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RESEARCH ARTICLE

Unrevealing of the Effect of Olfactory Bulb Lesion on Histomorphological Changes in Sublingual Glands: An Experimental Study

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HIGHLIGHTS

- The olfactory system has been shown to be essential for the functioning and structure of the salivary glands.
- The macroscopic inspection of the sublingual glands revealed that the mere presence of olfactory bulb lesion (OBL) adversely affects the volume of sublingual glands.
- The histological examination has shown that the number of follicles in the sublingual glands has reduced due to OBL.
- Parasympathectomy-like changes occur in the olfactory bulb lesion.

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ABSTRACT

This study was performed to examine basic neuropathological mechanisms of undetermined effects of olfactory bulb lesion in sublingual glands. Twenty-six adult rats were randomized in three groups as control group (Group I, n=8), Sham group (Group II, n=7) and olfactory bulb lesion (OBL) group (Group III, n=11), and they all observed for the duration of eight weeks and finally euthanized. The olfactory bulbs of rat brains and sublingual glands (SLG), were extracted bilaterally. Samplings stained with hematoxylin/eosin and TUNEL stains to assess the stereogical olfactory bulbs volume loss degrees, and follicular volume values (FVV) of SLG per cubic centimeters. The estimated mean volumes of olfactory bulbs were as 4.87±0.33/mm3 in group I, 4.32±0.43/mm3 in group II, and 2.12±0.19/mm3 in group III ($p<0.0001$ and $p<0.0005$; in Group III vs. I, Group III vs. II, respectively). The mean volumes of FVV and degenerated neuron density of SLG were 215±56×106µm3/cm3 and 13±3/mm3 in Group I, 193 ± 44×106µm3/cm3 and 62±11/mm3 in Group II ($p<0.005$), 134±27×106 µm3/cm3 and 346±83/mm3 in Group III ($p<0.0001$ and $p<0.0005$; in Group III vs. I, Group III vs. II, respectively). This study suggests that OBL may be responsible for sublingual gland degeneration, which has not been stated so far.

Contents

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1. Introduction

Human brain does not fully reveal an intrigue exploration due to presence of certain neuroanatomical pathways related to the interaction between the salivation center and forebrain structures, and the greater portion of information regarding the projections from brain cortex to brain stem available in the literature comes from animal studies [1, 2]. Brain cortex is activated by the transmission of ascending second and third order neurons coming from sensory nuclei, therefore, reflexes can be regulated by efferent inputs. Correspondingly, salivation is not only affected by chewing and taste afferent impulses but also influenced by thermoreceptive, nociceptive, psychic and olfactory stimuli [3, 4]. Sublingual glands supplied by secretory parasympathetic fibers of facial and glossopharyngeal nerves arise from sphenopalatine, otic, and geniculate ganglia [5] projecting to intrinsic ganglia of sublingual glands [6] and secretion breaking sympathetics arise from cervical sympathetic trunk [7]. Sympathetic stimulation or parasympathetic denervation causes decreased salivary secretion [8]. Therefore, salivary glands undergo atrophy following parasympathectomy [9]. Taste and olfactory stimulants could also change the functions of salivary glands [10]. Olfactory stimulants play a major role on secretion [11].

We hypothesized that disruption of olfactory nerve impulses may cause decrease in secretion and histomorphological degenerations in sublingual glands. In this study, the effect of olfactory bulb lesions on histomorphological structures in sublingual glands were evaluated and analyzed.

2. Material and Method

2.1. Ethical Statement

The Animal Experiments Local Ethics Committee of our university, has granted the permission for the conduction of this research and related activities thereof. The authors hereby declare that all guidelines brought in by the committee have been strictly followed.

2.2. Experimental Procedures

The study included a total of 26 rats kept in appropriate metal cages under suitable environmental and cage temperature of 22±2'C and a humidity of 50%. The subjects had artificial-day light for 12 h a day and kept under regular veterinary supervision. The rats were feed with a routine laboratory rat diet and water ad libitum.

