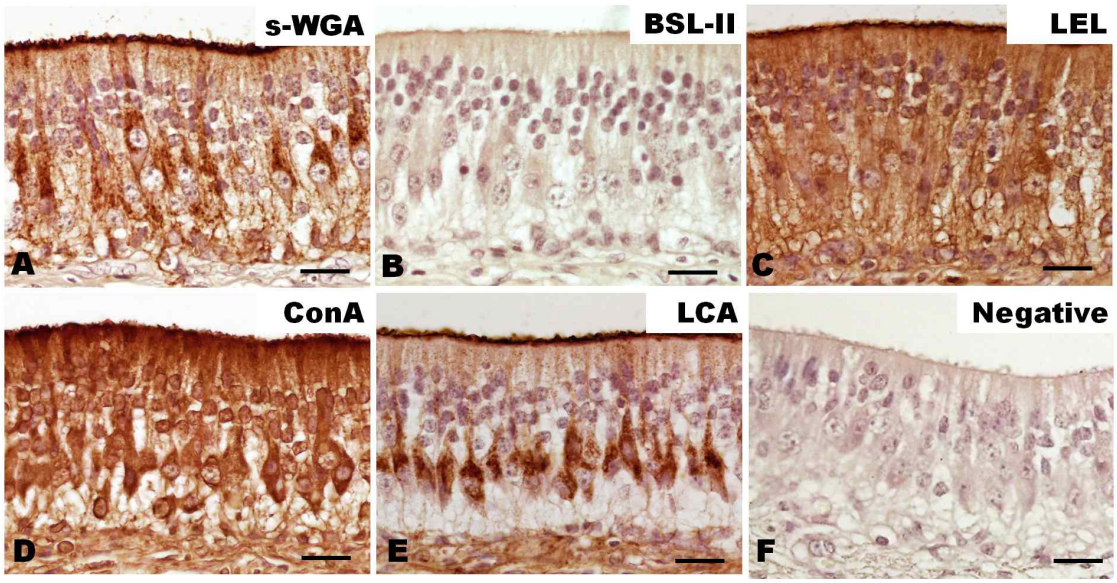


**Figure 12.** Lectin histochemistry with N-acetylglucosamine and mannose binding lectins in the horse VSE. (A) The free border and receptor cells show intense reactions for s-WGA. The supporting cells and the basal cells are moderately labeled by s-WGA. (B) No positive reaction for BSL-II was detected in the VSE. (C) The entire layer of the VSE exhibits intense LEL positivity. (D) Con A is strongly positive in the entire layer of the VSE. (E) Intense reactions for LCA were observed in the free border and in the receptor cells. The supporting cells show a moderate reaction for LCA. (F) Negative control. Scale bars = 20 μm.

**Table 6. Lectin binding patterns of the horse vomeronasal sensory epithelium**

| Lectin | Free border | Receptor cells | Supporting cells | Basal cells |
|---|---|---|---|---|
| N-acetylglucosamine binding lectins | | | | |
| s-WGA | +++ | +++ | ++ | +++ |
| WGA | +++ | +++ | +++ | +++ |
| BSL-II | - | - | - | - |
| DSL | +++ | +++ | +++ | +++ |
| LEL | +++ | +++ | +++ | +++ |
| STL | +++ | +++ | +++ | +++ |
| Mannose binding lectins | | | | |
| ConA | +++ | +++ | +++ | +++ |
| LCA | +++ | +++ | ++ | ++ |
| PSA | +++ | ++ | + | + |
| Galactose/ N-acetylgalactosamine binding lectins | | | | |
| $RCA_{120}$ | +++ | +++ | +++ | +++ |
| BSL-I | +++ | ++ | - | - |
| VVA | +++ | + | ++ | - |
| DBA | + | +++ | - | - |
| SBA | +++ | +++ | ++ | ++ |
| SJA | +++ | - | - | + |
| Jacalin | +++ | ++ | ++ | +~++ |
| PNA | +++ | +++ | - | - |
| ECL | +++ | ++ | +++ | ++ |
| Complex type N-glycan binding lectins | | | | |
| PHA-E | +++ | - | +++ | +++ |
| PHA-L | ++ | - | + | +++ |
| Fucose binding lectin | | | | |
| UEA-I | +++ | - | +++ | - |

–, negative staining; +, faint staining; ++, moderate staining; +++, intense staining.

#### 4.4.3. Galactose/N-acetylgalactosamine binding lectins

$RCA_{120}$ labeledthe free border and all layers of the VSE (Fig. 13A), and similar labeling patterns were detected for SBA, Jacalin, and ECL (Table 6). Staining with BSL-I was restricted to the free border and the receptor cells and did not label the supporting and basal cells (Fig. 13B). PNA labeling patterns are similar to that of BSL-I (Table 6). VVA labeled the free border and the supporting cells in the VSE (Table 6). Moreover, the supranuclear cytoplasm of the receptor cells was weakly stained with VVA, while no basal cells were stained (Table 6). Some receptor cells exhibited intense DBA labeling patterns, and their dendritic knobs reached the free border (Fig. 13C). SJA stained the free border and the basal cells of the VSE (Table 6).

#### 4.4.4. Complex type N-glycan binding lectins

Both PHA-E (Fig. 13D) and PHA-L (Fig. 13E) showed labeling patterns in the free border, the supporting cells, and the basal cells of the VSE, but PHA-E labeled more intensively the supporting cells than PHA-L did.

