A Thesis

For the Degree of Master of Veterinary Medicine

Histological and lectin histochemical studies on the olfactory mucosae of the Korean roe deer, *Capreolus pygargus*

Department of Veterinary Medicine

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

Changnam Park

2015. 8.

Histological and lectin histochemical studies on the olfactory mucosae of the Korean roe deer, *Capreolus pygargus*

Changnam Park
(Supervised by professor Taekyun Shin)

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

2015. 6.

This thesis has been examined and approved.

.....
Thesis director, Youngheun Jee, Prof. of Dept. of Veterinary Medicine

.....
Meejung Ahn, Research Prof. of School of Medicine

.....
Taekyun Shin, Prof. of Dept. of Veterinary Medicine

2015. 6.

Department of Veterinary Medicine
GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

CONTENTS

General introduction

Main olfactory and vomeronasal systems in mammals

Most mammals use olfaction to detect chemical substances for navigation and food source identification and to sense species-specific odors for social communication and reproduction [1]. Mammals possess two olfactory systems: the main olfactory system (MOS) and the vomeronasal system (VNS). In the MOS, the olfactory mucosa perceives various odors and projects olfactory nerves to the main olfactory bulb (MOB). The MOB transmits signals to specific areas of the brain, including the anterior olfactory nucleus, piriform cortex, olfactory tubercle and anterior cortical amygdala [2] (fig. 1). The VNS -specific substances the vomeronasal organ (VNO to the accessory olfactory bulb (AOB). The AOB conveys to the vomeronasal amygdala in the brain [3].

Morphological analysis of the olfactory system in Korean roe deer

Olfactory ability varies across mammal; indeed, there are morphological differences in the mammalian olfactory systems of even rats and mice, which have a close evolutionary relationship [4]. However, most morphological analyses of the MOS and VNS in mammals have focused on rodents living in an artificial laboratory environment or domesticated ungulates such as sheep and goats [5]. The Korean roe deer is a wild ungulate that inhabits the Korean Peninsula, particularly Mount Halla. Under natural condition, Korean roe deer use odors for communication, including marking

territory and sending reproductive information during mating season [6]. Determining the morphological characteristics of the MOS and VNS in Korean roe deer is important for understanding the MOS and VNS in wild ungulates.

Comparative morphology of the MOS in mammals

In mammals, the olfactory mucosa occupies the caudal roof of the nasal cavity, it covers the ethmoturbinates completely, and the degree of lamellar folding is related to the olfactory capacity of each species [7]. In contrast to sheep, in which the turbinates are short and form a scroll-like cylinder, the ethmoturbinates in dogs project more rostrally, and the surface is covered with complex folds of lamellae [8].

The olfactory epithelium, which lines the olfactory mucosa, consists of olfactory receptor cells, supporting cells, and basal cells. In mammals, the populations of supporting and receptor cells vary with development. In rats and dogs, the density of receptor cells is greater than that of supporting cells during maturation [8, 9]. However, in sheep, adults have a reduced density of receptor cells compared with suckling lambs [8].

Olfactory receptor cells are responsible for sensory transduction by forming a cilia-bearing knot [10]. The pattern of cilia on the receptor cells varies among mammals. A radial arrangement exists in dogs, horses, and humans [8, 11, 12] whereas a parallel orientation has been noted in members of the Bovidae family, including sheep, oxen, and goats [8, 13].

The olfactory receptor classification system in mammals

Two major types of olfactory receptors exist in the olfactory epithelium of mammals. Most receptor cells express Golf protein; they are called odorant receptors. They are encoded by up to 1,000 genes and are activated by various odor molecules [18] (fig. 2A). The second type of receptors is called trace amine-associated receptors. They are dispersed in the olfactory epithelium and are sensitive to biogenic amines [19] (fig. 2B).

There are major two receptor classes in the VNO of mammals: vomeronasal receptor type 1 (V1R) and vomeronasal receptor type 2 (V2R). They are located in two layers in the VNO and project their axonal bundle to the anterior and posterior regions of the AOB, respectively [3]. V1R cells located in the apical half of the VSE detect volatile pheromones via the activation of Gαi2 protein (fig. 2C). V2R cells in the basal layer of the VSE sense non-volatile pheromones via the activation of Gαo protein (fig. 2D). The VNO of mammals is classified as rodent, goat, or human type based on the VNO receptor expression pattern. Rodents have two types of VNO receptors [20], whereas goat-type mammals, such as sheep, horses, and cats, express only V1R in the VNO [18]. In humans, the VNO degenerates during fetal development, becoming a vestigial structure [21].

The roles of lectins in the mammalian olfactory system

Lectins are carbohydrate-binding proteins that protrude from glycolipids and glycoproteins [22]. They function in various biological processes through glycoconjugation, including cell-to-cell self-recognition, extracellular matrix interactions, embryonic development, cell growth, cell differentiation, cell signaling, cell adhesion, apoptosis, and inflammation [23, 24]. In the olfactory

system, odorant detection is related to the glycoconjugate composition of the mucus and olfactory receptor membranes [25]. Furthermore, their role in cell–cell recognition is associated with the establishment of synaptic connections [26] (fig. 3). For these reasons, glycoconjugates detected by lectin histochemistry are considered important discriminants of olfactory receptor subpopulations [27].

Reference

1. Bargmann, C.I., *Comparative chemosensation from receptors to ecology*. Nature, 2006. 444(7117): p. 295-301.
2. Breer, H., J. Fleischer, and J. Strotmann, *The sense of smell: multiple olfactory subsystems.* Cell Mol Life Sci, 2006. 63(13): p. 1465-75.
3. Halpern, M. and A. Martinez-Marcos, *Structure and function of the vomeronasal system: an update.* Prog Neurobiol, 2003. 70(3): p. 245-318.
4. Salazar, I. and P. Sanchez Quinteiro, *Lectin binding patterns in the vomeronasal organ and accessory olfactory bulb of the rat.* Anat Embryol (Berl), 1998. 198(4): p. 331-9.
5. Keller, M. and F. Levy, *The main but not the accessory olfactory system is involved in the processing of socially relevant chemosignals in ungulates.* Front Neuroanat, 2012. 6: p. 39.
6. Danilkin, A.A., et al., *[The social organization, behavior and ecological parameters of a migrating population of Siberian roe deer (Capreolus pygargus Pall.)]*. Izv Akad Nauk Ser Biol, 1995(1): p. 48-61.
7. Pihlstrom, H., et al., *Scaling of mammalian ethmoid bones can predict olfactory organ size and performance.* Proc Biol Sci, 2005. 272(1566): p. 957-62.
8. Kavoi, B., et al., *Comparative functional structure of the olfactory mucosa in the domestic dog and sheep.* Ann Anat, 2010. 192(5): p. 329-37.
9. Chuah, M.I., A.I. Farbman, and B.P. Menco, *Influence of olfactory bulb on dendritic knob density of rat olfactory receptor neurons in*

vitro. Brain Res, 1985. 338(2): p. 259-66.

10. Herrera, L.P., et al., *Ultrastructural study of the primary olfactory pathway in Macaca fascicularis.* J Comp Neurol, 2005. 488(4): p. 427-41.
11. Kumar, P., et al., *Light and scanning electron microscopic studies of the nasal turbinates of the horse.* Anat Histol Embryol, 2000. 29(2): p. 103-9.
12. Lenz, H., *[Surface of the olfactory region in man observed by scanning electron microscope].* Acta Otolaryngol, 1977. 84(1-2): p. 145-54.
13. Menco, B.P., et al., *Bovine olfactory and nasal respiratory epithelium surfaces. High-voltage and scanning electron microscopy, and cryo-ultramicrotomy.* Cell Tissue Res, 1978. 193(3): p. 503-24.
14. Hagino-Yamagishi, K., *Diverse systems for pheromone perception: multiple receptor families in two olfactory systems.* Zoolog Sci, 2008. 25(12): p. 1179-89.
15. Liberles, S.D. and L.B. Buck, *A second class of chemosensory receptors in the olfactory epithelium.* Nature, 2006. 442(7103): p. 645-50.
16. Ichikawa, M., et al., *Differential development of binding sites of two lectins in the vomeronasal axons of the rat accessory olfactory bulb.* Brain Res Dev Brain Res, 1994. 78(1): p. 1-9.
17. Witt, M., et al., *On the chemosensory nature of the vomeronasal epithelium in adult humans.* Histochem Cell Biol, 2002. 117(6): p. 493-509.
18. Bies, C., C.M. Lehr, and J.F. Woodley, *Lectin-mediated drug*

targeting: history and applications. Adv Drug Deliv Rev, 2004. 56(4): p. 425-35.

