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# Antimicrobial protein secretion in the rat submandibular gland induced by aroma inhalation

**Takayuki Izutani, Kenji Uchihashi and Yasuo Nishikawa**

*Department of Physiology, Osaka Dental University, 8-1 Kuzuhahanazono-cho, Hirakata-shi, Osaka 573-1121, Japan*

**Application of aroma inhalation is likely to enhance antimicrobial action. We studied the influence of sympathetic nerve stimulation and aroma inhalation on both SIgA and lactoferrin secretion in the rat submandibular gland. We found no spontaneous secretion from the submandibular gland in the control rats. Although the flow of the saliva evoked by black pepper and cardamom was very small in volume, a marked saliva secretion was found evoked when isoprenaline stimulation was combined with black pepper or cardamom inhalation. Significant secretion of SIgA and lactoferrin was not obtained with black pepper or cardamon inhalation alone. However, there was considerable secretion of SIgA and lactoferrin induced when isoprenaline stimulation was combined with black pepper inhalation.**

**The salivary amylase activity induced by aroma inhalation was approximately 10% that of isoprenaline stimulation alone. Combined use of the two enhanced salivary amylase activity compared with stimulation by isoprenaline alone. Their combined use had a synergistic effect.**

**These results suggest that the use of aroma inhalation enhances salivary antibacterial activity, and that this activity may be induced by the sympathetic nervous system. In addition, aroma inhalation imparts a greater effect on SIgA and lactoferrin secretion than does solitary sympathetic stimulation alone. We hypothesized that aroma inhalation might positively influence the intraoral environment. (J Osaka Dent Univ 2017 ; 51 : 81-88)**

**Key words : Microbial protein secretion ; Aroma therapy ; Sympathetic nerve ; Submandibular gland**

## INTRODUCTION

Saliva plays an essential physiological role in normal upper gastrointestinal tract function and oral health.[1] Human saliva contains a large variety of proteins, peptides, epithelial cells and leukocytes, each of which carries several significant biological functions. Because of the advancement of novel technologies, such as bioinformatics, metabolomics, genomics and proteomics, the study of saliva has become an increasingly attractive option because of its ability to mirror both oral and systemic health.[2] Saliva is secreted by acinar cells in the three major salivary glands, as well as the minor glands.[3, 4] Secretion of saliva is dependent upon stimuli from autonomic nerves that are the effector arms of reflexes activated predominantly by taste and chewing.[5] Nerve mediated stimulus evokes saliva secretion of water and proteins by different mechanisms.

Secretion of submandibular saliva is controlled by the autonomic nervous system. The parasympathetic nervous system is the main controller of this secretion via impulses in the chorda tympani nerve that innervates it and releases acetylcholine and substance P. Both can evoke copious salivary secretion by activating muscarinic and tachykinin-1 receptors. In contrast the sympathetic nervous system also controls salivary secretion by acting on α-

and β-adrenergic receptors. Sympathetic nerve stimulation induces a relatively low flow of saliva that is rich in protein and is accompanied by extensive degranulation from both acinar and granular duct cells.[6]

Saliva contains many antimicrobial proteins such as SIgA, which is a specific immunity substance, and lactoferrin, which is a nonspecific immunity substance. SIgA is the main component of the adaptive immune system. Polymeric, J-chain-containing IgA, which is secreted by plasma cells, binds to the polymeric immunoglobulin receptor (pIgR) present on the basolateral membrane of epithelial cells and is transcytosed to the apical membrane.[7] The pIgR-IgA complex is then cleaved with the release of SIgA into the epithelial cell secretion. In rat submandibular glands, SIgA is increased by stimulation from sympathetic nerves.[8] SIgA, together with other glycoproteins such as mucin, lactoferrin and peroxidase, are responsible for helping to maintain the integrity of mucosal surfaces against infectious agents.[9] Several studies in rat salivary glands have shown that secretion of SIgA is increased by stimulation from sympathetic nerves.[8] Because increasing SIgA secretion in the oral cavity helps prevent oral diseases, we examined how both sympathetic nervous stimulation and olfactory stimulation affect secretion of SIgA by the rat submandibular gland as compared with other proteins.

