Original Article

The expression of pulpal substance P after dentinal application of *Escherichia coli* lipopolysaccharide

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Abstract

Purpose: The purpose of the research was to quantify the expressions of substance P (SP) in sensory nerve cells contained in the dental pulp of Wistar rats following the application of *Escherichia coli* lipopolysaccharide (LPS).

Materials and Methods: Forty-two male Wistar rats were divided into six groups ($n = 7$): Groups C1 and C2 received no treatment and served as negative controls; Groups C3 and C4 only had cavity preparation; and Groups T1 and T2 received cavity preparation and treated with LPS. All teeth were subjected to immunohistochemistry analysis of SP expression at 24 h (C1, C3, and T1) and at 72 h (C2, C4, and T2).

Results: There was a significant increase in the SP of the T1 group compared to the C1 and C3 groups and of the T2 group compared to the C2 and C4 groups. While there was no significant difference in the expression of SP between the C1 and C2 groups, a significant increase was found in T1 group compared to that of the T2 group and in the C3 group compared to the C4 group ($P < 0.05$).

Conclusion: The application of LPS to teeth resulted in the highest expression of SP after 24 h which then decreased after 72 h.

Keywords: Lipopolysaccharides, pulpitis, sensory neurons, substance P, Wistar rats

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INTRODUCTION

Pulpitis occurs in response to inflamed tooth pulp and is associated with painful clinical symptoms which usually indicate a patient presenting with pulpal diseases. Clinical studies have shown that application of lipopolysaccharide (LPS) to the pulp induced the inflammation and sensitization in dental pulp.[1] LPS can diffuse through open dentinal tubules by fluid movement to reach the pulp chamber, triggering a cascade of pathological changes.[2] It also indirectly activates nociceptors via the release of prostaglandins, leukotrienes, and cytokines.[3,4] However, neither the nature of the mechanism for applying LPS to open dentinal tubules without exposing the pulp and inducing acute pulpal inflammation nor the modulation of the quantity expression of substance P (SP) in pulpal afferents is completely understood. Therefore, an understanding of these will help clinicians to preserve pulp vitality during conservation procedures and also solve the problems associated with the anesthesia of inflamed teeth.

Access this article online	
Quick Response Code:	Website: www.saudiendodj.com
	DOI: 10.4103/sej.sej_92_18

How to cite this article: Ismiyatin K, Wahluyo S, Soetojo A, Rahayu RP, Utomo H, Anindya C. The expression of pulpal substance P after dentinal application of *Escherichia coli* lipopolysaccharide. Saudi Endod J 2019;9:169-73.

The dental pulp is innervated with primary sensory neurons branching out from the largest cranial nerves of the trigeminal (TG) nerve, while the tooth is stimulated by a large number of myelinated and unmyelinated axons. The dental pain signal can be conducted via unmyelinated C fibers before entering predentin and dentin tubules which are responsible for the slow, dull, drawling dental pain. Alternatively, it can be directed via myelinated A-δ fibers located on the border of the pulp and dentin, especially in the pulp horn area, which are responsible for the rapid, sharp, lancinating, well-localized nociception pain.[1,5] Neuropeptide is located inside large dense core vesicles in a synaptic space and will be released in response to membrane depolarization, resulting in neurotransmitter exocytosis. Neurotransmitter peptide binds with specific receptors of neuropeptide to produce new electrical impulses conducted by myelinated nerves though changes in voltage-gated sodium channels (Na+).[6] Pulpal nerve fibers contain neuropeptides such as calcitonin gene-related peptide (CGRP), SP, neuropeptide Y, neurokinin A, and vasoactive intestinal peptide. SP, the first neuropeptide to be detected in dental pulp, plays a key role in the pulpal inflammatory process, modulates pulpal circulatory and immune system responses, while also promoting wound healing and maintaining the homeostasis of the pulp.[7-9] SP is largely responsible for the development and prolongation of dental pain and inflammation.[10]

However, it is not known whether *Escherichia coli* LPS application for periods of 24 and 72 h modulates the expression of SP in pulpal afferents. Applying *E. coli* LPS to the freshly cut dentin cavity without exposing the pulp can induce pulpitis in acute conditions.[11] SP was selected as a marker for neuronal activation since previous studies have demonstrated that it mediates the initiation of neurogenic inflammation and is released from the pulpal neurons. To subject these findings to further examination, a quantity of SP expression in the sensory nerve cells of Wistar rats following *E. coli* LPS application to the dentin surface was assessed using immunohistochemistry (IHC) methods.

