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Full Length Research Paper

Morphological study of the infraorbital gland of the male barking deer, *muntiacus muntjak*

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The morphology of the infraorbital gland of the barking deer (*Muntiacus muntjak*) was examined using lectin histochemistry, immunohistochemistry and scanning electron microscopy. The glands consisted of sebaceous and apocrine glands, with proportion of apocrine glands was larger than the sebaceous gland. Sebaceous glands consisted of many lobules with polyhedral cells. The apocrine glands were lined by cuboidal to columnar cells with typical apical protrusions. Seven lectins applied showed various binding intensities in the sebaceous and apocrine glands. Peanut agglutinin (PNA) and soybean agglutinin (SBA) were strongly positive in the sebaceous gland, while the others lectins showed various positive reactions from weak to strong intensities in the membrane and cytoplasm areas. In the apocrine gland portion, positive reaction with various intensities was observed for all the seven lectins. In the myoepithelial cells of the apocrine glands, positive reactions were found in moderate intensity only for SBA and dolichos biflorus agglutinin (DBA). Immunoreactivity for α-smooth muscle actin was observed in the myoepithelial cells in the basal membrane of the apocrine gland. These findings suggest that the secretion of the infraorbital gland of the barking deer contains lipid from the sebaceous gland and glycoconjugates secreted by both the sebaceous and apocrine glands.

Key words: Barking deer, infraorbital gland, lectin histochemistry.

INTRODUCTION

Scent glands are present in many artiodactyla and vary in its morphology (Gosling, 1985). The morphology of the scent glands has been studied in a few ruminant species including the serow (Kodera et al., 1982; Atoji et al., 1987, 1988, 1993, 1995, 1996; Atoji and Suzuki, 1990), fallow deer (Parillo and Diverio, 2009) and the lesser mouse deer (Agungpriyono et al., 2006). Osborn et al. (2000) reported that the reproductive communication in fallow deer is thought to be accomplished by odours associated with skin glands localized to specific areas, such as infraorbital, tarsal and interdigital areas. The infraorbital gland has been debated on its functions for some time now. Research indicates that the scent released from this gland is used for communication when a deer rubs its face against brush or trees. Others remark that it is used for visual communication purposes between all deer. Gosling (1985) stated the scent-marking function of the infraorbital gland. The barking deer is a ruminant animal belonging to the family Cervidae and is regarded as pre-historic and the oldest known species of deer. The barking deer is a relatively small animal, has a solitary lifestyle and is named for the sound it emits that resembles a large dog barking. These animals inhabit areas of tropical forest and have a wide distribution, being found from India eastwards across southeastern Asia as far as Indonesia

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Table 1. List of lectins used, corresponding carbohydrate binding or sugar residue and their optimal concentration.

Lectins	Acronym	Source	Specificity	Concentration (ug/ml)
Concanavalin A	Con A	*Canavalia ensiformis*	a-D-Man, > a-D-Glc	5
Soybean agglutinin	SBA	*Glycine max*	a-D-GalNAc > B-D-GalNAc	5
Wheat germ agglutinin	WGA	*Triticum vulgaris*	GlcNAc > Sialic acid	5
Dolichos biflorus agglutinin	DBA	*Dolichos biflorus*	a-D-GalNAc	5
Ulex europeus agglutinin I	UEA	*Ulex europeus*	a-L-Fuc	5
Ricinus communis agglutinin I	RCA	*Ricinus communis*	B-D-Gal-(1,3)-D-GalNAc	5
Peanut agglutinin	PNA	*Arachis hypogaea*	B-D-Gal-(1,4)-D-GalNAc	5

(Lekagul and McNeely, 1977; Ohtaishi and Gao, 1990; Long, 2003). To date, no data were documented regarding the morphology of the infraorbital glands in the barking deer. Thus, in the present study we report the detailed morphology of the infraorbital gland of the barking deer using histochemical, immunohistochemical and scanning electron microscopy.