19. Nimrichter, L., et al., *Intact cell adhesion to glycan microarrays.* Glycobiology, 2004. 14(2): p. 197-203.
20. Opdenakker, G., et al., *Concepts and principles of glycobiology.* FASEB J, 1993. 7(14): p. 1330-7.
21. Ibrahim, D., et al., *Histological and lectin histochemical studies on the olfactory and respiratory mucosae of the sheep.* J Vet Med Sci, 2014. 76(3): p. 339-46.
22. Bettini, S., et al., *Immunohistochemical and histochemical characteristics of the olfactory system of the guppy, Poecilia reticulata (Teleostei, Poecilidae).* Anat Rec (Hoboken), 2009. 292(10): p. 1569-76.
23. Plendl, J. and F. Sinowatz, *Glycobiology of the olfactory system.* Acta Anat (Basel), 1998. 161(1-4): p. 234-53.

Figures

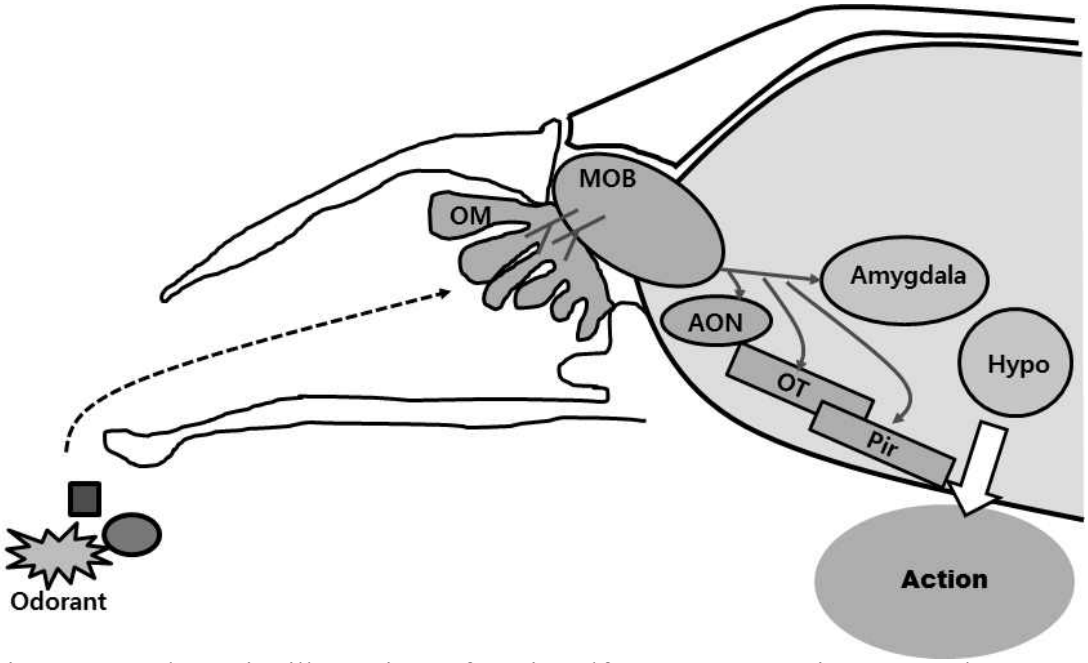

Figure 1. Schematic illustration of main olfactory system in mammal. AON, anterior olfactory nucleus; Hypo, hypothalamus; MOB, main olfactory bulb; OE, olfactory epithelium; OT, olfactory tubercle; Pir, piriform cortex.

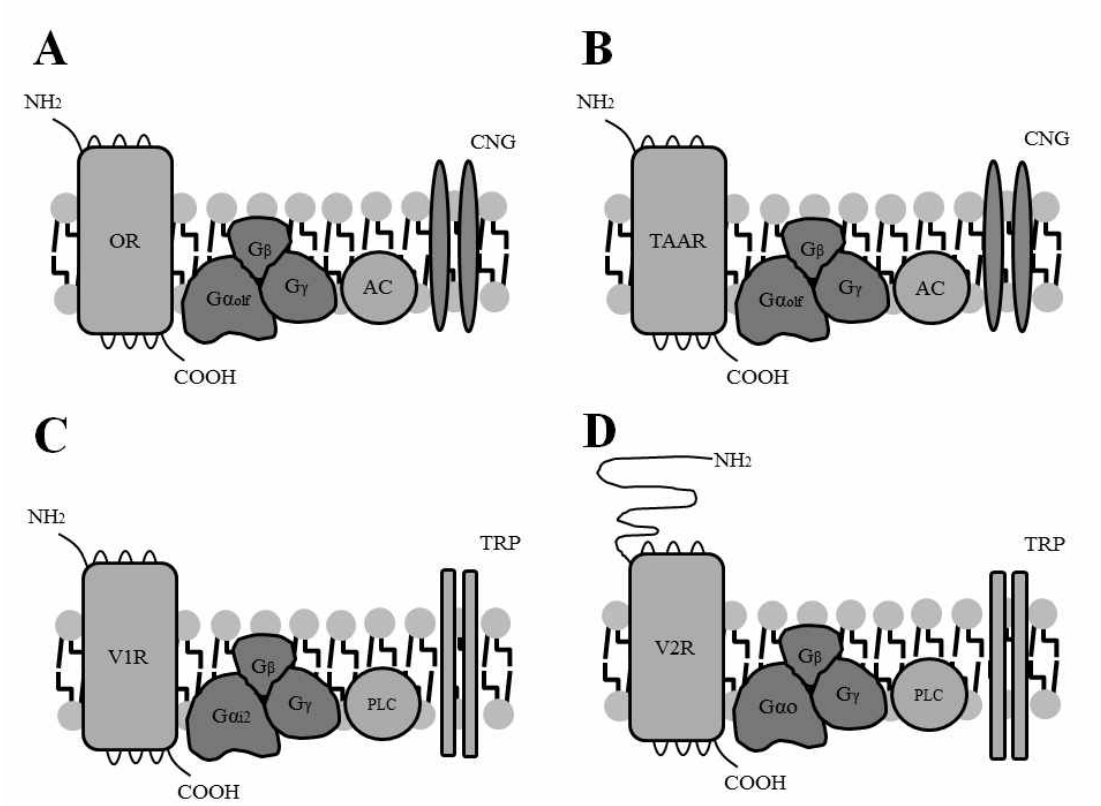

Figure 2. Four types of vertebrate olfactory receptor proteins. All have a nitrogen (N) terminal on the cell exterior and a carboxylic acid (C) terminal extending into the cell interior. (A) odorant receptor (OR), (B) trace amine associate receptor, (C) vomeronasal receptor 1 and (D) vomeronasal receptor 2. AC, adenylyl cyclase CNG channel, cyclic nucleotide-gated cation channel; PLC, phospholipase C; TRP, transient receptor potential channel.

