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Comparative study of substance P and neurokinin A in gingival crevicular fluid of healthy and painful carious permanent teeth

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ABSTRACT

Background: It is shown that neuropeptides can be transported from pulp chamber to periodontal ligament through apical foramen and accessory canals. Therefore, clinical pulpal pain leads to expression of preinflammatory neuropeptides such as substance *P* (SP) and neurokinin A (NKA) in gingival crevicular fluid (GCF). This study aimed to evaluate levels of SP and NKA in GCF of carious and healthy permanent teeth, comparatively.

Materials and Methods: This cross-sectional study was performed on twenty children referred to Department of Pediatric Dentistry, Tehran University of Medical Sciences, who had a painful permanent first molar. Sampling was done by sterile paper cone from GCF of the mentioned teeth and the intact tooth of the other side of the jaw in the same patient. Values of SP and NKA were measured by ELISA test.

Results: The mean concentration of SP in GCF of painful carious and healthy teeth was 2.65 ± 0.56 and 1.83 ± 0.65 pcgr/ml, respectively. This value was 2.29 ± 0.29 and 1.61 ± 0.35 pcgr/ml for NKA concentration in carious and healthy teeth as well.

Conclusion: Significant higher levels of both SP and NKA in GCF of painful carious teeth were observed, which is in line with previous studies' findings.

Key Words: Gingival crevicular fluids, neurokinin A, neuropeptide, substance P

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INTRODUCTION

Dental caries is the most common chronic disorder in children, and toothache is a usual complication.[1] Odontogenic pain occurs as a result of physical stimulation or inflammatory mediators provoking nociceptive efferent neural fibers. Nociceptive fibers are richly distributed in trigeminal nerve fibers which are responsible for periapical and pulp tissues innervations.[2] It has been shown in studies that irritation of the affected tooth initiates inflammatory reactions in gingival mucosal tissues.[3]

Pulp damages lead to cell apoptosis and inflammation. Depending on duration and intensity of stimuli and body reactions, these damages may lead to a variety of changes from reversible pulpitis or progression through to irreversible pulpitis and necrosis.[4]

Stimulation of the pulp results in different biologic reactions due to a nonspecific inflammatory response in body, which are presented with polymorphonuclear leukocytes degranulation, protease inhibitor activation,

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and histamine, bradykinin, and arachidonic acid metabolites release.[5]

It has been shown that neuropeptides can enter from pulp to periodontal ligament (PDL) space through communication pathways such as apical foramen and accessory canals. Thus, pulp-related pain leads to expression of pro-inflamatory neuropeptides in adjacent gingival crevicular fluid (GCF).[6-8]

Many studies have highlighted the role of neuropeptides in molecular mechanisms of dental pain.[9,10] These Neuropeptides are found in dental pulp and the periodontium, and their raised levels have been reported in decayed painful teeth.[2,11] Different studies have used various methods including radioimmunoassay, enzyme immunoassay (EIA or ELISA), and immunohistochemistry to measure these mediators, but among these methods, ELISA is known as a significantly safe and effective method.[11]

The present study aimed to compare the level of SP and NKA as pain mediators available in GCF, between intact and decayed permanent teeth, using ELISA method.

Evaluating the mediators and clarifying their role in the neurophysiology of pulpitis may lead the research into more effective ways to alleviate the symptoms of pulpitis.

MATERIALS AND METHODS

This survey was a cross-sectional study conducted on 20 children (13 girls and 7 boys) aged between 7 and 12 years referred to Pediatric Dentistry Department of Dentistry Faculty of Tehran University of Medical Sciences, Tehran, Iran, between 2012 and 2013.

Inclusion criteria were the presence of a painful first molar tooth diagnosed with irreversible pulpitis by clinical or radiographic examination. The control tooth was an intact molar in the opposite quadrant in the same jaw. Exclusion criteria were considered to be antibiotic use during the last month, taking analgesic or anti-inflammatory drugs during the last month, positive history of any systemic disease which may affect oral tissues, any evidence of gingivitis, abscess, bleeding during periodontal probing, any radiographic evidence for periapical or periodontal defect, carious, painful, or previously restored the first molar in the opposite side of the patient's jaw, uncooperative patient, and parents' disconsent with taking part in the study.

Then, eligible patients were asked about their child's demographic information (name and family name, date of birth, and gender), medical and dental histories, and the presence of dental pain. The location of the carious and intact molar teeth (upper or lower jaw) was also documented. After giving brief information about the aims and method of the study, written consent form was obtained from parents. Patients without recent radiographs underwent radiographic examination to rule out any proximal decay in the test and control teeth. The intensity of the pain was evaluated by visual analog scale (VAS) which is one of the most reliable tools to assess and report pain in children age group.

