A Thesis for the degree of Doctor of Philosophy

Glycoconjugate-specific Developmental Changes in the Horse Vomeronasal Organ

Department of Veterinary Medicine

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

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List of Abbreviations

BSL-I	*Bandeiraea simplicifolia lectin-I*
BSL-II	*Bandeiraea simplicifolia lectin-II*
ConA	*Concanavalin A*
DBA	*Dolichos biflorus agglutinin*
DSL	*Datura stramonium lectin*
ECL	*Erythrina cristagalli lectin*
LCA	*Lens culinaris agglutinin*
OMP	Olfactory marker protein
PBS	Phosphate-buffered saline
PGP9.5	Protein gene product 9.5
PHA-E	*Phaseolus vulgaris erythroagglutinin*
PHA-L	*Phaseolus vulgaris leucoagglutinin*
PNA	*Arachis hypogaea (peanut) agglutinin*
PSA	*Pisum sativum agglutinin*
RCA_{120}	*Ricinus communis agglutinin-1*
SBA	*Glycine max (soybean) agglutinin*
SJA	*Sophora japonica agglutinin*
STL	*Solanum tuberosum lectin*
s-WGA	*Succinylated wheat germ agglutinin*
UEA-I	*Ulex europaeus agglutinin-I*
VNO	Vomeronasal organ
VNSE	Vomeronasal non-sensory epithelium
VSE	Vomeronasal sensory epithelium
VVA	*Vicia villosa agglutinin*
WGA	*Wheat germ agglutinin*

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Glycoconjugate-specific Developmental Changes in the Horse Vomeronasal Organ

1. Abstract

Vomeronasal organ (VNO) is a tubular pheromone sensing organ in which the lumen is covered with sensory and non-sensory epithelia. This study used immunohistochemistry and lectin histochemistry techniques to evaluate developmental changes, specifically of the glycoconjugate profile, in the horse VNO epithelium. Immunostaining analysis revealed protein gene product 9.5 (PGP9.5) expression in some vomeronasal non-sensory epithelium (VNSE) cells and in the vomeronasal receptor cells of the vomeronasal sensory epithelium (VSE) in fetuses, young foals, and adult horses. Olfactory marker protein (OMP) expression was exclusively localized in receptor cells of the VSE in fetuses, young foals, and adult horses and absent in VNSE. To identify the glycoconjugate type, lectin histochemistry was performed using 21 lectins. Semi-quantitative analysis revealed that the intensities of glycoconjugates labeled with WGA, DSL, LEL, and RCA_{120} were significantly higher in adult horse VSE than those in foal VSE, whereas the intensities of glycoconjugates labeled with LCA and PSA were significantly lower in adult horse VSE. The intensities of glycoconjugates labeled with s-WGA, WGA, BSL-II, DSL, LEL, STL, ConA, LCA, PSA, DBA, SBA, SJA, RCA_{120}, jacalin, and ECL were significantly higher in adult horse VNSE than those in foal VNSE, whereas the intensity of glycoconjugates labeled with UEA-I was lower in adult horse VNSE. Histochemical analysis of each lectin revealed that various glycoconjugates in the VSE were present in the receptor, supporting, and basal cells of foal and adult horses. A similar pattern of lectin histochemistry was also observed in the VNSE of foal and adult horses.

In conclusion, these results suggest that there are increase in the level of N-acetylglucosamine (labeled by WGA, DSL, LEL) and galactose (labeled by RCA_{120}) in horse VSE during postnatal development, implying that they may influence the function of VNO in adult horses.

Key words: Development, glycoconjugate, horse, lectin, vomeronasal organ

2. Introduction

Flehmen response appears to be associated with the functioning of the vomeronasal organ. A response in male cattle, goats and horses commonly stimulated by the smell of urine or genital area of female, even by other males or male's own urine. It is also appeared by females, especially with the birth fluids of newborn animals (Hart, 1985). Thus, age related study for flehmen response in semi-wild cattle herd has also found that flehmen occurred in calves for both gender after 1 week old and became frequent after 4 month of age (Reinhardt, 1983). The analyses of VNOs in pheromone-recognizing mammals (Johnston, 1998; Silva and Antunes, 2017; Choi and Yoon, 2021) have suggested that the flehmen response in horses is closely associated with pheromone perception in the VNO (Ma, 2012; Silva and Antunes, 2017).

In horses, the vomeronasal organ is a sensory tubular organ, located at the base of the nasal septum, involved in pheromone detection (Halpern and Martinez-Marcos, 2003). The VNO comprises sensory epithelium in medial and non-sensory epithelium in lateral encased in cartilage. VNO receptor cell axons terminate in the caudal part of the accessory olfactory bulbs (Halpern et al., 1998; Halpern and Martinez-Marcos, 2003). The receptor cells in these epithelia contain both the mature neuronal marker olfactory marker protein and immature/mature neuronal marker protein gene product 9.5 (Lee et al., 2016b).

Several glycoconjugates have recently been identified in the VSE of adult horses (Lee et al., 2016b), implying that they are involved in VSE integrity and recognition of odorant molecules by the olfactory mucosa (Takami et al., 1994; Trinh and Storm, 2004). Glycoconjugates involved in cell proliferation and differentiation detected via lectin histochemistry reveal their importance in biological processes such as embryonic development and signal transduction (Plendl and Sinowatz, 1998; Nimrichter et al., 2004). Therefore, there is a presumption that the glycoconjugate profile in the VNO dynamically changes during both fetal and postnatal development.

This needs to be examined further to better understand the pheromone detection capabilities of adult horses. Additionally, while there is adequate knowledge about morphological characteristics and glycoconjugate profiles of adult horses (Lee et al., 2016b), information on developmental changes in horse VNO (from fetal to adult stages) have not been semi-quantitatively analyzed.

This study was conducted to evaluate the VNO sensory receptor cells express of PGP9.5 and OMP in fetal horses and to investigate the changes in glycoconjugate intensities in both VSE and VNSE of horse VNO during postnatal development.

3. Materials and Methods

3.1. Animals and tissue preparation

Thoroughbred horse VNO samples were obtained from the local abattoir in Jeju, Republic of Korea. Tissue collection was performed at the Jeju National University Equine Teaching Hospital after the owners had consented to organ donation. The sample tissues belonged to two fetuses (4 and 7 months of gestation), five foals, (i.e., young horses aged < 1 year), and five adult horses (3~4 years old). The foals had been euthanized after severe accidents, between 2020 and 2021. While, the sex of the fetuses was unknown, all foals and three adult horses were male. The other two adult horses were gelded. Fetal age was calculated based on the crown-rump length. Based on the gross and histological findings in the nasal cavity, none of the sample animals had any underlying respiratory system related diseases.

Some adult horse samples from a previous study (Lee et al., 2016b) were reevaluated via a different immunohistochemical approach. For light microscopy analysis, VNO was removed from the skull immediately after death and fixed in 4% paraformaldehyde for 48 h. They were then dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin, and sectioned to a thickness of 5 μm. All experimental procedures were conducted in accordance with Jeju National University Guidelines for the Care and Use of Laboratory Animals (approval no. 2021-0059).

3.2. Immunohistochemistry

For histological analysis, paraffin-embedded sections were deparaffinized and stained with hematoxylin and eosin as described in previous studies (Lee et al., 2016b; Yang et al., 2021). Immunohistochemical techniques were used for the analysis of all the obtained samples, whereas lectin histochemical techniques were only used for the analysis of foals and adult horse samples. Immunohistochemistry was performed via the avidin–biotin complex method using the Vector ABC Peroxidase kit (Vector Laboratories, Newark, CA, USA) as previously described (Park et al., 2012a; Yang et al., 2021). Briefly, the deparaffinized sections were immersed in citrate buffer (0.01 M, pH 6.0) and heated for 3 min in a microwave (700 W). After the sections were cooled for 20 min, they were incubated with 0.3% aqueous hydrogen peroxide in methanol for 20 min to block endogenous peroxidase activity. Non-specific binding was blocked with blocking serum, and the sections were washed in phosphate-buffered saline (PBS, pH 7.4) for 1 h and incubated with primary antibodies, including mouse anti-OMP (1:200, sc-365818; Santa Cruz, Dallas, TX, USA) and mouse anti-PGP9.5 (1:1000, ab72911; Abcam, Cambridge, UK) for 1 h at room temperature. For negative control samples, primary antibody staining was omitted. The sections were washed three times in PBS, incubated for 45 min with biotinylated secondary antibodies (Vector Kits; Vector Laboratories, Newark, CA, USA), washed again in PBS, and incubated for 45 min with an avidin–biotin–peroxidase complex that had been prepared in accordance with the manufacturer's instructions. After the sections were washed in PBS, signals were developed using a diaminobenzidine substrate kit (Vector Laboratories, Newark, CA, USA), prepared in accordance with the manufacturer's instructions.

3.3. Lectin histochemistry

Lectin histochemistry was performed as described in previous studies (Park et al., 2012b; Park et al., 2013; Lee et al., 2016b; Yang et al., 2021) using a Vector Lectin Kit (Vector Laboratories, Newark, CA, USA; Table 1). Briefly, the sections were deparaffinized with xylene and rehydrated in a graded ethanol series. The sections were incubated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. After the sections were washed three times in PBS, they were incubated with 1% bovine serum albumin in PBS to block non-specific binding. Subsequently, each section was incubated overnight with lectin at 4 °C in a humidification chamber and then washed again three times with PBS. Signals were developed using a diaminobenzidine kit (Vector Laboratories, Newark, CA, USA), and sections were counterstained with hematoxylin for 10s before mounting. Negative controls were generated for lectin histochemistry by preincubating the lectins with appropriate inhibitors in Tris buffer for 1 h at room temperature, prior to staining with the lectins (Lee et al., 2016b).

