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ORIGINAL ARTICLE

Nerve growth factor concentration in human saliva

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OBJECTIVE: The aims of this study were to measure the normal concentration of nerve growth factor (NGF) in healthy human saliva and to investigate the effects of age and gender differences on saliva NGF level.
MATERIALS AND METHODS: Resting whole, stimulated parotid, and stimulated submandibular/sublingual saliva were collected from 127 healthy volunteers with ages ranging from 20 to 81 years. The saliva NGF concentration was measured by enzyme immunoassay.
RESULTS AND CONCLUSIONS: The mean concentrations of NGF were 901.4 ± 75.6 pg ml^{-1} in resting whole saliva, 885.9 ± 79.9 pg ml^{-1} in stimulated parotid saliva, and 1066.1 ± 88.1 pg ml^{-1} in stimulated submandibular/sublingual saliva. The stimulated submandibular saliva showed lower NGF concentrations with increasing age ($\rho = -0.296$, $P = 0.001$). The NGF concentrations of resting whole saliva ($P = 0.025$) and stimulated parotid saliva ($P = 0.005$) were significantly higher in women than men. The NGF concentration of stimulated submandibular saliva was significantly higher than stimulated parotid saliva ($P = 0.005$) and significantly correlated with stimulated parotid saliva NGF level ($\rho = -0.244$, $P = 0.008$). We found measurable concentrations of NGF in all three sources of saliva; the concentration was affected by the source for the stimulated parotid and submandibular saliva, age for stimulated submandibular saliva, and gender difference for resting whole saliva and stimulated parotid saliva.
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Keywords: enzyme immunoassay; nerve growth factor; saliva; age; gender; salivary gland

Introduction

Nerve growth factor (NGF) is a peptide known as a neurotrophin. As the name implies, interest in this molecule arose from its effectiveness on the survival and differentiation of sensory and sympathetic neurons (Levi-Montalcini *et al*, 1975; Varon, 1975). In addition to the neurotrophic activity, NGF has been reported to exert broader biological activities on non-neuronal cells. NGF is known to stimulate the hypothalamus–pituitary–adrenal axis increasing the secretion of ACTH and glucocorticoid hormone (Otten *et al*, 1979; Scaccianoce *et al*, 1993), and exerts a crucial effect on the cells of the immune system (Otten *et al*, 1989; Kannan *et al*, 1991; Ehrhard *et al*, 1993). Recently, it has been suggested that NGF plays an important role in hyperalgesia as the concentration of this molecule was found to be increased by inflammatory injury and up-regulated in response to noxious stimuli (Donnerer *et al*, 1992; Woolf *et al*, 1994). NGF has been thought to be related to pain, behavioral changes, and neuropsychiatric disorders affected by the endocrine mechanism (Lakshmanan, 1986; Cirulli, 2001; Hadjiconstantinou *et al*, 2001). A number of studies were performed on NGF in chronic pain disorders for which the cause and pathophysiological mechanisms were not well understood (Fabricant and Todaro, 1981; Parkhouse *et al*, 1992; Koo *et al*, 1993; Anand, 1995).

Nerve growth factor was originally isolated from the mouse submandibular gland (Cohen, 1960). This protein is produced by the granular convoluted tubule cells of the gland, stored within secretory granules, and secreted from the salivary gland into the circulation under certain conditions (Watson *et al*, 1985; Mathison *et al*, 1994). NGF levels in normal human sera have been reported in several studies and variations with age and gender are contradictory (Serrano *et al*, 1996; Hadjiconstantinou *et al*, 2001; Lang *et al*, 2002, 2003).

Although the salivary gland has been regarded as a major source of NGF and high concentrations of NGF in the submandibular gland were reported in the animal studies (Watson *et al*, 1985; Mathison *et al*, 1994), there are currently very few reference values for NGF levels in

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human saliva (Fischer *et al*, 1998; Ruhl *et al*, 2004). In addition, no studies exist regarding the normal concentration of NGF in saliva samples obtained from a large population group. As saliva carries out a number of biological functions to maintain oral health and salivary glands are integrated into the neuroendocrine system through complex regulatory pathways (Mathison *et al*, 1994; Sabbadini and Berczi, 1995), the accurate determination of NGF levels in saliva may be of interest. The data of the NGF concentration in normal saliva may provide valuable information for research into pathophysiological mechanisms of neurodegenerative diseases or chronic pain conditions of the oral cavity in which this factor appears to be involved.