Lactoferrin, an 80-kDa iron-binding glycoprotein of the transferrin family, is a component of exocrine secretions such as milk and saliva, and is present in neutrophil granules.[10] It has been studied for its antimicrobial activity against periodontopathic bacteria and its relationship with periodontal diseases.[11] Furthermore, lactoferrin is thought to play a role in innate defense and exhibits a diverse range of biological activities, including antimicrobial activities, antiviral activities, antioxidant activities, immunomodulation, modulation of cell growth, and binding of several bioactive compounds, such as lipopolysaccharide.[12] Therefore, lactoferrin is an important host defense factor in the oral cavity.

Aroma therapy aims to promote health by lowering stress and making the mind and body relax through the use of aromatic components of plant origin. It has been demonstrated to have various benefits, such as the promotion of blood circulation, suppression of muscle spasms, antibacterial action, promotion of immunity, and antidiuretic properties. On the other hand, saliva has various functions in maintaining healthy oral tissues. This quality has a big influence on dental caries and periodontal diseases, and influences quality of life. Therefore, we hypothesized that aroma therapy might positively influence the intraoral environment. During food intake, flavor perception results from the simultaneous activation of the gustatory, olfactory, and trigeminal nerves. In particular, olfactory stimulation through the retronasal pathway greatly contributes to the perception of flavor in foods.[13, 14] The perception of specific flavors typically results from specific aromas.[15, 16]

The primary function of salivary alpha amylase is to break down high molecular weight carbohydrates to lower molecular weight sugars.[17] In addition, it seems to play a role in maintaining mucosal immunity.[18] Studies have suggested that amylase inhibits streptococcal bacterial adherence, which interferes with the propagation and colonization of bacteria and may help regulate normal bacterial flora in the mouth. Salivary amylase has been shown to increase rapidly during acute stress, and it has been suggested that it may even be used as a marker of sympathetic nervous system activity, although this concept is still debated.[19] We utilized salivary amylase activity as a measure of the excitation level of the sympathetic nervous system.

Olfactory stimulation by fragrance inhalation exerts various physiological effects on humans. Quantitative measurements of the physiological effects of fragrance include physical measurements such as electroencephalograms,[20] blood pressure[21] and heart rate.[22] Aromatic plant-derived essential oils exhibit a variety of biological properties, such as mood enhancement, pain relief, and improved cognitive function. They are used in traditional medicine and as complementary treatment in primary care settings.[23] The inhaled aromas from the essential oils are believed to be useful for a vast array of symp-

toms and conditions. A large body of published literature has focused on the human brain and emotions,[24, 25] blood pressure,[26] and heart rate measurements.[27]

We used aromatic oils to study the effect of olfactory stimulation on the promotion of salivation, because their use can easily be applied to patients who have difficulty following instructions and opening their mouth. We attempted to elucidate the effects of aroma inhalation on the secretion of microbial protein in the rat submandibular gland saliva.

## MATERIAL AND METHODS

### *Experimental animals*

Approval was obtained from the Animals Research Committee of Osaka Dental University (No.15-04008), and the experiments were performed according to the Ethical Guidelines of the International Association for the Study of Pain.[28] We used the Laboratory Animal Facilities of Osaka Dental University. Eighteen adult male Wistar rats weighing 250-280 g were housed in the animal care center under controlled light and environmental conditions under dark/light cycles of 12 hr each, at 23 ± 1°C, and at 55% relative humidity. Dry food and water were available *ad libitum.* The experiment began with one day of familiarization with the enclosure. The eighteen experimental animals were divided into three groups. There were three animals in the control group (the non-inhalation group). Three animals received IPR only. Three were twelve animals in the aroma group. Three received BPP alone, three received CAL alone three received IPR + BPP, and three received IPR + CAL.

### *Study design*

We collected saliva after aroma inhalation for 2 hours. After anesthesia, we collected saliva with and without sympathetic stimulation. Collected saliva was analyzed for SIgA concentration, lactoferrin concentration, amylase activity and the total amount of protein (Fig. 1).