MATERIALS AND METHODS

Animals and tissue preparation

Infraorbital glands from three adult and healthy male barking deer (*Muntiacus muntjak*; aged two to four years, mean weight of 22.4 ± 0.57 kg) were used in this study. The animals were obtained from their habitat in central Java, Indonesia under license (based on permit SK.23/Menhut-II/2011 to SW). Tissue samples measuring about 1 cm^3 were obtained from certain parts of the dorsal infraorbital gland and were fixed in the Bouin's solution for 24 h and then transferred to 70% ethanol. The samples were dehydrated in graded series of ethanol, cleared with xylene and embedded in paraffin. The paraffin blocks were cut serially at 4 µm thickness using a microtome (LEICA RM-2155 Leica Microsystem Inc. Bensheim, Germany). Sections were stained with haematoxylin and eosin (HE staining) for observation of general histological structure, immunohistochemically for detection of presence of α-smooth muscle actin and with lectin histochemistry for detection of distribution of glycoconjugate. Stained sections were observed under a light microscope equipped with image analyzer (Olympus BX51, Olympus optical Co. Tokyo, Japan).

Immunohistochemistry

The sections were stained immunohistochemically by an avidin-biotin-peroxidase complex (ABC) procedure (Hsu and Raine, 1981). Sections were dewaxed in xylene and rehydrated in descending grades of ethanol and washed under tap water. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide (H_2O_2) in absolute methanol for 15 min at room temperature and then incubated in 10% normal goat serum for 30 min to minimize non-specific background staining. The sections were then ready for overnight incubation at 4°C with antibody monoclonal to α-smooth muscle actin (5 µg/ml, A5228, Sigma Aldrich Inc. Saint Louis. Missouri, USA). After washing in phosphate buffered saline (PBS) pH 7.4, the sections were incubated with biotinylated goat anti mouse (1:600, BA-1000, Vector Lab. Inc. Burlingame, USA) for 30 min, followed by Vectastain Elite ABC Kit (1:2, PK-6100, Vector Lab. Inc. Burlingame, USA) for 30 min. Immunoreactive sites were visualized with ImmPACT™ DAB peroxidase substrate (SK-4105, Vector Lab. Inc. Burlingame, USA). Sections were lightly counterstained with Harris's haematoxylin, dehydrated and mounted with Entellan® (Merck, Germany).

Lectin histochemistry

Seven kinds of biotinylated lectins obtained from Vector Laboratories (BK 1000, Vector Lab. Inc. Burlingame, USA) were used in the present study. The sections were deparaffinized, rehydrated and treated with 0.01 M PBS pH 7.4 (P8313, Sigma Aldrich Inc. Saint Louis. Missuori, USA) and incubated with 3% H_2O_2 for 15 min to destroy activity of endogenous peroxidase, rinsed in distilled water and dipped in 10% goat normal serum in 0.01 M PBS pH 7.4. The sections were incubated with seven biotinylated lectins respectively (Table 1), in a moist chamber overnight at 4°C temperature. The optimal concentration chosen for each lectin was 5 µg/ml). After washing in PBS pH 7.4, the sections were incubated with Vectastain Elite ABC Kit (1:2, PK-6100, Vector Lab. Inc. Burlingame, USA) for 30 min followed by visualization with ImmPACT™ DAB peroxidase substrate (SK-4105, Vector Lab. Inc. Burlingame, USA). Sections were lightly counterstained with Harris's hematoxylin, dehydrated and mounted with Entellan® (Merck, Germany). The common names, sugar specificity and optimal concentration chosen for each lectin are summarized in Table 1.