The rats randomized into three groups, eight rats were picked for the control group (Group I, n = 8). Second and third groups were the Sham and study groups. In order to reduce pain in rats and to decrease mortality rats, injectable anesthetics medications were preferred in 2nd and 3rd groups. Anesthesia was triggered with isoflurane via a face mask, and 0.2 mL/kg of anesthetic injections of xylazine hydrochloride (30 mg/1.5 mL), ketamine hydrochloride (150 mg/1.5 mL), and distilled water (1 mL) were subcutaneously applied before surgery. In order for providing access to the olfactory bulb (OB), the anterior cranium had to be shaved and drilled to access the olfactory bulb on frontal bone. In Group II, the Sham group (n = 7), only a hole was drilled, but OB was left untouched. In Group III (n=11), bilateral olfactory bulbs were damaged with a micro clamp to obtain OB lesion.

All groups were observed for eight weeks without giving any medical treatments. They were followed for noticing their vital signs with 10-min periods at two times a day during the observation. All rats were euthanized under general anesthesia after the observation period of eight weeks. Both macroscopically and microscopically, evaluations were done to olfactory bulbs to examine if OBL was done and sublingual glands specimens were evaluated stereologically. The brain tissues and SL glands were removed and placed in 10% formalin solutions for one week for further histological examinations.

2.3. Histopathological and Stereological Methods

The sections from SL glands in 5 μm thickness and in 30 μm lengths were prepared for histological evaluation. All 30th and 31st samples were picked for the estimation of the volumes of SLG follicles. The fractionator method was used to estimate the total number of follicles of the SL glands [6, 12, 13]. Similarly, brain tissues were sectioned through the long axis of the Olfactory nerve for examining the olfactory bulb lesions. The brain and SLG tissues were dehydrated by a series of alcohol in certain grade, and then immersed into liquid paraffin. Prepared OBs and SLG sections were stained by using hematoxylin–eosin and TUNEL stain and examined with a light microscope to take the photos at different magnification levels between 20X and 40X.

Stereological methods were used for experimental analyses. The first pair sections were picked at a random position beyond the 30th section. Later, every 30th section and the

next neighbor section (31st) were chosen for the analysis. Hence this section fraction (f1) was 1/30. The sections without any SLGs and ganglia tissues were excluded. The average of this sampling fraction had 10–11 section pairs. The sampling fraction area (f2), has a scale of 1/1. To evaluate the number of SLG follicles, physical dissector method was applied. Two consecutive sections (dissector pairs) taken from tissue samples were mounted on each slide and named as reference section. In order to double the number of dissector pairs without taking new ones, reference and look-up sections were reversed.

For the total number of follicles in each specimen, the Cavalieri volume estimation method was utilized. By multiplication of the volume (mm^3) and the numerical density of follicles in each SLG was calculated to assess the total number of follicles.

The calculation of follicle numbers of SLG follicles (N) in SLG glands was achieved by ΣQ^- Equation 1.

$$N = \Sigma Q^- \times \frac{1}{f1} \times \frac{1}{f2} \quad (1)$$

After the calculation procedure, total sublingual gland volume (TSLGV) was assessed via summation of the volume of ellipsoid shaped vesicle volumes, which are calculated by using Equation 2.

$$\sum_1^n TSLGV = \sum_{f=1}^{n} n[\![\frac{4}{3}\pi\langle\frac{a+b+c}{3}\rangle^3]\!]^* \quad (2)$$

Whereas a, b, and c are halves of the ellipsoid axes in x, y, and z axis.

The Total Olfactory Bulb Volume (TOBV) was estimated as yielded by Equation 3:

$$TOBV = \sum_{N=1}^{N=N} NxV\, n \quad (3)$$

The formula of the mean numerical density of the SLG follicles per/cm^3 / SLG tissue (Nt/Gt) per/mm^3 was given below:

$$\frac{Nt}{Gt} = \frac{\frac{\Sigma Q^-}{\Sigma A}}{xd} \quad (4)$$

Where, d is the section thickness, ΣQ^-N is the total number of calculated neurons existing only in the reference sections, and A is the counting frame area.