### *Aroma inhalation*

We used two essential oils that are known to have opposite effects on the behavior of the autonomic nervous system which induces the sympathetic nervous system, black pepper (BPP) and cardamom (CAR) (both from Dr. Eberhardt GmbH, Trausdorf, Austria). Each aroma (10 μL/100 mL $H_2O$) was diffused into the rat's enclosure using a commercially available aroma diffusion device (Aroma Lamp Diffuser, Global Product Planning, Tokyo) (Fig. 2).

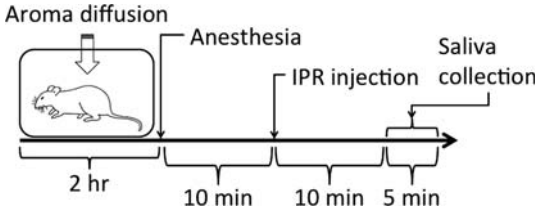


**Fig. 1** Experimental design.

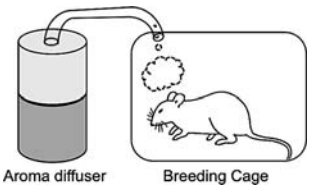


**Fig. 2** Aroma diffusion apparatus.

### *Saliva collection*

After anesthetization by intraperitoneal injection of sodium pentobarbital (65 mg/kg, body weight), a saliva sample from the submandibular gland was collected through a polyethylene cannula (Intramedic PE-10 ; Becton Dickinson, Franklin Lakes, NJ, USA) inserted into the oral opening of the submandibular gland duct.

### *Secretory stimulation induced by sympathetic secretagogue*

The rats that had inhaled the aroma and those that had not were further divided into two groups, the sympathetic stimulation and non-stimulation animals. Sympathetic stimulation was performed with an intraperitoneal injection of 2 mg/kg body weight of isoprenaline hydrochloride (IPR) (Wako Pure Chemical Industries, Osaka, Japan). Five minutes after stimulation, the secreted saliva was collected

for 5 min (Fig. 1).

### *Assay of salivary proteins*

The concentration of salivary SIgA was measured by enzyme-linked immunosorbent assay (ELISA). Primary (anti-human IgA) and secondary antibodies (peroxidase-conjugated anti-human IgA) were purchased from Sigma (Poole, UK) using a rabbit anti-rat IgA (Serotec, Oxford, UK). The assay was calibrated using serial dilutions of human colostrum IgA (Sigma). The concentration of salivary lactoferrin (SLF) was measured using a commercial ELISA assay kit (DRG Diagnostics, Marburg, Germany) according to the manufacturer's instructions. Salivary α-amylase activity (SAA) was measured using a hand-held salivary amylase monitor (Nipro, Osaka, Japan). This analyzer enables automatic measurement of salivary amylase activity using a dry-chemical system, within one minute from collection to completion of the measurement. The tip of the testing strip was set under the tongue for 30 sec to collect saliva. The strip was then immediately inserted into the analyzer, which displays the result automatically. The total protein (TP) content of the fluid responses was analyzed by the method of Lowry.[29]

## RESULTS

### *Salivary flow rate*

There was no spontaneous secretion from the submandibular gland. Successive intraperitoneal injections of IPR at 9 ± 1.1 μL/min/100 mg gland elicited increased secretory responses. A slight saliva secretion response was observed with aroma inhalation alone of only BPP (0.2 μL/min/100 mg gland) or CAR (0.1 μL/min/100 mg gland). There was significant difference between the IPR stimulation group and the aroma inhalation rats. However, the salivary flow rate with the combined use of IPR and BPP was significantly increased to 13 ± 1.9 μL/min/100 mg gland when compared with the use of IPR alone ($p < 0.05$) (Fig. 3).