Sampling

Sampling was done in each patient in the eight following steps:

1. Isolation of the tooth with the use of cotton roll and suction
2. Drying the area by air tooth dryer for 10 s
3. Removing the biofilm with using sterile cotton
4. Putting the absorbent paper points in gingival crevice.

For this purpose, gamma-sterilized paper number 30 was used. The paper (Aria Dent Co. Tehran, Iran) was placed in gingival crevice of buccal surface of the tooth along the linear axis till a resistance was felt and it remained there for 30 s. In case, the paper was contaminated with saliva or blood during or before its placement; it was changed with a new one.

All samples were taken between 9 and 11 am from all patients.

5. Placing every paper in a propylene microtube containing 300 μl of phosphate-buffered saline (PBS) right after sampling.

PBS is a water-based saline solution which is used in biologic studies for its isotonic and nontoxic nature. It is made up of sodium chloride and sodium phosphate. The phosphate group helps keep a constant pH (almost 7.4). Utilizing PBS tablets is one of the ways for preparing this solution (43).

6. Placing samples in a freezer immediately after sampling and transferring frozen samples to Immunology Laboratory of Tehran University of Medical Sciences
7. Keeping samples in −70°C until laboratory analysis was done
8. Analyzing the GCF samples in laboratory.

In this study, we used ELISA method and ELISA reader machine (Dana, DA3200) for measuring SP (Cyman kit, USA) and NKA (Phoenix kit, USA) concentrations.

Samples were placed in room temperature for an hour and were then centrifuged for 20 min for complete solvation of absorbed particles in buffer solution.

To measure SP levels, we first prepared EIA buffer, washed buffer, and standards were created according to the ELISA kit instructions. Samples were incubated at 4°C overnight.

Unlinked proteins were washed the next day, and a junctional substance was added to the wells which would bind to the intended antibody. Unbound molecules of this substance were then washed, out and a color-producer substance was added to allow the use of optical densitometry and software analysis to determine the level of antigen.

For measuring NKA levels, all the same steps were performed, and standard density solutions were prepared by following the manufacturer's instructions as well. Five test tubes were prepared, and 900 µl of standard attenuator solution was poured in the first laboratory tube, and each of other laboratory tubes contained 500 µl of it. Five hundred microliters of the high density standard solution was then added to the first test tube and mixed by shaker. Five hundred microliters of the first laboratory tube solution was poured to the second one, and it continued to the 5th one. Final achieved densities were 10, 5, 2.5, 1.2, and 0.6 pg/ml, respectively. Wells of the microplate were previously covered by anti-NKA antibody; then, 50 µl of the prepared standard solution from the five test tubes was poured in the first five wells of the plate, and the same procedure was done for the rest wells with 50 µl of each sample. Twenty-five microliters of primary antibody and biotinylated peptide were added to each well afterward, and samples were incubated in room temperature for 2 h.

Unlinked proteins were washed, and 100 mcl of SA-HRP solution (junctional substance) was added to the wells. Samples were incubated in room temperature for an hour, washed again, and the color-producer substance was added in the last step. The plate was transferred to ELISA reader machine (Dana, 3200 model) to measure NKA level according to color densitometry. Final measures were reported as level of SP and NKA (in picogram) in each milliliter of GCF.

RESULTS

Twenty patients enrolled in the present study, 7 (35%) patients were male and 13 (65%) were female. The patients' average age was 9.4 ± 1.4 (with a range of 7-12 years). Demographic data of participants are demonstrated in Table 1. Among 40 evaluated teeth, 26 belonged to the lower jaw, and the remaining teeth (14 teeth) were located in the upper jaw.

The pain intensity described by VAS was reported severe (≥5) by all patients.

Mean concentration of SP mediator were 2.65 ± 0.56 and 1.83 ± 0.65 in GCF of painful decayed and healthy intact teeth, respectively. These measures were reported for NK level 2.29 ± 0.29 and 1.61 ± 0.35, respectively.

As demonstrated in Table 2, Kolmogorov–Smirnov test showed a normal distribution for SP and NKA level variables among decayed and intact teeth ($P > 0.05$). Paired *t*-test was then used for comparison both of these variables between decayed and healthy teeth. The mean levels of SP and NKA difference in GCF were significantly different between healthy and painful decayed teeth ($P < 0.05$). Table 3 shows related values comparatively.

To test the relevance between SP and NKA, correlation test was performed which showed no significant relation between two variables in GCF.