The aims of this study were to measure the NGF concentration of resting whole saliva, stimulated parotid saliva, and stimulated submandubular/submaxillary saliva in a large population of healthy individuals and to investigate the effects of age and gender differences on saliva NGF level.

Materials and methods

Subjects

A total of 127 gender- and age-matched healthy volunteers participated in this study. The subjects consisted of 64 men (mean age ± s.d. of 48.7 ± 18.0 years) and 63 women (mean age ± s.d. of 49.3 ± 18.4 years) with ages ranging from 20 to 81 years (mean age ± s.d. of 49.0 ± 18.1 years). Subjects were excluded if they had any history of psychiatric disorders or metabolic diseases. They had no smoking habits, major illnesses, infections, treatment with drugs, and oral mucosal lesions. After a complete description of the study, informed consent was obtained from each subject.

Saliva collection

Resting whole saliva was collected from all 127 subjects by the spitting method with a sterilized cap-tube (Birkhed and Heintze, 1989; Mandel, 1990). The resting saliva was collected for 10 min and salivary flow rate of each subject was calculated. The mean and s.d. of salivary flow rate of resting whole saliva was 0.61 ± 0.39 ml min^{-1}.

Stimulated parotid saliva was collected from 118 subjects with the aid of a plastic suction cup (modified Lashley cup) placed directly over the orifice of Stensen's duct. Secretion of the parotid saliva was stimulated with the application of 2% citric acid solution on the tongue. Stimulated submandibular/sublingual saliva was collected from 124 subjects by an individualized silicon collection device on the orifice of the submandibular/sublingual gland with 2% citric acid stimulation. The sublingual area was isolated with cotton rolls and the orifices of the parotid glands were blocked with the plastic suction cup to prevent the influx of other glandular saliva during the collection of the submandibular/sublingual saliva sample (Birkhed and Heintze, 1989; Mandel, 1990). Each stimulated parotid saliva and submandibular/sublingual saliva was collected until the volume of the sample was reached to 3 ml.

All saliva samples were collected between 2:00 p.m. and 4:00 p.m. after lunch to minimize the possible effect of diurnal variability in saliva NGF levels (Siminoski *et al*, 1993). Whole saliva samples were always collected before collecting the parotid and submandibular/sublingual saliva samples. The saliva samples were centrifuged at 10 000 ***g*** for 5 min and supernatants were aliquoted and frozen at −70°C.

Nerve growth factor quantification

The concentration of NGF in saliva samples was measured by means of a commercially available enzyme-linked immunoassay kit according to the manufacturer's instructions (Promega Corp., Madison, WI, USA). Plates were measured by a plate reader (Power Wave, Bio-Tek Instrument Inc., Winooski, VT, USA) at a wavelength of 450 nm. All saliva samples were assayed in duplicate and NGF concentrations were determined from the regression line for the NGF standard incubated under same conditions for each assay.

Statistical analysis

The Spearman's correlation coefficients between age and NGF concentration in each type of saliva were computed and tested. The statistical differences between genders in each type of saliva were tested by the Wilcoxon rank sum test.

Multiple linear regression analyses were used to examine the impacts of explanatory variables including age and gender on the NGF concentration of each type of saliva. As the Kolmogorov–Smirnov test for normality of NGF levels had a significant result ($P < 0.01$), NGF levels were log-transformed. The transformed data were normally distributed ($P = 0.08$), and used for the multiple linear regression.

To compare the NGF concentrations between stimulated parotid saliva and stimulated submandibular saliva, Wilcoxon rank sum test was used. To investigate the correlation between stimulated parotid and stimulated submandibular saliva NGF levels, Spearman's correlation test was performed.

