Nerve Growth Factor in Saliva Stimulated by Mastication

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Abstract: The aim of the present study was to measure the amount of nerve growth factor (NGF) present in saliva, and investigate the relationship with aging, gender, and number of retained teeth. The subjects were 93 healthy volunteers (35 males and 58 females) aged 18–87 years. Stimulated saliva was collected during gum chewing, and saliva secretion per minute and NGF concentration per unit volume were measured by enzyme-linked immunosorbent assay. We found that NGF in saliva was measurable in all subjects. The mean NGF concentration in saliva was 55.70 ± 38.27 pg/ml, and no relationship was found between NGF concentration and aging, gender, and the number of retained teeth. The mean NGF amount in saliva was 84.38 ± 71.06 pg/min. The NGF amount in saliva decreased with age, especially for the group aged 70 years and older. It was higher in male than female subjects, and was significantly higher in the group without tooth loss compared to the edentulous group. In addition, regarding the relationship between the saliva flow rate per minute and NGF amount in saliva, the latter increased significantly with a rise in the former. These results indicate that the NGF amount in saliva is influenced by aging, masticatory function, and saliva amount. It is speculated that human salivary glands play an important role in the synthesis and expression of NGF.

Key words: human, nerve growth factor, stimulated saliva, retained teeth

Introduction

The function of mastication is to crush food, stimulate the secretion of digestive fluids including saliva, and aid digestion and absorption. It is known that tooth loss due to aging decreases the masticatory function¹,². Epidemiological study reports indicate that mastication changes cerebral blood flow³, and tooth loss can be a risk factor for Alzheimer’s disease⁴. These findings suggest that tooth loss and a decrease in masticatory function markedly affect higher brain functions, and the relationship between dementia, which is a sign of aging of the brain, and masticatory function has been attracting attention.

On the other hand, the decreased function of the central cholinergic system has been pointed out as a cause of decreased function of the brain⁵. This
system forms a complicated circuit in the brain. NGF is necessary to maintain all of these mechanisms, and it has been closely linked with dementia. NGF is a neurotrophic factor, and has been demonstrated to be essential for the maintenance of life, including the growth and maintenance of nerve cells, and repair of damaged cells. There have been several reports on the NGF concentration in serum since NGF was first extracted from human placental tissue by Goldstein et al. in 1978. Although it is known that the NGF concentration decreases with age, and fluctuates due to emotional stress, the clinical significance of NGF remains to be elucidated. NGF in human saliva was extracted by Lipps et al. in 2000. However, there have been few reports on salivary NGF, and the significance of NGF in saliva remains unknown. Saliva secretion is influenced by many factors such as aging and masticatory function, and the level of NGF in saliva is also considered to be affected by these factors. However, there have been only two reports describing the concentration of NGF in saliva.

In the present study, we proposed the hypothesis that tooth loss and decreased masticatory function reduce saliva secretion, followed by a decrease in the level of NGF in saliva, and measured the NGF concentration in human saliva to investigate the relationship with aging, gender, and number of retained teeth.

Materials and Methods

1. Subjects

The subjects were 93 volunteers who agreed to provide saliva samples between April 2005 and October 2006, and were aged 18–87 years (35 males and 58 females, average age: 54.6 ± 19.1). Subjects were divided into age groups: group A (18–29 years, n = 14), group B (30–49 years, n = 15), group C (50–69 years, n = 43, and group D (70 years and older, n = 21), and we compared the results. Subjects with severe conditions, difficulty in communication, and those with a history of radiation treatment due to submandibular gland, parotid gland or systemic diseases were excluded. No subject under medication with a drug that may affect saliva secretion was included in this study.

The present study was approved by the ethical committee of the school before initiation (authorization number 506).

2. Saliva collection

Saliva was collected between 9:00 am and 1:00 pm. A saliva collection kit (BML Corp., Japan) was employed, and paraffin-containing chewing gum in the kit was used to stimulate saliva production. All subjects with complete dentures chewed gum using them. As for partial dentures, subjects who usually wear dentures also chewed gum using them. The complete absence of paired teeth contact was not noted in any subject. They were instructed to swallow any saliva in the mouth before measurement, and to chew gum for 10 minutes, when saliva was collected into spit bottles through funnel tubes, followed by measurement of the volume of saliva produced. Collected saliva was temporarily frozen at −80°C, then thawed in 40°C water, followed by centrifugal separation at 3,000 r.p.m. for 10 minutes, collection of the supernatant, and measurement of the NGF concentration.

