

Neurogenic Inflammation Pathway on the Up-Regulation of Voltage-Gated Sodium Channel NaV1.7 in Experimental Flare-Up Post-Dental Pulp Tissue Extirpation

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Abstract

Dental caries is one of infectious diseases that still has a serious problem and causes irreversible pulpitis. In endodontic treatment, pain or swelling can occur immediately during the treatment in the root canal of teeth. This study aimed to explain the role of neurogenic inflammation and immunology in the occurrence of post-root canal flare-ups based on the vitality of pulp tissue and the expression of CGRP, NaV1.7 in nerve cells, and HSP70 in macrophages.

The experimental study was conducted to the 15 Sprague Dawley rats divided into three groups: the control group, pulp tissue extirpation group, and LPS group. The pulp tissue was extirpated, and the collected samples were obtained from the apical field of the mandibular incisor. Examination was done with immunohistochemical methods. *Significant differences were found* in NaV1.7, HSP70, and NaV1.7 expressions. The expression of CGRP and HSP70 experienced a significant increase while the expression of Nav1.7 experienced a significant decrease.

The extirpation of the pulp tissue increased the expressions of CGRP, HSP70, and NaV1.7 in the pulp tissue. Increased CGRP in nerve cells and HSP70 in macrophage cells could enrich TNF, thereby causing NaV1.7 to increase. An increase in CGRP can directly incline NaV1.7 through the AC and PLC pathways.

The administration of LPS followed by the pulp tissue's extirpation causes over-expression of HSP70, which will inhibit TRAF6. Consequently, it decreases TNF, which then decline NaV1.7.

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Introduction

Calcitonin gene-related peptide (CGRP), as well as its receptors, are widely distributed and expressed in the human's peripheral and central nervous system in nociceptive pathways in the human peripheral and central nervous system.¹ Nerve fibers in dental pulps produce neuropeptides such as Substance P (SP) and CGRP. Inflammation of the dental pulps can increase the number of SP and CGRP released, especially in irreversible pulpitis. Neuropeptides

serve as an indicator of pathological activity in teeth with symptomatic irreversible pulpitis.² Increasing evidence suggests that CGRP plays an important role in the development of peripheral sensitivity and pain enhancement. CGRP is involved in the development of neurogenic inflammation and is upregulated in inflammatory and neuropathic conditions.³

Trauma due to harmful pulp extirpation can increase HSP-70. Increased HSP-70 from macrophages can increase TNF α expression which occurs after the pulp extirpation responds to physical damage.⁴⁻⁸ Some HSP bands increase concentration shortly after being exposed to the noxious stimulus.⁹ Protein expression of HSP70 was significantly enhanced after 24 hours of heat shock, and the phosphorylation of AKT was also confirmed. Because HSP70 is critical in tissue repair and

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regeneration, mild short-term heat shock may enhance tissue repair in hDDPF.¹⁰

The trigeminal ganglion (TG) is a sensory neuron body that expresses heat shock proteins in response to mild short-term heat shock in human deciduous dental pulp fibroblast-like cells. These proteins innervate the spinal cord, while peripheral axons innervate the teeth. The tetrodotoxin-sensitive sodium (NA) channel (Nav1.7) plays an important role in the pathophysiology of pain. Meanwhile, Nav1.7 and ERK play an important role in the induction of inflammatory pain caused by pulpitis. It is suggested that there is a correlation between the expression levels of Nav1.7 and ERK and the level of inflammatory pain. The ERK signaling pathway is promoted by Nav1.7 in the TG after pulpitis.¹¹ Several ligand-gated and voltage-gated ion channels expressed in the main afferent nerves of teeth are associated with potential contributions to dental pain.¹² Voltage-Gated Sodium Channels (VGSC) are responsible for the generation of action potentials and excitability of cell membranes. Nav1.7 is the most common VGSC family found in nociceptive neurons. Nav1.7 has an important role in inflammatory tooth pain.¹²