Results

The distribution of data for the NGF concentration of each type of collected saliva is shown in Figure 1, and the descriptive results of NGF concentrations in resting whole saliva and stimulated glandular saliva according to the gender and age groups are shown in Table 1. The mean concentrations of NGF were 901.4 ± 75.6 pg ml^{-1} in resting whole saliva, 885.9 ± 79.9 pg ml^{-1} in stimulated parotid saliva, and 1066.1 ± 88.1 pg ml^{-1} in stimulated submandibular/sublingual saliva. The stimulated submandibular saliva showed lower NGF concentrations with increasing age ($\rho = -0.296$, $P = 0.001$). There were no significant correlations between age and resting whole saliva or stimulated parotid saliva NGF levels. The NGF concentrations of resting whole saliva ($P = 0.025$) and stimulated parotid saliva ($P = 0.005$) were significantly higher in women than in men. There was no significant

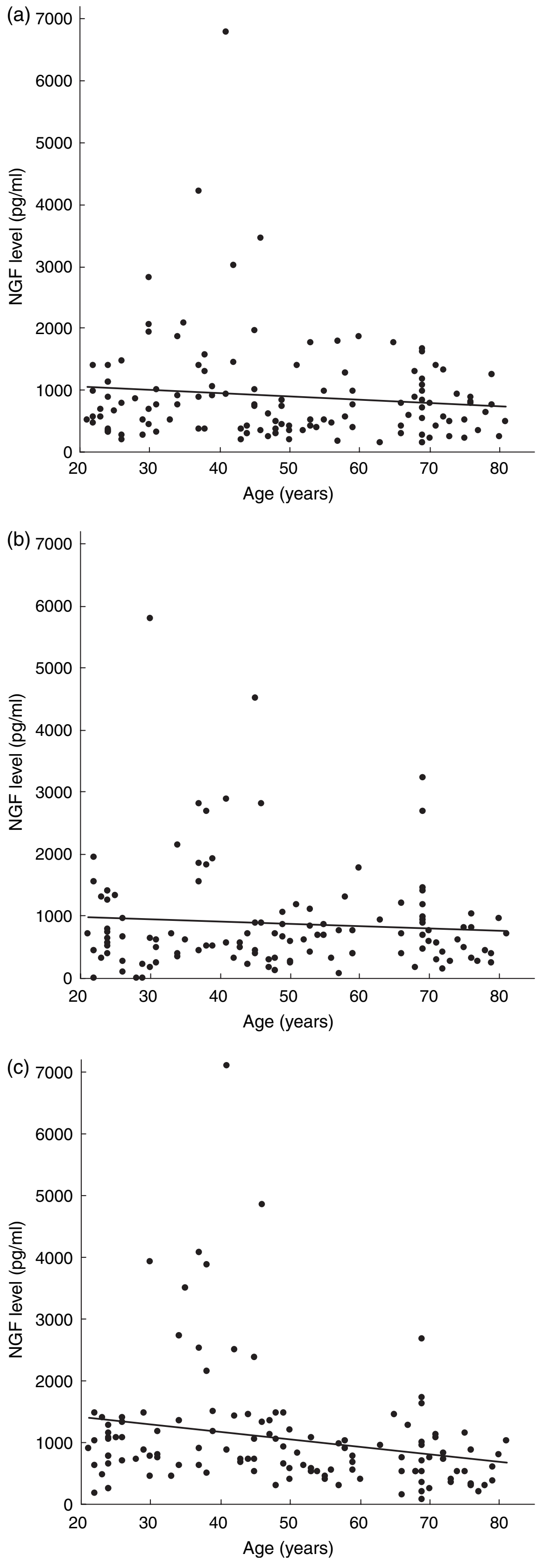

Figure 1 Diagram of the distribution of nerve growth factor concentration in (**a**) resting whole saliva, (**b**) stimulated parotid saliva, and (**c**) stimulated submandibular/sublingual saliva

difference between genders in stimulated submandibular NGF level (Table 1).

