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EFFECT OF INFLAMED PULP TISSUE EXTIRPATION ON LEVELS OF INFLAMMATORY NEUROPEPTIDES IN HUMAN SALIVA

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ABSTRACT

Aim: To measure and compare the salivary levels of substance P (SP), calcitonin gene-related peptide (CGRP), and neurokinin A (NKA) before and after inflamed pulp tissue extirpation. Also, the differences between pre- and postoperative visual analog scale (VAS) pain scores were evaluated.

Methods: Twelve patients with irreversible pulpitis related to a mandibular molar tooth were enrolled in this study after fulfillment of the selection criteria. Patients rated their level of pre- and postoperative pain on a visual analog scale. Saliva samples were collected from each patient before and 5 days after the first endodontic treatment visit. All samples were analyzed using (ELISA) kits. The differences between the pre- and postoperative levels of SP, CGRP, and NKA were analyzed using the one-way ANOVA test while the differences between the pre- and postoperative VAS scores were evaluated using the paired t-test. Any correlation between levels of SP/CGRP/NKA and VAS pain scores was examined using the bivariate analysis.

Results: There was a statistically significant difference between pre- and postoperative levels of SP and also between pre- and postoperative levels of CGRP. No significant difference was found between pre- and postoperative levels of NKA. Regarding VAS scores, the preoperative pain was significantly higher than postoperative pain. The VAS score of subjective pain was significantly correlated with levels of SP and CGRP but not NKA.

Conclusions: SP and CGRP can be employed as beneficial determinants of the degree of pain in inflammatory pulpal diseases. NKA plays an insignificant role in neurogenic inflammation and pain.

KEYWORDS: Substance P; Calcitonin gene-related peptide; Neurokinin A

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INTRODUCTION

Activation of the neural system was observed to be essential to the inflammatory process many years ago when Celsus characterized inflammation as involving four cardinal signs: Dolor (pain), Calor (heat), Rubor (redness), and Tumor (swelling). Since that time, however, pain has primarily been viewed as a symptom rather than a contributor to the development of inflammation [1]. Inflammation that is caused by the local release of inflammatory neuropeptides that serve as mediators by afferent neurons is known as neurogenic inflammation. The central and peripheral nervous systems contain a collection of tiny peptides called neuropeptides that range in size from 4 to more than 40 amino acids[2]. This study focused on neuropeptides that, upon neural activation, are released into peripheral tissues during acute inflammation. When nociceptive fibers, particularly C-fibers, are activated, a variety of neuropeptides are released [3]. Rapid plasma extravasation and edema follow the activation of nociceptors on a timescale that is quicker than that of immune cell infiltration. But it is important to remember that nociceptors can have a positive modulatory effect in other scenarios, for example, the release of calcitonin gene-related peptide (CGRP) by nociceptors which has been found to regulate inflammation in bacterial infections [4].

Human salivary fluid is secreted from different types of salivary glands. Salivary glands are supplied with nerve fibers that contain neuropeptides, such as substance P, calcitonin gene-related peptide, and Neurokinin A[5]. A member of the tachykinin neuropeptide family, substance P (SP) is an undecapeptide of 11 amino acids. The most well-known roles of substance P are as a neurotransmitter and as a pain modulator through modifying cellular signaling pathways [6,7]. Substance P also contributes to angiogenesis, memory processing, cell growth, proliferation, and vasodilation[8]. Substance P is involved in several bodily functions, including inflammation, chemotaxis, and pain perception [9]. Pain perception is one of substance P's earliest and best-documented functions [10].

Another neuropeptide that is thought to play a role in neurogenic inflammation is calcitonin gene-related peptide (CGRP). In mammalian primary afferents, CGRP is the most prevalent peptide and is found in sensory neurons of all sizes. In contrast to its well-established role in peripheral neurogenic inflammation, CGRP is also thought to play an excitatory function in the spinal cord. CGRP has a very delayed onset and a protracted half-life when depolarizing dorsal horn neurons [11].

A neuroactive peptide produced by the preprotachykinin A gene is called neurokinin A (NKA), formerly known as substance K [12]. Both substance P and neurokinin A are transcribed by the same mRNA, which can be translated into either molecule when alternatively spliced [12].