#### 4.4.5. Fucose binding lectin

UEA-I produced strong reactivity in the free border and in the apical cytoplasm and basilar process of the slender type supporting cells of the VSE. The receptor cells and basal cells were negative for UEA-I (Fig. 13E).

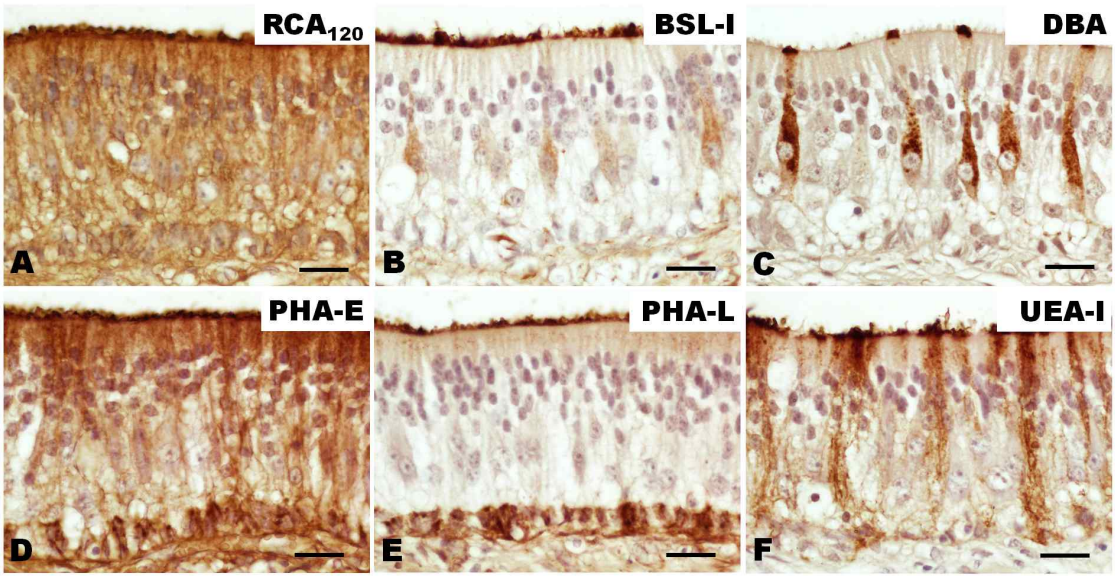


**Figure 13.** Lectin histochemistry with galactose/N-acetylgalactosamine, complex type N-glycan, and fucose binding lectins in the horse VSE. (A) $RCA_{120}$ stained the free border and all cell types of the VSE. (B) BSL-I labeled the receptor cells moderately, but not the supporting cells or the basal cells. (C) Some receptor cells exhibit a strong reaction for DBA and their dendritic knobs reach the free border. (D) PHA-E was observed in the supporting cells and basal cells, but not in the receptor cells. (E) Only the basal cell layer shows PHA-L reactivity in the VSE. (F) UEA-I stained some supporting cells from the basement membrane to the free border. Scale bars = 20 μm.

### 4.5. Lectin histochemistry in the VNSE and vomeronasal glands

#### 4.5.1. N-acetylglucosamine binding lectins

There was no reaction seen for BSL-II in the VNSE (Fig. 14A). WGA, LEL, and STL labeling patterns are similar to that of DSL, while s-WGA showed moderate reactivity (Table 7). Strong labeling by DSL was seen in the free border and ciliated cells of the VNSE (Fig. 14C). STL showed a similar labeling pattern to DSL (Table 7). Moderate staining was seen in the ciliated cells for s-WGA, WGA, and LEL (Table 7). The basal cells of the VNSE were labeled with varying intensities for s-WGA, WGA, BSL-II, DSL, LEL, and STL. Acinar cells of the vomeronasal glands exhibited intense reactivity for BSL-II (Fig. 14B). s-WGA, WGA, LEL, and STL labeling patterns were similar to DSL (Table 7).

#### 4.5.2. Mannose binding lectins

The free border and all layers of the VNSE were intensively labeled with Con A (Fig. 14E), and ConA reacted with moderate intensity in the vomeronasal glands (Fig. 14F). LCA and PSA showed a similar labeling pattern to ConA in the VNSE and vomeronasal glands (Table 7).

#### 4.5.3. Galactose/N-acetylgalactosamine binding lectins

Jacalin showed varying intensities in the free border and all layers of the VNSE (Fig. 14G). $RCA_{120}$, BSL-I, PNA, and ECL labeling patterns are similar to that of Jacalin. VVA showed a faint signal, while DBA was negative in the VNSE (Table 7). SBA (Fig. 14I) and SJA exhibited similar

labeling patterns for the free border and basal cells of the VNSE, but only SBA produced weak staining in the ciliated cells (Table 7). SBA intensively labeled some acinar cells (Fig. 14J). VVA and DBA labeling patterns are similar to that of SBA, while most acinar cells of the vomeronasal glands were stained by $RCA_{120}$, Jacalin, ECL, PHA-E, and PHA-L (Table 7). PNA stained the apical region of the acinar cells in the vomeronasal glands (Table 7). BSL-I and SJA were negative in the vomeronasal glands (Table 7).