The estimation of ΣA for the set of dissector is via

$$\Sigma A = \Sigma Pa \quad (5)$$

Where a is a constant area and ΣP is the total number of counting set frame points.

2.4. Statistical Methods

The differences between the TSLGV and OB volume values were statistically analyzed. by using statistics package program (SPSS® for Windows v. 12.0). The determination of normal distribution was analyzed by Kolmogorov–Smirnov and Shapiro–Wilk test ($p < 0.05$). Mann–Whitney-U test was applied for these two independent study groups, and then for three groups by using the Kruskal–Wallis variance test. The statistical significance was accepted as the p value <0,05.

3. Results

3.1. Clinical Results

Two rats of the study group were dead during the experiment. In clinical examination, scar formation, osteophytes in the burr-hole region, adhesive bands between dura mater and inner tabula, decreased size of olfactory bulbs and olfactory cell over the dura mater, and closed ethmoidal foramina were determined in the gross anatomical examination.

3.2. Histopathological Findings

Figure 1 shows the macroscopical and histological appearances of normal olfactory bulbs. Whereas, the macroscopical and histopathological appearances of lesioned olfactory bulb are shown in Figure 2. It is clearly understandable from this photos, the lesioned tissue sections were shrinked and atrophied both macroscopically and microscopically.

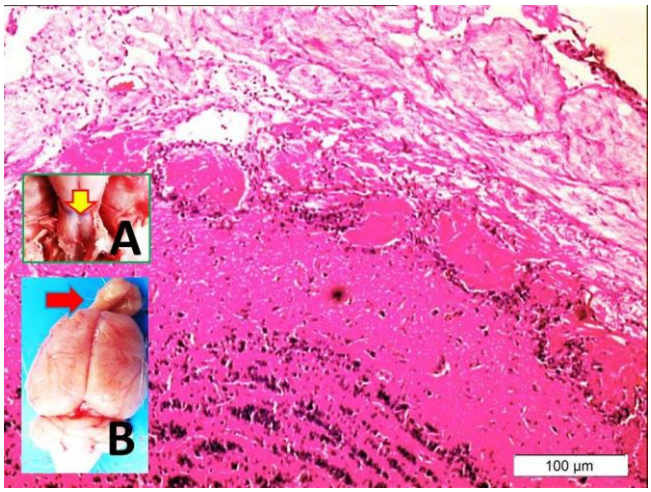

Figure 1 Olfactory bulbs seen in macroscopically, pointed with yellow arrow in cranium (A), after extraction of brain (B) and histological appearance of olfactory bulb (LM, H&Ex10) at the base with normal animal.

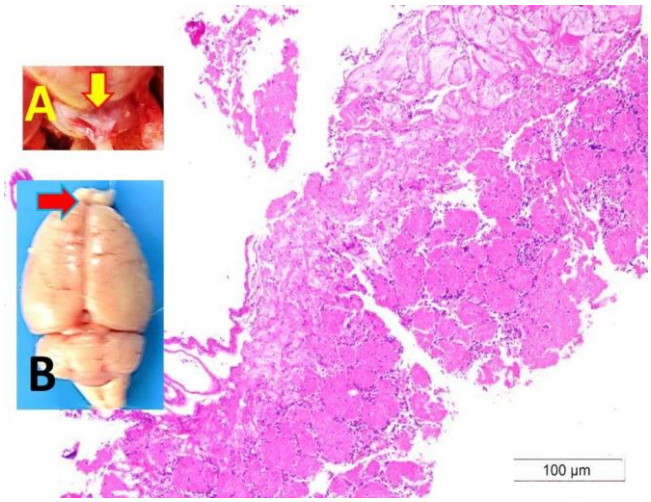

Figure 2 Lesioned Olfactory bulbs seen in macroscopically, pointed with yellow arrow in cranium (A), after extraction of brain (B) and histopathological appearance of lesioned olfactory bulb (LM/H&Ex10) at the base with OBL animal.

Histological appearances serous and mucous parts with intraglandular ganglia of sublingual gland and macroscopical appearances of gland are shown in Figure 3.

