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ORIGINAL ARTICLE

Plasma and saliva levels of nerve growth factor and neuropeptides in chronic migraine patients

M-U Jang, J-W Park, H-S Kho, S-C Chung, J-W Chung

Orofacial Pain Clinic, Department of Oral Medicine and Oral Diagnosis, School of Dentistry and Dental Research Institute, Seoul National University, Seoul, Korea

OBJECTIVES: To investigate the level and interrelationship of nerve growth factor (NGF) and sensory neuropeptides [substance P (SP), calcitonin gene-related peptide (CGRP)] in plasma and saliva of chronic migraine patients, and to analyze the association between pain intensity and their concentration.

MATERIALS AND METHODS: Plasma and resting whole saliva were collected from 33 chronic migraine patients and 36 control subjects. NGF, SP, and CGRP concentrations were measured by enzyme immunoassay and pain intensity of each subject was measured using the Graded Chronic Pain Scale.

RESULTS: Chronic migraine patients showed higher NGF and neuropeptide levels in both plasma and saliva compared to the control subjects. Plasma NGF, and plasma and saliva levels of SP and CGRP were highly associated with pain intensity. There was a significant positive correlation between NGF and both neuropeptide levels in plasma, and between the neuropeptide levels in both plasma and saliva. Plasma levels of SP and CGRP were significantly correlated with their saliva level.

CONCLUSIONS: The increased production of NGF and sensory neuropeptides may play an important role in the maintenance of pain in chronic migraine and analysis results of human saliva could act as an index of disease state and therapeutic outcome.

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Keywords: chronic migraine; plasma, saliva; nerve growth factor; substance P; calcitonin-gene related peptide

Introduction

Daily or near-daily headache is a widespread problem in clinical practice. Chronic daily headache (CDH) has been defined as a primary headache presenting more than 15 days per month for more than 3 months and includes chronic migraine (CM), chronic tension-type headache, new daily persistent headache, and hemicrania continua (Silberstein *et al*, 2008). Among the types of CDH, it is reported that 87% of CDH patients were diagnosed as CM (Bigal *et al*, 2002) and approximately 2% of the general population are known to have CM (Scher *et al*, 1998; Castillo *et al*, 1999).

CM was originally termed transformed migraine because patients with CM occasionally have a past history of episodic migraine (Silberstein *et al*, 2008). Patients with episodic migraine often develop a pattern of daily or near-daily headaches that clinically resemble a mixture of tension-type headache and migraine (Solomon *et al*, 1992; Silberstein *et al*, 2008).

The pathologic substrate causing the chronic pain of CM is an important yet controversial issue. Many models have been proposed such as functional impairment of the endorphinergic and serotoninergic pathway, and intracellular transduction mechanisms as well as an increased nitric oxide synthase activity in platelets (Hering *et al*, 1993; Srikiatkhachorn *et al*, 1998; Sarchielli *et al*, 1999). Other proposed mechanisms involve NMDA receptor activation, and sustained increase in neuropeptides and nerve growth factor (NGF) production.

Calcitonin gene-related peptide (CGRP) and substance P (SP) are one of the most abundant neuropeptides in nervous tissue. A role for CGRP has been implicated in migraine based on its increased plasma concentration during and outside of active pain periods (Goadsby *et al*, 1990; Ashina *et al*, 2000). The evidence shows that SP level increases with noxious stimulation suggesting that SP may play a role in central sensitization associated with chronic headaches (Greco *et al*, 2008). CGRP and SP immunoreactive sensory fibers were seen along the blood vessel walls and within nerve bundles in skeletal muscles (Tsukagoshi *et al*, 2002). These findings indicate that ongoing activity in sensory neurons may be reflected in the change of plasma CGRP and SP concentration.

