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Glycohistochemistry of the lateral tympanic membrane in the syrinx of the Denizli cock

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Abstract: The Denizli cock is a special race that has harmonious long crowing. In this study, the syrinx of the Denizli cock was studied by using glycohistochemical analyses. Paraffin sections were stained with Alcian blue (AB) at pH 1 and pH 2.5 for demonstration of sulfated and carboxylated carbohydrates, respectively. At AB-pH 1, the lateral tympanic membrane (LTM) and also the medial tympanic membrane (MTM) exhibited a weak reactivity, whereas they were stained intensely at AB-pH 2.5. The frozen sections were incubated with 5 digoxigenin-labeled lectins for lectin histochemistry: peanut agglutinin, *Maackia amurensis* leucoagglutinin, *Galanthus nivalis* agglutinin, *Datura stramonium* agglutinin, and *Sambucus nigra* agglutinin. In LTM, positive stainings were observed for the 5 lectins. These results suggest that LTM exhibits an intense reactivity with AB-pH 2.5, indicating carboxylated carbohydrates, and it also has terminal saccharide moieties consisting of β(1→3) linked galactose, α(2→3) linked sialic acid, mannose, β(1→4) linked galactose, and α(2→6) linked sialic acid.

Key words: Denizli cock, syrinx, tympanic membrane, glycostructures

1. Introduction

The syrinx, the avian vocal organ, is located at the point where the trachea divides into the 2 primary bronchi. It consists of cartilage (or bony) rings, the medial tympanic membrane (MTM), the lateral tympanic membrane (LTM), and the labia, which are loose connective tissue masses and syringeal muscles. The MTM is on the medial wall of the bronchus on either side of and behind the pessulus. The LTM is located between the end of the tympanum and the first cartilago bronchosyringealis on either side of the syrinx walls. The pessulus, a triangular bar, is located in the division part of the bronchus primarius at the end of the syrinx (1). The LTM and MTM are lined by stratified epithelium, which surrounds the connective tissue. Several studies have shown the functions of tympanic membranes and the syringeal muscles of the syrinx for sound-generating mechanisms in birds (2–7). The membranes are activated by the syringeal muscles during phonation (8–10). It has been reported that the LTM is the main sound generator in pigeons (4). Furthermore, Goller and Larsen (11) suggested that the labia play an important role as principal sound generators in the songbirds. Riede and Goller (12) also reported that laterally positioned labia are passively set into vibration, thus interrupting a passing air stream. Together with subsyringeal pressure, the size and tension of the labia determine the spectral characteristics of the primary sound. Larsen et al. (7) concluded that the MTM is not required for song production in zebra finches, but may play a role in adjusting the tension of the labia.

Glycoconjugates (glycoproteins, glycolipids, proteoglycans) are sugar-containing molecules that have many important structural and modulatory functions in biological systems (13). The extracellular matrix (ECM), the product of connective tissue, is mainly composed of proteins and proteoglycans. In this study, we aimed to demonstrate the glycohistochemical characteristics of the LTM in the syrinx of the Denizli cock.

2. Materials and methods

The Denizli cock is a special race of bird that has been bred in Denizli, Turkey. It has long, harmonious crowing and a beautiful appearance. Syrinx samples were studied using histochemical techniques in 15 Denizli cocks between 9 and 12 months old at the light microscopic level. They were isolated from ether-anesthetized cocks (ethical approval was obtained from the Pamukkale University Animal Ethics Committee) and divided longitudinally into 2 parts. The samples were fixed in 10% formaldehyde solution for 3 days and then processed for histology and embedded in paraffin. The blocks were cut in serial longitudinal sections (5 µm), which were then stained with the cationic dye toluidine blue (TB-metachromatic dyes) in an aqueous

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solution of 0.1% (14) and Alcian blue (AB) (15,16) at pH 1 for sulfated carbohydrates and at pH 2.5 for acidic carbohydrates.

Lectins are carbohydrate-binding proteins that have been widely used in many biological assays. For lectin histochemistry, the samples were embedded within an optimal cutting-temperature compound. They were snap-frozen in liquid nitrogen and stored at –86 °C for the cryostat technique. Lectin histochemical studies were performed on the frozen sections (6 µm). In order to identify the localization of some of the specific carbohydrate structures, 5 different digoxigenin (DIG)-labeled plant lectins (Cat: 11210238001, Roche Applied Science) were used: peanut agglutinin (PNA) directed to galactose β(1→3) N-acetylgalactosamine; *Maackia amurensis* leucoagglutinin (MAL), which recognizes sialic acid linked α(2→3) to galactose; *Galanthus nivalis* agglutinin (GNA) specific for terminal mannose residues; *Datura stramonium* agglutinin (DSA) specific for galactose β(1→4) N-acetylglycosamine; and *Sambucus nigra* agglutinin (SNA) specific for sialic acid α(2→6) galactose.