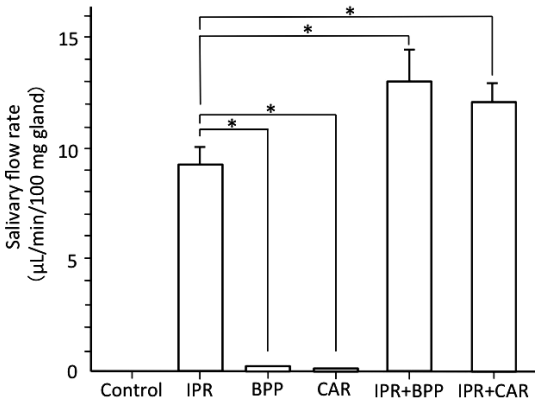


**Fig. 3** Salivary flow rate. (*$p < 0.05$)

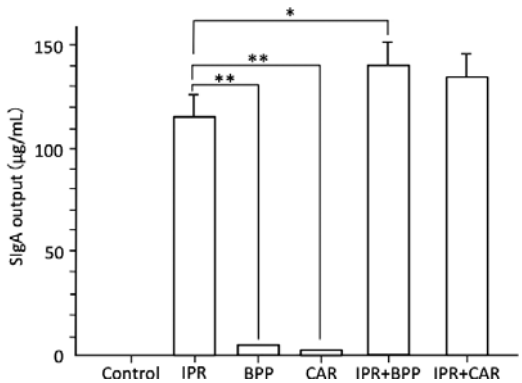


**Fig. 4** SIgA concentration in collected saliva. (**$p < 0.01$)

### *Concentration of salivary SIgA*

SIgA secretion of 114 ± 11 μg/mL was induced by IPR stimulation, 5.0 μg/mL by BPP inhalation, and 2.0 μg/mL by CAR inhalation. Aroma inhalation alone resulted in a very small secretion of SIgA. SIgA secretion was significantly increased with the combined use of IPR (140 ± 11 μg/mL when IPR was combined with BPP, and 133 ± 9 μg/mL when it was combined with CAR). The SIgA concentration resulting from the IPR stimulation alone was significantly different from that when IPR was combined with BPP inhalation ($p < 0.05$) (Fig. 4).

### *Concentration of salivary lactoferrin*

SLF secretion induced by IPR stimulation was 3.85 ± 0.5 μg/mL, 0.9 μg/mL by BPP inhalation alone, and 0.5 μg/mL by CAR inhalation alone. Very little SLF was secreted by aroma inhalation alone. However, the secretion was significantly increased when IPR and aroma therapy were used in combination. It was 6.55 ± 0.4 μg/mL when IPR was combined

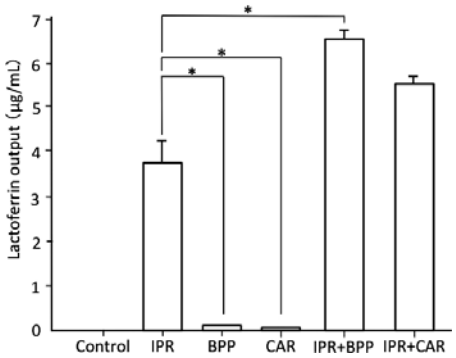


**Fig. 5** SLF concentration in collected saliva.

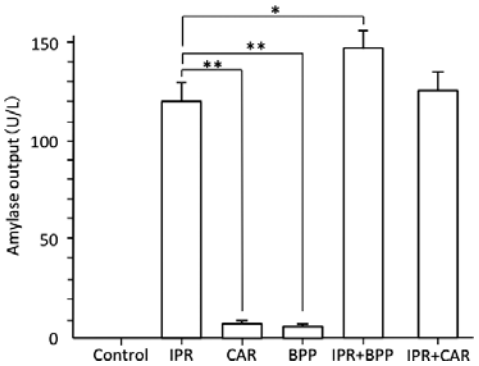


**Fig. 6** SAA in collected saliva.

with BPP, and 5.50 ± 0.5 μg/mL when IPR was combined with CAR. There was a significant difference in the SLF concentration when IPR stimulation was compared with IPR in combination with BPP inhalation (*$p < 0.05$). IPR combined with aroma therapy had a synergistic effect on SLF secretion (Fig. 5).