Another important factor, NGF is known to be released by several cell types in response to tissue inflammation and it also induces hyperalgesia (Shu and

Correspondence: Jin-Woo Chung, DDS, PhD, Orofacial Pain Clinic, Department of Oral Medicine and Oral Diagnosis, School of Dentistry and Dental Research Institute, Seoul National University, 28 Yunkeun-Dong, Chongro-Ku, Seoul 110-749, Korea. Tel: +82 2 2072 3021, Fax: +82 2 744 9135, E-mail: jwchung@snu.ac.kr
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Mendell, 1999; Hao *et al*, 2000). Interestingly, NGF-induced hyperalgesia seems to involve an enhanced production of SP and CGRP from nociceptive peripheral nerve endings and vice versa, neuropeptides released from cutaneous nerves after an injurious stimulus are also able to influence the expression and secretion of proNGF/NGF (Donnerer *et al*, 1992; Dallos *et al*, 2006). These findings suggest that CGRP, SP, and NGF may have a putative role in the generation and maintenance of pain in CM and moreover, the activity of each component appears to be closely interrelated.

Most of the past studies on the role of neuropeptides in chronic pain have assessed their plasma concentration. However saliva has advantages such as easy non-invasive nature of collection and the close relationship between saliva and plasma levels of various substances makes saliva a valuable clinical tool (Choo and Huestis, 2004). But, studies that evaluate the values of saliva as a diagnostic tool for headache assessment are scarce.

The aims of this study were to investigate NGF, SP, and CGRP levels and their relationships in plasma and saliva of CM patients, and to analyze their association with pain intensity. Through this study we expected to elucidate the role of NGF, SP, and CGRP and their relationship in CM and evaluate potentialities of saliva as a diagnostic tool for headache evaluation.

Materials and methods

Subjects

Thirty-three consecutive patients (12 men and 21 women, mean age 43.7 ± 18.1 years) diagnosed with CM who had visited the Orofacial Pain Clinic of Seoul National University Dental Hospital were evaluated. Inclusion criteria were patients who were classified as having CM according to the Revised Criteria of the International Classification of Headache Disorders (ICHD-IIR) for CM (Headache Classification Committee, 2006).

Thirty-six age- and sex-matched healthy control subjects (17 men and 19 women, mean age 44.3 ± 14.2 years) were also evaluated. The control subjects did not have any history of orofacial pain symptoms within the previous six months including headache.

The exclusion criteria for both groups were smokers, patients with severe periodontal disease including gingival bleeding, mucosal pain or ulceration, and tooth pain. And also patients that had taken anti-inflammatory medication on a regular basis during the past 2 months or were currently taking medication were excluded. The project was approved by the Institutional Review Board at the Seoul National University Dental Hospital, and each subject gave informed consent.

The characteristics and demographic features of both the CM patient group and control group are shown in Table 1.

Characteristic pain intensity

The characteristic pain intensity of CM was assessed using a structured questionnaire of the Graded Chronic Pain Scale (Von Korff *et al*, 1992). The pain intensity was calculated by averaging 0–10 ratings of present pain, average pain, and worst pain in the past 6 months. This average was multiplied by 10 to yield a 0–100 score.

Table 1 Characteristics of chronic migraine (CM) patients and control subjects

	CM patients (n = 33)	*Control subjects (n = 36)*
Age (years)	43.7 ± 18.1	44.3 ± 14.2
Gender (women)	21 (63.6%)	19 (52.8%)
Saliva flow rate (ml min^{-1})	0.41 ± 0.22	0.45 ± 0.23
Duration of CDH (years)	10.2 ± 4.6	NA
Number of headache days/month	20.9 ± 5.1	NA
Pain intensity	50.63 ± 23.91	NA

Collection of saliva and plasma

Resting whole saliva samples were obtained between 9 and 11 a.m. Subjects were prohibited from eating and drinking for an hour before collection. Subjects were seated under observation for 5 min, and right before starting the collection process the mouth was prepared by rinsing with diluted water and swallowing the residual saliva. Samples were collected through self-drainage into a sterilized tube for 10 min. The salivary flow rate was measured in ml min^{-1}.

Plasma samples were collected from the subject's antecubital vein at the same time and transferred to Lavender tubes (Becton Dickinson Vacutainer System, Rutherford, NJ, USA) coated with EDTA.

All the samples were centrifuged at 2000*g* at 4°C for 10 min and supernatants were immediately stored at −70°C.