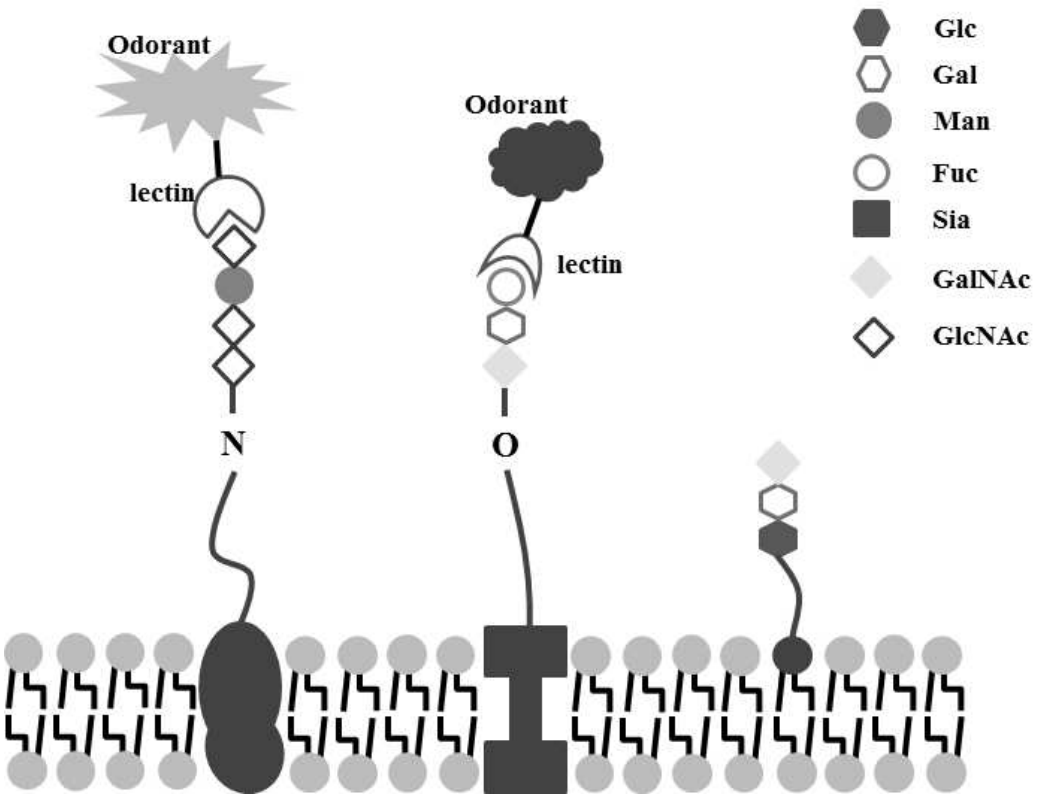

Figure 3. Schematic illustration of relationship between odorant-carbohydrate heterogeneity on cell surface. Glc, glucose; Gal, galactose Man, mannose; Fuc, fucose GalNAc, N-acetylgalactosamine GlcNAc, N-acetylglucosamine.

Histological and lectin histochemical studies on the olfactory mucosae of the Korean roe deer, *Capreolus pygargus*

1. Abstract

The morphological features of the olfactory mucosae of Korean roe deer, *Capreolus pygargus*, were histologically studied using the ethmoid turbinates containing the olfactory mucosae from six roe deer (males, 2-3 years old). The ethmoid turbinates were embedded in paraffin, and were histochemically evaluated to see the mucus characteristics. Lectin histochemistry was performed to investigate the carbohydrate binding specificity on the olfactory mucosa. Lectins including *Triticum vulgaris* wheat germ agglutinin (WGA), *Ulex europaeus* agglutinin I (UEA-I), soybean agglutinin SBA) were one of representative lectins for N-acetylglucosamine, fucose and N-acetylgalactosamine groups of carbohydrates, respectively. Histologically, the olfactory mucosa, positioned mainly in the caudal roof of the nasal cavity, consisted of the olfactory epithelium and the lamina propria. The olfactory epithelium consisted of protein gene product (PGP) 9.5-positive olfactory receptor cells, galectin-3-positive supporting cells, and basal cells. Bowman's glands in the lamina propria were stained by both the periodic acid Schiff reagent and alcian blue (pH 2.5). Two types of lectins including WGA and SBA were labelled in free border, receptor cells, supporting cells and Bowman's glands with the exception of basal cells, while UEA-I was labelled in free border, supporting cells and Bowman's glands, but not in receptor cells and basal cells, suggesting that carbohydrate terminals on olfactory mucosa of roe deer varied depending on cell types. Collectively, this is a first morphological study of the olfactory mucosa of the Korean roe deer, in which carbohydrate terminals on olfactory mucosa of roe deer were first evaluated.

Keywords: lectin; olfactory epithelium; olfactory mucosa; roe deer.

2. Introduction

Olfaction in mammals plays important roles in exploration of the outer environment for odorous signals from food sources and habitats and in detection of chemical substances controlling social interaction and reproductive behavior [1-3]. The olfactory mucosa is a major nasal chemosensory organ that perceives odorous substances [4], and it is responsible for chemical signal reception in the main olfactory system [5], sending the information to the main olfactory bulb, its primary brain center [6]. Recent studies have shown that the main olfactory system in ungulates is also involved in reproduction [3] and social behavior [1].

For the chemoreception in olfactory systems, carbohydrate terminals have been known to play important roles in the process of the perception of odorous substance [7], in which lectin histochemistry has been used to discriminate carbohydrate terminals on chemosensory systems [8-10].

Over the past several decades, the morphological characteristics of the olfactory mucosae of various animals have been studied, including those of mice [11], rats [12], dogs [13, 14], horses [15], cattle [16, 17], pigs [18] and sheep [13, 19]. However, little is known about the main olfactory system tissues of wild ruminants, except deer [20]. The Korean roe deer, *Capreolus pygargus*, an ungulate ruminant, inhabits fields and meadows, particularly on the Halla mountain in Jeju island, Republic of Korea [21]. Little is known about its morphological characteristics and carbohydrate terminals of main olfactory systems, except for those of the chemosensory vomeronasal system [22].

The aim of this study was to examine the morphological characteristics of the chemosensory olfactory mucosae in the roe deer, and also to discriminate the carbohydrate terminals on the olfactory mucosae.

3. Materials and Methods

Tissue preparation

Roe deer (six males and one female, 2-3 years old) samples were obtained from the Jeju Wildlife Rescue Center. Their ages were estimated based on the number of horn branches (spikes). The vomeronasal organs from the same roe deer used in this study have been examined previously [22]. All six deer were sexually mature. For light microscopic evaluation of the olfactory mucosa, the ethmoturbinates 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.

Histological examination

Formalin-fixed ethmoturbinates were trimmed and decalcified in a sodium citrate–formic acid solution, with several changes of the solution, until the bony pieces softened, as described in our previous study [23]. The decalcified ethmoid turbinates 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 ethmoid turbinates focused on male roe deer, because only one sample of female ethmoturbinates was obtained and was histologically similar to males.

Antibodies

To confirm the presence of receptor cells and supporting cells in the OE, immunohistochemistry was performed using a rabbit polyclonal antibody to protein gene product 9.5 (PGP 9.5) and a rat anti-galectin-3 monoclonal antibody, respectively. Using affinity chromatography, the rat anti-galectin-3 monoclonal antibody was purified from supernatant derived from hybridoma cells (clone TIB-166™, M3/38.1.2.8. HL.2; American Type Culture Collection, Manassas, VA, USA) and used at a final concentration of 1–5 μg/mL. This antibody has been used previously to detect the supporting cells of vomeronasal organs of some ungulates, including Korean native black goats [24] and pigs [23]. A rabbit polyclonal antibody targeting PGP 9.5 (Biotrend, Cologne, Germany) was used to detect receptor cells in the olfactory mucosa as described in a previous study on rats [25]. PGP 9.5 has also been used as a marker of receptor cells in the chemosensory vomeronasal organ of Korean roe deer [22].