In the multiple linear regression, the impacts of explanatory variables on the NGF concentration of each type of saliva were tested after adjusting for the other effect (Table 2). The stimulated submandibular saliva NGF level was decreased with increasing age (coefficient = −0.012, $P = 0.001$), but resting whole saliva and stimulated parotid gland saliva NGF level were not affected by age. The resting whole saliva (coefficient = 0.316, $P = 0.016$) and stimulated parotid gland saliva (coefficient = 0.413, $P = 0.005$) NGF level increased in women, but stimulated submandibular saliva NGF level was not affected by gender difference.

Stimulated submandibular saliva NGF level was significantly higher than stimulated parotid saliva ($P = 0.005$) and significantly correlated with stimulated parotid NGF level ($\rho = -0.244$, $P = 0.008$, data not shown).

Discussion

Most saliva NGF values in the literature were based on small groups of control subjects, where the possible influences of age and gender were not taken into account. Furthermore, only one report gave saliva NGF levels according to the glandular secretion of different types of salivary glands (Ruhl *et al*, 2004). We examined NGF concentrations from three sources, from a large number of normal subjects, and considered age and gender in each type of saliva.

The mean NGF concentration in human serum has been reported ranged from 3.8 to 194 pg ml^{-1} (Aloe *et al*, 1994; Bonini *et al*, 1996; Serrano *et al*, 1996; Lang *et al*, 2003; Hadjiconstantinou *et al*, 2001). Our results for NGF concentration in saliva are higher than these reports for serum, and were consistent with previous reports of other neuropeptide levels in saliva (Fischer *et al*, 1998). Previous studies showed unequivocal evidence for the physiological role of salivary glands as a major source of blood NGF (Aloe *et al*, 1986; Lakshmanan, 1986). Higher NGF level in saliva indicates that the salivary gland may play an important role in the synthesis or secretion of NGF. Many studies have demonstrated that salivary glands are an important source of neuropeptides with a variety of biological activities such as epidermal growth factor, kallikrein, transforming growth factor, insulin-like growth factor, and NGF and play a role in neuroendocrine control of homeostasis in tissue injury (Hansson and Tunhall, 1986; Miller *et al*, 1989; Berg *et al*, 1990; Amano *et al*, 1993; Penschow and Coghlan, 1993; Mathison *et al*, 1994; Zelles *et al*, 1995).

Our data of saliva NGF concentration are lower than those of a recent study by Ruhl *et al* (2004) which reported 9644 pg ml^{-1} as a median concentration of whole saliva from 10 subjects. When we measured the serum NGF concentration in a subset of 36 individuals among our subjects, the mean and standard deviation of serum NGF concentration of the subjects was 38.36 ± 51.33 pg ml^{-1} (data were not shown in the

Table 1 Means and standard error means of nerve growth factor (NGF) concentration (pg ml^{-1}) of each type of saliva and differences in age and gender

Variables	*Resting whole*	*Stimulated parotid*	*Stimulated submandibular*
Total	901.4 ± 75.6 (n = 127)	885.9 ± 79.9 (n = 118)	1066.1 ± 88.1 (n = 124)
Age			
Correlation coefficient[a]	−0.089	−0.034	−0.296
P-value	0.322	0.717	0.001
Gender			
Men	750.6 ± 80.5 (n = 64)	649.6 ± 64.6 (n = 60)	966.3 ± 87.2 (n = 61)
Women	1054.7 ± 126.4 (n = 63)	1130.3 ± 141.8 (n = 58)	1162.7 ± 151.3 (n = 63)
P-value[b]	0.025	0.005	0.930

[a]Spearman's rho correlation coefficients between age and NGF concentration of each saliva.
[b]Gender differences were determined by Wilcoxon rank sum test.

Table 2 Regression parameters of the explanatory variables on the log nerve growth factor level of each type of saliva in the multiple linear regression model

Variables	*Coefficient*	*95% CI*	P-*value*
Resting whole[a]			
Age	−0.005	−0.012, 0.002	0.199
Gender	0.316	0.059, 0.572	0.016
Stimulated parotid[b]			
Age	−0.004	−0.012, 0.004	0.319
Gender	0.413	0.127, 0.699	0.005
Stimulated submandibular[c]			
Age	−0.012	−0.019,−0.005	0.001
Gender	−0.076	−0.174, 0.326	0.547

[a]Adjusted $R^2 = 0.042$.
[b]Adjusted $R^2 = 0.061$.
[c]Adjusted $R^2 = 0.074$.