Scanning electron microscopy (SEM)

For scanning electron microscopy, samples of infraorbital gland were fixed in 2.5% glutaraldehyde at 4°C for 4 h. After washing with 0.1 M cacodylate buffer, the samples were post fixed in 1% osmium tetroxide at room temperature for 2 h. The specimen was dehydrated in graded series of acetone and dried at critical-point dryer (CPD Baltec-030 Bal-tec. Canonsburg. Pennsylvania, USA) using CO_2. The specimens were carefully mounted on aluminum stubs using double-sided carbon tape, sputtered with gold coat (Sputter Coater Baltec SCD 005, Bal-tec. Canonsburg. Pennsylvania, USA) and observed under a SEM (JEOL-JSM 6400, Jeol Ltd. Tokyo, Japan) at the accelerating voltage of 15 kV.

RESULTS

Macroscopically, the preorbital gland was a small pocket located in front of the deer's eyes. At most times the pocket was closed. The gland measured 2.3 to 2.7 cm in length and 2.0 to 2.3 cm in width (Figure 1). Histological examination revealed that the infraorbital gland consisted of

Figure 1. Photograph showing the infraorbital gland of the male barking deer, *Muntiacus muntjak.*

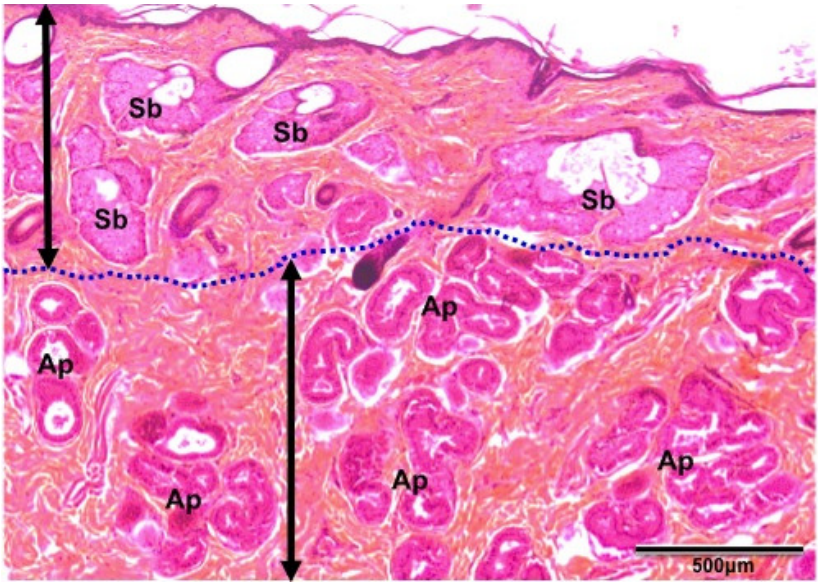

Figure 2. Light micrographs showing the histological structure of the infraorbital gland of the barking deer. The gland consists of sebaceous gland portion (Sb) and apocrine gland portion (Ap). Haematoxylin and eosin (HE) staining. Scale bar: 500 µm.

sebaceous gland portion and apocrine gland portion. The sebaceous gland portion was located in the superficial region below the skin and the apocrine gland portion was deeper in the sub mucosa, with proportion of sebaceous glands larger than the apocrine gland (Figure 2). Sebaceous gland was composed of polyhedral cells and necrotic cells were secreted through the opening of the gland. The apocrine gland was simple tubular gland. Scanning electron microscopic examination revealed that the apocrine glands consisted of tubules that were lined with secretory cells. The luminal surface of the secretory cells appeared hexagonal shaped and the secretion was found on the surface of the secretory cells (Figure 3). Immunohistochemical examination revealed that 'immunoreactivity' to α-smooth muscle actin was observed in the myoepithelial cells in the basal membrane of the apocrine gland (Figure 4). The α-smooth muscle actin was absent in the sebaceous gland portion. In the present study, we did not find comparable levels of immunostaining intensity in the myoepithelial cells. Seven lectins applied showed various binding intensities in the sebaceous and apocrine glands (Table 2). Peanut agglutinin (PNA) and soybean agglutinin