Table 1. List of plant lectins

Lectins	Source	Concentration (mg/ml)	Inhibitor or eluting sugar*
N-acetylglucosamine-binding lectins			
s-WGA	*Succinylated-Wheat germ agglutinin*	1.0×10^{-2}	0.2M GlcNAc
WGA	*Wheat germ agglutinin*	1.0×10^{-2}	0.2M GlcNAc
BSL-II	*Bandeiraea simplicifolia lectin-II*	4.0×10^{-3}	0.2M GlcNAc
DSL	*Datura stramonium lectin*	4.0×10^{-3}	0.5M chitin hydrolysate
LEL	*Lycopersicon esculentum*	2.0×10^{-2}	0.5M chitin hydrolysate
STL	*Solanum tuberosum lectin*	1.0×10^{-2}	0.5M chitin hydrolysate
Mannose-binding lectins			
ConA	*Concanavalin A*	3.3×10^{-3}	0.2M MeαMan/0.2M MeαGlc
LCA	*Lens culinaris agglutinin*	4.0×10^{-3}	0.2M MeαMan/0.2M MeαGlc
PSA	*Pisum sativum agglutinin*	4.0×10^{-3}	0.2M MeαMan/0.2M MeαGlc
N-acetylgalactosamine-binding lectins			
VVA	*Vicia villosa agglutinin*	4.0×10^{-3}	0.2M GalNAc
DBA	*Dolichos biflorus agglutinin*	1.0×10^{-2}	0.2M GalNAc
SBA	*Glycine max (soybean) agglutinin*	1.0×10^{-2}	0.2M GalNAc
SJA	*Sophora japonica agglutinin*	2.0×10^{-2}	0.2M GalNAc
BSL-I	*Bandeiraea simplicifolia lectin-I*	4.0×10^{-3}	0.2M GalNAc
Galactose-binding lectins			
RCA_{120}	*Ricinus communis agglutinin-1*	2.0×10^{-3}	0.2M lactose
Jacalin	*Artocarpus integrifolia*	5.0×10^{-4}	0.2M melibiose
PNA	*Arachis hypogaea (peanut) agglutinin*	4.0×10^{-3}	0.2M βGal
ECL	*Erythrina Cristagalli Lectin*	2.0×10^{-2}	0.2M lactose
Complex type N-glycan (Complex oligosaccharide)-binding lectins			
PHA-E	*Phaseolus vulgaris Erythroagglutinin*	5.0×10^{-3}	0.1M acetic acid
PHA-L	*Phaseolus vulgaris Leucoagglutinin*	2.5×10^{-3}	0.1M acetic acid
Fucose-binding lectins			
UEA-I	*Ulex europaeus agglutinin-I*	2.0×10^{-2}	0.1M L-fucose

Abbreviations: Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine. MeαMan, α-Methylmannoside; MeαGlc, α-Methylglucoside

3.4. Semi-quantitative analysis of lectin and/or antibody-positive area

Before semi-quantitative analysis, lectin-positive sections (n = 5 per group) were photographed using a digital camera (ProgRes C5 or DP72; Olympus Corp., Tokyo, Japan) attached to a light microscope (BX53/U-LH 100HG; Olympus Corp., Tokyo, Japan). Each section was photographed at three points. Semi-quantitative analysis of lectin-positive areas was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) as described in a previous study (Park et al., 2020). Finally, the ratio of the lectin-positive area to the total area [(positive area/total area) × 100] was calculated.

4. Results

4.1. Histological analysis

At low magnification (Fig. 1), VNOs were classified as either VSE or VNSE, and the VNO was encapsulated by the vomeronasal cartilage in each tissue sample from different age groups. In some cases, the VSE comprised free borders, receptor cells, supporting cells, and basal cells; in other cases, it comprised free borders, ciliated cells, and basal cells. VNSE had no receptor cells. The vomeronasal glands were located under the blood vessel-containing areas of the VNSE. Both the VSE and VNSE were more developed in adult horses than in fetuses or foals. The cytoarchitectural features of the adult VNO were consistent with previous findings (Lee et al., 2016b).

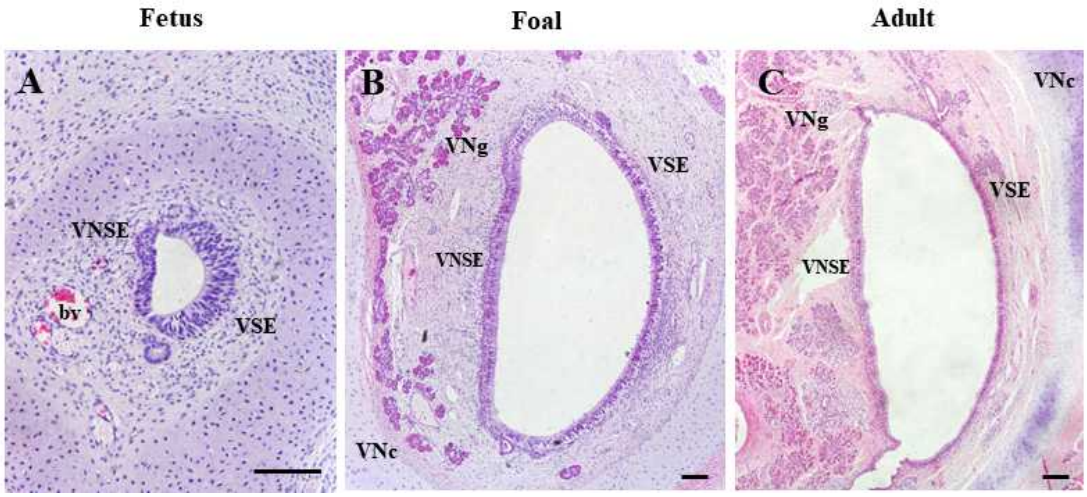

Figure 1. Histochemical analysis of the equine vomeronasal organ during development in fetuses (A), foals (B), and adult horses (C). The VNO was encapsulated by vomeronasal cartilage (VNc) and composed of vomeronasal sensory epithelium (VSE), vomeronasal non-sensory epithelium (VNSE), vomeronasal glands (VNg), and blood vessels (bv) in adult horses (C). The VNg, VSE, and VNSE were not fully differentiated in fetal horses, but were similar between foals and adult horses. Hematoxylin and eosin staining. Scale bars = 100 μm (A and B), 200 μm (C).

4.2. The thickness of horse VNO epithelium in the different age group

The average thickness of the VNSE increased during the early development (Fig. 2C). The VNSE was thicker in foal (49.79 ± 1.22 μm) than in fetus (28.28 ± 2.73 μm, Fig. 2D). However, the average thickness of the VSE did not significantly change from fetus to foal and foal to adult horses (Fig. 2A). The thickness of VSE of the fetus was 56.74 ± 1.55 μm, that of foal was 55.62 ± 1.59 μm, and that of adult horse was 56.24 ± 3.3 μm (Fig. 2B).

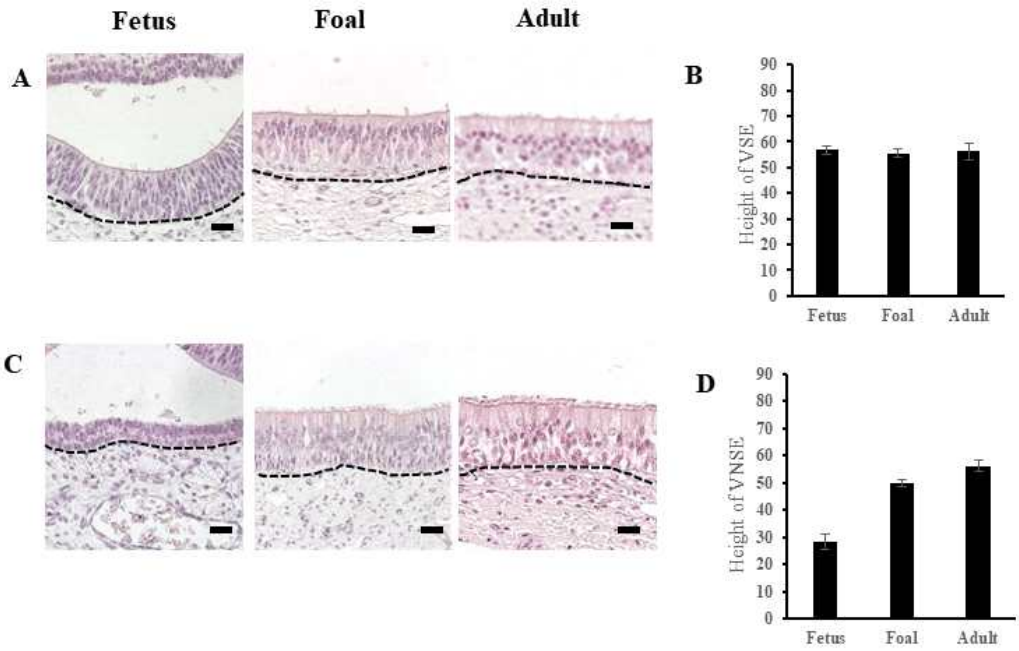

Figure 2. The height of the VSE (A) and quantitative assessment of the mean VSE heights (B) in fetus, foal and adult horse in paraffin-embedded tissue stained with H&E. The height of the VNSE (C) and quantitative assessment of the mean VNSE heights (D) in fetus, foal and adult horse in paraffin-embedded tissue stained with H&E. The dashed line is the basement membrane. The average thickness of VNSE in foal is thicker than that of fetus (D). Scale bars = 20 μm.