3. NGF quantification

The NGF concentration in saliva was measured by enzyme-linked immunosorbent assay (ELISA) using the NGF Emax ImmunoAssay System (Promega Corp., USA). The NGF concentration in saliva was measured at 450 nm absorbance using a Plate Reader (Model 550 Microplate Reader, Bio-Rad Laboratories, USA). The following equation was used to calculate the rate of NGF appearance in saliva:

NGF amount in saliva per minute (pg/min) = saliva flow rate during stimulation (ml/min) × NGF concentration in saliva (pg/ml)

4. Statistical analysis

Spearman rank correlation was used to determine correlations among age, number of retained teeth, and saliva flow rate. A t-test was performed to compare mean values between the two groups, and a one-way analysis of variance was performed to examine differences in the NGF amount in each
The least significant difference (LSD) was used to compare means among groups, and $p < 0.05$ was considered significant.

**Results**

1. **Age and retained teeth**
   Concerning the relationship between age and number of retained teeth, the average number of retained teeth was $20.6 \pm 9.9$, and decreased with age ($p < 0.001$) (Fig. 1). The average number of retained teeth was $19.6 \pm 8.5$ in males, and $21.2 \pm 6.9$ in females, with no significant gender difference.

2. **Age, gender, retained teeth, and saliva flow rate**
   Regarding the relationship between age and the saliva flow rate per minute, the rate decreased significantly with age ($p < 0.01$) (Fig. 2). The average saliva flow rate per minute in group D (70 years or older) was $1.3 \pm 0.9$ ml/min, and showed a significant decrease ($p < 0.05$) compared to $2.0 \pm 0.7$ ml/min in group A (18–29 years).
   
   As for gender differences, the average saliva flow rate per minute was $2.2 \pm 1.2$ ml/min in males, and $1.4 \pm 0.8$ ml/min in females, being significantly higher in males ($p < 0.001$).
   
   For the relationship between number of retained teeth and saliva flow rate, the rate per minute significantly increased as the number of retained teeth increased ($p < 0.05$) (Fig. 3). The average saliva flow rate per minute in the group without tooth loss (subjects with 28 teeth, $n = 9$) was $2.0 \pm 0.9$ ml/min, and significantly increased compared to $0.9 \pm 0.7$ ml/min in the edentulous group ($n = 32$) ($p < 0.001$).

3. **Age, gender, retained teeth, saliva flow rate and NGF concentration**
   The average NGF concentration in saliva was $55.70 \pm 38.27$ pg/ml, and there was no correlation between aging and the NGF concentration in saliva. The NGF concentrations of each group were as follows: group A (18–29 years): $59.9 \pm 47.4$ pg/ml, group B (30–49 years): $47.5 \pm 33.9$ pg/ml, group C (50–69 years): $59.6 \pm 40.4$ pg/ml, and group D (70 years and older): $50.8 \pm 30.2$ pg/ml. No significant difference was observed between groups.
   
   The average NGF concentrations were $51.1 \pm 37.9$ pg/ml in males, and $58.5 \pm 38.5$ pg/ml in females. The results showed that the NGF concentration was higher in females, but no significant difference was observed.
   
   No significant correlation was observed between number of retained teeth and NGF concentration in saliva.
As for the relationship between the saliva flow rate per minute and NGF concentration in saliva, the latter decreased significantly with a rise in the former ($p < 0.01$) (Fig. 4, Table 1).

4. Age, gender, retained teeth, saliva flow rate and NGF amount

The average NGF amount in saliva per minute was $84.38 \pm 71.06$ pg/min. The results showed that the NGF amount decreased with age, but no significant difference was observed. The NGF amounts in each group were as follows: group A: $117.1 \pm 119.5$ pg/min, group B: $75.2 \pm 51.2$ pg/min, group C: $85.9 \pm 60.5$ pg/min, and group D: $66.1 \pm 57.1$ pg/min. The results showed that the NGF amount decreased significantly in group D compared to group A ($p < 0.05$), but no significant difference was observed between the other groups.

The average NGF amounts per minute in saliva were $107.0 \pm 94.5$ pg/min in males, and $70.7 \pm 48.2$ pg/min in females, showing a significantly higher value in males ($p < 0.05$).

No significant correlation was observed between number of retained teeth and NGF amount per minute in saliva; however, the NGF amount was significantly higher in the group without tooth loss ($n = 9$) compared to the edentulous group ($n = 32$) ($p < 0.05$) (Fig. 5).

As for the relationship between the saliva flow rate per minute and NGF amount in saliva, the latter increased significantly with a rise in the former ($p < 0.001$) (Fig. 6, Table 1).
**Discussion**

**Saliva secretion:**

Saliva secretion is controlled by autonomic nerves. The saliva flow rate differs in each individual, and fluctuates under the influence of mental condition such as stress, excitement, and medication. It also fluctuates by day and season. Saliva collection methods include: 1. Measurement of the resting saliva flow rate, and 2. Collection of stimulated saliva under taste stimulation or masticatory stimulation by chewing gum.