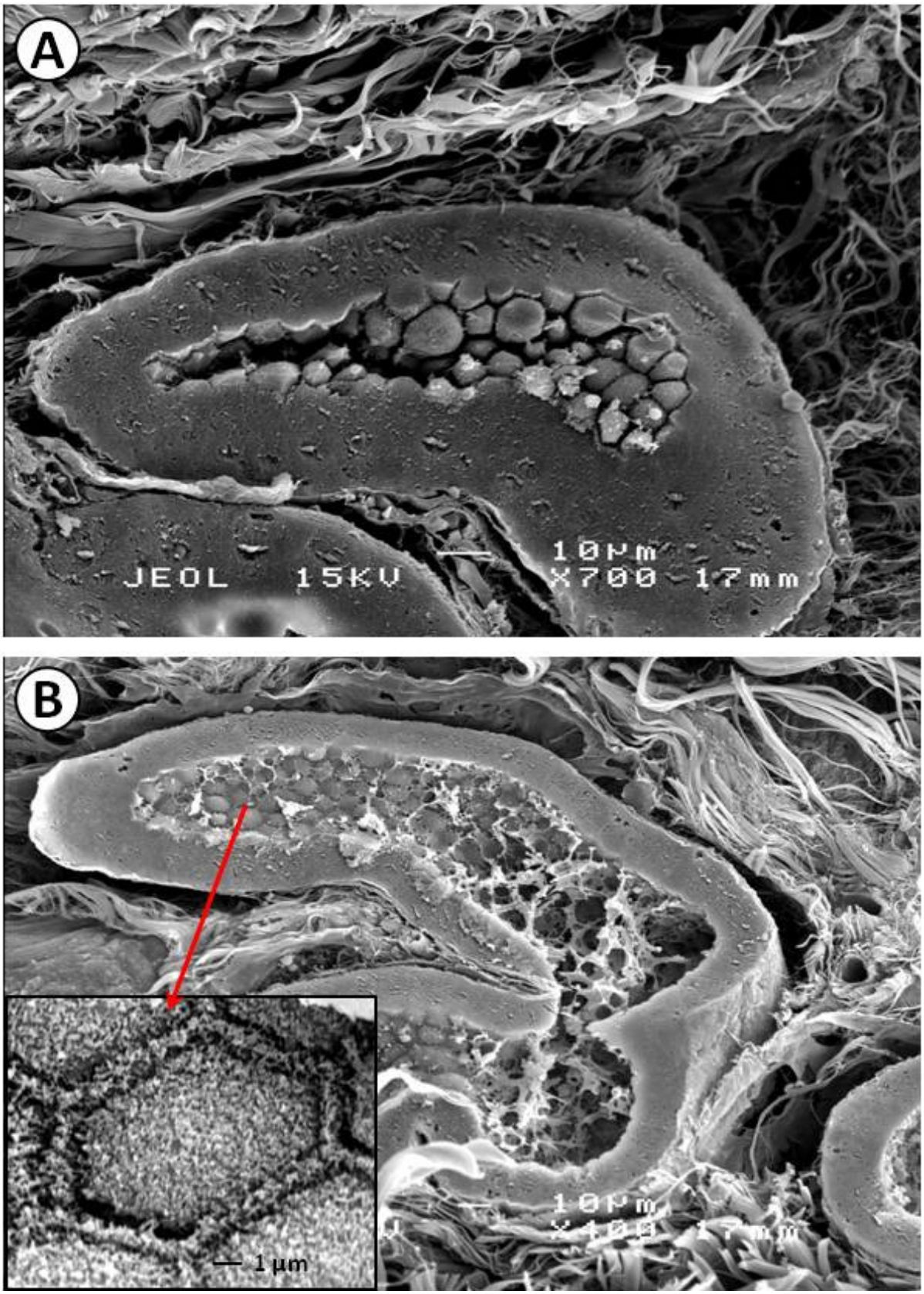

Figure 3. Scanning electron micrographs of apocrine gland portion in the infraorbital gland of the barking deer. A, on the apical surface of the secretory cells; B, higher magnification of the secretory cells surface shows hexagonal in shape (inset).