4.3. Immunohistochemical localization of PGP9.5 and OMP

To investigate the development of vomeronasal receptor cells, we performed immunohistochemical staining of PGP9.5 and OMP, which are markers of immature and mature vomeronasal receptor cells, respectively (Lee et al., 2016b; Yang et al., 2021). In the VSE, intense PGP9.5 immunoreactivity was detected in the free borders and receptor cells of the VNO tissue (Fig. 3A, B and C) from fetuses (Fig. 3A, arrows), foals (Fig. 3B, arrows), and adult horses (Fig. 3C, arrows). PGP9.5 immunoreactivity was also occasionally localized in ciliated and basal cells of the VNSE (Fig. 3D, E and F). However, it was not localized in these cells in the vomeronasal glands. OMP was exclusively localized in receptor cells in the VSE (Fig. 4A, B and C). OMP was not detected in the VNSE (Fig. 4D, E and F). These results suggest that OMP signaling is activated in the fetal VNO of horses.

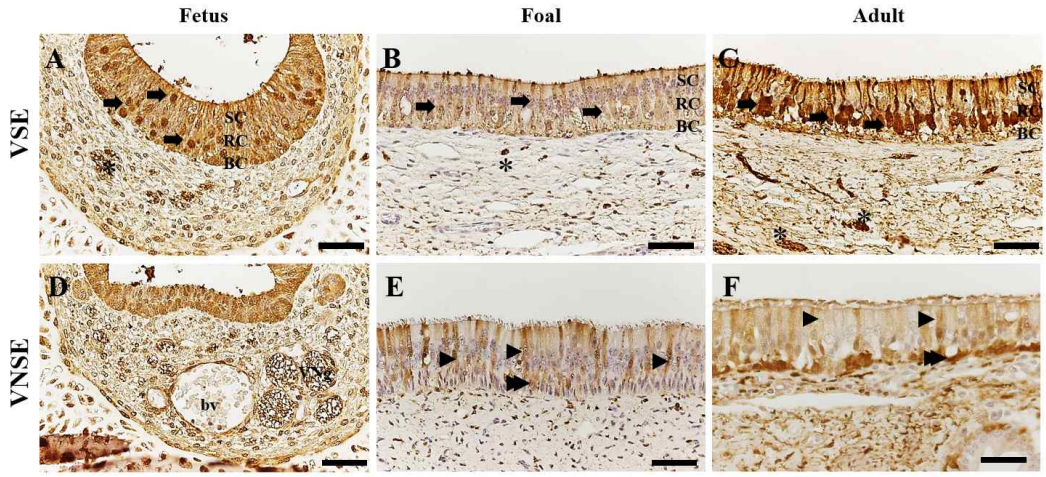

Figure 3. Immunohistochemical localization of protein gene product 9.5 (PGP9.5) in the VSE (A, B and C) and VNSE (D, E and F) during development. Fetus (A and D), foal (B and E), and adult horse (C and F). In the VSE, PGP9.5 immunoreactivity was strongly detected in immature receptor cells in fetuses, foals, and adult horses (arrows in B and C), whereas PGP9.5-positive receptor cells were detected in all layers in fetuses (A). PGP9.5 immunoreactivity was detected in nerve bundles (asterisks in A –C) in fetuses, foals, and adult horses. In the VNSE, PGP9.5 immunoreactivity was detected in some ciliated cells (arrowheads) and basal cells (double arrowheads) in fetuses, foals, and adult horses (D, E and F). BC, basal cells; RC, receptor cells; SC, supporting cells. Scale bars = 40 μm.

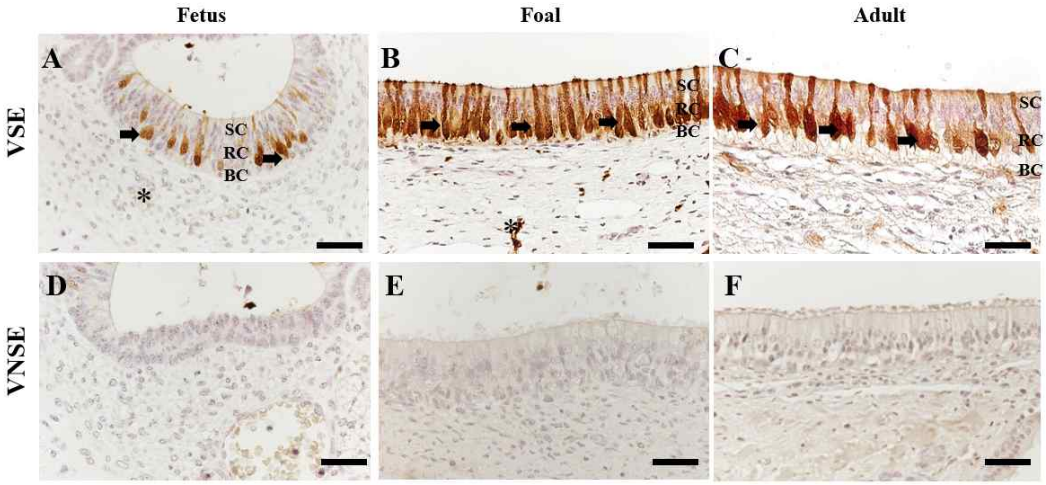

Figure 4. Immunohistochemical localization of olfactory marker protein (OMP) in the VSE (A, B and C) and VNSE (D, E and F) during development. Fetus (A and D), foal (B and E), and adult horse (C and F). In the VSE, OMP immunoreactivity was strongly detected in mature receptor cells in all age groups (arrows in A, B and C). OMP immunoreactivity was not detected in the VNSE in any age group (D, E and F). Asterisks indicate OMP-positive nerve bundles. BC, basal cells; RC, receptor cells; SC, supporting cells. Scale bars = 40 μm.

4.4. Lectin histochemistry analysis of the VSE in foals and adult horses

4.4.1. N-acetylglucosamine-binding lectins

Among N-acetylglucosamine-binding lectins, the reactivity of WGA, DSL, and LEL were significantly greater in adult horses than in foals (Fig. 5N), while the reactivity of s-WGA, BSL-II, and STL did not significantly differ between the groups. Histological analysis of lectin reactivity revealed that s-WGA was localized in free borders, receptor cells, supporting cells, and basal cells in the VSE of foals (Fig. 5A) and adult horses (Fig. 5B, Table 2); similar reactivity patterns were observed for WGA, DSL, LEL, and STL. BSL-II reactivity was detected in the free borders, receptor cells, and basal cells of foals and adult horses (Fig. 5F and G, arrows); however, this was not detected in the supporting cells.

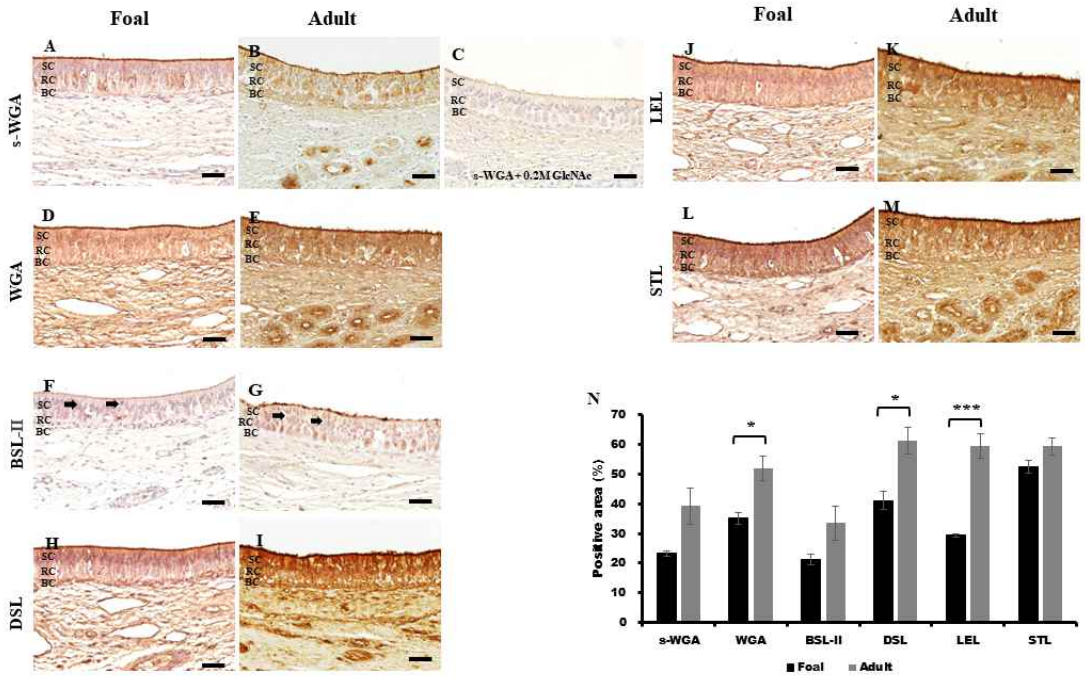

Figure 5. N-acetylglucosamine-binding lectin histochemistry (A-M) and semi-quantitative analysis of the positive area in the VSE of foals and adult horses (N). The entire structures in VSE including free border, receptor cells, supporting cells and basal cells were stained with varying intensity with s-WGA in foal (A) and adult horse (B), WGA in foal (D) and adult horse (E), BSL-II in foals (F) and adult horse (G), DSL in foal (H) and adult horse (I), LEL in foal (J) and adult horse (K), STL in foal (L) and adult horse (M). The reactivities of WGA, DSL, and LEL were significantly greater in adult horses than foals (N). *$p < 0.05$, ***$p < 0.001$, foal vs. adult horse. (C) Negative control. BC, basal cells; RC, receptor cells; SC, supporting cells. Scale bars = 40 μm.