In the present study, we collected saliva produced by chewing gum since it has been demonstrated to have high reproducibility. This method is also simple and places less of a burden on the subjects. Furthermore, the total amount of NGF, as well as NGF concentration, was calculated, since it is speculated that the saliva rate varies due to masticatory function, and so the amount of NGF appearing in the mouth may also vary.

As for the relationships between gender, aging, and saliva flow rate, the rate was higher in males than females. This is considered to be due to the different size of the salivary glands. There have been many reports that the saliva flow rate decreases with age due to the atrophy of salivary glands, systemic disease, medication, and menopause in women. However, there have been mixed opinions regarding these reports, and a consensus has yet to be reached. In the present study, the saliva flow rate was significantly higher in males than females, and decreased with age, especially in the group of 70 years and older.

Concerning the relationship between aging and number of retained teeth, the number decreases with age due to an increased incidence of dental caries and periodontal diseases. The same relationship was observed in the present study. As for the relationship between number of retained teeth and saliva flow rate, the rate decreased with age, suggesting that the decrease in the number of retained teeth may cause the decline in the occlusal force and masticatory function.

**NGF appearance in saliva, aging, and number of retained teeth:**

There have been many reports on human NGF, and the average NGF concentration in human serum is 3.8–110.4 pg/ml. NGF levels decrease with age, fluctuate due to emotional stress, and increase in patients with allergic disease. However, there have also been contradictory reports, and a consensus has not been reached.

NGF in human saliva was isolated by Lipps in 2000, but there are few reports on this subject. Aside from our reports, there have been only two reports by Ruhl and Nam regarding measurement of the NGF concentration. Ruhl et al. measured proteins in the saliva of 31 gingiva-overgrowth cases, and reported that the average NGF concentration of the cases was 9644 pg/ml. Nam et al. measured NGF in saliva at rest and during taste stimulation in 127 volunteer subjects, and reported that the average NGF concentration was 901 pg/ml. In the present study, NGF in saliva was detectable in all cases. The results showed that the average NGF concentration in saliva while chewing gum was 55.70 pg/ml. This figure was much lower than those of previous reports. The exact cause has not been identified, but it is speculated that this variation was caused by differences in measurement procedures. The measurement methods differed between those of Ruhl et al. and ours. Also, it is presumed that the procedure before measurement was different, although the measurement methods were the same between Nam et al. and us. The premeasurement procedure and measurement method need to be standardized to make a comparison. It is also considered important to standardize the saliva collection method since proteins in saliva are markedly influenced by the saliva flow rate.

Regarding the relationships between aging, gender, and NGF in saliva, Nam reported that the NGF concentration decreased significantly with age in saliva from the submandibular and sublingual glands during stimulation, but no significant decrease was observed in saliva at rest or in saliva from the parotid gland during stimulation. In the present study, no correlation was observed...
between aging and the NGF concentration in saliva. However, since the saliva flow rate per minute decreased with age, NGF in saliva decreased as a result, especially in the group of 70 years and older. No significant gender difference was observed in the NGF concentration of saliva, but the NGF amount in saliva was higher in males than females. This result differed from the report byNam et al.13

No reports have investigated the relationship between the number of retained teeth, saliva flow rate, and NGF amount in saliva. The number of retained teeth is a parameter for the evaluation of masticatory function2. A decrease in number of retained teeth causes a decline in the masticatory function, resulting in a decrease in the NGF amount in saliva. There has been a report that a decline in masticatory function may accelerate the deterioration in brain function3,4. However, the relationship between NGF in saliva and the brain function remains unclear, and further epidemiological surveys are awaited.

The significance of NGF in saliva remains unclear. However, the application of NGF to wounds in mice directly accelerates healing25. Therefore, it is presumed that NGF is involved in the accelerated healing of damaged mucosa of the upper gastrointestinal tract including the oral cavity. It is an interesting topic whether or not NGF in saliva enters the blood circulation. It has been reported that EGF, another cell growth factor, is absorbed from the gastrointestinal tract and sublingual membrane26. This report suggests that NGF might follow the same absorption path despite its different molecular weight. It is known that the submandibular gland of the mouse acts like an endocrine gland, and secreted NGF enters the blood circulation27.

In conclusion, the relationship between the mastication-induced amount of NGF in saliva and salivary volume suggests that the salivary gland plays an important role in the synthesis and expression of NGF in humans. The expression and absorption of and metabolic processes occurring in human saliva remain to be elucidated in the future.

Reference