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 3 min. After cooling, the sections were exposed to aqueous 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. Non-specific binding was blocked using 10% normal goat serum (ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA). The sections were washed in phosphate-buffered saline (PBS, pH 7.4) for 1 h and then reacted with rat anti-galectin-3 antibody (1:1,000) for 1 h at room temperature. After washing in PBS, the sections were reacted for 45 min with biotinylated rabbit anti-rat IgG (1:100;

Vector Laboratories). A rabbit polyclonal antibody targeting PGP 9.5 (1:800) was reacted with a biotinylated goat anti-rabbit IgG (1:100; Vector Laboratories). After another wash in PBS, the sections were incubated for 45 min with the avidin–biotin peroxidase complex kit (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).

Lectins used in this study

WGA, belonging to N-acetylgalactosamine group, labels both N-acetylglucosamine and N-acetylneuraminic acid. UEA-I, belonging to fucose group, recognize fucose, while SBA have been known to belong to N-acetylgalactosamine group, binds to both galactose and N-acetylgalactosamine. All lectins were purchased from Sigma–Aldrich (St. Louis, MO, USA), and its reactivity on neurosensory epithelium was reported in previous studies [22, 23, 26].

Lectin histochemistry

Lectin histochemistry was performed as described in previous studies [22, 23, 26]. Briefly, the paraffin-embedded ethmoid turbinates 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 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 either WGA-peroxidase (1:100), UEA-I-peroxidase (1:200) or SBA-peroxidase (1:100) for 3 h at room temperature. Signals 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.

4. Results

Gross anatomy of the nasal cavity

The nasal cavity, the first air passage cavity surrounded by bones, extended from the nostril to the nasopharynx. In the rostral two third of the nasal cavity, the air passage was divided by the nasoturbinate and maxilloturbinate, while the vomeronasal organ was located below the nasal septum (Fig. 1.). The ethmoturbinates were positioned at caudal one third of nasal cavity and had more complex lamella folding (Fig. 1, marked as I-VI) and branching pattern than nasoturbinate (Fig. 1, NT) and maxilloturbinate (Fig. 1, MT).

Histological features of the olfactory mucosa

Histologically, it was found that the olfactory mucosa was covered by all of ethmoturbinates and the caudal portion of nasoturbinate and maxilloturbinate. Briefly, the olfactory mucosa was shown to consist of the olfactory epithelium (Fig. 2A, OE) and the underlying lamina propria (Fig. 2A, LP). The olfactory epithelium consisted of the olfactory supporting cells (Fig. 2B, arrows), receptor cells (Fig. 2B, arrowheads) labeled with PGP9.5 by immunohistochemistry, and basal cells (Fig. 2B, hollow arrowheads). The free border of the olfactory epithelium was adjacent to the nasal cavity (Fig. 2B, asterisk). Precisely, at the apical region of the olfactory epithelium, the nuclei of the olfactory supporting cells were oval shaped (Fig. 2B, arrows), while the olfactory receptor cells had globular nuclei and were positioned

between the olfactory supporting and basal cells (Fig. 2B, arrowheads). Below the olfactory receptor cells, the globose basal cells (Fig. 2B, hollow arrows) and horizontal basal cells (Fig. 2B, hollow arrowheads) are lined with the basal lamina. The shape of the horizontal basal cells was irregular, and these cells were arranged in one row just above the basal lamina (Fig. 2B, hollow arrowheads).

The lamina propria contained the Bowman's glands (Fig. 2A, arrows) and olfactory nerve bundles (Fig. 2A, arrowheads). Bowman's glands were mainly located in the upper half of the lamina propria. The secretory acinar cells of Bowman's glands were pyramidal in shape (Fig. 2A, inset, arrows). The secretary ducts consisting of simple cuboidal ductal epithelium (Fig. 2A, hollow arrows) were interposed vertically in the olfactory epithelium to the luminal surface. The secretory materials in the ducts (Fig. 2C and 2D, arrowhead) and acinar cells in the Bowman's glands (Fig. 2C and 2D, arrow) were both positive for PAS (Fig. 2C) and alcian blue staining (pH 2.5) (Fig. 2D), respectively.

Immunohistochemical analysis of the olfactory epithelium and the lamina propria

Olfactory supporting cells, with galectin-3 immunoreactivity, were mainly observed in apical one third of the olfactory epithelium (Fig. 3A-C). Galectin-3 immunoreactivity was recognized at intra-nuclei (Fig. 3B, arrow) and non-nuclear zone of the apical portion of supporting cells (Fig. 3A, arrows), nerve bundles in the lamina propria (Fig. 3A, hollow arrows). In addition, thin processes of galectin-3-positive cells extend to the basal lamina

(Fig. 3A and 3B, arrowheads) and the free border. In the underlying lamina propria, galectin-3 was immunostained in the Schwann cells in the olfactory nerve bundles (Fig. 3C, hallow arrows) and some ductal epithelium. In contrast, no galectin-3 reactivity was observed in the olfactory receptor or basal cells.

Olfactory receptor cells, with expression of PGP 9.5, was found in the middle part of the olfactory epithelium (Fig. 3D, arrows), while PGP9.5 was not immunostained in both supporting cells and basal cells. PGP 9.5-positive cells showed extension of some dendrites to the free border (Fig. 3E, arrow). The axons of PGP 9.5-positive cells were bundled below the basal lamina (Fig. 3D, arrowheads), forming axonal bundles in the lower region of the lamina propria (Fig. 3D and 3F, hollow arrows).

Lectin histochemistry of the olfactory epithelium and the lamina propria

WGA reactivity was detected in the free border and all phenotype of the olfactory mucosa including olfactory epithelial cells (Fig. 4A). WGA was stained at the non-nuclear zone and nuclear surface of the olfactory supporting cells (Fig. 4B, arrows). Also, the dendrites and cell body of the olfactory receptor cells show WGA reactivity (Fig. 4B, arrowheads). The olfactory basal cells were positive for WGA at cell surface (Fig. 4B, hollow arrow). In the lamina propria, WGA was labeled in the acini of the Bowman's glands (Fig. 4C, hollow arrowhead) and olfactory nerve bundle (Fig. 4C, asterisk).

UEA-I staining was intense on the free border along the luminal surface of the olfactory epithelium (Fig. 4D).The olfactory supporting cells showed UEA-I reactivity at the non-nuclear zone (Fig. 4E, arrows). In addition, UEA-I was labeled at the cell surface of the olfactory receptor cells

(Fig. 4E, arrowheads). But no reactivity for UEA-I was seen in the olfactory basal cells (Fig. 4E, hollow arrow). Below the basal lamina, some acinar cells of the Bowman's glands showed UEA-I reactivity (Fig. 4F, hollow arrowhead). The olfactory nerve bundles were not stained by UEA-I (Fig. 4F, asterisk).

SBA labeling was intensely recognized at the free border (Fig. 4G). SBA was faintly detected on the cell membrane of some olfactory receptor cells (Fig. 4H, arrowheads) while SBA reaction was neither detected in olfactory supporting cells (Fig. 4H, arrow) nor olfactory basal cells (Fig. 4H, hollow arrow). In contrast to the weak reaction of SBA on the olfactory epithelium, SBA was intensely reacted in all of acini of the Bowman's glands (Fig. 4I, hollow arrowhead), but not in the olfactory nerve bundles (Fig. 4I, asterisk).

The lectin reactivities in the olfactory mucosa described above was summarized in Table 1, in which its reactivity was largely similar to those in the vomeronasal organ in the same roe deer [22].