Briefly, sections were washed in Tris-buffered saline (TBS) and blocked with a blocking buffer (10% blocking reagent (DIG Glycan Differentiation Kit, Roche), 90% TBS (pH 7.5)). They were then washed with TBS and twice with buffer 1 (TBS, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.5). Afterwards, they were incubated with PNA (20 mg/mL), MAL (10 mg/mL), GNA (20 mg/mL), DSA (10 mg/mL), and SNA (10 mg/mL) in a humidified chamber for 1 h. Thereafter, they were washed twice with TBS and incubated with 1 mL/mL antidigoxigenin-alkaline phosphatase (Roche Applied Science) in TBS for 1 h. After being washed twice with TBS, they were incubated with a staining solution that contained 20 mL/mL nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (Roche Applied Science) in buffer 2 (0.1 M Tris-HCl, 0.05 M $MgCl_2$, 0.1 M NaCl, pH 9.5) until a dark color reaction appeared. The reaction was stopped by washing with ultrapure water. Slides were then mounted with glycerin.

2.1. Controls of lectin histochemistry

Lectin histochemistry controls were performed by omitting the PNA, MAL, GNA, DSA, and SNA lectins and incubating slides with TBS. Cryostat sections were also stained with hematoxylin and eosin (H&E) for general histology. An Olympus BX50 light microscope and Olympus DP2-BSW microscope digital camera system were used for photography.

3. Results

3.1. Histology

In the longitudinal sections of the tracheobronchial part of the syrinx, the triangular pessulus, tympanic membranes, and cartilage rings were observed (Figure 1a). The pessulus contains ossified and cartilaginous tissues. Cartilage tissues, which were stained metachromatically with TB, are present at the upper tip of the pessulus (Figure 1b). The LTM was positioned in the lateral wall, and the MTM is just on both sides of the pessulus (Figures 1a and 1b). In a part of the syrinx, the MTM consists of connective tissue between 2 simple squamous epithelial layers, whereas the connective tissue underlies the stratified squamous epithelial tissue in the LTM (Figure 1c). The AB reactions in the LTM and also in the MTM were varied at pH 1 and pH 2.5. At AB-pH 1, the LTM and MTM exhibited a weak reactivity, whereas they were stained intensely at AB-pH 2.5. These results indicated that the LTM and MTM have denser carboxylated carbohydrates than sulfated carbohydrates (Figures 1d and 1e).

3.2. Lectin histochemistry

The patterns of lectin staining were demonstrated in the LTM of the syrinx from the Denizli cock (Figures 2 and 3). In general, the LTM were positive for binding with PNA, MAL, GNA, DSA, and SNA. This result indicated that galactose β(1→3) N-acetylgalactosamine, sialic acid α(2→3) galactose, mannose, galactose β(1→4) N-acetylglycosamine, and sialic acid α(2→6) galactose structures demonstrated PNA, MAL, GNA, DSA, and SNA binding patterns, respectively, in the LTM.

4. Discussion

The morphological structure (17–23) and the histological structure (8,12,24–26) of the syrinx have been extensively studied. The tympanic membranes have also been investigated in several birds (4,20,26–28). In the Denizli cock, the LTM lied between the caudal part of the tympanum and the first cartilago bronchialis syringis, and the MTM covered the medial ends of the first 3 bronchial C-shaped rings of the bronchus primarius (23). Contraction of syringeal muscles constricts the syringeal lumen and thus reduces airflow by adducting connective tissue masses, the medial labia, and lateral labia (6). Sound production is always accompanied by vibratory motions of both labia, indicating that these vibrations may be the sound source (11). Additionally, it has been reported that sound production is initiated by almost full adduction of the LTM into the tracheal lumen in the tracheal syrinx of the pigeon (7). The labia contain the ECM, which provides elasticity to the labia for their biomechanical alterations during phonation (11). ECM and cell-surface glycostructures serve as functional and structural roles in biological systems. They determine the physical characteristics of tissues and many of the biological properties of cells. The major components of the ECM are fibrous proteins that provide tensile strength (e.g.,