Quantification of NGF, SP, and CGRP

The plasma and saliva concentrations of NGF, SP, and CGRP were measured by means of a commercially available enzyme-linked immunoassay (EIA) kit according to the manufacturers' instructions (NGF: Promega Corp., Madison, WI, USA; SP: R&D system Inc., Minneapolis, MN, USA; CGRP: Spi-Bio Inc., Montigny le Bretonneux, France). Plates were measured with a plate reader (Power Wave, Bio-Tek Instrument Inc., Winooski, VT, USA). Each plasma and saliva sample was assayed in duplicate.

Statistical analyses

Independent *t*-test was conducted to compare the NGF, SP and CGRP level in plasma and saliva between CM and control subjects. Correlations among NGF and each sensory neuropeptide level in both plasma and saliva samples and correlations between saliva flow rate and each NGF and neuropeptide level in saliva were examined using Pearson's correlation coefficient. Multiple linear regression analysis was done to show the associations between explanatory variables (age, gender, pain intensity, saliva flow rate) and each plasma and saliva NGF, SP, and CGRP level.

Results

NGF and neuropeptide levels in plasma and saliva

The descriptive results of NGF and neuropeptide levels in plasma and saliva of both groups are shown in Table 2.

CM patients showed significantly higher NGF (plasma: $P = 0.001$; saliva: $P = 0.034$), SP (plasma: $P = 0.002$; saliva: $P = 0.031$), and CGRP (plasma: $P = 0.003$; saliva: $P = 0.026$) levels in both plasma and saliva compared to the control subjects.

Saliva flow rate and concentration of NGF and neuropeptides in whole saliva

The saliva concentration of NGF and sensory neuropeptides showed negative correlations with the flow rate of resting whole saliva. NGF, SP, and CGRP levels in saliva decreased as the salivary flow rate increased (NGF: $r = -0.480$, $P < 0.01$; SP: $r = -0.270$, $P < 0.05$; CGRP: $r = -0.261$, $P < 0.05$).

Associations between explanatory variables and each NGF and neuropeptide level

The impacts of explanatory variables on the NGF and sensory neuropeptide levels in plasma and saliva of CM patients were tested after adjusting for other effects through multiple linear regression.

Descriptive results of associations between explanatory variables (age, gender, pain intensity) and plasma concentrations of NGF and each neuropeptide are shown in Table 3. The plasma levels of NGF (standardized coefficient = 0.516, $P = 0.004$), SP (standardized coefficient = 0.699, $P = 0.001$) and CGRP (standardized coefficient = 0.442, $P = 0.018$) all significantly increased with increasing pain intensity. Age and gender did not significantly affect the plasma levels of NGF and sensory neuropeptides.

Descriptive results of associations between explanatory variables (age, gender, saliva flow rate, pain intensity) and the saliva concentration of NGF and each neuropeptide are shown in Table 4. The saliva NGF level appeared to be unaffected by pain intensity after adjusting for the effect of other variables. The saliva levels of NGF significantly decreased as the salivary flow rate increased (standardized coefficient = -0.371, $P = 0.034$) and women tended to have a higher salivary concentration of NGF (standardized coefficient = 0.375, $P = 0.037$). The saliva level of SP (standardized coefficient = 0.483, $P = 0.007$) and CGRP (standardized coefficient = 0.458, $P = 0.012$) significantly increased with pain intensity, but was not significantly affected by other variables.

Correlations among NGF and each neuropeptide level in plasma and saliva

Both the plasma levels of SP and CGRP showed significant positive correlation with their saliva concentration (SP: $r = 0.579$, $P < 0.01$; CGRP: $r = 0.459$, $P < 0.01$, Figure 1a,b). NGF levels of plasma and saliva did not show significant correlations.

The plasma NGF level was significantly correlated with plasma SP ($r = 0.527$, $P < 0.01$) and CGRP ($r = 0.547$, $P < 0.01$) levels (Figure 2a,b). The saliva NGF level did not show significant correlations with saliva SP or CGRP levels.