DISCUSSION

SP and NKA are two neuropeptides among tachykinins with a same origin which are released from trigeminal nerve endings due to physical or chemical stimuli and are the main pain-related neurotransmitters.[12] Existence of these mediators in GCF of intact teeth may indicate their role in physiologic processes.[11] Pulpitis causes SP and NKA release from sensory endings of pulp which are vasodilators and increase inner pressure of pulp.[7,13] Communicating canals between pulp cavity and PDL including apical foramen, accessory canals, canals of the furcation area, and dentinal tubules direct these neuropeptides

Table 1: Demographic data of participants

Gender	Number	Frequency (%)	SD	Mean age
Female	13	65	1.49	9.54
Male	7	35	1.24	9.12
Total	20	100	1.4	9.4

SD: Standard deviation

Table 2: Comparison of substance P and neurokinin A concentrations between gingival crevicular fluid of decayed and intact teeth

Pain mediators	Mean concentration in GCF of intact teeth (pg/ml)	Mean concentration in GCF of decayed teeth (pg/ml)	Average of differences	*P*
SP	1.83±0.65	2.65±0.56	0.825±0.385	0.0001
NKA	1.61±0.35	2.29±0.29	0.687±0.397	0.0001

SP: Substance P; NKA: Neurokinin A; GCF: Gingival crevicular fluid

Table 3: Correlation test between substance P and neurokinin A levels

Pain mediators	Correlation coefficient	*P*
SP and NKA in intact teeth GCF	−0.65	0.785
SP and NKA in decayed teeth GCF	0.123	0.6

SP: Substance P; NKA: Neurokinin A; GCF: Gingival crevicular fluid

into PDL space and into GCF, in turn. On the other hand, since sensory nerves of pulp and periodontal tissues have the same origin, sensory stimulation of pulp may have a provoking effect on periodontal tissue innervations too.[5,11,14]

Since it has been previously shown in studies that gingivitis and periapical or periodontal defects can alter the level of SP and NKA in GCF, the study did not include patients with evidence of the above-mentioned disorders.[11,15-17] Samples also were taken in the same time of the day from all participants to avoid probable changes of GCF amount secretion affected by circadian rhythm.

SP and NKA levels were shown to have significant higher concentrations in GCF of decayed teeth which were affected by pulpitis. Considering above clarification of the mechanism and cause of this additional concentration around decayed teeth, obtaining such a result seems rational and is also supported by some previous surveys. Avellán *et al.* and Giannopoulou *et al*. in their two distinct studies reported higher level of SP found in GCF of decayed teeth in comparison to the control group. The nature of pain was different in these studies as it was induced by pushing elastic separators and using simulator machine, which varies in origin with the pain caused by pulpitis and pulp disorders.[6,18]

Another group of studies has also compared the level of pain mediators such as SP between painful and intact teeth and supported its role in toothache by finding its higher level in GCF of decayed teeth.[19-21] While it has been shown that local anesthetic ingredients can affect the result of such studies by slowing the release of neuropeptides from dental pulp as well as lowering SP concentration in pulp tissue and diluting it.[22,23] Some of these studies were performed on samples of coronal pulp tissue taken after local anesthesia administration. Among evaluated researches, Awawdeh *et al.*'s study was the nearest to ours in regard to methodology but was carried out in adult setting.[2] Reported results of the study were in line with our findings both for SP and NKA. They had found higher levels of these mediators in GCF of decayed teeth than what we did, and this quantitative difference is attributable to the sampling method as they used periopapers which have larger absorbing surface and thus higher rate of absorption. The duration of sampling was also different in both studies as it was 30 s in ours and 1 min in Awawdehal's. Variability in the laboratory methods was another cause.

Besides presenting SP and NKA as effective mediators contributing in dental pain induction, this study highlighted the fact that each of these mediators participates in pain induction independently.

Our study includes some limitations. These include the small sample size as many of the eligible patients had used medications stated in our exclusion criteria. It was not possible to collect longitudinal data on the changes in these mediators following treatment due to limited resources in our busy public clinic. Further studies are suggested to support the pivotal role of SP and NKA in dental pain process with larger populations and applying a variety of changes in sampling-related factors such as number of times and duration of sampling as well as evaluating after-treatment conditions. Making a clearer understanding of the exact role of these important mediators in dental pain process can lighten the path for future studies on drugs and methods used for dental pain alleviation.

For better understanding of responsible substances and mechanisms of dental pain and better finding of dental pain-reducing ways, this study aimed to evaluate levels of SP and NKA in GCF of carious and healthy permanent teeth, comparatively.

CONCLUSION

Significant higher levels of both SP and NKA in GCF of painful carious teeth were observed, which is in line with previous studies' findings.

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Conflicts of interest
The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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