MATERIALS AND METHODS

Experimental design

This research represented a laboratory-based experimental study involving 42 male Wistar rats (*Rattus norvegicus*), approximately 2.5 months old and weighing 220–300 g, as subjects.

The sample size was defined using the Higgins formula outlined by Lemeshow *et al*.[12] In total, 42 rats were randomly divided into six groups (n = 7) as follows: the maxillary first molars of the negative control groups (C1 and C2) received no treatment; those of the positive control groups (C3 and C4) were prepared without LPS application; and those of the treatment group were prepared and administered with *E. coli* LPS for 24 h (T1) and 72 h (T2) at a concentration of 10 mg/ml (LPS derived from *E. coli*, serotype 0111: B4; Sigma Chemical Company, St. Louis, MO, USA; product number L2630).[11] Ethical approval was granted, and the study was supervised by the Health Research Ethical Clearance Commission, Faculty of Dental Medicine, No. 018/HRECC. FODM/III/2018.

Cavity preparation and dentinal applications of lipopolysaccharide

The research models in groups C3, C4, T1, and T2 were anesthetized intramuscularly with 0.2 cc of a mixture of 0.5cc ketamine (Ketalar®, PT. Pfizer, Indonesia) and 0.5cc of xylazine base (Xyla®, PT. Tekad Mandiri Citra, Indonesia). The maxillary left first molar was prepared to a depth of 1 mm and a diameter of 2 mm using a new sterile 0.3 mm fissurotomy bur (SS White Burs Inc., Lakewood, NJ, USA), incorporating a high-speed drill and continuous water spray. This process was carried out with minimal damage to the pulp. Fine paper points were used to dry the cavity and detect the presence of bleeding indicative of pulp horn exposure. These cavities were subsequently irrigated with sterile saline solution and dried with sterile cotton rolls. LPS was applied using a fine paper point (~0.5 mm) (VDW GmbH, Munich, Germany) to deliver the liquid to the freshly cut dentin of the cavity which was then allowed to dry. This wet and dry procedure was repeated five times.[11] The cavities were then sealed with glass ionomer cement (Fuji 7, GC Corp, Tokyo, Japan).

Immunohistochemistry staining

The C1, C3, and T1 groups were sacrificed after 24 h and their C2, C4, and T2 counterparts at 72 h. To obtain analysis specimens, the maxillary left first molar tooth together with the upper jaw was surgically removed before being fixated with 10% paraformaldehyde at room temperature for 24 h. After the decalcification of the upper jaw and all its teeth in ethylenediaminetetraacetic acid solution 10% at pH 7.4 for 30 days, the specimens were embedded in paraffin before the pulp tissues were sliced in series to ± 4–5 μm thickness using a rotary microtome (Leica, RM2125RTS, Leica Biosystems Nussloch GmbH, D-69226 Nussloch, China). Slicing was performed parallel to the axis of the teeth which were mounted on the slides for fixation. Conventional IHC staining procedures were conducted using peroxidase staining mouse monoclonal anti-SP antibody (ab14184; Abcam, Cambridge, UK). The slides were subsequently rinsed three times in phosphate-buffered

saline (PBS) for 5 minutes on each rinsing and incubated with biotinylated secondary antibodies (Dako, Ely, UK). The positive SP expression in sensory nerve cells was indicated by brown staining in the cytoplasm. The counting procedure relating to SP expression was observed by two examiners, both specialists in oral pathology, who had been trained in calibration by an expert in that discipline to achieve the highest possible level of reliability. These observers examined blank specimens without the provision of grouping or interventional information. To avoid biased results, both the first examiner and second examiner observed the section from 10 perspective points with a light microscope at ×400 magnification (E 100 Dr; Nikon, Tokyo, Japan). Images captured by a digital camera (A7; Sony, Tokyo, Japan) were used to calculate the surface area of the cells. The presence of SP sensory nerve cell expression in each group was noted. The results obtained from the two examiners were then counted to calculate the mean value.