The SP and CGRP levels showed significantly positive correlations in both plasma ($r = 0.448$, $P < 0.01$) and saliva ($r = 0.394$, $P < 0.01$) samples (Figure 3a,b).

Table 2 NGF, SP, and CGRP levels in plasma and saliva of both groups

		CM patients	*Control subjects*	P-*value*
NGF	Plasma	41.1 ± 21.5	21.6 ± 13.5	0.001
(pg ml^{-1})	Saliva	1123.1 ± 471.6	891.8 ± 415.9	0.034
Substance P	Plasma	168.8 ± 89.5	105.0 ± 67.1	0.002
(pg ml^{-1})	Saliva	236.1 ± 114.1	177.5 ± 106.2	0.031
CGRP	Plasma	253.6 ± 195.2	136.2 ± 92.5	0.003
(pg ml^{-1})	Saliva	431.6 ± 272.8	301.5 ± 188.9	0.026

P-values were obtained from independent *t*-test.

Discussion

Even though there are several previous studies which investigated the sensory neuropeptide levels in migraine patients, our study is the first to investigate the NGF levels in both plasma and saliva of CM patients diagnosed with well defined ICHD criteria (Headache Classification Committee *et al*, 2006), and to investigate both NGF and sensory neuropeptide levels simultaneously in plasma and saliva samples. In addition, we investigated their associations with characteristic pain intensity.

Both plasma and saliva levels of NGF, SP, and CGRP are all elevated in our CM patients when

Table 3 Associations between explanatory variables (age, gender, pain intensity) and each NGF and neuropeptide level in plasma of chronic migraine patients by multiple linear regression analysis

	Plasma NGF[a]		*Plasma SP*[b]		*Plasma CGRP*[c]	
Explanatory variables	*Standardized coefficient*	P-*value*	*Standardized coefficient*	P-*value*	*Standardized coefficient*	P-*value*
Age (years)	-0.113	0.494	-0.235	0.108	0.010	0.954
Gender (women)	0.082	0.629	-0.062	0.672	0.045	0.800
Pain intensity	0.516	0.004	0.699	0.001	0.442	0.018

[a]Adjusted $R^2 = 0.221$.
[b]Adjusted $R^2 = 0.412$.
[c]Adjusted $R^2 = 0.128$.

Explanatory variables	*Saliva NGF*[a]		*Saliva substance P*[b]		*Saliva CGRP*[c]	
	Standardized coefficient	P-*value*	*Standardized coefficient*	P-*value*	*Standardized coefficient*	P-*value*
Age (years)	0.031	0.851	0.156	0.333	-0.070	0.668
Gender (women)	0.375	0.037	-0.042	0.805	-0.143	0.411
Saliva flow rate (ml min^{-1})	-0.371	0.034	-0.189	0.253	-0.296	0.086
Pain intensity	-0.110	0.522	0.483	0.007	0.458	0.012

[a]Adjusted $R^2 = 0.222$.
[b]Adjusted $R^2 = 0.265$.
[c]Adjusted $R^2 = 0.225$.

Table 4 Associations between explanatory variables (age, gender, saliva flow rate, pain intensity) and each NGF, and neuropeptide level in resting whole saliva of chronic migraine patients by multiple linear regression analysis

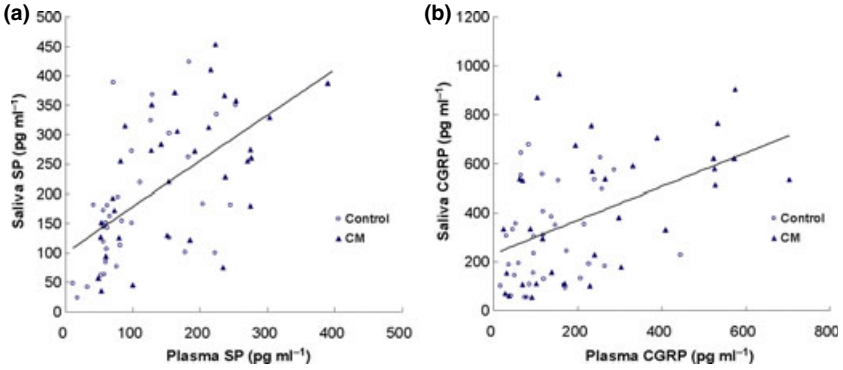

Figure 1 Correlations between (**a**) SP levels in plasma and saliva ($r = 0.579$, $P < 0.01$), and (**b**) CGRP levels in plasma and saliva ($r = 0.459$, $P < 0.01$)