Results). Our data agree with the previous reports cited above. The reasons for the difference of saliva NGF concentrations between our own and Ruhl's data are unknown, but the different ages of the subjects, collection times, and collection methods might contribute to the different concentrations. Future research dealing with NGF secretion is needed to reveal the effects of each of these factors.

Our results, showing a significantly lower NGF concentration in submandibular saliva with increasing age, concur with previous studies on serum NGF level (Serrano *et al*, 1996; Lang *et al*, 2003) and NGF receptor immunoreactivity (Gómez-Pinilla *et al*, 1989; Antonelli *et al*, 2003). A decrease in plasma NGF and low expression of NGF receptors on the peripheral lymphocytes in the elderly could indicate altered functional activity of the lymphocytes (Gómez-Pinilla *et al*, 1989; Antonelli *et al*, 2003). Previous studies indicate that older people have a reduced number of T cells, as well as a low response to T-cell and B-cell mitogens (Min *et al*, 2005). Immunohistochemical studies reveal that immunoreactivity for NGF in the granular convoluted tubule cells of the mouse submandibular gland was decreased with age (Gresik and Azmitia, 1980).

As shown in Figure 1, the variability of saliva NGF values was higher at younger ages and these data are similar to a previous study of saliva basic fibroblast growth factor (Westermark *et al*, 2002). A possible explanation is that the younger individuals could respond more actively to various physiological or pathological conditions as growth factors play an important role in the control of homeostasis. Thus high and varied NGF concentrations could be observed in this age group.

Gender difference in human serum NGF level has been controversial (Serrano *et al*, 1996; Lang *et al*, 2003) and no reports exist for NGF concentration in human saliva. Our result of higher saliva NGF level in women is contrary to the case of mouse submandibular gland. The NGF concentration in the mouse submandibular gland has been reported to be higher in male, but we should consider the known sexually dimorphic character of the female mouse submandibular gland.

Nerve growth factor has been reported to be produced and stored by a variety of cells outside the nervous system, but little is known about the distribution of NGF synthesis or about its regulation in different tissue. The mouse submandibular gland is well known for its high concentration of NGF ranged from 0.85 to 35 mg g^{-1} (Thoenen and Barde, 1980; Shooter, 2001) and immunohistochemical studies have revealed that the granular convoluted tubules of the submandibular gland are one of the locations for the synthesis and storage of NGF (Watson *et al*, 1985; Mathison *et al*, 1994). The submandibular gland has been reported to have a significant role in the regulation of inflammatory and immune reactions, but this phenomenon is still poorly defined (Sabbadini and Berczi, 1995). We do not know whether the results obtained in one species can be extrapolated to other species such as humans. However, our findings demonstrated that the saliva NGF level of stimulated submandibular/sublingual saliva was significantly higher than stimulated parotid saliva, and these two were significantly correlated.

Three limitations should be mentioned. First, in our study we did not collect the whole saliva and glandular saliva by same stimulatory conditions, so we could not compare the NGF concentration between them. We considered that the measurement of resting whole saliva might be more relevant to the ordinary intraoral conditions than the measurement of stimulated whole saliva, and collection of whole saliva by stimulation could increase certain glandular saliva component such as parotid saliva. Secondly, our study did not consider the separation of luminal effect and the systemic effect. Further investigations might consider the systemic effects such as the emotional status, pain prevalence, and endocrine effect. Thirdly, we did not measure the total protein concentration in saliva. Further studies including NGF proportion of total protein concentration may help to compare the secretion of different components of saliva. However, we believe the methods that we used were suitable to meet the objectives of this study and the results give some helpful information for the knowledge about NGF in human saliva.

In conclusion, we found measurable concentrations of NGF in all three sources of saliva. The concentration was affected by the source for the stimulated parotid and submandibular saliva, age for stimulated submandibular saliva, and gender difference for resting whole saliva and stimulated parotid saliva.

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