#### 4.5.4. Complex type N-glycan binding lectins

PHA-E was intensely bound on free border, ciliated cells as well as basal cells (Fig. 15A), while PHA-L labeled the free border and the basal cells of the VNSE, but did not label ciliated cells (Fig. 15C). Most acinar cells of the vomeronasal glands were labelled by PHA-E (Fig. 15B), but not PHA-L (Fig. 15D).

#### 4.5.5. Fucose binding lectins

Labeling for UEA-I was observed in the free border and in the ciliated cells of the VNSE with strong reactivity (Fig. 15E), while the basal cells were negative and some acinar cells were intensively stained for UEA-I (Fig. 15F).

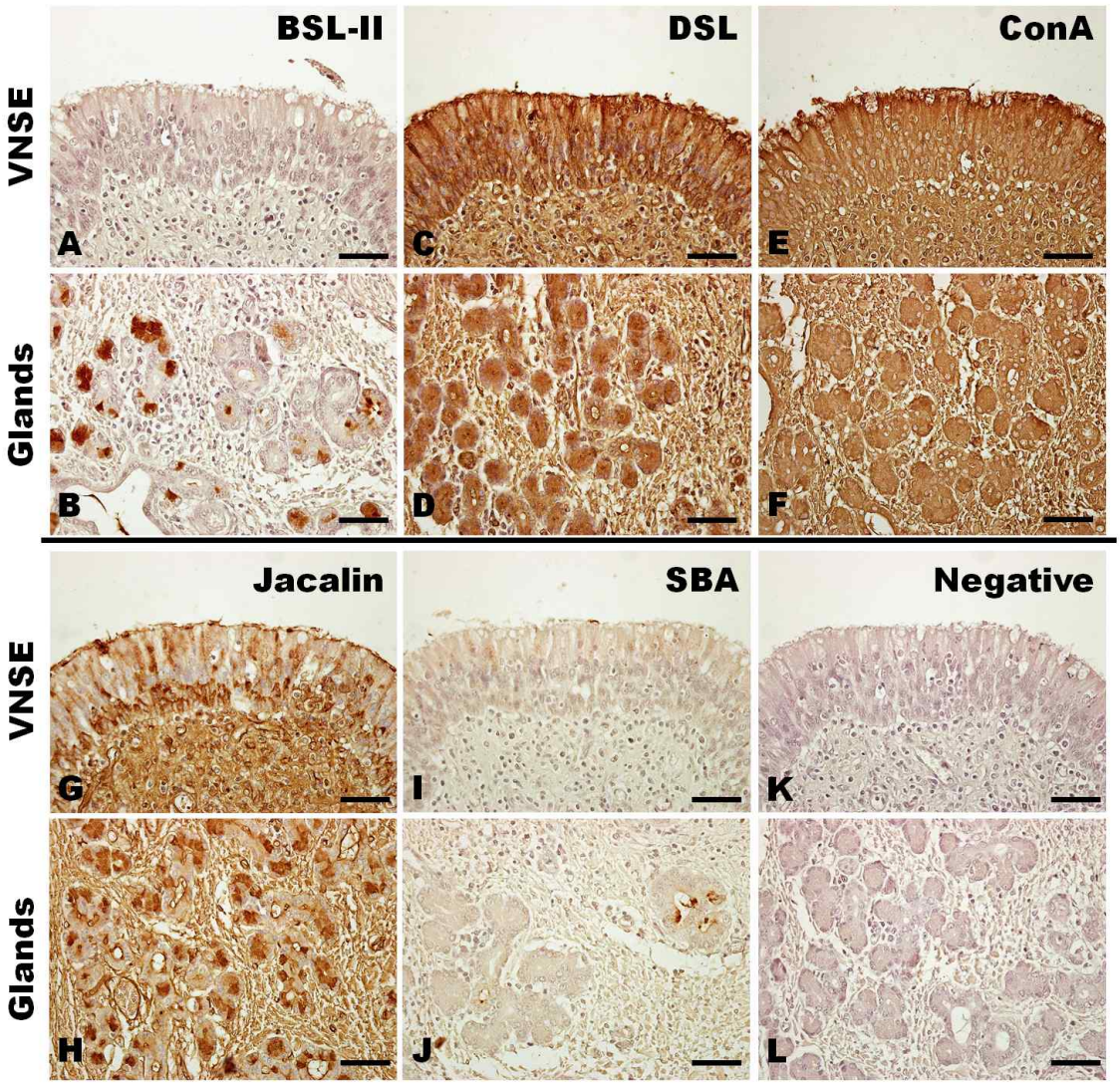


**Figure 14.** Lectin histochemistry with N-acetylglucosamine, mannose, and galactose/N-acetylgalactosamine binding lectins in the horse VNSE (A, C, E, G, I, and K) and vomeronasal glands (B, D, F, H, J, and L). (A and B) BSL-II. (C and D) DSL. (E and F) ConA. (G and H) Jacalin. (I and J) SBA. (K and L) Negative control. Scale bars = 40 μm.