### *Salivary amylase activity (SAA)*

We measured SAA as an indicator of sympathetic nerve excitability. SAA induced by IPR stimulation was 120 ± 11 U/L, 8 U/L by BPP inhalation, and 2 U /L by CAR inhalation. Both BPP and CAR were found to increase SAA. SAA was significantly increased when IPR and aroma therapy were used in combination (**$p < 0.01$). It was 144 ± 13 U/L when IPR was combined with BPP, and 125 ± 10 U/L when IPR was combined with CAR. The amylase level was significantly increased when IPR was used in combination with aroma therapy (*$p < 0.05$) (Fig. 6). We found that the excitation of the sympathetic nerve was increased by aroma inhalation.

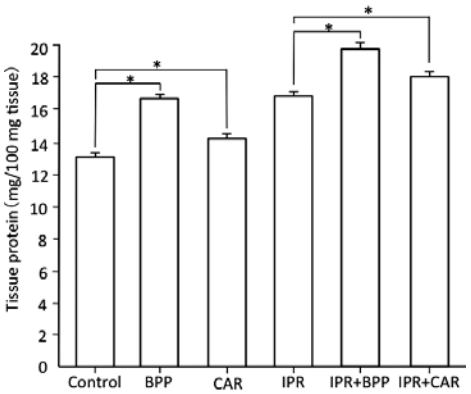


**Fig. 7** Amount of gland tissue protein.

### *Amount of protein in submandibular gland tissue*

The amount of tissue protein (TP) in the control gland was 13.5 ± 1.5 mg/100 mg tissue. It was 16.7 ± 2.0 mg/100 mg tissue after IPR stimulation, 15.6 ± 1.4 mg/100 mg tissue after BPP inhalation, and 14.2 ± 1.5 mg/100 mg tissue after CAR inhalation. There was a significant difference between the control gland and gland exposed to BPP inhalation. TP was significantly increased when IPR and aroma therapy were used in combination ($p < 0.05$) (Fig. 7). It was 19.3 ± 1.8 mg/100 mg tissue when IPR was combined with BPP and 17.9 ± 1.7 mg/100 mg tissue when it was combined with CAR.

## DISCUSSION

The control rats did not produce a measureable quantity of saliva. Sympathetic nerve stimulation induced a relatively low flow of saliva that was rich in protein and was accompanied by extensive degranulation from both acinar and granular duct cells.[6] We used IPR as a β 1, 2 -adrenergic agonist, which induced saliva secretion in this study. Rats stimulated with IPR at 2 mg/kg body weight, secreted 9 ± 1.1 μL/min/100 mg gland of saliva. This administered volume of IPR is the threshold of salivation in the rats. Salivary secretion induced by aroma inhalation was about 10 to 20% of IPR stimulation alone. However, when IPR and BPP were used in combination, the salivary flow rate in-

creased to 1.4-fold compared with IPR stimulation alone. Thus, BPP inhalation has a synergistic effect on the sympathetic nervous system when combined with IPR stimulation. The sympathetic nervous system controls salivary secretion by acting on α- and β-adrenergic receptors. In other words, it is thought that aroma inhalation stimulates the autonomic nervous system. We may consider BPP an agonist of salivary secretion.

SIgA secretion was slight with BPP or CAR inhalation alone. However, IPR stimulation alone increased the SIgA secretion by more than 22-fold that of BPP or CAR inhalation alone. IPR combined with aroma therapy increased the SIgA secretion by more than 1.2-fold that of IPR alone. We found that the greatest secretion of SIgA was evoked by IRP, and this was further increased by aroma therapy. The major antibody in saliva is SIgA, which is actively transported by parenchymal cells within the salivary glands. The autonomic nerves supplying the glands *in vivo* regulate the rate of SIgA secretion into saliva. SIgA is transported into saliva by salivary cells expressing the polymeric immunoglobulin receptor (pIgR).[30] In rat salivary glands, sympathetic nerves may be enhanced in this process.