4.4.2. Mannose-binding lectins

The reactivity of LCA and PSA were significantly greater in foals than in adult horses, while ConA reactivity did not significantly differ between the groups (Fig. 6H, Table 2). ConA reactivity was detected in all VSE structures of foals and adult horses (Fig. 6A and B). LCA reactivity was detected in all VSE structures in the foals (Fig. 6C). In adult horses, LCA reactivity was detected in free borders, basal cells, and some receptor cells (Fig. 6D, arrows), although it was not detected in supporting cells. A similar reactivity pattern was observed in PSA (Fig. 6E and F, arrows).

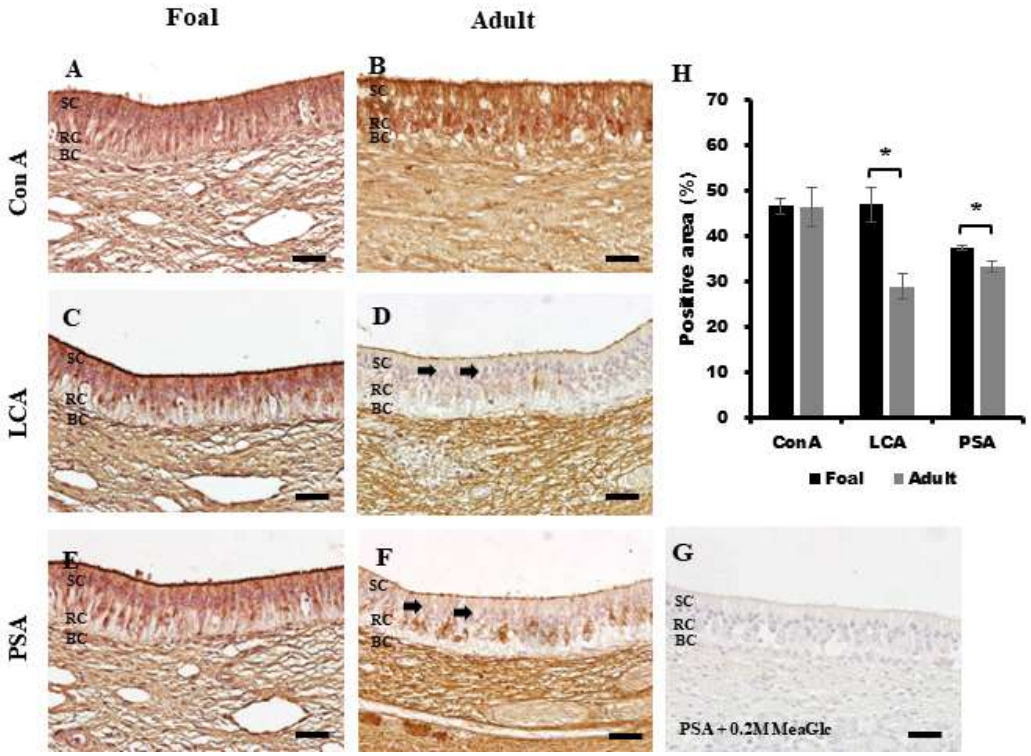

Figure 6. Mannose-binding lectin histochemistry (A-G) and semi-quantitative analysis of the positive area in the VSE of foals and adult horses (H). Lectin histochemistry for ConA, LCA, and PSA in the VSE of foals (A, C, and E) and adult horses (B, D, and F). The receptor cells of VNO in foals and adult horses were labeled with varying intensity by ConA, LCA and PSA but LCA and PSA did not correspond to the supporting cells of adult horse (arrows). The reactivities of LCA and PSA were significantly weaker in adult horses than foals (H). *$p < 0.05$, foal vs. adult horse. BC, basal cells; RC, receptor cells; SC, supporting cells. (G) Negative control. Scale bars = 40 μm.

4.4.3. N-acetylgalactosamine-binding lectins

The reactivity of N-acetylgalactosamine-binding lectins did not significantly differ between foals and adult horses (Fig. 7L). VVA reactivity was detected in the free borders and receptor cells in both foals and adult horses (Table 2). VVA reactivity was also detected in the supporting cells of adult horses (Fig. 7B) but not in the foals (Fig. 7A). VVA reactivity was not detected in the basal cells of foals or adult horses. DBA reactivity was detected in receptor cells of both foals and adult horses (Fig 7C and D); however, it was not detected in the supporting or basal cells.

SBA reactivity was not detected in the receptor, supporting cells, and basal cells in the VSE of foals (Fig. 7E). In adult horses, SBA reactivity was detected in free borders, receptor cells, basal cells, and some supporting cells (Fig. 7F, Table 2). SJA reactivity was not detected in the receptor or supporting cells in the VSE of foals or adult horses (Fig. 7G and H, Table 2). In contrast, BSL-I reactivity was detected in receptor cells in the VSE of both foals and adult horses (Fig. 7I and J, Table 2).

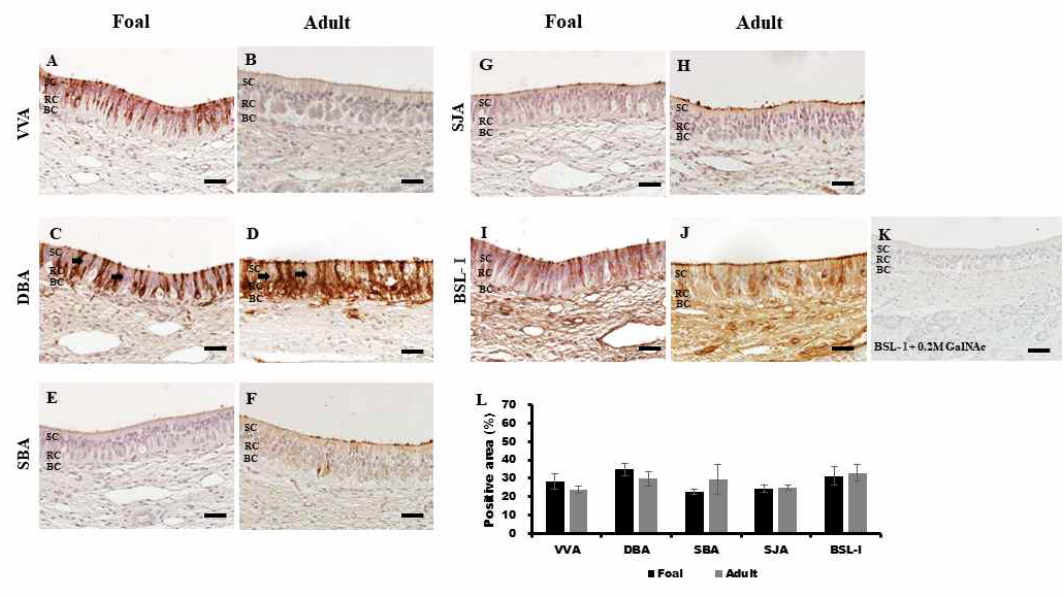

Figure 7. N-acetylgalactosamine-binding lectin histochemistry (A-K) and semi-quantitative analysis of the positive area in the VSE of foals and adult horses (L). All lectins including VVA, DBA, SBA, SJA and BSL-I in N-acetylgalactosamine-binding group stained the free border of VNO in foals and adult horses with varying intensity. DBA did not correspond to supporting cells in VNO both of foals and adult horses (C and D, arrows). BC, basal cells; RC, receptor cells; SC, supporting cells. (K) Negative control. Scale bars = 40 μm.

4.4.4. Galactose-binding lectins

Among the galactose-binding lectins (Fig. 8J), RCA_{120} reactivity was significantly greater in adult horses (Fig. 8B) than in foals (Fig. 8A). RCA_{120} reactivity was detected in all VSE structures of both foals and adult horses (Fig. 8A and B). Jacalin reactivity was not detected in the receptor, supporting cells, or basal cells in the VSE of foals (Fig. 8D). However, jacalin reactivity was detected in all VSE structures in adult horses (Fig. 8E).

PNA reactivity was detected in the free borders in the VSE of both foals (Fig. 8F) and adult horses (Fig. 8G); it was also detected in the receptor, supporting, and basal cells in adult horses (Table 2). ECL reactivity was detected in all VSE structures of both foals and adult horses (Table 2). This reactivity, in free borders and supporting cells, was stronger in foals than in adult horses (Fig. 8H and I, Table 2).