In Figure 4, sublingual gland and its degenerated ganglia neurons are shown in OBL applied animals. Figure 5 shows sublingual gland with serous and mucous parts. In Figure 6, volume estimation method of sublingual gland follicles is given with Equation 2 in the overlay.

Figure 7 shows histopathological appearance of degenerated sublingual gland with serous and mucous parts and magnified form of degenerated sublingual gland belongs to the OBL applied animal. Histopathological examinations of SLG ganglia showed neuronal angulation, shrinkage, cytoplasmic condensation, and apoptosis in neuronal structures.

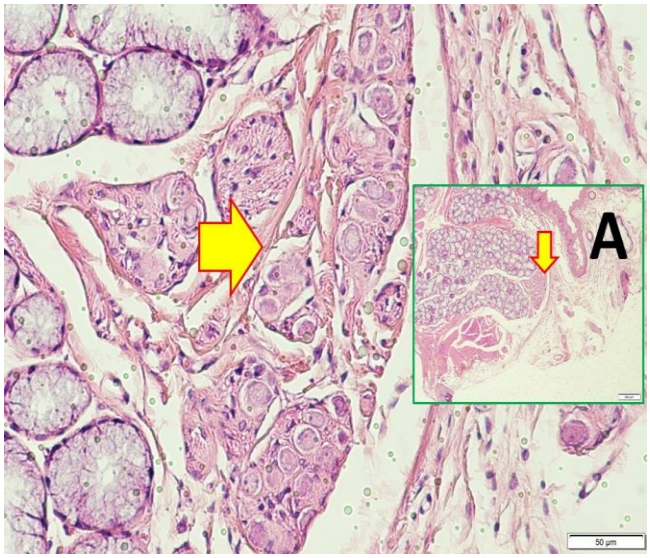

Figure 3 Sublingual gland with serous/mucous parts with intraglandular ganglia (yellow arrow (A) (LM, H&E, x4) and magnified form of glandular ganglia and neurons (LM, H&E, x10/Base).

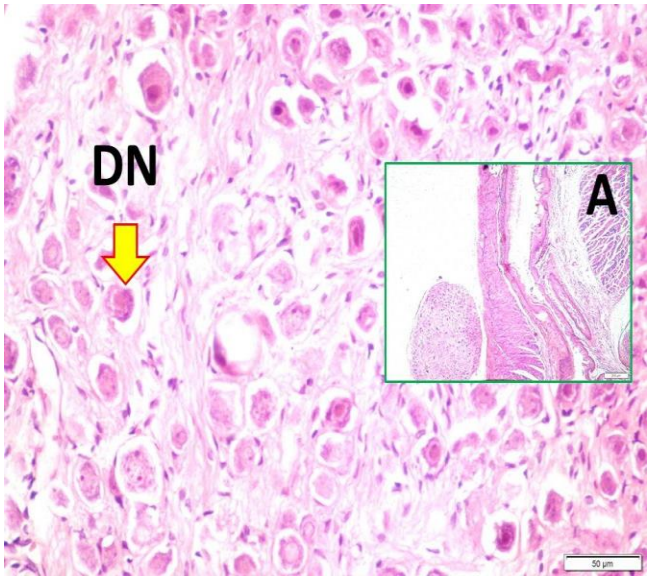

Figure 4 Degenerated sublingual gland ganglia (A) (LM, H&E, x4), magnified form of degenerated ganglia and neurons (LM, H&E, x10/Base) in OBL applied animals.