In the literature, it is becoming increasingly evident that periodontitis and indeed other orofacial inflammatory disorders may be modulated by imbalances in these neuropeptides [13,14,15] However, limited information is available on the evaluation of levels of neuropeptides in human saliva in inflammatory pulpal disease. Therefore, this pilot study aimed to measure and compare the salivary levels of substance P (SP), calcitonin gene-related peptide (CGRP), and neurokinin A (NKA) in human salivary fluid before and after inflamed pulp tissue extirpation. This study also aimed to evaluate the difference between pre- and postoperative visual analog scale (VAS) scores of patients' subjective pain. The null hypothesis stated that there would be no statistically significant difference between pre- and postoperative salivary levels of SP, CGRP, and NKA.

MATERIALS AND METHODS

Ethical regulation

The ethical approval number (REC-D 536-3) was obtained from the Research Ethical Committee at the Faculty of Dentistry, MSA University according to the rules and guidelines of research on humans.

Patient eligibility and selection criteria

Patients were referred to the endodontic clinic at MSA University in Cairo, Egypt in June 2023 and assessed for eligibility in this study. All patients shared the same chief complaint of irreversible pulpal inflammation including throbbing pain involving a mandibular molar tooth that is also sensitive to hot and cold stimuli. A thorough clinical examination and periapical radiographic imaging were performed on the patients as an integral part of the diagnostic process before initiating endodontic therapy.

Inclusion criteria

Patients must have met all of the following inclusion criteria to be eligible for participation in this study.

1. The presence of symptomatic irreversible pulpitis involving a mandibular molar.
2. The presence of spontaneous throbbing pain.
3. Age range (21 to 49 years old).
4. Patients were able to rate and record their pain levels.

Exclusion criteria

Patients with any of the following exclusion criteria were not eligible for participation in this study.

1. The presence of any chronic systemic disease.
2. The presence of any periodontal involvement.
3. Presence of more than one affected tooth.
4. Use of analgesics in the last 3 days before the first endodontic visit.

Measurement of pain severity

Patients rated their subjective assessment of the level of preoperative pain on a visual analog scale (VAS) ranging from 0 to 10. While a “10” number denoted the most painful scenario, a “zero” value denoted the absence of pain. Pain which was rated above “6” on the scale was considered severe and included in this study.

Clinical endodontic procedure

Saliva samples were collected from each patient twice. The first collection procedure was carried out prior to local anesthetic administration for endodontic intervention. The first visit of root canal treatment included complete extirpation of the pulp tissue and root canal preparation under strict rubber dam isolation. After administration of profound anesthesia, caries was excavated and a straight-line access cavity was gained using carbide round bur and Endo-Z bur. Inflamed coronal pulp tissues were amputated and canal orifices were located. Stainless steel K-files #10 and # 15 (MANI Inc, Tochigi, Japan) were used for canal negotiation and glide path preparation. This is followed by working length determination using an electronic apex locator (Morita Corp, Osaka, Japan) and confirmed radiographically. Mechanical preparation was carried out using EdgeFile X7 rotary system (EdgeEndo, Albuquerque, New Mexico, USA) in the following sequence: (#17/0.04 - #20/0.04 - #25/0.04 - #30/0.04 - #35/0.04). When the distal root had only one distal canal, #40/0.04 was additionally used. All rotary files were operated at 400 RPM and 3 N.cm. Root canal irrigation was performed between every rotary file. The canals were copiously and passively irrigated using 2.5 % sodium hypochlorite with side-vented needle tips to minimize the possible risk of apical extrusion of the root canal irrigant. Temporary filling material (Coltosol F, Coltene/Whaledent, Switzerland) was used to seal the coronal access cavity.

Ibuprofen 400 mg was prescribed to all patients only as a rescue analgesic drug. Patients were carefully instructed to take it only when needed in case of persistent pain following the first treatment visit. They were also asked to report such incidents if happened. Patients were then recalled for a second visit after 5 days for another sample collection and resumption of endodontic therapy. All patients reported that analgesic medication was not required following the first treatment visit. Additionally, the patients subjectively rated and recorded their pain on a VAS scale on the fifth day following the first visit and before initiating the second visit. By collecting the saliva samples twice, each participating patient served as his/her own control.