5. Discussion

This is the first descriptive morphological study of the olfactory mucosa of Korean roe deer, the morphological characteristics of which are similar to those of other ruminants, including deer [20], sheep [19] and cattle [16, 17]. The olfactory mucosa and the vomeronasal organ have been found to be morphologically independent [27], and their functional roles differ based on the odorous substance [28]. In particular, the vomeronasal organ is closely involved in sexual behavior through the detection of pheromones [28], but recent studies show that the olfactory mucosa is also related to pheromone perception [3]. In this study, we found that there were similarities between the two chemosensory organs with regard to lectin binding patterns in the receptor cells. If lectin binding is involved in pheromone perception, as discussed in a review article [3], it is possible that some odorous substances could be detected by both the olfactory mucosa and the vomeronasal organ, because certain lectins are present in both the vomeronasal [22] and olfactory receptor cells in roe deer in the present study.

Galectin-3, a beta-galactoside binding animal lectin, has been used as a marker of supporting cells in the vomeronasal sensory epithelium in humans [29], pigs (Park et al. 2012a, 2012b), roe deer [22] and goats [24]. Galectin-3 has been immunostained in olfactory supporting cells, but not in olfactory receptor cells in this study, even though the present finding differs from the human case that galectin-3 was immunostained in the olfactory receptor cells reported in the human olfactory epithelium [29]. Thus we postulated that galectin-3 immunostaining is exclusively found in the supporting cells, but not in receptor cells, in the both olfactory and vomeronsal epithelia in animals

with an exception of galectin-3 immunostaining in human olfactory receptor cells [29].

As for the lectin binding pattern in the present study, we postulated that three different kinds of carbohydrates teriminals exist in the olfactory mucosa, which were N-acetylglucosamine, fucose and N-acetylgalactosamine groups identified by WGA, UEA-I and SBA, respectively. The present findings that WGA, UEA-I and SBA are labeled on receptor cells with varying intensity suggest that some carbohydrate terminals including N-acetylglucosamine, fucose and N-acetylgalactosamine groups plays an important role in odor processing in roe deer.

As for the source of carbohydrates on the free border of olfactory mucosae, we postulate that carbohydrate terminals are mainly originated from Bowman'g glands because all lectins were positive for acini of Bowman's glands. We do not exclude a possibility that some are originated from supporting cells and/or receptor cells because WGA, UEA-I and SBA labels receptor cells as well.

Odorous signals has been known to be captured by lectins in which each lectin recognizes different odorous signals [7]. The present study showed that the odorous signal perception and transduction is achieved by all lectins including WGA, UEA-I and SBA in this study as far as olfaction is involved in Korean roe deer even though the capacity of odor capture varied on the type of each lectin.

As for the role of lectins in receptor cells, internalization of WGA -horseradish peroxidase by some olfactory receptor cells has been resulted in their death by apoptosis and a subsequent deafferentation of the olfactory bulb [30]. In other reports, alpha-extended lactoseries carbohydrates, present on

novel glycoforms of the neural cell adhesion molecule, has been found in rat olfactory receptor cells, in which they play a role in the axonal sorting toward their targeting [31]. Thus it is postulated that each lectin depending on binding conditions plays a diverse role including axonal sorting and apoptosis in addition to odor perception.

In the supporting cells of olfactory epithelium, three types of lectins including WGA, UEA-I and SBA were identified in Korean roe deer, even though minor differences exit in sheep because WGA, but not SBA and UEA-I, was present in the olfactory supporting cells of sheep [19]. In addition to the labeling of WGA, UEA-I and SBA in olfactory epithelium, we have consistently found that galectin-3, a beta–galactoside binding protein, was localized in supporting cells of chemoreceptor vomeronasal organ in pigs [23] and other ungulates including goats [24] and Korean roe deer [22]. Because galectin-3 plays a role in cell proliferation and cell adhesion [32] and axon sorting [31], we postulated that galectin-3 and other carbohydrate terminals synergistically promote the survival of supporting cells, leading to regeneration of olfactory epithelia [33] as well as to axonal sorting in nerve fiber layers [31].

Even though both receptor cells and supporting cells in olfactory mucosa contains carbohydrate terminals recognized by lectins, unexpected is that lectins including WGA, UEA-I and SBA in the present study were not labeled on basal cells, a possible source of stem cells in olfactory epithelia [34, 35]. The link between lectin reactivity and stem cell character remains to be further studied.

Collectively, we postulated that this is a first descriptive study of the olfactory mucosa in Korean roe deer, and that carbohydrate terminals on olfactory mucosa were evaluated by lectin histochemistry.

6. References

1. Keller, M. and F. Levy, *The main but not the accessory olfactory system is involved in the processing of socially relevant chemosignals in ungulates.* Front Neuroanat, 2012. 6: p. 39.
2. Breer, H., J. Fleischer, and J. Strotmann, *The sense of smell: multiple olfactory subsystems.* Cell Mol Life Sci, 2006. 63(13): p. 1465-75.
3. Baum, M.J. and J.A. Cherry, *Processing by the main olfactory system of chemosignals that facilitate mammalian reproduction.* Horm Behav, 2014.
4. Takami, S. and K. Toida, *Structure and function of the olfactory system: overview.* Anat Sci Int, 2008. 83(4): p. 183-5.
5. Anholt, R.R., *Molecular neurobiology of olfaction.* Crit Rev Neurobiol, 1993. 7(1): p. 1-22.
6. Carr, V.M. and A.I. Farbman, *Ablation of the olfactory bulb up-regulates the rate of neurogenesis and induces precocious cell death in olfactory epithelium.* Exp Neurol, 1992. 115(1): p. 55-9.
7. Plendl, J. and F. Sinowatz, *Glycobiology of the olfactory system.* Acta Anat (Basel), 1998. 161(1-4): p. 234-53.
8. Shapiro, L.S., P.L. Ee, and M. Halpern, *Lectin histochemical identification of carbohydrate moieties in opossum chemosensory systems during development, with special emphasis on VVA-identified subdivisions in the accessory olfactory bulb.* J Morphol, 1995. 224(3): p. 331-49.
9. Malz, C.R., P. Schwartz, and H.J. Kuhn, *Lectin binding sites in the vomeronasal organ and the olfactory epithelium of the tree shrew*

tupaia belangeri. J Hirnforsch, 1999. 39(4): p. 481-7.

10. Saito, H., K. Ogawa, and K. Taniguchi, *[Lectin-binding patterns of the olfactory receptors (olfactory epithelium, vomeronasal organ and septal olfactory organ of Masera) in the rat]*. Jikken Dobutsu, 1994. 43(1): p. 51-60.
11. Taniguchi, K. and K. Taniguchi, *Embryonic and postnatal differentiation of olfactory epithelium and vomeronasal organ in the Syrian hamster.* J Vet Med Sci, 2008. 70(1): p. 57-64.
12. Kwon, B.S., et al., *Age-related changes in microvillar cells of rat olfactory epithelium.* Neurosci Lett, 2005. 378(2): p. 65-9.
13. Kavoi, B., et al., *Comparative functional structure of the olfactory mucosa in the domestic dog and sheep.* Ann Anat, 2010. 192(5): p. 329-37.
14. Adams, D.R. and D.K. Hotchkiss, *The canine nasal mucosa.* Anat Histol Embryol, 1983. 12(2): p. 109-25.
15. Krueger, K. and B. Flauger, *Olfactory recognition of individual competitors by means of faeces in horse (Equus caballus).* Anim Cogn, 2011. 14(2): p. 245-57.
16. Aceto, A., et al., *Glutathione transferase isoenzymes in olfactory and respiratory epithelium of cattle.* Biochem Pharmacol, 1993. 46(12): p. 2127-33.
17. Wenisch, S. and S. Arnhold, *NADPH-diaphorase activity and NO synthase expression in the olfactory epithelium of the bovine.* Anat Histol Embryol, 2010. 39(3): p. 201-6.
18. Nadol, J.B., Jr. and A.J. DeLorenzo, *Fine structural localization of acetylcholinesterase in the olfactory mucosa in guinea pig and rabbit.*

Ann Otol Rhinol Laryngol, 1974. 83(1): p. 55-64.