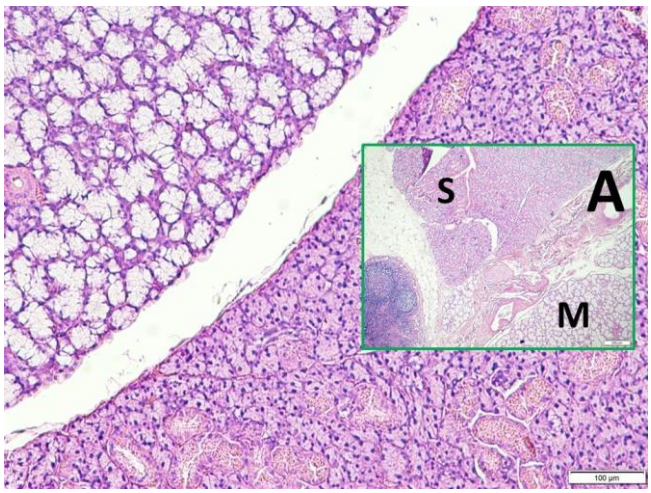

Figure 5 Histological appearance of normal sublingual gland with serous (S) and mucous (M) parts (A) (LM, H&E, x4), magnified form of sublingual gland (LM, H&E, x10/Base)

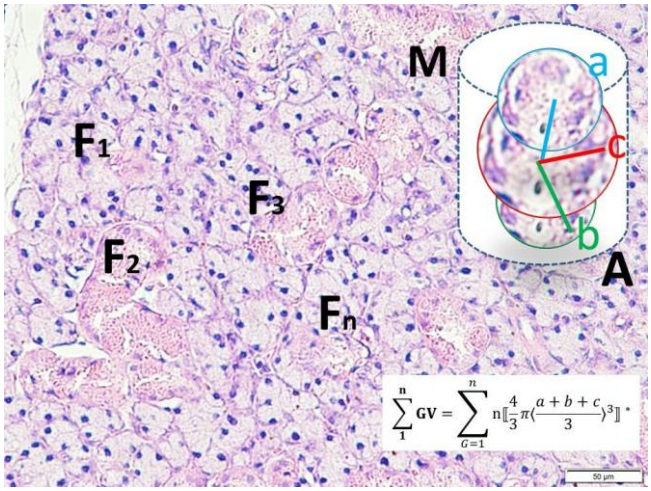

Figure 6 Sublingual gland follicles volume estimation method (A) and mucous follicles (LM, H&E, x10/Base).

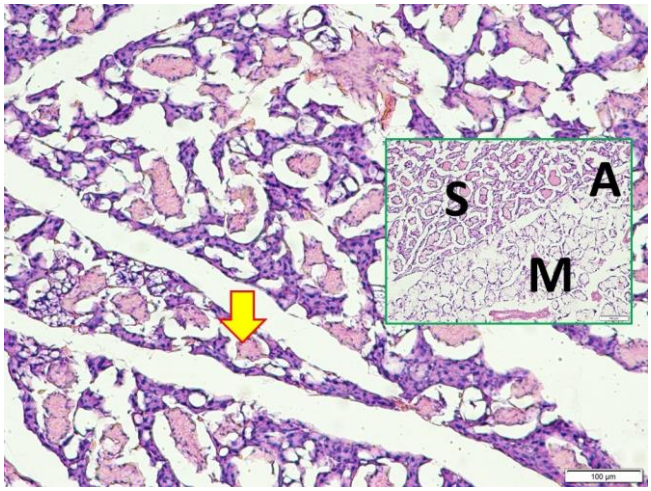

Figure 7 Degenerated sublingual gland with serous (S) and mucous (M) parts (A) (LM, H&E, x4), magnified form of degenerated sublingual gland belongs to the OBL applied animal (LM, H&E, x10/Base)

3.3. Numerical Results

The mean olfactory bulb, FVV and degenerated neuron density of SLG for each group are shown in Table 1. The estimated mean olfactory bulb volumes were

4.87±0.33/mm3 in group I, 4.32±0.43/mm3 in group II, and 2.12±0.19/mm3 in group III (Group III vs. I-II, p<0.0005). The mean FVV were 215±56×106µm3/cm3, and degenerated neuron density of SLG was 13±3/mm3 in Group I, 193 ± 44× 106µm3/cm3 and 62±11/mm3 in the Group II (p<0.005), and 134±27×106 µm3/cm3 and 346±83/mm3 in Group III (p<0.0001, p<0.0005 in Group III vs. Group I, Group III vs. Group II, respectively).