Collection of samples

All patients were instructed to refrain from food intake, liquid intake, and teeth brushing for 3 hours before the sampling procedure. To obtain salivary fluid samples, patients were asked to expectorate into sterile polypropylene tubes. Salivary fluid samples containing 500 µL were pipetted into a different set of polypropylene tubes. After centrifuging the salivary samples at 10,000 rpm for 6 minutes, the supernatant fluid was poured into sterile polypropylene tubes and kept in the freezer until analysis.

Measurement of salivary levels of neuropeptides:

All pre- and postoperative frozen samples were defrosted until they reached room temperature. All samples were analyzed by measuring levels of SP, CGRP, and NKA using enzyme-linked immune assay (ELISA) kits.

For the measurement of salivary levels of SP, CGRP, and NKA, 50 µL of each saliva sample was pipetted into a pre-coated microplate well followed by the addition of 50 µL of acetylcholinesterase (AChE) tracer and ELISA antiserum then incubated overnight at 4°C. Ellman's reagent (DTNB) was added for the determination of cholinesterase activity (original Ellman's spectrophotometrical method) and the absorbance levels were read with a microplate reader at 412 nm. The concentrations of the tested neuropeptides were calculated in accordance with ELISA standards.

STATISTICAL ANALYSIS

The Statistical Package for the Social Sciences (IBM.SPSS) software, version 26, was used to evaluate the collected data. The differences between the pre- and postoperative levels of SP, CGRP, and NKA in saliva were analyzed using the one-way ANOVA test. The differences between the pre- and postoperative VAS pain scores were evaluated using the paired t-test. Any correlation between the levels of SP/CGRP/NKA in the salivary fluid and (VAS) scores of patients' subjective pain was examined using the bivariate analysis. ($P < 0.05$)

RESULTS

Fifty-three patients were referred for endodontic therapy and the purpose of the study was clearly explained in simple words yet in detail to all targeted patients. Thirty-eight patients were excluded because they declined to take part, and 15 patients were assessed for eligibility. Three patients were also disqualified for not meeting the previously mentioned inclusion criteria of the study design. Therefore, 12 patients (4 males and 8 females) with symptomatic irreversible pulpitis related to a mandibular molar tooth were enrolled in this study after complete fulfillment of the criteria. The minimum age and maximum age of participating patients were 23 years and 46 years, respectively and the mean age of patients was 29.67 ± 6.89 years.

Results revealed that there was a statistically significant difference between preoperative (68.51±11.37) and postoperative salivary levels of SP (24.79±15.45) ($P<0.05$) and also between preoperative (29.83±15.58) and postoperative salivary levels of CGRP (13.98±10.81) ($P<0.05$). However, no statistically significant difference was found between preoperative (33.16±17.41) and postoperative salivary levels of NKA (34.55±14.54) ($P = 0.83$). **Table (1) Figure (1)**

TABLE (1) Means and standard deviations of preoperative and postoperative salivary levels of SP, CGRP, and NKA.

Inflammatory Neuropeptide	*Preoperative level*	*Postoperative level*	*P value*
SP	68.51 ± 11.37	24.79 ± 15.45	<0.05
CGRP	29.83 ± 15.58	13.98 ± 10.81	<0.05
NKA	33.16 ± 17.41	34.55 ± 14.54	0.83

SP, substance P; CGRP, calcitonin gene-related peptide; NKA, neurokinin A

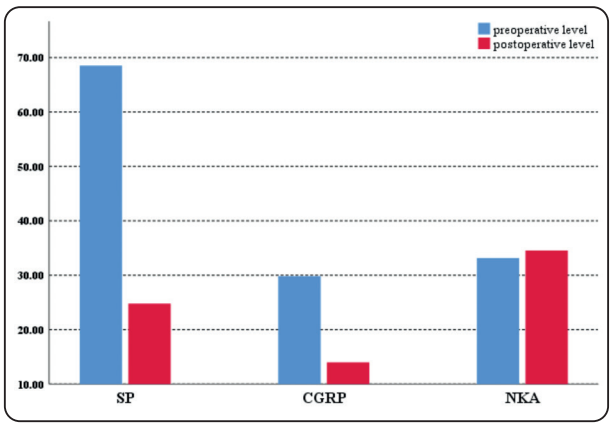

Fig. (1) Bar chart showing preoperative and postoperative salivary levels of SP, CGRP, and NKA.