19. Ibrahim, D., et al., *Histological and lectin histochemical studies on the olfactory and respiratory mucosae of the sheep.* J Vet Med Sci, 2014. 76(3): p. 339-46.

20. Kolb, A., *[Light microscopy studies on the olfactory epithelium of the deer]*. Anat Anz, 1975. 137(5): p. 417-28.

21. Han, S., et al., *Identification of Species and Sex of Korean Roe Deer (Capreolus pygargus tianschanicus) Using SRY and CYTB Genes.* Animal Cells Syst, 2007. 11(1): p. 165-168.

22. Park, C., et al., *A morphological study of the vomeronasal organ and the accessory olfactory bulb in the Korean roe deer, Capreolus pygargus.* Acta Histochem, 2014. 116(1): p. 258-64.

23. Park, J., et al., *Developmental changes affecting lectin binding in the vomeronasal organ of domestic pigs, Sus scrofa.* Acta Histochem, 2012. 114(1): p. 24-30.

24. Park, C., et al., *A morphological study of vomeronasal organ of Korean black goat (Capra aegagrus hircus).* Korean J Vet Res, 2013. 53(1): p. 55-60.

25. Oikawa, T., H. Saito, and K. Taniguchi, *Immunohistochemical studies on the differential maturation of three types of olfactory organs in the rats.* J Vet Med Sci, 2001. 63(7): p. 759-65.

26. Lee, W., et al., *Histochemical study of lectin-binding patterns in the rat vomeronasal organ during postnatal development.* Korean Journal of Veterinary Research, 2012. 52(1): p. 1-8.

27. Halpern, M., *The organization and function of the vomeronasal system.* Annu Rev Neurosci, 1987. 10: p. 325-62.

28. Brennan, P.A., *The vomeronasal system.* Cell Mol Life Sci, 2001. 58(4): p. 546-55.

29. Witt, M., et al., *On the chemosensory nature of the vomeronasal epithelium in adult humans.* Histochem Cell Biol, 2002. 117(6): p. 493-509.

30. Moon, Y.W. and H. Baker, *Lectin-induced apoptosis of mature olfactory receptor cells.* J Neurosci Res, 2002. 68(4): p. 398-405.

31. Storan, M.J., et al., *Expression and putative role of lactoseries carbohydrates present on NCAM in the rat primary olfactory pathway.* J Comp Neurol, 2004. 475(3): p. 289-302.

32. Matarrese, P., et al., *Galectin-3 overexpression protects from apoptosis by improving cell adhesion properties.* Int J Cancer, 2000. 85(4): p. 545-54.

33. Magrassi, L. and P.P. Graziadei, *Cell death in the olfactory epithelium.* Anat Embryol (Berl), 1995. 192(1): p. 77-87.

34. Murrell, W., et al., *Multipotent stem cells from adult olfactory mucosa.* Dev Dyn, 2005. 233(2): p. 496-515.

35. Suzuki, J., et al., *Neural crest-derived horizontal basal cells as tissue stem cells in the adult olfactory epithelium.* Neurosci Res, 2013. 75(2): p. 112-20.

7. Figures

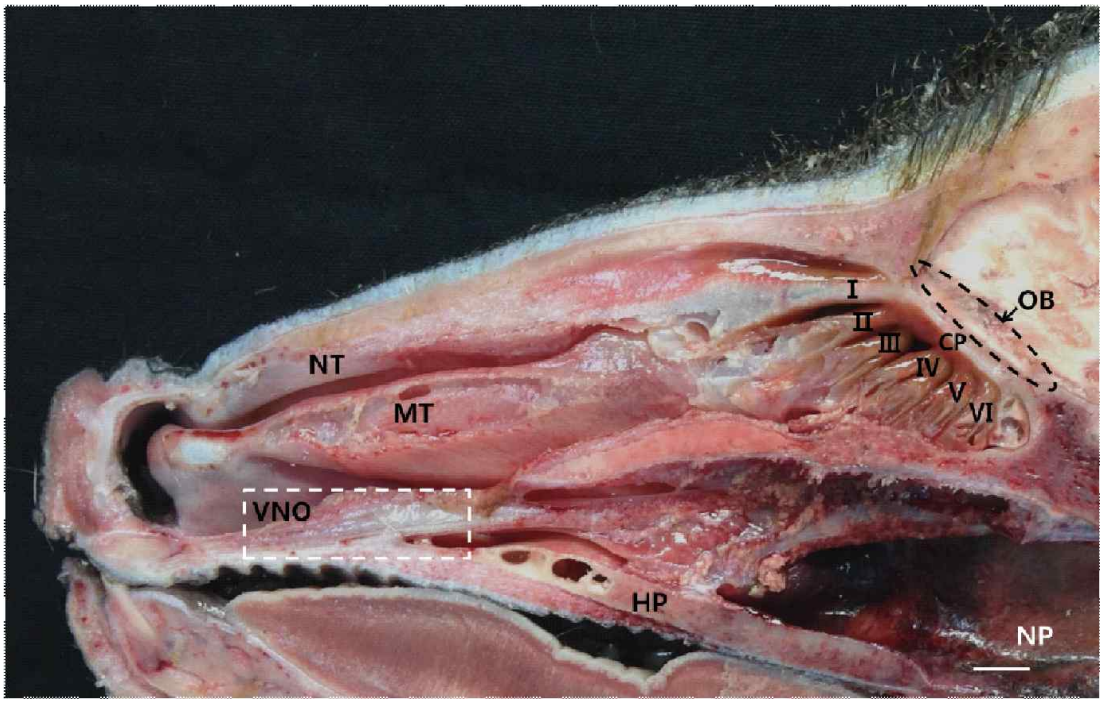

Figure 1. Gross anatomy of the rostral part of the head of the adult Korean roe deer (*Capreolus pygargus*) on the right lateral side. The ethmoturbinates (Ⅰ-Ⅵ), located in the caudal portion of the nasal cavity, are covered by the olfactory mucosa. CP, cribriform plate; HP, hard plate; MT, maxilloturbinate; NP, nasopharynx; NT, nasoturbinate; OB, olfactory bulb; VNO, vomeronasal organ. Scale bar represents 1cm.

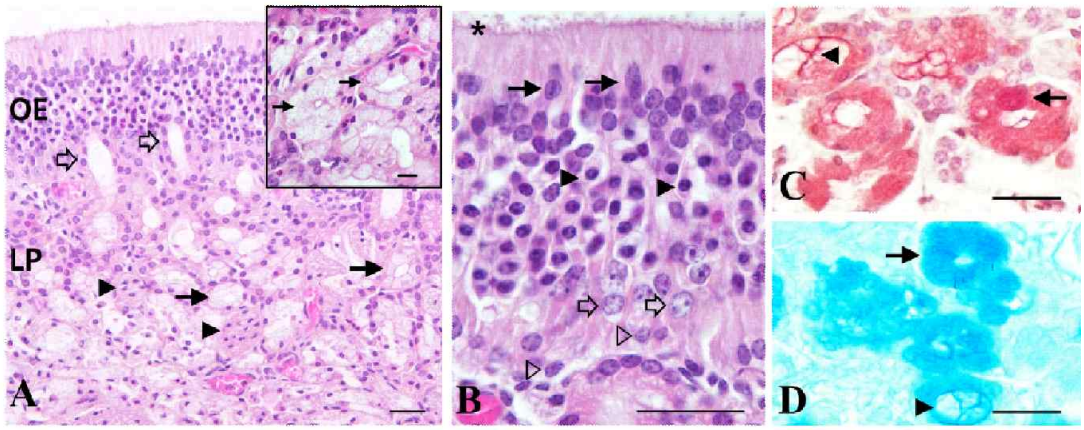

Figure 2. Histological characteristics of the olfactory mucosa of Korean roe deer.