Table 1 The mean olfactory bulb, follicular volume values (FVV) and degenerated neuron density of Sublingual Glands (SLG)

	Group I	Group II	Group III
Olfactory bulb (mm3)	4.87 ± 0.33	4.32 ± 0.43	2.12 ± 0.19 α, β
FVV (106 µm3/cm3)	215 ± 56	193 ± 44*	134 ± 27α, β
Degenerated Neuron density of SLG (mm3)	13±3	62 ± 11	346 ± 83 α, β

* p < 0.005 (Group I vs. II) α p < 0.0005 (Group II vs. III) β p < 0.0001 (Group I vs. III)

Group I: Control Group, II: Sham group, III: Study group,

4. Discussion

In Parkinson's disease, salivary secretion decreases with anosmia. This suggests a relationship between the olfactory system and salivary secretion levels as seen in Parkinson's disease. There are studies in the literature that multiple stimuli play a role in saliva secretion.

The stimuli from the superior and inferior salivatory nuclei which are localized in the brain stem are controlling the SLG mainly by parasympathetic nervous innervation [14]. The chorda tympani carry parasympathetic stimulus to all salivary glands below the level of the oral fissure. Various parasympathetic impulses increase in volume of salivary glands [15]. In contrast, parasympathectomy, bilateral extraction [16] or damages of chorda tympani [17], and parasympathetic nerve blocking drugs could result in salivary glands atrophy [18–20]. All these studies explain the various stimuli innervation of salivary glands which can shrinkage or hypertrophy of the gland. However, none of the studies in the literature searched the relationship of olfactory impulses with the volume changes of salivary glands.

We claimed that, disruption of olfactory nerve impulses will cause histomorphological degenerations in sublingual glands. In order to investigate this topic, we conducted an experimental olfactory bulb lesion in rats to evaluate the changes in salivary gland volumes for the first time in literature. Three different groups, namely, the study group, the sham and control group were settled. By the results we obtained, we found SLG degeneration in the study group. We determined that SLG was diminished in size in the study group, in comparison to the control and Sham groups, due to the fact that efferent stimuli from olfactory nerves were hindered. This suggests that olfactory nerve stimuli contributed the growth of SLG and prevented the atrophy of SLG. We also observed that not only does these stimuli become effective in the growth of SLG, but also it has an effect on the follicle count and volume included in histopathological context. This depicted us that the reduction occurred in follicle count and volume of SLG is caused by absence of olfactory nerve stimuli.

We have concluded that olfactory information must have important roles on salivary gland morphology and functions because decussation of olfactory network causes cellular degeneration in salivary glands. In our study, we detected SLG atrophy in the rats applied by OBL similar to the ones used in parasempatectomy. This leads us to assess the effects of anosmia on salivary glands to develop its structural atrophy and loss of function, which can also impair oral hygiene and arise many diseases.

4.1. Limitations

Although essential data have been collected and current neurohistological methods have been used sufficiently, olfactory examination was not performed scholarly because of insufficient odorant detectors. Olfactory nerve–facial nerve relations were proven only relying on literature data and information but in contrast, neural pathways should be shown via modern neurohistological methods. Some enzymes and hormones should be measured by biochemical analysis.

5. Conclusion

Bilateral OBL cause histomorphological degenerations in sublingual glands. When there is no transmission of stimuli coming from olfactory nerve, both SLG is atrophied in size and follicle count is reduced.

In this study, in which we made olfactory bulb injury in rats, it was found that sublingual salivary glands were shrinked in follicle size and volume due to absence of olfactory stimulus. Parasympathectomy-like changes occurred in the olfactory bulb lesion. This suggests that the olfactory nerve stimulus serve a function in the activation of the salivary glands, essential for the function and structure of the salivary glands. As a hypothetical conclusion, there can be a reflex neuronal web between olfactory system and salivary glands. For the future studies, neuroanatomical researches should be conducted to determine the probable neural network system between the olfactory system and saliva system.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Compliance with Ethical Standards

The Local Ethics Committee for animal experiments approval was obtained before the study settled.

For the care and use of animals, all applicable international, national, and/or institutional guidelines were followed carefully. All procedures performed in animal studies were in accordance with the ethical standards of the international and national practice guidelines.

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