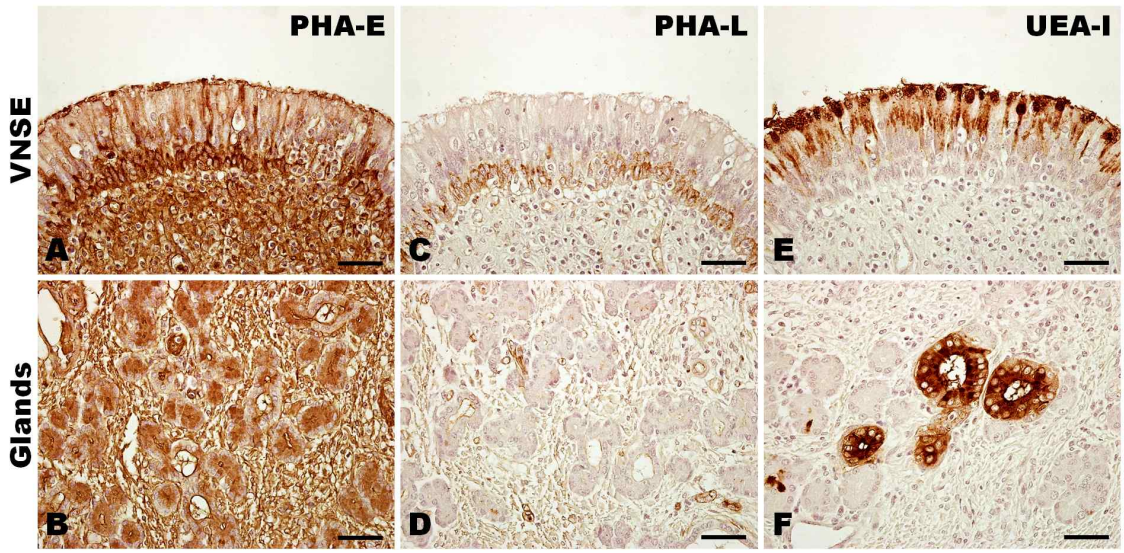


**Figure 15.** Lectin histochemistry with galactose/N-acetylgalactosamine and fucose binding lectins in the horse VNSE (A, C, and E) and vomeronasal glands (B, D, and F). (A and B) PHA-L. (C and D) PHA-L. (E and F) UEA-I. Scale bars = 40 μm.

**Table 7. Lectin binding patterns of the vomeronasal non-sensory epithelium and the vomeronasal gland in the horse**

| Lectin | Free border | Ciliated cells | Basal cells | Vomeronasal glands |
|---|---|---|---|---|
| N-acetylglucosamine binding lectins | | | | |
| s-WGA | ++ | ++ | + | +++ |
| WGA | +++ | ++ | ++ | +++ |
| BSL-II | - | - | - | +++* |
| DSL | +++ | +++ | +++ | +++ |
| LEL | +++ | ++ | + ~ ++ | +++ |
| STL | +++ | +++ | + | +++ |
| Mannose binding lectins | | | | |
| ConA | +++ | +++ | +++ | ++ |
| LCA | ++ | ++ | ++ | + |
| PSA | ++ | ++ | ++ | + |
| Galactose/ N-acetylgalactosamine binding lectins | | | | |
| $RCA_{120}$ | +++ | +++ | ++ | ++ |
| BSL-I | ++ | + | - ~ ++ | - |
| VVA | - ~ + | - ~ + | - | +++* |
| DBA | - | - | - | +++* |
| SBA | + | + | - | +++* |
| SJA | + | - | - | - |
| Jacalin | +++ | +++ | +++ | +++ |
| PNA | ++ | + | ++ | +++* |
| ECL | +++ | ++ | ++ | +++ |
| Complex type N-glycan binding lectins | | | | |
| PHA-E | +++ | +++ | +++ | +++ |
| PHA-L | ++ | - | +++ | - ~ + |
| Fucose binding lectin | | | | |
| UEA-I | +++ | +++ | - | +++* |

*Some acinar cells are positive.

–, negative staining; +, faint staining; ++, moderate staining; +++, intense staining.

## 5. Discussion

To our knowledge, this is the first study to examine both the sensory and non-sensory mucosa of the horse VNO using receptor cell specific antibodies against OMP and PGP 9.5 and to histochemically assess the mucosa and VNO gland glycoconjugate sugar residues using a variety of lectins. We investigated the general morphological structure of a cross-section of the horse VNO, and determined that it is encased by cartilage, similar to sheep (Ibrahim et al., 2013), pigs (Salazar et al., 2000), Korean black goats (Park et al., 2013), cows (Salazar et al., 1995), and wild roe deer (Park et al., 2014). In contrast, the VNO of rodents is encased by bone (Lee et al., 2012).

Histologically, the horse VNO consists of both sensory and non-sensory epithelia, as seen in other domestic animals, including pigs (Park et al., 2012a, Park et al., 2012b), cows (Salazar et al., 1995), and goats (Park et al., 2013). In this study, we first confirmed that the receptor cells of the horse VNO sensory epithelium are immune-positive for OMP and PGP 9.5, which are representative markers for olfactory receptor cells. Further, VNO receptor cells were positive for G protein $G_{\alpha i2}$ (goat type, vomeronasal receptor type 1 marker) (Takigami et al., 2000), but not $G_{\alpha o}$ (rodent type, vomeronasal receptor type 2 marker) (Hagino-Yamagishi, 2008, Yokosuka, 2012), suggesting that VNO signaling in horses transmits information to the accessory olfactory bulb through a V1R pathway.

With regard to the mucus specificity of the VNO gland, both PAS and Alcian blue pH 2.5 were occasionally positive in gland acini. These results suggest that these glands secrete neutral and seromucous components over the

surface of the VNO epithelium, which includes some carbohydrates detected by lectins, as seen in other animals, such as sheep (Ibrahim et al., 2013). We postulate that mucous secretions may be involved in the protection of the mucosa and/or may contribute to the perception of odors, including pheromones.