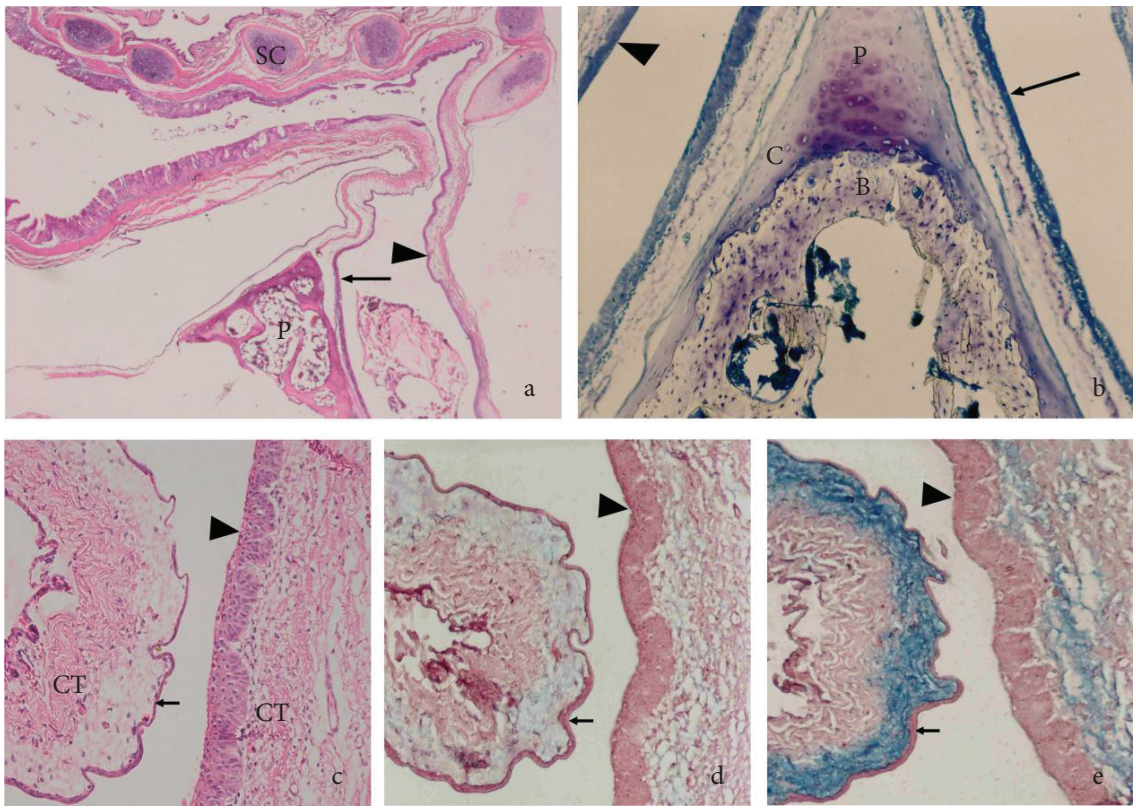

Figure 1. Longitudinal sections of the syrinx: a) H&E staining, 40×; b) TB staining, 400×. Medial (arrow) and lateral (arrowhead) tympanic membranes: c) H&E staining, 200×; d) AB-pH 1, 200×; e) AB-pH 2.5, 200×. Pessulus (P), cartilage (C), bone (B), syringeal cartilage ring (SC), and connective tissue (CT).

various collagen and elastin), adhesive glycoproteins (e.g., fibronectin, laminin, and tenascin), and proteoglycans that provide a hydrated gel for resisting compressive forces (13). Goller and Larsen (4) reported that the LTM has relatively thick masses of connective tissue. Collagen, elastin, and hyaluronan as ECM components have also been demonstrated in the labia (12). Since the ECM is rich in carbohydrates, lectins can serve as structural probes to analyze carbohydrates in the connective tissue of the LTM and MTM in the syrinx. ECM glycostructures in the labia may have functions in the interactions between labial morphology and their sound-producing mechanism. In this study, the glycohistochemical characteristics of the syrinx in the Denizli cock were demonstrated. It was observed that there was intense staining of carboxylated carbohydrates, probably hyaluronic acid, with the cationic dye AB at pH 2.5 both in the LTM and in the MTM. Furthermore, PNA, MAL, GNA, DSA, and SNA staining