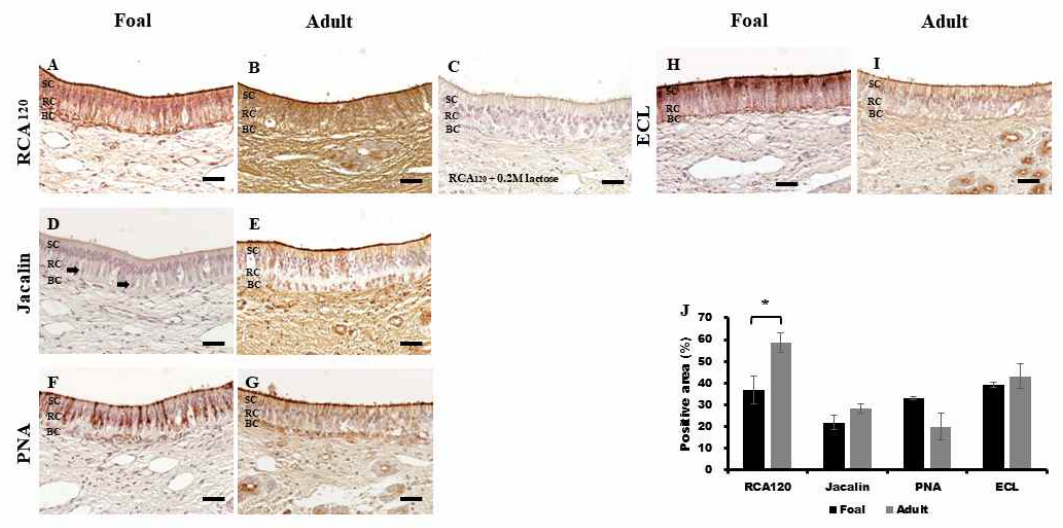

Figure 8. Galactose-binding lectin histochemistry (A-I) and semi-quantitative analysis of the positive area in the VSE of foals and adult horses (J). Free border and receptor cells in VNO of foals (A, D, F and H) and adult horses (B, E, G and I) were stained with Galactose-binding group lectins including RCA_{120}, jacalin, PNA and ECL with varying intensity while the receptor cells in VNO of foal did not correspond to Jacalin (D, arrows). The reactivity of RCA_{120} was significantly greater in adult horses than foals (J). *$p < 0.05$, foal vs. adult horse. BC, basal cells; RC, receptor cells; SC, supporting cells. (C) Negative control. Scale bars = 40 μm.

4.4.5. Complex type N-glycan (Complex oligosaccharide)-binding lectins

PHA-E reactivity was similar between foals and adult horses (Fig. 9F); no PHA-E reactivity was detected in the receptor cells in the VSE of foals or adult horses (Fig. 9A and B, Table 2). PHA-L reactivity was not detected in the receptor cells in the VSE of foals or adult horses (Fig. 9C and D); some PHA-L reactivity was detected in supporting cells in the VSE in foals (Fig. 9C) but not in adult horses (Fig. 9D, Table 2).

4.4.6. Fucose-binding lectin

UEA-I reactivity was detected in the free borders of the VSE of both foals and adult horses (Fig. 9G and H); it was also detected in the receptor, supporting, and basal cells in adult horses (Table 2). Fucose-binding lectin reactivity did not differ significantly between foals and adult horses (Fig. 9J).

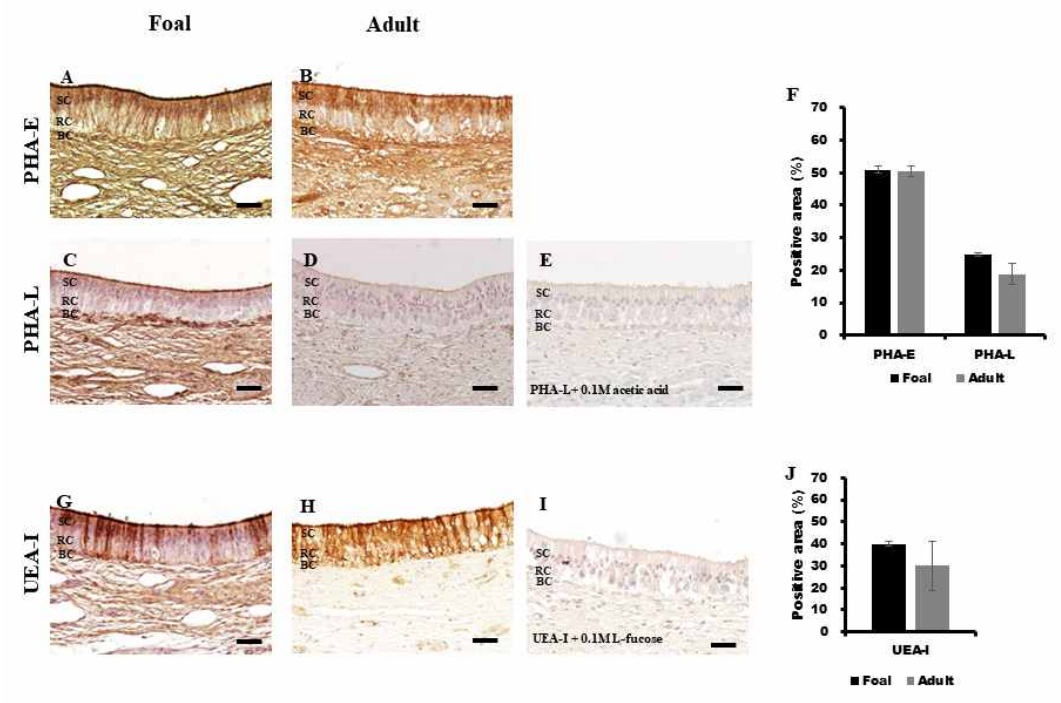

Figure 9. Complex type N-glycan-binding lectin histochemistry (A-E) and semi-quantitative analysis of the positive area in the VSE of foals and adult horses (F) and Fucose-binding lectin histochemistry (G-I) and semi-quantitative analysis of the positive area in the VSE of foals and adult horses (J). PHA-E and PHA-L stained free border and basal cells with varying intensity while those lectins did not labeled receptor cells in VSE of foals (A and C) and adult horses (B and D). UEA-I labeled free border in VSE of foals (G) and adult horses (H). (E) and (I) Negative control. The reactivities did not significantly differ between foals and adult horses ($p > 0.05$, F and J). BC, basal cells; RC, receptor cells; SC, supporting cells. Scale bars = 40 μm.

Table 2. Lectin binding patterns in the VSE of horse

Lectins	Stage	Structures			
		Free border	Receptor cells	Supporting cells	Basal cells
N-acetylglucosamine-binding lectin					
s-WGA	Foal	+	+	±	+
	Adult	+	+	+	+
WGA	Foal	+	+	+	+
	Adult	+	++	++	+
BSL-II	Foal	+	±	-	±
	Adult	++	±	-	±
DSL	Foal	+	+	+	+
	Adult	+	++	++	+
LEL	Foal	+	+	+	+
	Adult	+	++	++	+
STL	Foal	+	+	+	+
	Adult	+	+	++	+
Mannose-binding lectins					
ConA	Foal	+	+	+	+
	Adult	+	+	+	+
LCA	Foal	++	++	+	+
	Adult	+	±	-	+
PSA	Foal	++	+	+	+
	Adult	+	+	-	+
N-acetylgalactosamine-binding lectins					
VVA	Foal	+	+	-	-
	Adult	+	+	+	-
DBA	Foal	±	+	-	-
	Adult	+	++	-	-
SBA	Foal	±	-	-	-
	Adult	+	+	±	+
SJA	Foal	±	-	-	-
	Adult	+	-	-	±
BSL-I	Foal	+	+	±	±
	Adult	+	+	±	±
Galactose-binding lectins					
RCA_{120}	Foal	+	+	+	+
	Adult	+	++	++	+
Jacalin	Foal	±	-	-	-
	Adult	+	+	+	+
PNA	Foal	+	+	±	±
	Adult	+	±	±	±
ECL	Foal	++	±	++	+
	Adult	+	+	+	+
Complex type N-glycan (Complex oligosaccharide)-binding lectins					
PHA-E	Foal	+	-	+	+
	Adult	+	-	+	+
PHA-L	Foal	+	-	±	+
	Adult	±	-	-	±
Fucose-binding lectin					
UEA-I	Foal	+	±	±	+
	Adult	+	±	±	±

Stained sections were scored as follows: -, negative; ±, some positive; +, moderate positive, ++, intense positive

4.5. Lectin histochemistry analysis of the VNSE in foals and adult horses

4.5.1. N-acetylglucosamine-binding lectins

The reactivity of all lectins (i.e., s-WGA, WGA, BSL-II, DSL, LEL, and STL) was significantly greater in adult horses than in foals (Fig. 10A-N).

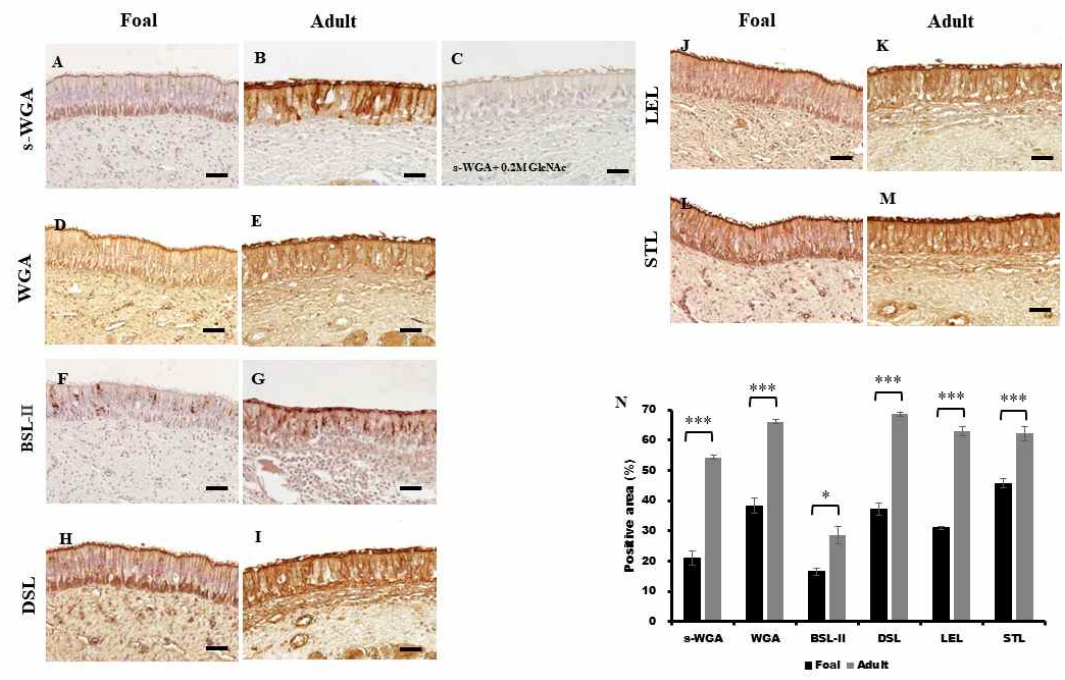

Figure 10. N-acetylglucosamine-binding lectin histochemistry (A-M) and semi-quantitative analysis of the positive area in the VNSE of foals and adult horses (N). The entire structures in VNSE including free border, ciliated cells, basal cells and vomeronasal gland were stained with varying intensity with s-WGA in foal (A) and adult horse (B), WGA in foal (D) and adult horse (E), BSL-II in foals (F) and adult horse (G), DSL in foal (H) and adult horse (I), LEL in foal (J) and adult horse (K), STL in foal (L) and adult horse (M). (C) Negative control. The reactivities of all N-acetylglucosamine-binding lectins were significantly greater in adult horses than foals (N). *p < 0.05, ***p < 0.001, foal vs. adult horse. Scale bars = 40 μm.