Using lectin histochemistry, we determined that four groups of lectins, including N-acetylglucosamine, mannose, galactose/N-acetylgalactosamine, and fucose, labeled the sensory epithelium with varying intensities. To better visualize each lectin binding, we used biotin-labeled lectins, followed by avidin-HRP, for which lectin reactivity was enhanced compared with results obtained from direct HRP-labeled lectin in our previous horse VNO study (Lee et al., 2003).

In brief, 20 lectins, but not BSL-II, labeled the mucomicrovillous free border, suggesting that the majority of the carbohydrates recognized by these lectins are present on the mucomicrovillous epithelium of the horse VNO. These findings differ in part from the study by Ibrahim et al. that investigated the VNO of sheep and found that SJA, Jacalin, PSA, and LCA were negative (Ibrahim et al., 2013). We highlight this species-specific difference because the same lectin source was employed in this study and the Ibrahim et al. study (Ibrahim et al., 2013).

The discrepancy in lectin binding seen in the receptor cells in the present study has also been recognized in sensory receptor, supporting, and basal cells of the horse VNO sensory epithelium. With regards to receptor cell UEA-I labeling, our findings differed from those of Ibrahim et al. and Carmanchahi et al., who found faint staining in sheep and intense staining in armadillos, respectively (Carmanchahi et al., 2000, Ibrahim et al., 2013).

In addition, galactose/N-acetylgalactosamine binding lectin BSL-I, DBA, and PNA labeled carbohydrates specific to the receptor cells, but were not present in the supporting and basal cells. These lectin binding carbohydrates are known to be involved in cell adhesion and transport of ions (Spicer and Schulte, 1992), suggesting that they may be involved in the perception of pheromones. Moreover, we found that DBA intensely labeled in receptor cells; however, DBA labeling was not found in sheep (Ibrahim et al., 2013) or armadillo VNO receptor cells (Carmanchahi et al., 2000). Thus, these findings highlight species-specific VNO lectin binding patterns and BSL-I, DBA, and PNA histochemistry could be useful markers for the receptor cells in horse VNO sensory epithelium.

Lectins in the mannose group, which include Con A, LCA, and PSA, labeled all cell types, including receptor, supporting, and basal cells. This result suggests that these lectins bind carbohydrates are involved in ion transport in all cells types, as reviewed previously (Spicer and Schulte, 1992).

N-acetylglucosamine group lectins are known to be involved in cell membrane and permeability processes (Spicer and Schulte, 1992). In the present study, all N-acetylglucosamine group lectins, apart from BSL-II, were intensely labeled in receptor cells, supporting cells, and basal cells, suggesting that these glycoconjugates detected by lectins play a role in cell-to-cell interaction in the VNO sensory mucosa.

Some lectins labeled the supporting and basal cells of the VNO sensory epithelium, except for BSL-II, BSL-I, DBA, SJA, and PNA. This finding is in contrast to results found in sheep (Ibrahim et al., 2013); only a limited number of lectins, including WGA, S-WGA, LEL, STL, DSL, BSL-II, VVA, $RCA_{120}$, UEA-I, Con A, PHA-E, and PHA-L, labeled the supporting cells

(Ibrahim et al., 2013). A similar pattern was also recognized in sheep VNO basal cells (Ibrahim et al., 2013), while additional lectins labeled horse VNO basal cells in the present study.

With regard to fucose group lectins, UEA-I specifically labeled the supporting cells, but not in receptor or basal cells. The oligosaccharide groups PHA-E and PHA-L were clearly labeled the supporting and basal cells, but not in receptor cells, suggesting that these are actively involved in cell adhesion and substrate diffusion in supporting cells and/or basal cells, as previously described (Spicer and Schulte, 1992).

In the non-sensory epithelium of the horse VNO, we found that some, but not all, lectins used in this study labeled in the free border, ciliated cells, and VNO glands with varying intensities. We postulate that the majority of the lectin reactions on the free border originated from secretions of the VNO gland, apart from BSL-I and SJA. Previous studies have shown that a variety of lectins, including those used in the present study, bind to the acini of VNO glands in sheep (Ibrahim et al., 2013) and armadillos (Carmanchahi et al., 2000), with minor differences in the positivity of each lectin. Thus, a variety of glycoconjugates are produced in the VNO glands, which are secreted into the VNO lumen, leading to mucosal protection of both the sensory and non-sensory epithelia. However, we do not exclude the possibility that minor modifications in lectin intensity may occur due to seasonal and hormonal changes, as well as species differences. In the present study, we found that lectins labeled a variety of horse VNO cell types with varying functions, including neural transmission, cell-to-cell adhesion, and proliferation. Because of the diverse reactivity (negative, weak, moderate, and intense) of lectins with the VNO sensory and non-sensory epithelia, we postulate that

carbohydrate sugar residues are partly involved in pheromone perception, as well as protection of both the sensory and non-sensory mucosa covering the free border of the VNO. Further investigation of the precise role of lectin binding carbohydrates on pheromone perception is warranted.

## References

Barrios, A.W., Nunez, G., Sanchez Quinteiro, P., Salazar, I., 2014a. Anatomy, histochemistry, and immunohistochemistry of the olfactory subsystems in mice. Front. Neuroanat. 8, 63.