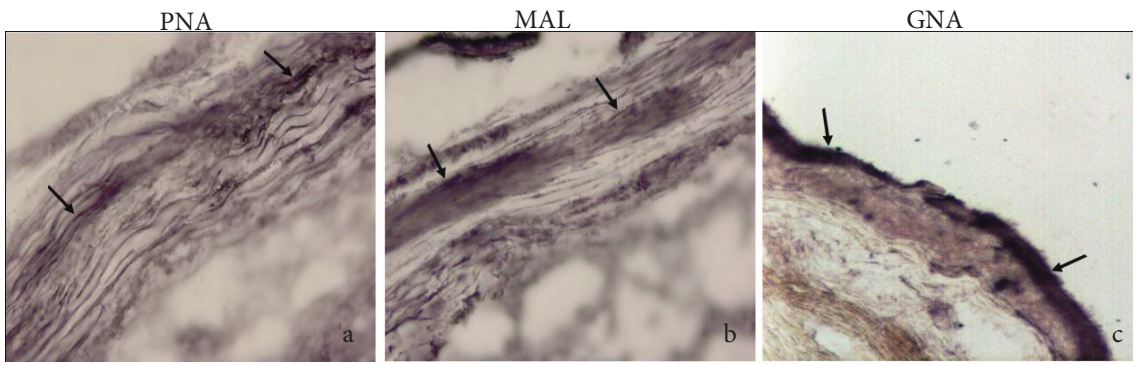

Figure 2. The PNA, MAL, and GNA reactivities (arrows) of the LTM in the syrinx of Denizli cock: a, b) 1000×; c) 200×.

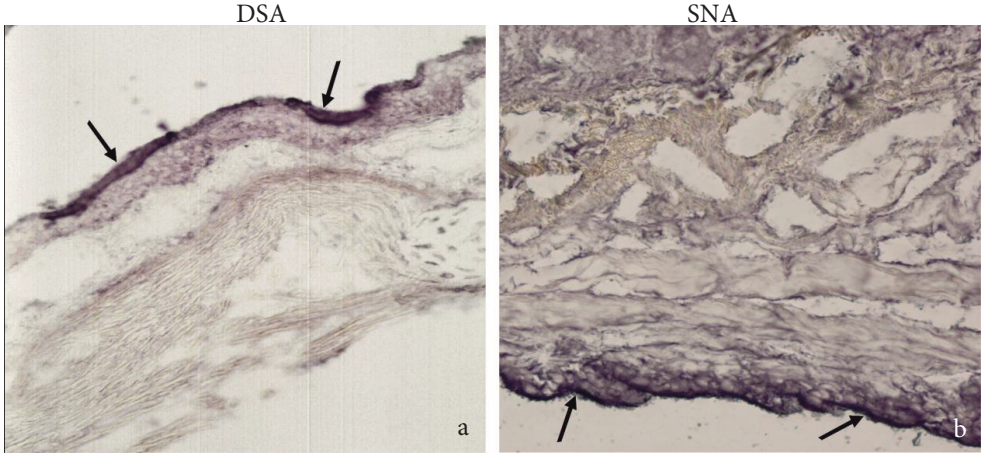

Figure 3. The DSA and SNA reactivities (arrows) of the LTM in the syrinx of Denizli cock. a) 200×, b) 400×.

patterns were detected in the LTM. These results allow the conclusion that β(1→3) linked galactose, α(2→3) linked sialic acid, mannose, β(1→4) linked galactose, and α(2→6) linked sialic acid containing glycoconjugates are present in the LTM of the syrinx in Denizli cocks. These observations can suggest that the glycostructures may be important for the viscoelastic functions of the tympanic membranes during phonation. However, further molecular analyses are needed to understand the correlation between the glycostructures and sound-generating mechanisms in the syrinx.