SLF is one of the antimicrobial factors in saliva.[10] Takakura *et al.* previously studied its effects on the oral pathogen Candida albicans.[31] Moreover, SLF has been studied for its antimicrobial activity against periodontopathic bacteria and its relationship with periodontal diseases.[10] Under inflammatory conditions, SLF is also secreted into the gingival crevicular fluids from neutrophils.[12] Previous studies have reported that SLF concentrations are modulated immediately after strenuous exercise.[13, 14] IPR caused a 25-27-fold greater secretion of SLF than BPP inhalation alone or CAR inhalation alone. However, IPR combined with aroma therapy significantly increased the secretion by 1.1-fold compared with IPR alone. Although SLF secretion was increased by CAR inhalation, the increase was less than that by BPP inhalation. Although IPR alone enhanced SLF secretion, this secretion was further increased by the combined use of IPR and of aroma therapy. We found that the combination of IPR and aroma therapy had a synergistic effect on both SIgA and SLF secretion. The secretion of both SIgA and SLF were proportional to the total protein secretion induced by BPP.

The autonomic nervous system regulates the secretion of bioactive proteins from salivary glands which are important in systemic physiological responses. As well, the autonomic nervous system regulation of exocrine functions of glands is an important response to physiological insults and stress.[32] We found that IPR stimulation increased the total protein secreted from $13.5 \pm 1.5$ mg/100 mg tissue in the control to $16.7 \pm 2.0$ mg/100 mg tissue. The above results suggest that aroma inhalation may enhance the excitation of the sympathetic nerves that dominate the submandibular glands, and there is a possibility that the sensory afferent path of the aroma stimulation is the upper centroid.[33]

The microbial protein composition in the saliva induced by sympathetic stimulation had different profiles. The secretion of SIgA is up regulated to a much greater extent by sympathetic stimulation, while SLF secretion induced by sympathetic stimulation was at a lower level than with SIgA. SLF is a component of saliva and is suspected of being a defense factor against oral pathogens. SIgA did not exceed its resting level in our study. Increases in both the SLF levels were observed lagging increases in SAA levels. The effect on SLF secretion increase by aroma inhalation was found to be lower than that on SIgA secretion.

It is unclear what kind of specific autonomic receptors transduce aroma stimulation, and what kind of reflex route exists to promote IgA and LSF secretion with aroma. Several studies suggest the sympathetic neurotransmitters 5-hydroxytryptamine and dopamine may be modulated by aroma oils from rose, lavender, lemon and peppermint.[34] Other studies have investigated the effects of various plant-derived or synthetic odors on task performance, reaction time, and autonomic parameters, and have evaluated the direct effects of odors on the brain via electroencephalogram patterns and

functional imaging studies.[35] These studies have consistently shown that odors can produce specific effects on human neuropsychological and autonomic function, suggesting that aroma therapy has beneficial effects during stressful and adverse psychological conditions. This report is an autonomic functional study designed to evaluate the effect of aroma oil preparations on Wistar rats.[25] Our results suggest that aroma therapy augments hypothalamic—pituitary—adrenocortical (HPA) activity.

Since SAA is an uninvasive method of measuring the degree of excitation of the sympathetic nervous system,[36] we measured SAA as an indicator of sympathetic nerve excitability. We observed changes in the submandibular saliva that occurred when the SAA level was altered during both IPR stimulation and aroma inhalation. Although the SAA level was found to increase significantly even with small inflow rates, the large increases in SAA level observed during sympathetic discharge can be attributed to changes in flow rate.[37] Our results indicated that an increase in the SAA level resulted from IPR stimulation and aroma inhalation. This marker is sensitive enough to record sympathetic nerve activity. Marked increases in the SAA occurred in response to IPR stimulation, and its combined use with BPP. These results suggest that a different balance of efferent nervous stimuli from sympathetic and parasympathetic nerves regulates the protein secreted from the submandibular gland.

Our results indicated that SIgA and SLF secretion in the saliva is increased by aroma therapy, and that aroma therapy may enhance salivary antibacterial activity during sleep, and may have a positive effect on the sympathetic nervous system. We concluded that there is a reflex path for antimicrobial protein secretion in which the HPA system is activated by aroma inhalation.

This study was presented at the 554th meeting of the Osaka Odontological Society, February 11, 2017, Hirakata, Japan.

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