A flare-up refers to a period of intense pain and swelling or a combination of both during endodontic treatment which results in unscheduled patient visits.¹³ The incidence of endodontic flare-ups is an undesirable case for both patients and dentists.¹⁴ A flare-up can occur after root canal treatment and consist of an acute exacerbation of asymptomatic pulp and/or periradicular pathological conditions.¹⁵ A prospective study conducted in Israel for 8 months showed that 63.8% of 274 patients experienced postendodontic pain (PEP).¹⁶ A comparative study conducted at Liaquat Medical University Hospital Hyderabad from May 1 to November 30, 2010, showed that 58% of 60 patients experienced mild to severe pain the day after root canal treatment.¹⁷ A cross-sectional study conducted by the Dental Department of Aga Khan University Hospital, Karachi, Pakistan from January to December 2009 showed that 42.9% of 140 patients experienced postoperative pain after 24-hour obturation.¹⁸ According to a prospective clinical study at the Sao Paulo Dental Association (APDC) Clinic, Jardim Paulista branch, Sao Paulo, Brazil from June 2006 to June 2007 showed that 1.71% of 408 patients

experienced flare-ups after receiving endodontic treatment.¹⁹

The study also indicated that the incidence of flare-ups is higher in necrotic teeth than in vital teeth.¹³ Root canal treatment of vital pulps causes a significantly higher incidence and intensity of postendodontic pain compared to necrotic pulps or teeth which have already undergone root canal treatment.¹⁶ The relationship between the frequency of flare-ups after endodontic treatment, pain intensity, and the condition of the pulp (vital or necrotic) is still controversial.¹⁵

Objectives to follow up studies on this topic, the current research aimed to explain the role of neurogenic inflammation in post-root canal flare-ups based on the vitality of the pulp tissue and the expressions of CGRP, Nav1.7, and HSP70 in macrophages.

Materials and methods

Research Samples

This study was a laboratory experiment designed with a post-test-only control group. The analysis of the data was done using the t-test, ANOVA, and path analysis. This study used the 15 Sprague Dawley rats as experimental animals.

Research Methods

This study used 15 Sprague Dawley were divided into three groups (each group consisting of five rats). These three groups were categorized as the control group, the group with extirpated pulp tissue treatment, and the group with LPS administration followed by pulp tissue extirpation. Prior to intrapulpal injection of LPS in the mandibular incisors of Sprague Dawley rats, anesthesia was administered intraperitoneally with ketamine (80mg/kg) and xylazine (10mg/kg) which were injected in sterile PBS. Decapitation of the tooth with interdental papillae as high as 3 mm was performed using a high-speed handpiece with a fissure bur to obtain a flat plane. The unit of analysis was taken from the mandibular incisors and was examined using the immunohistochemical method.⁴

Prior to the extermination of the pulp tissue in the mandibular incisors of the Sprague Dawley rats, anesthesia was administered intraperitoneally with ketamine (80mg/kg) and xylazine (10mg/kg) injected in sterile PBS. After the pulp roof was exposed, the pulp tissue was

removed from the root canal using a needle extirpation as deep as 21 mm or less (based on the width of the root canal of the apical 1/3). The inserted needle was then rotated 360° and pulled out.⁴

After the pulp tissue extraction was completed in each group, each jaw piece was fixed in a 10% neutral formalin buffer for 24 hours and declassified at 4% EDTA for 30 days before paraffin block preparations were made. Tissue slices in paraffin blocks with a thickness of 4 μ were then placed on polysine slides and then heated at 56-58°C overnight. Endogenous peroxidase activity was removed by incubating the incision for 30 minutes at a room temperature in 3% hydrogen peroxide. The 4-μ periapical tissue sections were deparaffinized in xylol and rehydrated with graded alcohol and water (xylol for 20 minutes, absolute alcohol for 5 minutes, 95% alcohol for 5 minutes, and 70% alcohol for 5 minutes), then washed with running water for five minutes. Immunohistochemical staining was then performed using anti-rat monoclonal antibodies against TNFα, Nav1.7, and HSP70. The preparations were examined using a light microscope with 1,000 times magnification.²⁰

Results

Based on the examination of CGRP, HSP70, TNFα, and Nav1.7 expressions on post-extirpation neuroimmunology mechanisms of the dental pulp tissue, the data obtained can be seen in Table 1.