Barrios, A.W., Sanchez Quinteiro, P., Salazar, I., 2014b. The nasal cavity of the sheep and its olfactory sensory epithelium. Microsc. Res. Tech. 77, 1052-1059.

Baum, M.J., Cherry, J.A., 2015. Processing by the main olfactory system of chemosignals that facilitate mammalian reproduction. Horm. Behav. 68, 53-64.

Bies, C., Lehr, C.M., Woodley, J.F., 2004. Lectin-mediated drug targeting: history and applications. Adv. Drug Deliv. Rev. 56, 425-435.

Breer, H., Fleischer, J., Strotmann, J., 2006. The sense of smell: multiple olfactory subsystems. Cell. Mol. Life Sci. 63, 1465-1475.

Brennan PA. 2001. The vomeronasal system. Cell Mol Life Sci. 58, 546-55.

Carmanchahi PD, Ferrari CC, Marcos HJ, Affanni JM, Sonez CA, Paz DA. 2000. Characterisation of glycoconjugate sugar residues in the vomeronasal organ of the armadillo Chaetophractus villosus (Mammalia, Xenarthra). J Anat. 196 (Pt 3), 357-70.

Duan, D., Lu, M., 2015. Olfactory mucosa: a rich source of cell therapy for central nervous system repair. Rev. Neurosci. 26, 281-293.

Elsaesser, R., Montani, G., Tirindelli, R., Paysan, J., 2005. Phosphatidyl-inositide signalling proteins in a novel class of sensory cells in

the mammalian olfactory epithelium. Eur. J. Neurosci. 21, 2692-2700.

Ferrari, C.C., Carmanchahi, P.D., Aldana Marcos, H.J., Mugnaini, M.T., Affanni, J.M., Paz, D.A., 1999. Identification and localisation of glycoconjugates in the olfactory mucosa of the armadillo Chaetophractus villosus. J. Anat. 194 ( Pt 3), 395-405.

Garcia-Suarez O, Germana G, Naves FJ, Ciriaco E, Represa J, Vega JA. 1997. Sensory epithelium of the vomeronasal organ express TrkA-like and epidermal growth factor receptor in adulthood. An immunohistochemical study in the horse. Anat Rec. 247, 299-306.

Hagino-Yamagishi K. 2008. Diverse systems for pheromone perception: multiple receptor families in two olfactory systems. Zoolog Sci. 25, 1179-89.

Ibrahim, D., Abdel-Maksoud, F., Taniguchi, K., Yamamoto, Y., Taniguchi, K., Nakamuta, N., 2015. Immunohistochemical studies for the neuronal elements in the vomeronasal organ of the one-humped camel. J. Vet. Med. Sci. 77, 241-245.

Ibrahim, D., Nakamuta, N., Taniguchi, K., Taniguchi, K., 2013. Lectin histochemical studies on the vomeronasal organ of the sheep. J. Vet. Med. Sci. 75, 1131-1137.

Ibrahim, D., Nakamuta, N., Taniguchi, K., Yamamoto, Y., Taniguchi, K., 2014. Histological and lectin histochemical studies on the olfactory and respiratory mucosae of the sheep. J. Vet. Med. Sci. 76, 339-346.

Kaltner, H., Stippl, M., Knaus, M., El-Matbouli, M., 2007. Characterization of glycans in the developmental stages of Myxobolus cerebralis (Myxozoa), the

causative agent of whirling disease. J. Fish Dis. 30, 637-647.

Kavoi, B., Makanya, A., Hassanali, J., Carlsson, H.E., Kiama, S., 2010. Comparative functional structure of the olfactory mucosa in the domestic dog and sheep. Ann. Anat. 192, 329-337.

Keller, M., Levy, F., 2012. The main but not the accessory olfactory system is involved in the processing of socially relevant chemosignals in ungulates. Front Neuroanat 6, 39.

Kumar, P., Timoney, J.F., Southgate, H.H., Sheoran, A.S., 2000. Light and scanning electron microscopic studies of the nasal turbinates of the horse. Anat. Histol. Embryol. 29, 103-109.

Lee JY, Kang TY, Lee YD, Shin TK. 2003. Histochemical characterization of the lectin-binding sites in the equine vomeronasal organ. J Vet Sci. 4, 15-9.

Lee K, Ahn M, Lee Y, Ha T, Kim H, Shin T. 2001. Immunohistochemical localization of protein kinase C and nitric oxide synthase in the vomeronasal organ of the horse Korean J Vet Res. 41, 269-73.

Lee, W., Ahn, M., Park, C., Taniguchi, K., Moon, C., Shin, T., 2012. Histochemical study of lectin-binding patterns in the rat vomeronasal organ during postnatal development. Korean J. Vet. Res. 52, 1-8.

Luna, L.G. 1968. Manual of Histological Staining Methods of the Armed Forces Institute of Pathology. New York, Toronto, London, Sydney McGraw-Hill Book Company.

Montani, G., Tonelli, S., Elsaesser, R., Paysan, J., Tirindelli, R., 2006. Neuropeptide Y in the olfactory microvillar cells. Eur. J. Neurosci. 24, 20-24.

Murrell, W., Feron, F., Wetzig, A., Cameron, N., Splatt, K., Bellette, B., et al., 2005. Multipotent stem cells from adult olfactory mucosa. Dev. Dyn. 233, 496-515.