4.5.2. Mannose-binding lectins

The reactivity of ConA, LCA, and PSA was significantly greater in adult horses than in foals (Fig. 11H). All VNSE structures exhibited ConA, LCA, and PSA reactivity in both foals and adult horses (Fig. 11A-F, Table 3).

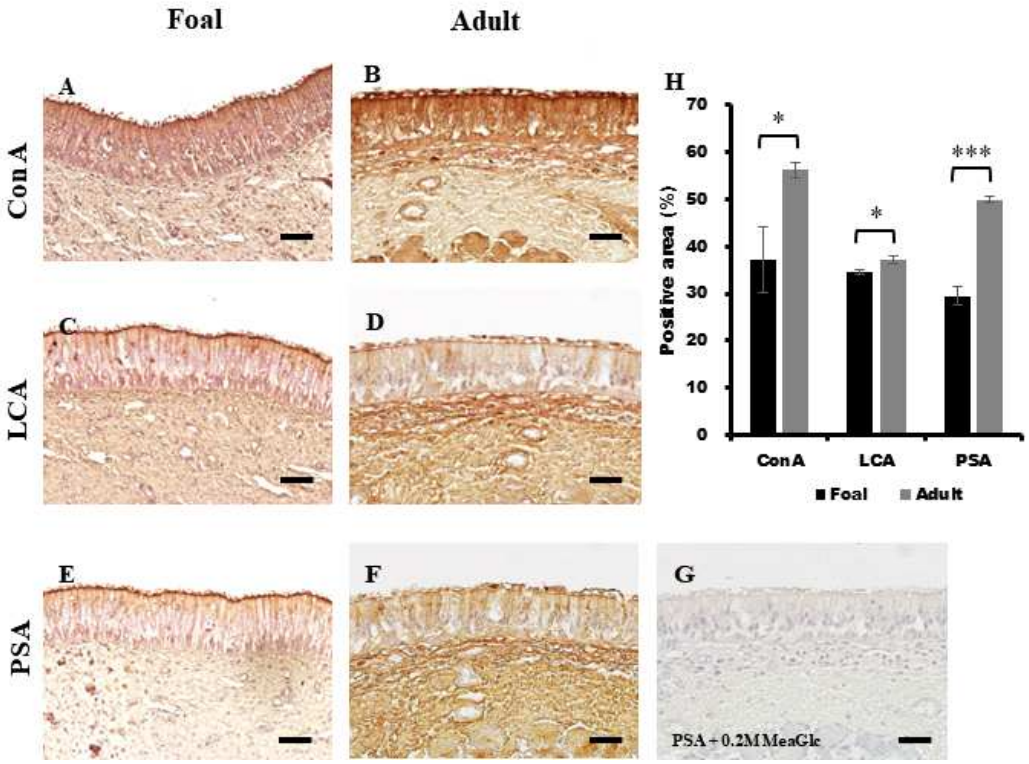

Figure 11. Mannose-binding lectin histochemistry (A-G) and semi-quantitative analysis of the positive area in the VNSE of foals and adult horses (H). All lectins in mannose-binding group including ConA, LCA and PSA correspond to all structures in VNSE of foals (A, C and E) and adult horses (B, D and F) with varying intensity. (G) Negative control. The reactivities of mannose-binding lectins were significantly greater in adult horses than foals (H). *$p < 0.05$, ***$p < 0.001$, foal vs. adult horse. Scale bars = 40 μm.

4.5.3. N-acetylgalactosamine-binding lectins

The reactivities of DBA, SBA, and SJA were significantly greater in adult horses than in foals (Fig. 12L). VVA reactivity was not detected in basal cells in the VNSE in foals or adult horses (Fig. 12A and B, Table 3). BSL-I reactivity was detected in free borders and basal cells in the VNSE of both foals and adult horses (Fig. 12I and J).

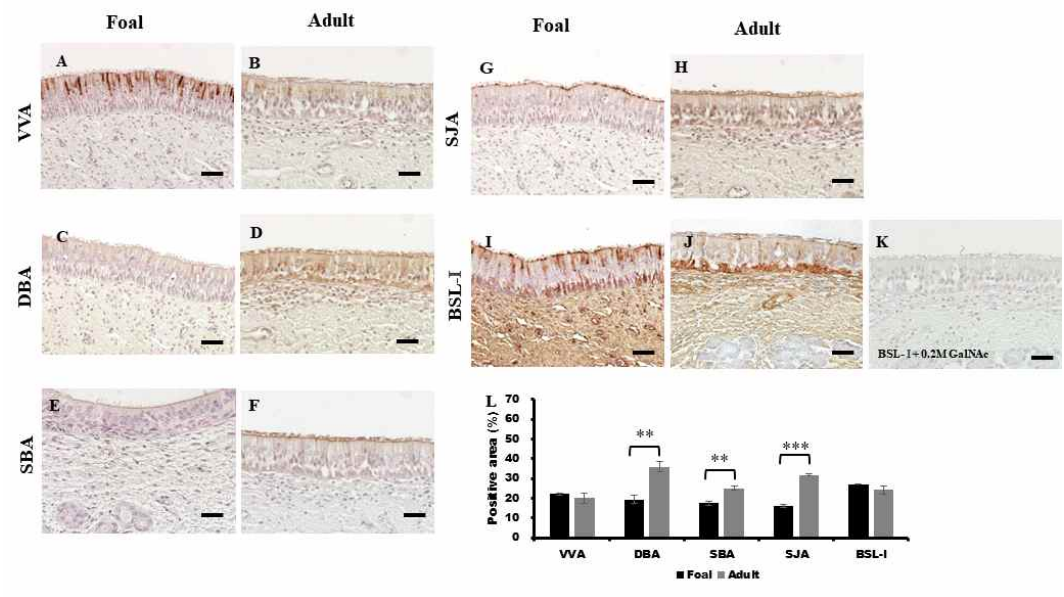

Figure 12. N-acetylgalactosamine-binding lectin histochemistry (A-K) and semi-quantitative analysis of the positive area in the VNSE of foals and adult horses (L). VVA did not correspond to basal cells in VNSE of foals (A) and adult horses (B) while BSL-I labeled basal cells in VNSE both of foals (I) and adult horses (J). SBA did not correspond to all structures of VNSE including free border, ciliated cells, basal cells and vomeronasal gland in foals (E). (K) Negative control. The reactivities of DBA, SBA, and SJA were significantly greater in adult horses than foals (L). **$p < 0.01$, ***$p < 0.001$, foal vs. adult horse. Scale bars = 40 μm.

4.5.4. Galactose-binding lectins

Among the galactose-binding lectins, the reactivity of RCA_{120}, jacalin, and ECL were significantly greater in adult horses than in foals (Fig. 13A-J). Jacalin reactivity was not detected in ciliated cells, basal cells, or vomeronasal glands in the foal VNSE (Fig. 13D, Table 3). PNA reactivity was detected in free borders and basal cells in the VNSE of both foals and adult horses (Fig. 13F and G, Table 3).

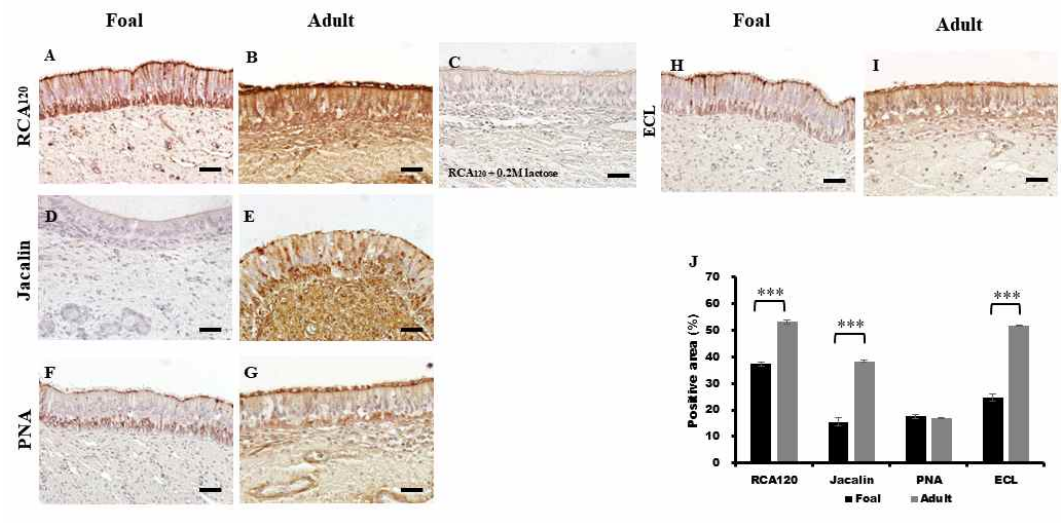

Figure 13. Galactose-binding lectin histochemistry (A-I) and semi-quantitative analysis of the positive area in the VNSE of foals and adult horses (J). All lectins in Galactose-binding group including RCA_{120}, jacalin, PNA and ECL stained VNSE both of foals (A, D, F and H) and adult horses (B, E, G and I) with varying intensity while jacalin did not labelled ciliated cells, basal cells and vomeronasal gland in VNSE of foals (D). (C) Negative control. The reactivities of RCA_{120}, jacalin, and ECL were significantly greater in adult horses than foals (J). ***$p < 0.001$, foal vs. adult horse. Scale bars = 40 μm.