Statistical analysis

All the data obtained were presented as mean ± standard deviation and were analyzed by one-way analysis of variance to identify the differences between the groups. A comparative analysis of least significance difference was subsequently performed to determine the groups with significant results. All tests were undertaken using the Statistical Package for the Social Sciences (SPSS) software, version 23 (IBM, NY, USA), with $P < 0.05$ being considered statistically significant.

RESULTS

Table 1 shows the number of SP expressions in the sensory nerve cells, while the results of IHC staining are shown in Figure 1. There was a 8.01-fold significant increase in SP expression in the T1 group compared to the C1 group, a 4.27-fold significant increase in SP expression in the T1 group compared to the C3 group, a 3.18-fold significant increase in the T2 group compared to the C2 group, a 1.5-fold significant increase in the T2 group compared to the C4 group, a 3.37-fold significant increase in SP expression in the T1 group compared to the T2 group, and a 1.18-fold significant increase in SP expression in the C3 group compared to the C4 group. There was no significant difference in SP expression in the C1 group compared to the C2 group.

DISCUSSION

The application of LPS to freshly cut dentin was performed in a noninvasive manner to minimize any potential effects of pulpal injury and separate it from unknown sources of inflammation. The application of *E. coli* LPS can induce pulpitis in acute conditions.[11] *E. coli* LPS was used to mimic pulpitis due to the agonist structure of hexa-acylated lipid A which is more immunogen compared to underacylated lipid A.[13,14]

The results of this study showed the presence of SP expression in the sensory nerve cells of normal teeth with healthy pulp without any significance increase between 24 h and 72 h. This result was supported by Caviedes-Bucheli *et al*., which confirmed the presence of SP expression in health human dental pulp from teeth without cavity preparation.[15] It showed that the immune response was active in healthy pulp, although in small quantities.

The expression of SP was significantly higher during the 24 h after cavity preparation compared to the negative

Table 1: Mean, standard deviation, and data distribution of substance P observed in the negative control groups (C1 and C2), positive control groups (C3 and C4), and treatment groups (T1 and T2)

Group	*n*	SP, mean±SD	ANOVA test (*P*)
C1	7	1.14^{a}±0.36	<0.001
C2	7	0.85^{a}±0.27	
C3	7	2.14^{b}±0.49	
C4	7	1.80^{c}±0.50	
T1	7	9.14^{d}±2.17	
T2	7	2.7^{b}±0.87	

^{a,b,c}Different superscript denotes a significant difference using LSD ($P<0.05$). ANOVA: Analysis of variance, SP: Substance P, SD: Standard deviation, LSD: Least significance difference

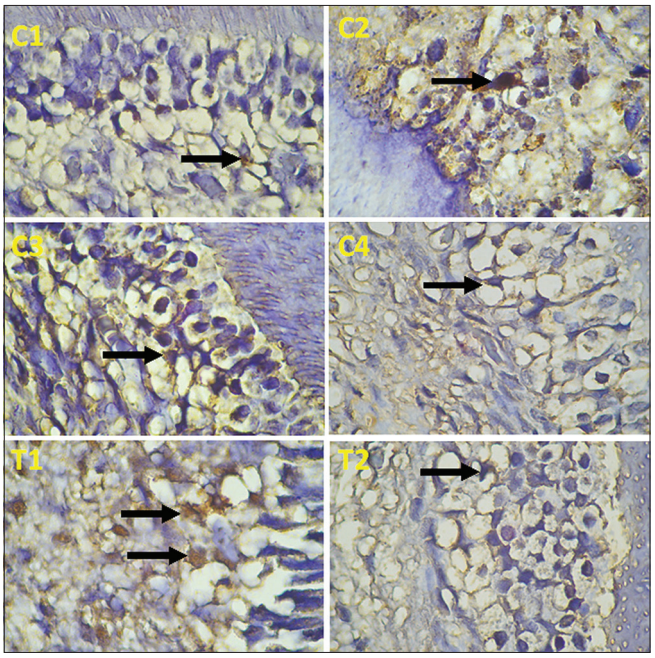

Figure 1: Figures C1 and C2 contain the representative images of substance P expression in sensory nerve cells for the negative control groups, while figures C3 and C4 contain those for the positive control groups and figures T1 and T2 those for the treatment groups. The black arrows indicate positive expressions of substance P and indicate examples of cells that were counted