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References

1. Hodges, R.D.: The Histology of the Fowl. Academic Press, London. 1974.
2. Brakenbury, J.: Control of sound production in the syrinx of the fowl *Gallus gallus*. J. Exp. Biol., 1980; 85: 239–251.
3. Gaunt, A.S., Gaunt, S.L.L.: Mechanics of the syrinx in *Gallus gallus*. 11. Electromyographic studies of *ad libitum* vocalizations. J. Morph., 1977; 152: 1–20.
4. Goller, F., Larsen, O.N.: *In situ* biomechanics of the syrinx and sound generation in pigeons. J. Exp. Biol., 1997; 200: 2165–2176.
5. Goller, F., Larsen, O.N.: Role of syringeal vibrations in bird vocalizations. Proc. R. Soc. Lond. B, 1999; 266: 1609–1615.
6. Larsen, O.N., Goller, F.: Direct observation of syringeal muscle function in song birds and a parrot. J. Exp. Biol., 2002; 205: 25–35.
7. Larsen, O.N., Goller, F., van Leeuwen, J.L.: Aspects of syringeal mechanics in avian phonation. Acta Zool. Sinica, 2006; 52 (Supplement): 478–481.
8. Frank, T., Walter, I., Probst, A., König, H.E.: Histological aspects of the syrinx of the male mallard (*Anas platyrhynchos*). Anat. Histol. Embryol., 2006; 35: 396–401.
9. Suthers, R.A., Goller, F., Pytte, C.: The neuromuscular control of birdsong. Phil. Trans. R. Soc. Lond. B, 1999; 354: 927–939.
10. Suthers, R.A.: Peripheral vocal mechanisms in birds: Are songbirds special? Neth. J. Zool., 2001; 51: 217–242.
11. Goller, F., Larsen, O.N.: A new mechanism of sound generation in songbirds. Proc. Natl. Acad. Sci. USA, 1997; 94: 14787–14791.
12. Riede, T., Goller, F.: Functional morphology of the sound-generating labia in the syrinx of two song birds species. J. Anat., 2010; 216: 23–36.
13. Varki, A., Cummings, R., Esko, J., Freze, H., Hart, G., Marth, J.: Essentials of Glycobiology. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 1999.
14. Cerri, P.S., Sasso-Cerri, E.: Staining methods applied to glycol methacrylate embedded tissue sections. Micron, 2003; 34: 365–372.
15. Lev, R., Spicer, S.S.: Specific staining of sulphate groups with alcian blue at low pH. J. Histochem. Cytochem., 1964; 12: 309.
16. Mowry, R.W.: Alcian blue techniques for the histochemical study of acidic carbohydrates. J. Histochem. Cytochem., 1956; 4: 407–408.
17. Cevik-Demirkan, A., Ozdemir, V.: A study on anatomical structures of the larynx, trachea and syrinx in Eurasian eagle owl (*Bubo bubo*). J. Anim. Vet. Adv., 2011; 10: 3218–3224.

18. Frank, T., Probst, A., König, H.E., Walter, I.: The syrinx of the male mallard (*Anas platyrhynchos*): special anatomical features. Anat. Histol. Embryol., 2007; 36: 121–126.

19. Gaban-Lima, R., Höfling, E.: Comparative anatomy of the syrinx in the tribe Arini (Aves: Psittacidae). Braz. J. Morphol. Sci., 2006; 23: 501–512.

20. Gezer İnce, N., Pazvant, G., Alpak, H.: Anatomical features of the syrinx in sea gulls. Ankara Üniv. Vet. Fak. Derg., 2012; 59: 1–3.

21. Miller, E.H., Seneviratne, S.S., Jones, I.L., Robertson, G.J., Wilhelm, S.I.: Syringeal anatomy and allometry in murres (Alcidae: Uria). J. Ornithol., 2008; 149: 545–554.

22. Pierko, M.: Structural analysis of upper respiratory tract in *Anas platyrhynchos* (L., 1758) and *Clangula hyemalis* (L., 1758). Elec. J. Pol. Ag. Univ., 2010; 13: 22.

23. Taşbaş, M., Hazıroğlu, R.M., Çakır, A., Özer, M.: Morphological investigations of the respiratory system of the Denizli cock. II. larynx, trachea, syrinx. Vet. J. Ankara Univ., 1994; 41: 135–153.

24. Cevik-Demirkan, A., Haziroglu, R.M., Kurtul, I.: Gross morphological and histological features of larynx, trachea and syrinx in Japanese quail. Anat. Histol. Embryol., 2007; 36: 215–219.

25. Yildiz, H., Bahadir, A., Akkoc, A.: A study on the morphological structure of syrinx in ostriches (*Struthio camelus*). Anat. Histol. Embryol., 2003; 32: 187–191.

26. Yildiz, H., Yilmaz, B., Arican, I.: Morphological structure of the syrinx in the Bursa roller pigeon (*Columba livia*). Bull. Vet. Inst. Pulawy., 2005; 49: 323–327.

27. Kabak, M., Orhan, I.O., Haziroglu, R.M.: The gross anatomy of larynx, trachae and syrinx in the long-legged buzzard (*Buteo rufinus*). Anat. Histol. Embryol., 2007; 36: 27–32.

28. Onuk, B., Hazıroğlu, R.M., Kabak, M.: The gross anatomy of larynx, trachae and syrinx in goose (*Anser anser domesticus*). Kafkas Univ. Vet. Fak. Derg., 2010; 16: 443–450.