4.5.5. Complex type N-glycan (Complex oligosaccharide)-binding lectins

The reactivity of Complex type N-glycan-binding lectins did not significantly differ between foals and adult horses (Fig. 14F). PHA-E reactivity was detected in all the VNSE structures of both foals and adult horses (Fig. 14A and B, Table 3). PHA-L reactivity was detected in free borders and basal cells in the VNSE of both foals and adult horses (Fig. 14C and D, Table 3).

4.5.6. Fucose-binding lectin

UEA-I reactivity in the VNSE was significantly weaker in adult horses than in foals (Fig. 14J). UEA-I reactivity was detected in free borders in the VNSE of both foals and adult horses (Fig. 14G and H, Table 3).

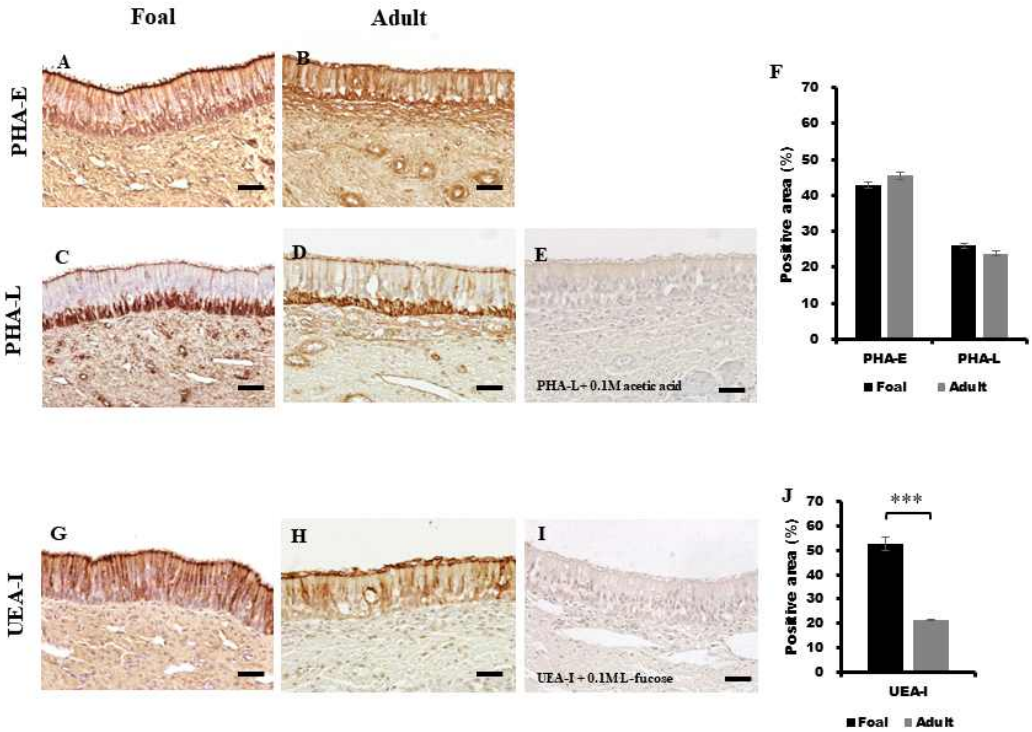

Figure 14. Complex type N-glycan-binding lectin histochemistry (A-E) and semi-quantitative analysis of the positive area in the VNSE of foals and adult horses (F) and Fucose-binding lectin histochemistry (G-I) and semi-quantitative analysis of the positive area in the VNSE of foals and adult horses (J). PHA-E and PHA-L stained all structures in VNSE including free border, ciliated cells, basal cells and vomeronasal gland both of foals (A and C) and adult horses (B and D) with varying intensity. Free border in VNSE of foals (G) and adult horses (H) reacted with UEA-I. (E) and (I) Negative control. UEA-I reactivity was significantly weaker in adult horses than foals (J). ***$p < 0.001$, foal vs. adult horse. Scale bars = 40 μm.

Table 3. Lectin binding patterns in the VNSE of horse

Lectins	Stage	Structures			
		Free border	Ciliated cells	Basal cells	Vomeronasal gland
N-acetylglucosamine-binding lectin					
s-WGA	Foal	+	±	+	+
	Adult	++	++	+	+
WGA	Foal	+	+	+	+
	Adult	++	+	+	+
BSL-II	Foal	±	±	±	+
	Adult	++	±	±	+
DSL	Foal	+	+	+	+
	Adult	+	++	+	+
LEL	Foal	+	+	+	+
	Adult	+	+	+	+
STL	Foal	+	+	+	+
	Adult	++	+	+	+
Mannose-binding lectins					
ConA	Foal	+	+	+	+
	Adult	++	++	+	++
LCA	Foal	+	+	+	+
	Adult	+	+	+	+
PSA	Foal	+	+	+	+
	Adult	+	+	+	++
N-acetylgalactosamine-binding lectins					
VVA	Foal	±	+	-	±
	Adult	±	±	-	±
DBA	Foal	±	-	-	±
	Adult	+	+	+	±
SBA	Foal	-	-	-	-
	Adult	+	±	+	±
SJA	Foal	±	-	-	-
	Adult	+	±	+	±
BSL-I	Foal	+	±	+	+
	Adult	+	±	+	-
Galactose-binding lectins					
RCA_{120}	Foal	+	+	+	+
	Adult	++	++	+	+
Jacalin	Foal	±	-	-	-
	Adult	+	±	+	+
PNA	Foal	+	±	+	-
	Adult	+	+	+	±
ECL	Foal	+	±	+	+
	Adult	+	+	+	+
Complex type N-glycan (Complex oligosaccharide)-binding lectins					
PHA-E	Foal	+	+	+	+
	Adult	+	+	+	+
PHA-L	Foal	+	±	+	+
	Adult	+	±	+	±
Fucose-binding lectin					
UEA-I	Foal	+	+	+	+
	Adult	+	±	±	±

Stained sections were scored as follows: -, negative; ±, some positive; +, moderate positive, ++, intense positive

5. Discussion

To the best of our knowledge, this is the first immunohistochemical analysis of the equine VNO at multiple stages of development (from the fetal stage to adulthood). It is also the first analysis of lectin reactivity in both the VSE and VNSE, including a semi-quantitative comparison between foals and adult horses.

Mucosae (including epithelia) are generally presumed to develop after birth. For example, pigs exhibit gradual increase in the thickness of both the VSE and VNSE after birth (Park et al., 2012a). In contrast, we found that horses did not exhibit increased thickness in either VSE or VNSE, suggesting that both VSE and VNSE are sufficiently differentiated in foals (aged < 1 year) and that no further epithelial growth occurs.

PGP9.5, is a marker for both immature and mature neurons in the VNO. We found some PGP9.5-immunopositive cells in the horse VNSE and VSE, similar to previous studies on goat VNO (Yang et al., 2021). PGP9.5-positive cells are presumed to participate in neuronal activity or unique signaling, as demonstrated in cancer cells (Day, 1992).

OMP is a marker of mature olfactory sensory neurons in the horse olfactory mucosa (Lee et al., 2016a) and horse VSE (Lee et al., 2016b). Although OMP has been widely used as a marker for mature olfactory receptor cells (Lee et al., 2016a), our observation of OMP in fetal horse VNO receptor cells was unexpected because both fetuses were in a stage of gestation and were not associated with odorant perception. This suggests that OMP signaling, rather than olfaction, may occur in fetal VNO receptor cells, similar to the ectopic expression of OMP in mouse non-neuronal tissue (Kang et al., 2015). OMP in the fetal horse VNO may thus be involved in uncharacterized signaling pathways, as well as the olfactory signaling cascade.

Lectin histochemistry revealed that the reactivity of WGA, DSL, LEL (all

in the N-acetylglucosamine-binding group), and RCA_{120} (galactose-binding group) in the VSE were significantly greater in adult horses than in foals. These results suggest that N-acetylglucosamine and galactose glycoconjugates are involved in VSE differentiation, implying that these glycoconjugates are associated with pheromone perception. In contrast, the reactivity of LCA and PSA (mannose-binding group) in the VSE was significantly weaker in adult horses than in foals, suggesting that mannose glycoconjugates are involved in cell proliferation during early postnatal development of horses.

Regarding glycoconjugate-specific changes in receptor cells in VSE, most lectin reactivity was detected in VNO receptor cells in foals and/or adult horses, with the exceptions of SJA (N-acetylgalactosamine-binding group), PHA-E (complex type N-glycan-binding group), and PHA-L (complex type N-glycan-binding group). The absence of SJA, PHA-E, and PHA-L reactivity in the VNO receptor cells in both foals and adult horses suggests that the corresponding glycoconjugates are less involved in odorant perception by receptor cells. The presence of SBA and jacalin reactivity in the VNO receptor cells of adult horses, but not in foals, suggest that the corresponding glycoconjugates are involved in the differentiation of receptor cells during the VNO development in horses.