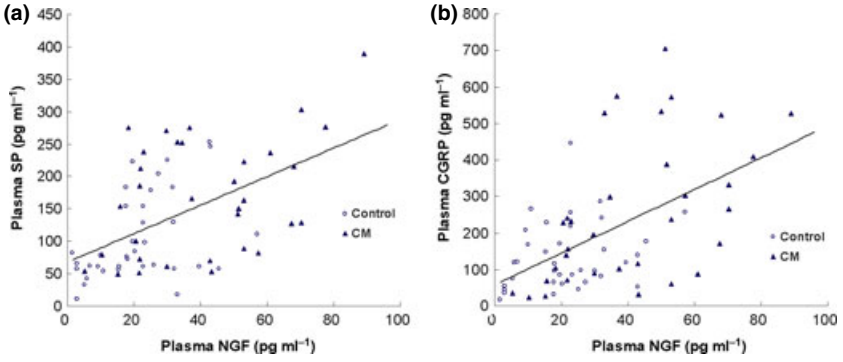

Figure 2 Correlations between (**a**) plasma NGF and SP ($r = 0.527$, $P < 0.01$) and (**b**) plasma NGF and CGRP ($r = 0.547$, $P < 0.01$) levels

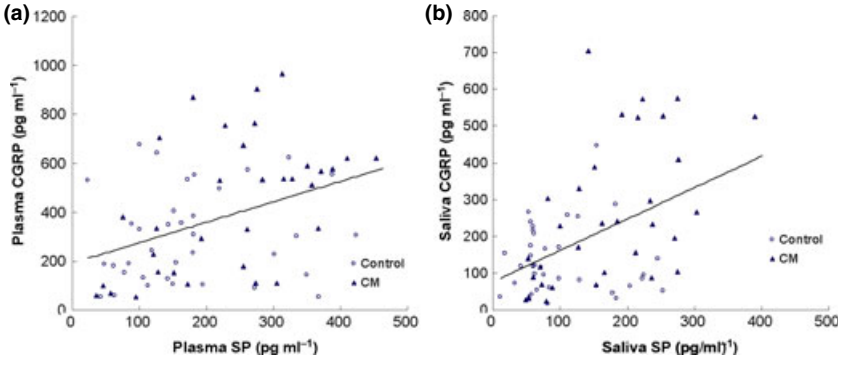

Figure 3 Correlations between (**a**) plasma SP and CGRP ($r = 0.448$, $P < 0.01$), and (**b**) saliva SP and CGRP ($r = 0.394$, $P < 0.01$) levels

compared to healthy controls. There have been several studies of SP and CGRP levels in plasma of patients with migraine, but these have provided contradictory results (Goadsby *et al*, 1990; Ashina *et al*, 2000; Tvedskov *et al*, 2005; Fusayasu *et al*, 2007). Especially, SP is not typically considered by some to be associated with migraine since trials in abortive and preventive therapy involved with SP receptor antagonists have failed

(Goldstein *et al*, 1996; Connor *et al*, 1998). The neurogenic inflammation alone caused by SP and CGRP is unlikely to explain the whole pain mechanism of migraine and most of these studies were based on patients with common episodic migraine and excluded patients with migraine of a more chronic nature. As the pain duration extends, other factors such as hyperexcitability of the cortex and disturbance of the blood-brain barrier (Kaube *et al*, 1993; Welch *et al*, 1993) get involved in causing migraine attacks which cannot be controlled through applying SP antagonists. However, our results of elevated SP and CGRP levels in CM patients are in accordance with some of the previous studies on the episodic migraine. Ashina *et al* reported on the elevated plasma levels of CGRP in migraine outside of attacks (Ashina *et al*, 2000). Fusayasu *et al* (2007) reported that patients with episodic migraine had increased plasma CGRP and SP concentrations, and a positive correlation between SP and CGRP levels existed.