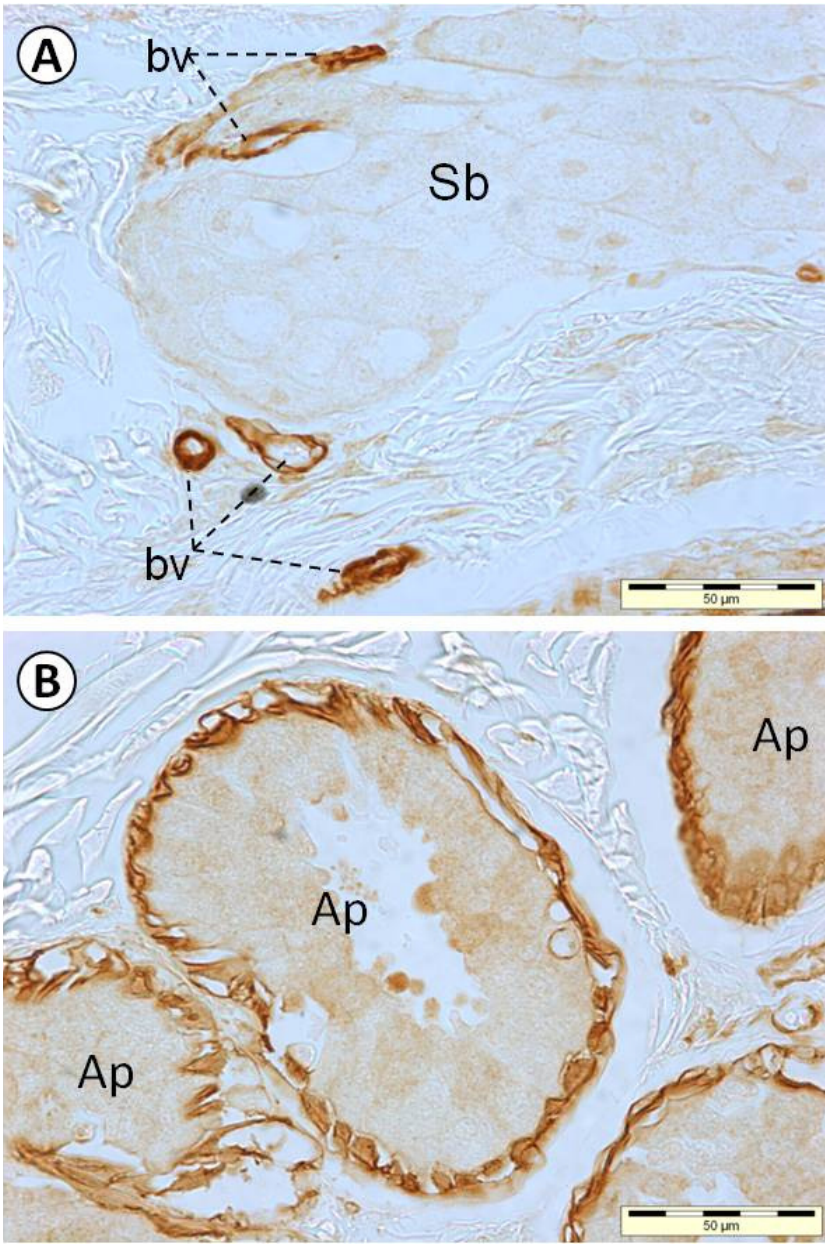

Figure 4. Microphotographs showing immunohistochemical distribution of α-smooth muscle actin in the sebaceous gland (A) and apocrine gland (B). In the sebaceous gland (Sb), positive reaction is observed in the smooth muscles around the blood vessel (bv). In the apocrine gland (Ap), α-smooth muscle actin is positive in the myoepithelial cell layer surrounding the apocrine tubules (Ap). Immunohistochemistry staining. Bars: 50 µm.