Notably, the reactivities of VVA, DBA, SBA, and SJA (all in the N-acetylgalactosamine-binding group) were not detected in basal cells in the VSE of foals, whereas the reactivities of both SBA and SJA were detected in basal cells of adult horses. The jacalin reactivity patterns were similar. These findings imply that lectin-binding specificities are distinct, even for a single glycoconjugate group.

Fucose is closely associated with interactions between epithelial and immune cells (Kononova et al., 2021). Furthermore, active neurogenesis in the olfactory mucosa of mice is reportedly associated with a higher number of fucose-containing cells (Ducray et al., 1999; Kondoh et al., 2017). In this study, fucose-containing cells (detected by UEA-I binding) were found in the VSE of

both foals and adult horses, suggesting that some cells exhibit continuous renewal. In terms of mucosal immunity in the VNO, fucose in the VSE and VNSE might provide protection against external pathogens (e.g., bacteria), as demonstrated in the intestinal tissue (Garber et al., 2021; Kononova et al., 2021).

Consistent with our VSE results, the lectin reactivity in the VNSE was significantly greater in adult horses than in foals. This suggests that the levels of various glycoconjugates increased in the mucosae during VNSE development of horses, likely because of the demand for glycoconjugates by the VNSE mucosae. Regarding VNSE-specific morphological and lectin histochemical changes in foals and adult horses (Figs. 10-14 and Table 3), there were no significant glycan-specific changes in any cell type (for a minor exception, see Table 3). The differentiation of epithelial cells in the VNSE was likely completed within 1 year after birth; the increased lectin reactivity was then maintained, with the exception of UEA-I reactivity (Fig. 14J, Table 3).

In conclusion, our findings suggest that the horse VNO achieved morphological differentiation during fetal growth, as indicated by the expression of both PGP9.5 and OMP in receptor cells (Fig. 15). Dynamic changes in some glycoconjugates occurred in both the VSE and VNSE during postnatal development, may contribute to pheromone perception of the horses. However, the functional significance of each glycoconjugate in the VNO requires further investigation.

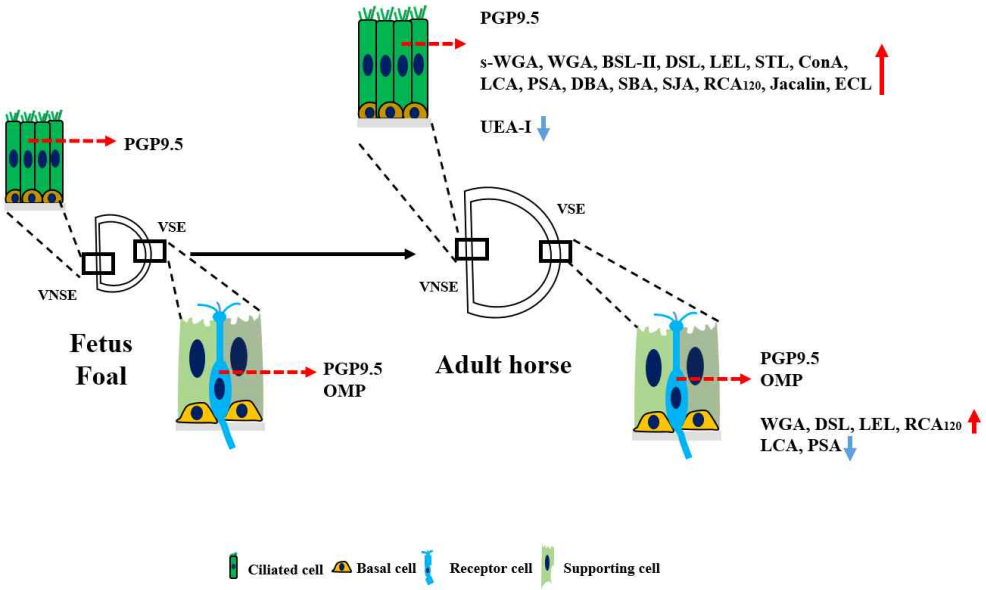

Figure 15. Schematic diagram showing the expression patterns of PGP9.5 and OMP during horse development (from fetus to adult), and changes in lectin reactivity during development (from foal to adult) in the VSE and VNSE. PGP9.5 is expressed in both the VSE and VNSE in all stages of horse growth. OMP is expressed in the VSE in all stages of horse growth, while it is not expressed in the VNSE during any stage. In the VSE, the reactivities of lectins WGA, DSL, LEL, and RCA_{120} increased during growth from foal to adult horse, while the reactivities of lectins LCA and PSA decreased during growth. In the VNSE, the reactivities of lectins s-WGA, WGA, BSL-II, DSL, LEL, STL, ConA, LCA, PSA, DBA, SBA, SJA, RCA_{120}, jacalin, and ECL increased during growth from foal to adult horse, while UEA-I reactivity decreased during growth.

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Abstract in Korean

말 보습코기관 발달단계에 따른 당접합체의 변화

(지도교수 : 신 태 균)

전 지 윤

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포유류에서 후각은 사회적 상호작용, 먹이와 서식지의 탐색, 생식 행동, 장소에 대한 기억 등에 관여하는 주요 감각 기관이고, 후각계는 주요후각계(main olfactory system)와 보조후각계(accessory olfactory system: Vomeronasal system)로 정보전달 경로 및 인지하는 냄새에 따라 구분된다. 주요후각계에서 후각점막의 후각수용체 세포(olfactory receptor cell)는 냄새를 탐지하고 정보를 후각망울에 전달하고, 후각망울은 이 신호를 뇌의 특정영역(후각피질)으로 전달하며, 보조후각계는 보습코기관(Vomeronasal organ, VNO)의 후각수용체세포를 통해 페로몬을 감지하고, 그 신호를 보조 후각망울을 경유하여 뇌로 전달한다. 말의 플레멘행동(Flehmen response)은 페로몬과 같은 후각정보를 보습코기관에서 더 세밀히 분석하기 위한 행동이다.

보습코기관은 연골에 둘러싸인 반달모양의 구조물로, 후각을 감지하는 수용체세포가 있는 내측의 감각상피와 수용체세포가 존재하지 않는 외측의 비감각상피인 호흡상피로 이루어져 있다. 후각상피는 후각수용체세포, 지지세포 그리고 바닥세포로 구성되어있고, 비감각상피는 거짓중층원주상피와 바닥세포로 구성되어있다. 후각에서 당접합체는 후신경의 발달과 증식, 신호 처리에 대한 역할을 하고 동물 종, 발생단계, 노화에 따라 발현에 차이가 있다고 알려져 있다. 성숙된 말의 보습코기관 감각상피에서 다양한 당접합체는 확인되었으나 말의 발달단계별 당접합체의 변화에 대해서는 밝혀진 바가 없다. 본 연구에서는

말의 출생 전 개체, 생후 1년 미만, 3~4년령의 성숙 개체를 이용하여 보습코기관의 발달과정에 따른 당접합체의 변화를 조사하였다.

동물의 후각점막과 보습코기관의 기능과 관련이 있으며, 미성숙/성숙 후각신경세포의 표지단백질인 Protein gene product 9.5 (PGP 9.5)와 성숙 후각신경세포의 표지단백질인 Olfactory marker protein (OMP)을 이용하여 면역염색을 실시하였다. 출생 전 개체와 생후 1년 미만, 그리고 성체 모두의 감각상피와 비감각상피에서 PGP9.5 양성반응이 확인되었다. OMP의 경우, 전 연령 감각상피의 후각수용체세포에서 강한 양성반응을 확인 할 수 있었다.

당접합체의 발달과정별 변화를 평가하기 위해 21가지의 렉틴을 이용해 렉틴조직화학염색을 실시하였다. 생후 1년 미만의 망아지 및 성체의 보습코기관 표면상피 각각의 렉틴 양성반응 면적을 정량하여 비교한 결과 감각상피의 경우 WGA, DSL, LEL, RCA_{120}의 렉틴반응도가 망아지에서 성체로 발달하며 유의적으로 증가하였고, LCA와 PSA는 유의적으로 반응도가 감소하였다. 비감각상피에서는 s-WGA, WGA, BSL-Ⅱ, DSL, LEL, STL, ConA, LCA, PSA, DBA, SBA, SJA, RCA_{120}, Jacalin, ECL의 렉틴반응도가 망아지에서 성체로 발달하며 유의적으로 증가하였고, UEA-Ⅰ은 반응도가 유의적으로 감소하였다.

본 연구를 통해 말의 발달단계에 따라 보습코기관 내 상피에서 일부 당접합체가 증가 또는 감소가 있음을 확인하였다. 특히 성체로 발달함에 따라 감각상피에서 증가하는 당접합체의 경우, 보습코기관의 주요 기능인 페로몬 탐지 및 말의 플레멘행동과 연관이 있을 것으로 추정된다.

주요어: 당접합체, 렉틴, 말, 발달단계, 보습코기관

감사의 글

대추 한 알 / 장석주

저게 저절로 붉어질 리는 없다
저 안에 태풍 몇 개
저 안에 천둥 몇 개
저 안에 벼락 몇 개
저 안에 번개 몇 개가 들어 있어서
붉게 익히는 것일 게다

저게 혼자서 둥글어질 리는 없다
저 안에 무서리 내리는 몇 밤
저 안에 땡볕 두어 달
저 안에 초승달 몇 날이 들어서서
둥글게 만드는 것일 게다

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