The few studies on the relationship between NGF level and chronic pain have been reported based on limited subjects and samples. All data obtained in experimental animal pain models support the role of NGF as a putative candidate intervening in the pathogenesis of chronic pain (Kitamura *et al*, 2005; Sabsovich *et al*, 2008). In spite of the evidence from animal studies, only one study has been performed to establish its role in humans with CM (Sarchielli *et al*, 2001) which was based on cerebrospinal fluid (CSF) samples obtained through lumbar puncture. This study reported that patients with CM showed higher NGF levels in CSF compared with control subjects and a positive correlation existed between NGF and both SP and CGRP levels in CSF. CSF is more likely to closely reflect the level of pain mediators causing CM pain but compared to blood collection, lumbar puncture is a far more complicated procedure that may cause side-effects including postural headache (Sand *et al*, 1987). The past literature did not investigate the relationship between headache and plasma NGF level. Our results showed that NGF levels in both peripheral circulation and saliva were also increased in CM patients.

Previous results suggest that neuropeptides such as CGRP and SP, and also NGF have a definite role in the pathophysiology of migraine generation and maintenance. The role of these neuropeptides and neurotrophins in pain generation in migraine patients are once more highlighted by our results from multiple linear regression analysis which show that the mean pain intensity of CM subjects increases with elevation of plasma NGF, SP, and CGRP and saliva SP, and CGRP levels. The hypothesis is that unknown triggers for headache activate perivascular trigeminal axons which release vasoactive neuropeptides such as SP and CGRP to promote neurogenic inflammation and spread the inflammatory response to adjacent tissues, and that the orthodromic conduction along trigeminovascular fibers transmits nociceptive information toward the trigeminal nucleus caudalis and higher brain centers for the registration of pain (Goadsby *et al*, 2002). NGF is released by several cell types in response to tissue inflammation and induces a pain like response. It is also responsible for inducing a hyperalgesic effect that can be prevented by anti-NGF antibodies (Chudler *et al*, 1997) and a more chronic effect involved in the development of allodynia and secondary pain response by potentiating nociceptive sensory input and NMDA-evoked responses (Lewin *et al*, 1994). Although the mechanisms are poorly clarified we could understand that endogenous substances including neuropeptides and NGF released during a headache attack sensitize trigeminal neurons to transmit nociceptive signals to the brainstem (Giniatullin *et al*, 2008).

An interesting variance is found with the relationship between pain intensity and NGF, SP and CGRP concentrations in this study. The NGF and sensory neuropeptide levels are significantly associated with pain intensity both in plasma and saliva with the exception of salivary NGF levels. Compared to the other substances, the salivary NGF level did not show a significant relationship with pain intensity, and instead was elevated in women and decreased with the increase of salivary flow rate. These results are in accordance with the results of our previous study which show NGF levels in saliva are highly gender dependent (higher in women) and also variable according to the glandular origin of the saliva (higher in submandibular gland secretions) (Nam *et al*, 2007). The proportion of each salivary gland secretion that constitutes whole saliva is highly affected by the flow rate (Miles *et al*, 2004). The mouse submandibular gland is especially well known for its high concentration of NGF (Thoenen and Barde, 1980; Shooter, 2001). There are no reports which show that salivary SP and CGRP share this characteristic. We may presume that the production and metabolism of NGF is orchestrated in a more complex manner that is easily influenced by various factors, resulting in an inconsistent salivary concentration.