Regarding VAS scores, the preoperative subjective pain (6.58±2.35) was significantly higher than postoperative subjective pain (2.25±1.83) (P<0.05). **Table (2)**

TABLE (2) Means and standard deviations of preoperative and postoperative VAS pain scores.

VAS Score	*Mean and Standard deviation*	*P* value
Preoperative subjective pain	6.58 ± 2.35	<0.05
Postoperative subjective pain	2.25 ± 1.83	

VAS, visual analog scale

The bivariate analysis showed that the VAS score of subjective pain was significantly correlated with salivary levels of SP (r = 0.38; P < 0.05) and CGRP (r = 0.35; P < 0.05). However, no statistically significant correlation was noted between the salivary level of NKA and the VAS score of subjective pain (r = 0.14; P = 0.27). **Table (3)**

TABLE (3) Correlation coefficient bivariate data of neuropeptides and pain.

Variable	*SP*	*CGRP*	*NKA*	*Pain*
SP	-	0.52	0.19	0.38*
CGRP	-	-	0.17	0.35*
NKA	-	-	-	0.14
Pain	-	-	-	-

SP, substance P; CGRP, calcitonin gene-related peptide; NKA, neurokinin A

****Significant correlation when (P<0.05)***

DISCUSSION

To our knowledge, few studies in the literature assessed and evaluated the levels of neuropeptides in human saliva and their correlation with pain[16,17]. However, this is the first study to measure and compare the salivary levels of neuropeptides before and after complete inflamed pulp tissue extirpation.

Irreversible pulpitis is a condition in which inflamed pulp tissue cannot be treated except by its removal. Cases with symptomatic irreversible pulpitis were selected in this study as an inclusion criterion since the pain of pulpal origin is considered one of the most undesirable scenarios among patients due to its severity. A specific age range (21 to 49 years old) was also selected as one of the inclusion criteria in this study to limit the difference in pain perception.

The null hypothesis was that no statistically significant difference would exist between preoperative and postoperative salivary levels of

SP, CGRP, and NKA. However, results clearly showed that there was a statistically significant difference between preoperative and postoperative salivary levels of SP and between preoperative and postoperative salivary levels of CGRP but not NKA. Hence, this null hypothesis was rejected.

The elevated preoperative salivary levels of SP and CGRP in comparison with their postoperative salivary levels could be related to the presence of an inflammatory state associated with pain. In both behavioral and electrophysiological studies, CGRP has been found to markedly enhance the excitatory effect of SP and other neuropeptides [11]. This finding is in harmony with **Schlereth et al 2016** [18] who demonstrated a complex interaction of the neuropeptides CGRP and SP and co-localization of both CGRP and SP. The findings of the present study are in agreement with the mentioned state of co-localization of both neuropeptides.

The present study surprisingly revealed no statistically significant difference between preoperative and postoperative salivary levels of NKA. Also, there was no significant correlation between NKA and VAS score of subjective pain. These findings could suggest that NKA has an insignificant or negligible role in the neurogenic inflammatory process and the state of pain in comparison to other evaluated neuropeptides.

Interestingly, this study included a number of patients that is similar to a previous one that evaluated almost similar neuropeptides in a different clinical scenario [16]. However, these relatively small sample sizes could remain the only limitation to be taken into consideration for future research.

CONCLUSIONS

Within the constraints of this study, it can be concluded that the salivary levels of SP and CGRP can be employed as beneficial determinants of the degree of pain in inflammatory pulpal diseases. It is also possible to infer that NKA plays an insignificant role in neurogenic inflammation and pain. It is worth noting that future studies with larger sample sizes are recommended to shed more light on the role of these neuropeptides in inflammatory conditions.

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