(A) Coronal section of the olfactory mucosa stained with HE. The olfactory mucosa comprises the olfactory epithelium (OE) and the lamina propria (LP). In the lamina propria, pyramidal shaped acinar cells (arrows) of the Bowman's glands, the ducts of the Bowman's glands intervening in the olfactory epithelium (hollow arrows) and the olfactory nerve bundles (arrowheads). Inset: higher magnification of acini of Bowman's glands.

(B) Higher magnification of the olfactory epithelium. The asterisk indicate the free border. The olfactory supporting cells have oval shaped nuclei (arrows). Below the olfactory supporting cells, the olfactory receptor cells were arranged (arrowheads). The globose basal cells (hollow arrows) and the horizontal basal cells (hollow arrowheads) were positioned above the basal lamina.

(C) Periodic acid-Schiff reaction in the olfactory mucosa. PAS reactivity was seen in the secretory materials in the ducts (arrowhead) and acini (arrow) of Bowman's glands.

(D) Alcian blue (pH 2.5) staining in the olfactory mucosa. Alcian blue reaction was seen in the secretory materials (arrowhead) and the acinar cells (arrow) of Bowman's glands.

Scale bars in (A) – (D) represent 25 μm. Scale bar in inset in (A) present 10μm.

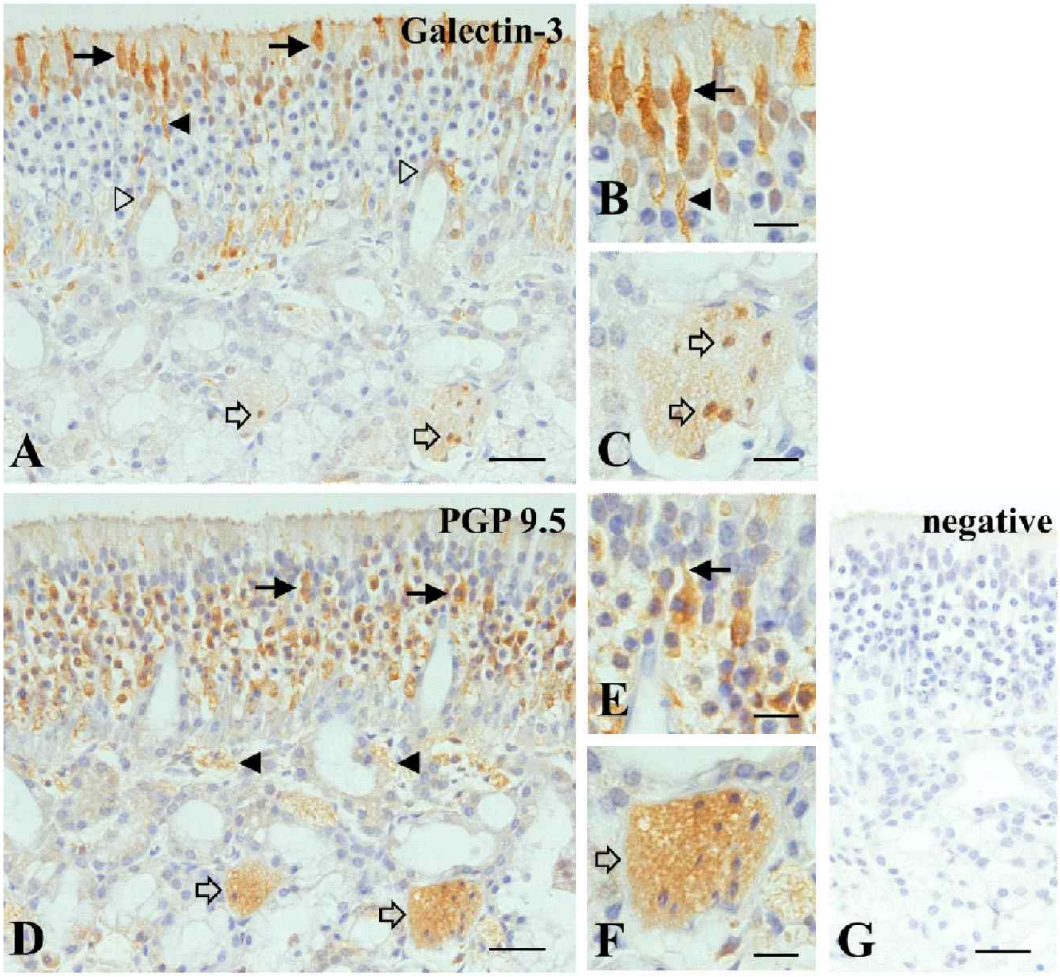

Figure 3. Immunohistochemical staining of galectin-3 and PGP 9.5 in the olfactory mucosa.

(A) In the olfactory epithelium, galectin-3 is immunostained in the olfactory supporting cells (arrows). Galectin-3 positive cells extend thin processes (arrowhead) to the basal lamina and free border. In the lamina propria, Schwann cells are positive for galectin-3 staining (hollow arrows).

(B) Higher magnification of olfactory epithelium. The olfactory supporting cells show galectin-3 reactivity at the non-nuclear zone (arrow) and thin process (arrowhead).

(C) Higher magnification of nerve bundle. Schwann cells in the olfactory nerve bundles are stained by galectin-3 (hollow arrows).

(D) PGP 9.5 was detected in lower two-thirds of the olfactory epithelium (arrows). The PGP 9.5-positive axons (arrowheads) were found in lamina propria adjacent to the basal lamina (arrowheads).

(E) Higher magnification of olfactory epithelium immunostained with PGP9.5. The dendrites of the olfactory receptor cells (arrow) are seen on the apical side of the nuclei of the olfactory receptor cells.

(F) Higher magnification of nerve bundle in the lamina propria immunostained with PGP9.5. All of the nerve bundles were intensely immunostained with PGP 9.5 (hollow arrow) in the lamina propria.

(G) Negative control.

Scale bars in (A), (B) and (G) represent 25 μm. Scale bars in (B), (C), (E) and (F) represent 10μm.