(SBA) were strongly positive in the sebaceous gland (Figures 5A and B), while others lectins showed various positive reaction from weak to strong intensities in the membrane and cytoplasm areas.

In the apocrine gland portion, positive reactions with various intensities were observed for all seven lectins while the strongest positive reactions were found in the SBA. In the myoepithelial cells of the apocrine glands, positive reactions were found in moderate intensity only for SBA and dolichos biflorus agglutinin (DBA) (Figures 5C and D).

DISCUSSION

The infraorbital glands of barking deer consisted of sebaceous and apocrine glands, with proportion of apocrine glands was larger than the sebaceous gland. These

Table 2. Lectin histochemistry of infraorbital gland of the barking deer.

Location	Con A	SBA	WGA	DBA	UEA	RCA	PNA
Sebaceous portion							
- Cells membrane	+	+++	++	++	+++	++	+++
- Cytoplasm	+	+++	++	+	++	+	+++
Apocrine portion							
- Secretion	++	+++	++ to +++	+	++	++	+
- Apocrine blebs	++	+++	++	++	++	++	++ to +++
- Cytoplasm	+	+++	+	+	+	+	++ to +++
- Golgi area	+	+++	++ to +++	+	+	++	+
- Myoepithelial cells	(-)	++	(-)	++	(-)	(-)	(-)

Con A, concanavalin A; SBA, soybean agglutinin; WGA, wheat germ agglutinin; DBA, dolichos biflorus agglutinin; UEA, lex europeus agglutinin I; RCA, ricinus communis agglutinin I; PNA, peanut agglutinin; -, negative; +, weak; ++, moderate and +++, strong.