Another interesting result is that plasma NGF level shows a positive correlation with plasma sensory neuropeptide levels. NGF-induced hyperalgesia seems to involve an enhanced production of SP and CGRP in nociceptive peripheral nerve endings (Donnerer *et al*, 1992). The levels of SP or CGRP have been shown to increase in the spinal cord and the peripheral nerves of animals with chronic inflammatory diseases and NGF plays a continuous dynamic role in the control of peptide neurotransmitter levels and synthesis in such situations. The regulatory role of NGF in the production of SP and CGRP is well shown in the fact that anti-NGF serum prevents the increase of neuropeptides caused by adjuvant inflammation (Donnerer *et al*, 1992). NGF, synthesized in larger quantities in inflamed tissue is thought to be taken up by NGF receptor-bearing sensory axon terminals, transported retrogradely to contribute to increased neuropeptide synthesis. Reversely, neuropeptides including SP and CGRP have been proven to be able to increase NGF mRNA expression (Dallos *et al*, 2006).

SP and CGRP are often co-localized in the trigeminal sensory fibers and it has been suggested that CGRP

enhances the action of SP when these peptides are co-administered in the central nervous system because of the ability of CGRP to inhibit an enzyme involved in SP degradation (Le Greves *et al*, 1985). Thus a correlation between SP and CGRP also exists and neuropeptides and NGF play a regulatory role on each other that will consequently modulate the production and maintenance of CM pain as it could be observed in our results.

The plasma SP and CGRP levels also showed close correlation with their saliva levels. Although few studies exist that handle the correlation between salivary and plasma levels of neuropeptides, there are numerous reports that show certain biochemical, immunological and endocrine analytes in oral fluid and plasma demonstrate good correlation forming the basis of using saliva as an effective diagnosis tests. The easy non-invasive nature of collection and the close relationship between oral fluid and plasma levels of such substances make oral fluid a valuable clinical tool (Choo and Huestis, 2004). Moreover, Parris *et al* reported that the salivary SP level of chronic pain patients were higher than its plasma level, showing that saliva may be a less invasive and more efficient diagnostic tool to measure markers that reflect pain states (Parris *et al*, 1993). The origin of salivary neuropeptides is insufficiently known. In rats the major part of circulating CGRP is released from perivascular nerve terminals. Thus, possible changes of neuropeptide levels in blood and subsequently in saliva may reflect changes in their expression in the inflamed PNS or CNS of CM patients.

The salivary concentrations of NGF, SP and CGRP showed negative correlations with salivary flow rates. Dawidson *et al* (1997) reported that neuropeptides in saliva decreased two- to fourfold when the volume of saliva increased 6 to 8 times due to stimulation. This is due to the diluting effect of the increased saliva volume and indicates the fact that neuropeptides are released in the saliva at a relatively constant rate. Our results also show that concentrations do fluctuate depending on the salivary flow rate and let us know that to gain reproducible results for follow-up studies, saliva must be collected under a repeatable standard measurement protocol.

Three limitations of our study should be mentioned. First, our data were based on a one-time measurement of plasma and saliva NGF, SP, and CGRP levels, which may not accurately reflect the status of the study participants. Second, the origins of the peptides in plasma and saliva and to what extent they reach the circulation from the central nervous system were not fully investigated. We cannot exclude the possibility that concentrations in the peripheral circulation and saliva differ from values in cranial circulation. Third, we selected patients diagnosed with CM and excluded other types of CDH. By including and differentiating subjects with various headache pain characteristics and comparing their neurotrophin and neuropeptide levels in future studies, we will be able to further discover the underlying mechanism of headache pain and apply plasma and salivary levels of such substances to the diagnosis and knowing of prognosis.

In conclusion, NGF, SP, and CGRP appear to be relevant to the pathophysiology of CM, and may have various interactions with each other. Moreover saliva concentrations of these neuropeptides showed excellent correlation with their plasma levels presenting the possibility of utilizing saliva as a less invasive diagnostic tool for measuring pain markers of CM in clinical situations.

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Author contributions

Min-Uk Jang and Ji-Woon Park contributed to this article by analyzing and collecting the data, and drafting the article. Hong-Seop Kho designed the study and revised the manuscript. Sung-Chang Chung provided funding, reviewed the collected data, and helped to prepare and finalize the manuscript. Jin-Woo Chung as a corresponding author designed the study, played a major role in the interpretation of data, and revised the manuscript critically.

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