Nakajima T, Shiratori K, Ogawa K, Tanioka Y, Taniguchi K. 1998. Lectin-binding patterns in the olfactory epithelium and vomeronasal organ of the common marmoset. J Vet Med Sci. 60, 1005-11.

Nimrichter L, Gargir A, Gortler M, Altstock RT, Shtevi A, Weisshaus O, et al. 2004. Intact cell adhesion to glycan microarrays. Glycobiology. 14, 197-203.

Oboti L, Ibarra-Soria X, Perez-Gomez A, Schmid A, Pyrski M, Paschek N, et al. 2015. Pregnancy and estrogen enhance neural progenitor-cell proliferation in the vomeronasal sensory epithelium. BMC Biol. 13, 104.

Ogura, T., Krosnowski, K., Zhang, L., Bekkerman, M., Lin, W., 2010. Chemoreception regulates chemical access to mouse vomeronasal organ: role of solitary chemosensory cells. PLoS One 5, e11924.

Opdenakker G, Rudd PM, Ponting CP, Dwek RA. 1993. Concepts and principles of glycobiology. FASEB J. 7, 1330-7.

Park, C., Ahn, M., Kim, J., Kim, S., Moon, C., Shin, T., 2015. Histological and lectin histochemical studies on the olfactory mucosae of the Korean roe deer, Capreolus pygargus. Tissue Cell 47, 221-227.

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,

258-264.

Park C, Choi S, Joo HG, Ahn M, Taniguchi K, Shin T. 2012a. Galectin-3 immunohistochemistry in the vomeronasal organ of the domestic pig, Sus scrofa. Acta Histochem. 114, 713-8.

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., 2012. Developmental changes affecting lectin binding in the vomeronasal organ of domestic pigs, Sus scrofa. Acta Histochem. 114, 24-30.

Plendl, J., Sinowatz, F., 1998. Glycobiology of the olfactory system. Acta Anat. (Basel) 161, 234-253.

Salazar I, Sanchez-Quinteiro P, Lombardero M, Cifuentes JM. 2000. A descriptive and comparative lectin histochemical study of the vomeronasal system in pigs and sheep. J Anat. 196 ( Pt 1), 15-22.

Salazar I, Sanchez Quinteiro P. 2003. Differential development of binding sites for four lectins in the vomeronasal system of juvenile mouse: from the sensory transduction site to the first relay stage. Brain Res. 979, 15-26.

Salazar I, Sanchez Quinteiro PS, Cifuentes JM. 1995. Comparative anatomy of the vomeronasal cartilage in mammals: mink, cat, dog, pig, cow and horse. Ann Anat. 77, 475-81.

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

Takami S. 2002. Recent progress in the neurobiology of the vomeronasal organ. Microsc Res Tech. 58, 228-50.

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, Mori Y, Tanioka Y, Ichikawa M. 2004. Morphological evidence for two types of mammalian vomeronasal system. Chem Senses. 29, 301-10.

Taniguchi K, Mikami S. 1985. Fine structure of the epithelia of the vomeronasal organ of horse and cattle. A comparative study. Cell Tissue Res. 40, 41-8.

Yokosuka, M., 2012. Histological properties of the glomerular layer in the mouse accessory olfactory bulb. Exp. Anim. 61, 13-24.

Zufall F, Kelliher KR, Leinders-Zufall T. 2002. Pheromone detection by mammalian vomeronasal neurons. Microsc Res Tech. 58, 251-60.

**Abstract in Korean**

# 말의 두 후각계의 형태학적 특성에 관한 연구

(지도교수 : 신태균)

이 광 협

제주대학교 일반대학원 수의학과

포유류에서 후각은 사회적 상호작용과 생식 행동을 결정하는 주요 감각이며, 그 경로는 주요 후각계(Main olfactory system)와 보조 후각계(Accessory olfactory system; Vomeronsasal system)로 구성된다. 주요 후각계에서 후각 점막의 후각 수용체 세포(olfactory receptor cell)는 냄새를 탐지하고 정보를 주요 후각망울에 전달하고, 후각망울은 앞쪽 후각핵, 조롱박엽 피질을 포함하여 뇌의 특정 영역으로 신호를 전송한다. 보조 후각계인 서골코기관 시스템은 후각 망울과 구별되는 경로로 서골코기관의 수용체 세포를 통해 페로몬을 감지하고 그 신호를 보조 후각망울을 경유하여 뇌로 전달한다.

본 연구에서는 성숙한 Thoroughbred종 숫말의 두 후각계의 위치 및 구조를 확인하기 위하여 Computed tomography (CT)촬영을 실시하였고, 육안해부학적 평가를 위해 정중단면으로 절단 후 구조를 확인하였다. 또한 두 후각계가 존재하는 위치인 벌집선반(ethmoturbinate)과 서골코기관을 조직학적으로 연구하였다. 말에서 채취한 벌집선반과 서골코기관은 탈회 작업을 한 후 파라핀에 포매하여, 절편을 제작한 후, 염색하여 후각 점막과 서골코기관 신경상피 및 점막에 대하여 평가하였다. 조직화학적 방법을 이용하여 탄수화물 결합 특이성을 조사하기 위해 렉틴을 이용하여 평가하였는데, 렉틴은 탄수화물 결합 단백질(carbohydrate binding protein)로 세포막 표면에 돌출되어 있으며, 복합당질화(glycoconjugation)를 통해 세포부착, 당단백 합성 등에 중요한 역할을 하며, N-acetylglucosamine, Mannose, Galatose/N-acetylgalactosamine, Complex type N-glycan, Fucose binding lectin 그룹과 같이 5가지 그룹으로 분류되며, 그 중 21종의 렉틴을 이용하여 평가하였다.