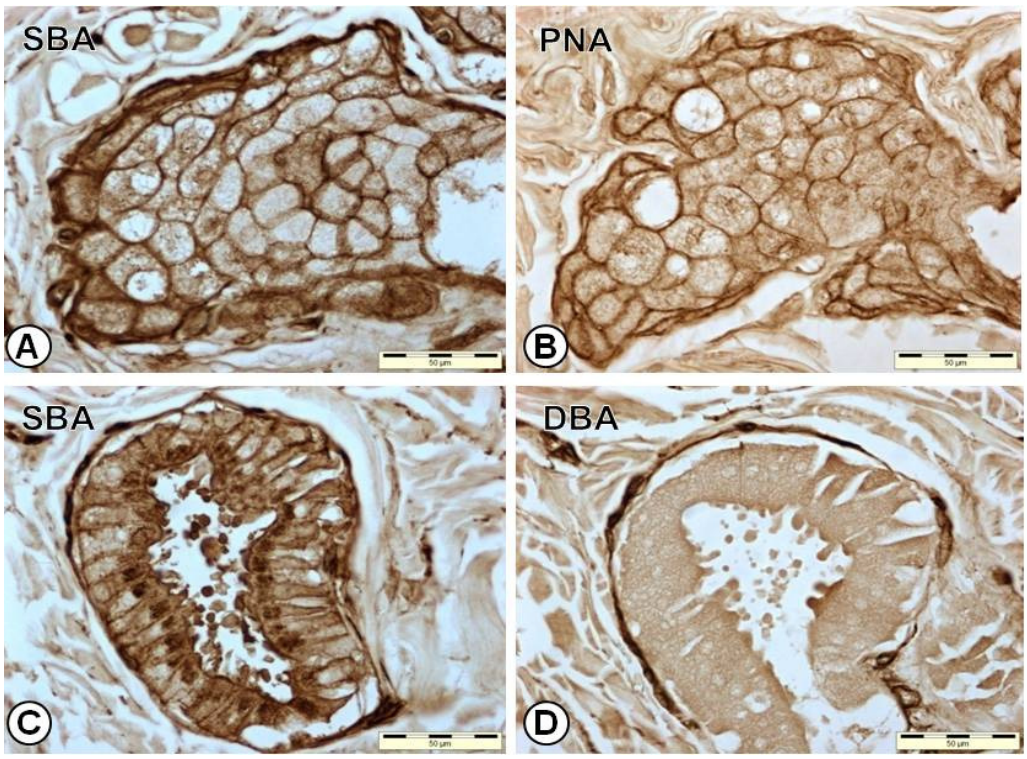

Figure 5. Microphotograph shows the distribution of glycoconjugate in the sebaceous gland portions (A and B) and the apocrine gland portions (C and D) in the infraorbital gland of the barking deer. Lectin histochemistry staining. Scale bars: 50 µm.

finding suggest that the secretions of the infraorbital gland of the barking deer were in combination of sebaceous and apo-crine glands. Similar compositions was also reported in the intermandibular gland of lesser mouse deer (Agungpriyono et al., 2006), but contradictive with the infraorbital gland of the Japanese and Formosan serow (Atoji and Suzuki, 1990; Atoji et al., 1996; Agungpriyono et al., 2006). The sebaceous gland portion was located in the superficial region below the skin and the apocrine gland portion was deeper in the submucosa. Similar findings have been reported in the intermandibular gland of the lesser mouse deer (*Tragulus javanicus*) (Agungpriyono et al., 2006), but contradictive with the results reported in the infraorbital gland of the Japanese serow (Atoji et al., 1987) and Formosan serow (Atoji et al., 1996). The glands consisted of an inner sebaceous portion and an outer apocrine portion. In this study, light microscopic analysis clearly demonstrates that the sebaceous gland

in the infraorbital gland of the barking deer is of the ordinary type. Similar finding had been reported in the infraorbital gland of the red duiker (Mainoya, 1978) and the infraorbital gland of the male Japanese serow (Atoji et al., 1989). The apocrine portion of the glands is composed of tubules in which the myoepithelial cells are stained intensely by immunohistochemical staining with antibody against α-smooth muscle actin. This finding suggests that the secretion processes from the apocrine portion of the gland was supported by the contraction of the myoepithelial cells surrounding the tubule of the gland. Similar finding had been reported in the intermandibular gland of the lesser mouse deer and infraorbital gland of the Japanese and Formosan serow (Atoji and Suzuki, 1990; Atoji et al., 1996; Agungpriyono et al., 2006).

Previous study that had been reported by Atoji and Suzuki (1990) and Atoji et al. (1996); the infraorbital gland of the Japanese and Formosan serows shows different levels of immunostaining intensity in the apocrine gland. However, in the present study we did not find comparable levels of immunostaining in the apocrine gland. All tubules of apocrine glands were stained intensely with α-smooth muscle actin immunostaining. Glycoconjugates are important for the structure and function of many biological processes including differentiation and maturation. Lectins are widely used to observe the distribution of glycoconjugates in various tissues, because their specific affinity to the sugar residue of glycoconjugates (Spicer and Schulte, 1988, 1992). The results show that the sebaceous and apocrine gland portions of the infraorbital glands of the barking deer stained with Con A, SBA, WGA, DBA, UEA, RCA and PNA. These findings suggest that the sebaceous and apocrine glands contain large amounts of glycoconjugates. Atoji et al. (1987) reported that the modified sebaceous gland contains large amounts of glycoconjugates and the apocrine gland shows a cyclic secretory process of apocrine secretion and exocytosis in the infraorbital gland of the Japanese serow. The findings of the current study suggest that the secretion of the infraorbital gland of the barking deer consists of lipid from the sebaceous gland and glycoconjugates secreted by both sebaceous and apocrine glands. The infraorbital glands of the barking deer may play an important role in marking the area and territory and in the production of odoriferous signals for their communication.

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