control group. However, it declined significantly over the subsequent 72 h. This finding was in accordance with that of a study undertaken by Takamori, which found that high-speed drilling performed on mice teeth was effective in stimulating neuropeptide release in dental pulp.[16] A research conducted by Kim and Trowbridge showed that preparation of the tooth cavity could affect pulp biology by causing enamel and dentin injury which, in turn, stimulates the pulp and activates the immune response.[17,18] Moreover, SP mediates the initiation of neurogenic inflammation on dental pulp.[19] These findings confirmed that cavity preparation protocol induces neurogenic inflammation in dental pulp.

In this study, it was demonstrated that the dentinal application of LPS for the periods of 24 h and 72 h induced SP expression in sensory nerve cells. This result was in accordance with those of previous research which showed an 8-fold increase of SP expression in symptomatic teeth and in pulp tissue diagnosed with irreversible pulpitis compared to clinically normal pulp tissue.[20,21] This indicated that SP may be involved in the inflammatory process in dental pulp via the sensory nerve fibers. Peptidase enzyme from LPS can cleave the receptors and weaken the toll-like receptor 4 (TLR4) and cluster of differentiation 14 (CD14) bonding, resulting in the activation of TLR4 and causing inflammation.[22] LPS, which is diffused through open dentinal tubules by fluid movement to the pulp chamber and triggers a cascade of pathological changes, increases the painful symptoms of pulpal invasion.[1,2] LPS can directly interact with the transient receptor potential (TRP) channel, regulating concentration and causing hyperalgesia under conditions of pulpitis.[23,24] It also indirectly activates nociceptors via the release of prostaglandins, leukotrienes, and cytokines.[3]

The results from the present study are consistent with those of previous research, which proved there to be an increase of SP expression in inflamed pulp.[5,19-21] There was an increase in the expression of TRP vanilloid (TRPV1), SP, and CGRP during the 24 h after injury.[8,11] The highest SP expression was found in the 24 h after LPS application, which was significantly higher compared to the 24-h control group. While the expression in the 72 h after LPS application groups decreased significantly compared to the 72-h control group, it showed no significant difference. Previous studies have postulated that TRPV1 activation can cause SP release 1 h after injury.[10] An increase in TRPV1 expression could contribute to tooth pain, especially during the acute time course. In mice, TRPV1 is expressed in pulpal afferents after LPS application following peripheral inflammatory. There was an 8-fold increase in TRPV1 expression after 24-h application of LPS compared to the saline group which, following an initial increase, experienced a decrease after 3 days to a level three times higher than its original figure.[11] Other studies reported that LPS sensitizes the function of TRPV1 in TG nociceptors and has been shown to increase capsaicin-induced neuropeptide release from rat TG neurons.[24] Based on this explanation, there is possibly a relationship between the worsening inflammation, the increase in TRPV1 expression, and the release of SP neuropeptide.

In inflamed tissue, TRPV1 is activated by prostaglandins E2 causing the ion channel in the membrane to open, resulting in membrane depolarization. Thus, the SP present in the synaptic spaces will be released.[5,10,15] Neuropeptides reduce the pain threshold of the pulp, accounting for symptoms associated with certain cases of pulpitis.[20]

CONCLUSION

It was demonstrated that the expression of SP was present in all groups, with the highest level being that at 24 h after LPS application which induced neurological inflammation of the dental cavity. It was also found that 72 h after LPS application, the SP expression was significantly reduced and this decrease was similar to that found in natural dental pulp inflammation. These findings could help researchers to observe and manipulate the process of pulp inflammation and pain in the future. LPS treatment is recommended for the use of dental pulp model protocol in rats.

Acknowledgment

The authors would like to thank to the Ministry of Research, Technology and Higher Education for funding the research.

Financial support and sponsorship

The study was financially supported by the Ministry of Research, Technology and Higher Education for an Excellence in Higher Education Institution Basic Research grant of 2018, No. 200/UN3.14/LT/2018.

Conflicts of interest

There are no conflicts of interest.

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