후각 점막은 후각 상피와 고유 점막판으로 구성되어 있으며, 서골코기관은 후각

수용체세포가 존재하는 신경상피와 수용체 세포가 존재하지 않는 호흡상피로 구성되어 있다. 후각 상피는 자유모서리, olfactory marker protein (OMP)과 protein gene product 9.5 (PGP 9.5) 양성 후각 수용체세포와 지지세포, 바닥세포로 구성되며, 서골코기관 신경상피 역시 같은 조직학적 특성을 보인다. 하지만, 서골코기관의 호흡상피는 수용체 세포가 존재하지 않고 자유 모서리, 지지세포와 바닥세포만으로 구성되어 있고 서골코기관의 신경상피, 호흡상피는 거짓중층원주상피의 형태를 나타낸다. 또한, 서골코기관 신경상피의 수용체 세포는 G protein $G_{\alpha i2}$ (서골코기관 수용체 1 형태)만 발현을 하였으며 이는 말에서 서골코기관의 vomeronasal type 1 receptor 경로를 통해 정보를 전달할 것으로 여겨진다. 후각 상피의 Bowman 샘과 서골코기관 샘은 중성 혹은 약산성의 당결합체와 반응하는 Periodic acid Schiff (PAS)와 황산염 에스테르기 및 카르복실기를 포함한 당결합체와 반응하는 alcian blue (pH 2.5)에 모두 양성 반응을 나타냈다.

렉틴조직화학 염색 결과 두 후각계의 자유 모서리에서는 맥아응집소(wheat germ agglutinin; WGA)를 포함한 16종의 렉틴이 결합하였고, 수용체 세포에서는 독말풀 유래 렉틴(Datura stramonium lectin; DSL)을 포함한 9종의 렉틴이 결합하였다. 또한, 지지세포에서는 바라밀 유래 렉틴(Jacalin)을 포함한 14종의 렉틴이 결합하였으며, 바닥세포에서는 토마토 유래 렉틴(Lycopersicon esculentum lectin; LEL)을 포함한 10종의 렉틴이 결합되었다. 샘에서는 말콩 유래 렉틴(Dolichos biflorus lectin; DBL)을 포함한 15종의 렉틴이 공통적으로 결합하였다. 이를 통해 일부 냄새 분자는 두 후각기 계통의 상피에서 동시에 감지할 것으로 여겨지며, 말의 후각 점막과 서골코기관 신경 상피에서 다양한 탄수화물 말단기를 보유하고 있음을 나타낸다. 이 연구는 말의 두 가지 후각계통에서 렉틴을 이용하여 탄수화물 말단기를 확인한 첫 형태학적 연구이다.

---

주요어 : 말, 렉틴, 호흡상피, 서골코기관.

# Acknowledgements

제가 말 임상 수의사를 목표로 제주대학교 수의학과에 입학한 것이 어느덧 20년이 되었습니다. 학부과정을 거쳐 석사과정을 마치고 새로운 목표를 위해 미국행을 선택한 이후, 다시 한국에 돌아와 이렇게 박사과정 졸업을 앞두게 되었습니다. 여기까지 올 수 있도록 제게 아낌없는 신뢰를 보내주시고 지켜 봐주신 모든 분들께 먼저 감사의 말씀을 드립니다.

제가 지금까지 인생을 살면서 제 삶에 큰 영향을 주신 분은 다름 아닌 지도 교수님이신 신태균 교수님입니다. 신태균 교수님과의 인연은 제가 대학입학 면접을 볼 때부터 시작되었습니다. 짧은 순간이었지만 교수님의 말씀을 통해 수의학도로서 제가 가야 할 길에 대한 생각을 할 수 있었습니다. 학부와 석사 과정 동안 학문의 다양한 방향에 대해 이야기를 해 주시고 언제나 아낌없는 가르침을 주셨습니다. 그러한 배움을 통해 말 임상 수의사로 한 걸음 더 나아가기 위해 과감히 미국행을 선택할 수 있었습니다. 다시 한 번 신태균 교수님께 감사의 말씀을 올립니다.

단순한 말의 질병만을 치료하는 말 임상 수의사가 아니라 전문가로 거듭나게 도와주신 Ruel Cowles Clinic의 대표 Dr. Ruel Cowles와 Gunston Hall Farm familly에게 감사드립니다. 그 분들의 도움 없이는 미국 생활을 무사히 마칠 수 없었습니다. 끊임없는 지도와 격려로 다양한 지식과 기술을 익힐 수 있었습니다.

제가 실험실 생활을 하면서 많은 시간과 추억을 공유한 해부학실험실 선후배님들에게도 감사의 말씀을 드리겠습니다. 이제는 단순한 실험실 선후배가 아닌 제 가족이라고 생각합니다.

마지막으로 사랑하는 부모님과 형제들, 소중한 딸을 믿고 맡겨 주신 장인어른, 장모님께 감사드립니다. 늘 아낌없는 조언과 격려를 해준 사랑하는 아내 지수, 제 삶의 이유인 딸 서원, 효원에게 감사의 마음을 전합니다.

2016. 12. 23.

이광협