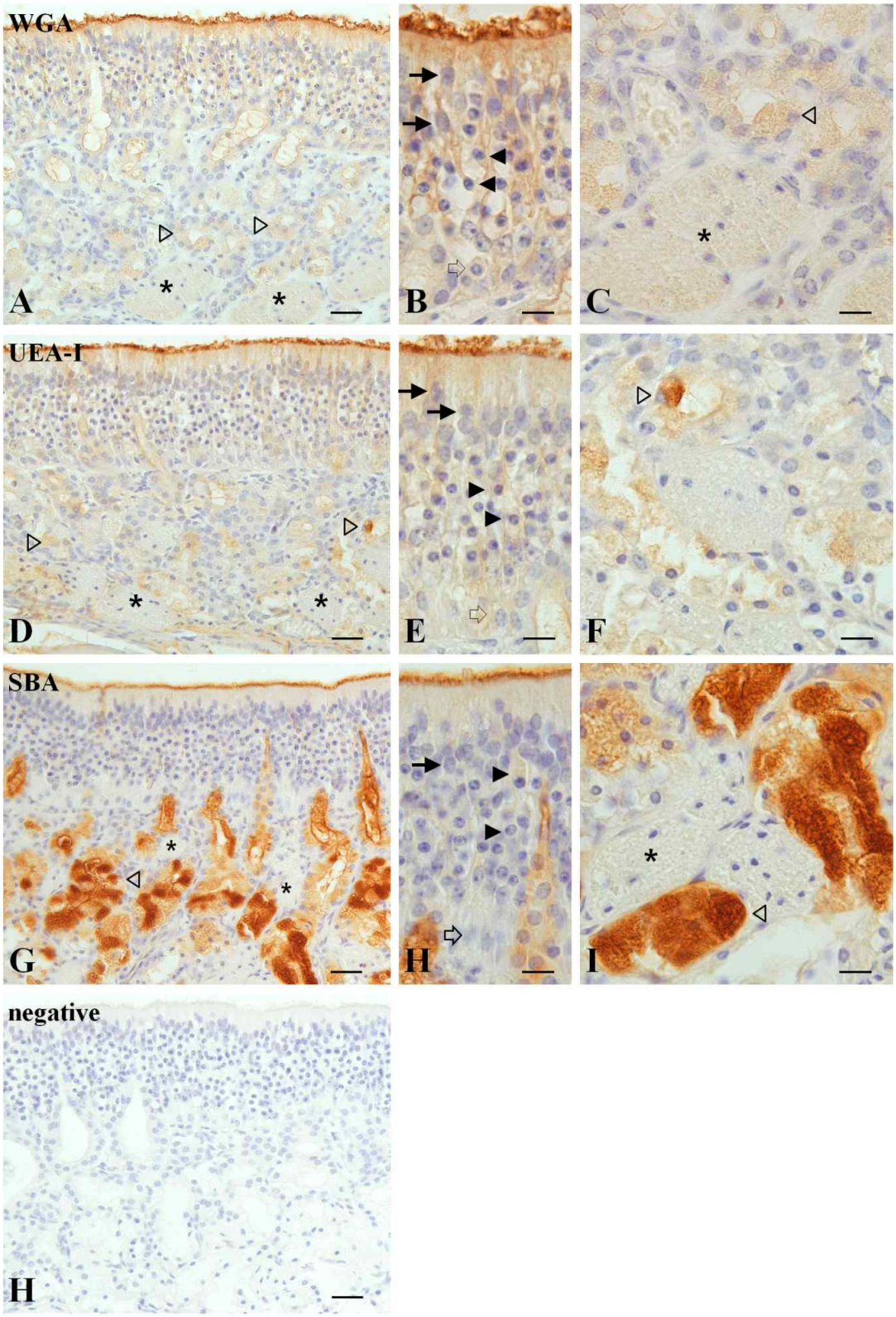
WGA
A
B
C
UEA-I
D
E
F
SBA
G
H
I
negative
H

Figure 4. Lectin histochemistry of WGA (A-C), UEA-I (D-F) and SBA (G-I) in the olfactory mucosa of Korean roe deer. (J) is negative control. A-I: Arrow, the olfactory supporting cell; arrowhead, the olfactory receptor cell; asterisk, the olfactory nerve bundle; hollow arrow, the olfactory basal cell; hollow arrowheads, the acinus of the Bowman's gland. Counterstaining with hematoxylin. Scale bars in (A), (D) and (G) represent 25 μm. Scale bars in (B), (C), (E), (F), (H) and (I) represent 10μm.

8. Table

Table 1. A comparison of lectin binding patterns in the olfactory mucosa with those in the vomeronasal organ of the same Korean roe deer [22]

Lectin	WGA[a]		UEA-I[b]		SBA[c]	
Structure	OM	VNO[d]	OM	VNO[d]	OM	VNO[d]
Free border	+	+	+	+	+	+
Supporting cell	+	-	+	+	-	-
Receptor cell	+	+	+	-	+	+
Basal cell	+	-	-	-	-	-
Nerve bundle	+	+	-	-	-	+
Glands	+	-	+	-	+	-

Abbreviations: OM, olfactory mucosa; VNO, vomeronasal organ; WGA, *Triticum vulgaris* wheat germ agglutinin; UEA-I, *Ulex europaeus* agglutinin I; SBA, soybean agglutinin.

[a-c.] WGA[a], UEA-I[b] and SBA[c] are lectins belonging to N-acetylglucosamine, fucose and N-acetylgalactosamine groups, respectively.

[d.] The lectin reactivity of the VNO was cited from Figure 2 and Table 2 of a previous report [22].

-, negative; +, positive; ND, not determined

초록

한국노루(Capreolus pygargus) 후각점막에서 조직학적 및 렉틴조직화학적 연구

(지도교수 : 신태균)

박 창 남

제주대학교 일반대학원 수의학과

한국노루(*Capreolus pygargus*) 후각점막의 형태학적 특징을 6마리의 수컷 노루에서 채취한 벌집선반을 이용하여 조직학적으로 연구하였다. 벌집선반을 파라핀에 포매하여 후각점막 특징에 대해 조직화학적으로 평가하였다. 렉틴조직화학법은 후각점막의 탄수화물 결합 특이성을 조사하기 위해 진행하였다. *Triticum vulgaris* wheat germagglutinin (WGA), *Ulex europaeus* agglutinin I (UEA-I), soybean agglutinin (SBA)을 포함한 렉틴은 각각 N-acetylglucosamine, fucose, N-acetylgalactosamine 탄수화물기를 확인하기 위해 사용하였다. 후각점막은 주로 비강의 뒤천장을 덮고 있으며, 조직학적으로 후각상피와 고유점막판으로 구성되었다. 후각상피는 protein gene product

9.5 양성 후각수용체세포, galectin-3 양성 지지세포, 바닥세포로 구성되었다. 고유점막판의 Bowman 샘은 periodic acid Schiff reagent와 alcian blue (pH 2.5)에 모두 양성을 띄었다. 두 종류의 렉틴인 WGA와 SBA는 후각상피의 바닥세포를 제외한 자유모서리, 수용체세포, 지지세포, Bowman 샘에 양성을 보였으나, UEA-I은 자유모서리, 지지세포, Bowman 샘에 양성을 보였다. 이러한 결과는 노루 후각점막 세포형에 따른 탄수화물 말단기 다양성을 제시하고 있다. 이 연구는 한국노루의 후각점막에서 탄수화물 말단기를 확인한 최초의 형태학적 연구이다.

주요어 : 렉틴, 후각상피, 후각점막, 노루

감사의 글

학부 때 실험실에 들어와 대학원을 통해 실험실생활을 이어간 것이 엊그제 같은데, 돌이켜 생각해보니 벌써 시간이 흘러 졸업이 얼마 남지 않았음을 새삼 느끼고 있습니다. 부족하지만 지면을 빌어 실험실에 몸담고 있었던 시간동안 저에게 가르침과 격려를 보내주셨던 분들에게 감사한 말씀을 드리려 합니다.

우선 2010년 겨울, 철없고 모자랐던 학부생을 실험실원으로 받아주신 대부이시자 스승이신 신태균 교수님께 감사드립니다. 대학에 입학하고 교수님을 뵐 수 있었던 건 저에게 큰 행운이자 축복이라 생각합니다. 그리고 학위 논문이 완성되기까지 많은 조언과 가르침을 주신 지영흔 교수님, 안미정 박사님께 감사의 말씀 올립니다.

수의학을 통해 생명의 소중함과 학문의 정수를 가르쳐주신 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, 박현정 교수님, 한창훈 교수님, 서종필 교수님께 감사의 말씀 올립니다.

실험실 생활동안 아낌없는 격려를 보내주시고 물심양면으로 도와주신 이용덕 박사님, 김승준 교수님, 강재윤 선배님, 김황룡 선배님, 김철 선배님, 진재광 박사님, 문창종 교수님, 강종철 선배님, 허승담 선배님, 이기현 선배님, 이광협 선배님, 김희철 박사님, 정경숙 선배님, 정찬우 선배님, 김정태 박사님, 정진우 선배님께 진심으로 감사드립니다. 그리고 실험실에서 희노애락을 함께한 석원형, 준우형, 원호형, 성영형, 원준형, 한슬이누나, 연지, 철호, 소희, 윤형, 효진, 지환, 민호, 윤호에게 고마운 마음을 전합니다.

끝으로 지금의 제가 설 수 있도록 바탕이 되어주신 부모님, 힘든 시간을 같이 보내준 동생 용남, 앞으로 평생을 함께 할 동반자 민진이에게 감사의 말을 남깁니다.