Variables	Groups with Treatment	N	X	SD
CGRP	Control	5	0.8	0.8367
	Pulp tissue extirpation	5	5.4	1.8166
	LPS and pulp tissue extirpation	5	13	1.8708
HSP70	Control	5	0.6	0.8944
	Pulp tissue extirpation	5	5.2	2.8636
	LPS and pulp tissue extirpation	5	13.4	1.3416
Nav1.7	Control	5	0.8	0.8367
	Pulp tissue extirpation	5	10.6	1.1402
	LPS and pulp tissue extirpation	5	5.4	1.1402

Table 1. Mean value, standard deviation, and the samples of CGRP, HSP70, and Nav1.7 expressions.

Before the difference test was carried out to each group, the Kolmogorov Smirnov and Levene tests were done to examine the distribution and homogeneity of each group. Table 2 demonstrates the results of the Kolmogorov Smirnov and Levene tests.

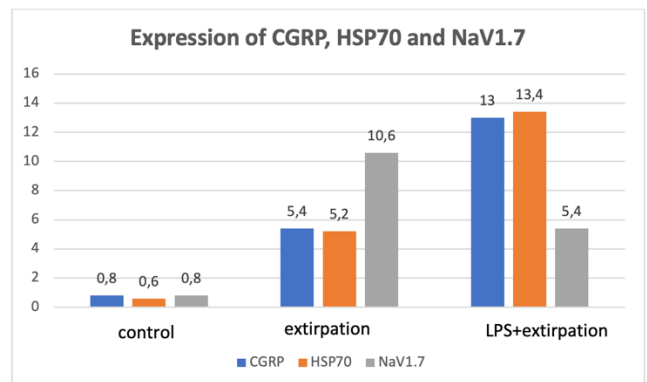


Figure 1. The measurement results on CGRP, HSP70, and Nav1.7 expressions

Variables	Groups with Treatment	Kolmogorov Smirnov Test	Levene Test
CGRP	Control	p = 0.953	p = 0.170
	Pulp tissue extirpation	p = 0.977	
	LPS and pulp tissue extirpation	p = 0.746	
HSP70	Control	p = 0.577	p = 0.046
	Pulp tissue extirpation	p = 0.997	
	LPS and pulp tissue extirpation	p = 0.851	
Nav1.7	Control	p = 0.953	p = 0.720
	Pulp tissue extirpation	p = 0.941	
	LPS and pulp tissue extirpation	p = 0.941	

Table 2. The results of the Kolmogorov Smirnov test and Levene test on CGRP, HSP70, and Nav1.7 expressions.

To determine the differences in the expressions of CGRP, HSP70, and Nav1.7 between each group, the ANOVA test was performed. The results showed there were differences in the expressions of CGRP, HSP70, and Nav1.7 between each group (p = 0.001). To further examine the differences in each group, the Tukey HSD test was carried out. The results of the Tukey HSD test are presented in Table 3.

Variables	Groups with Treatment	Pulp Tissue Extirpation	LPS + Pulp Tissue Extirpation
CGRP	Control	p = 0.002	p = 0.001
	Pulp tissue extirpation	-	p = 0.001
HSP70	Control	p = 0.044	p = 0.001
	Pulp tissue extirpation	-	p = 0.003
Nav1.7	Control	p = 0.001	p = 0.001
	Pulp tissue extirpation	-	p = 0.001

Table 3. The results of the Tukey HSD test on CGRP, HSP70, and Nav1.7 expressions

Table 3 suggests that the expressions of CGRP, HSP70, and Nav1.7 between each group have a p-value of less than 0.05, respectively. This indicated that there were significant differences in the expressions of CGRP, HSP70, and Nav1.7 between each group.

Discussion

The increase in CGRP, HSP70, and NaV1.7 after pulp tissue extirpation

The increase in CGRP expression was caused by the inflammation of the pulp tissue after extirpation. CGRP can be recognized by CALCRL on the surface of GPCR. The activation of GPCR via CGRP can stimulate AC that produces cAMP. After that, this cAMP can activate PKA as an adapter which subsequently activates transcription factors to cause protein synthesis and increases CGRP expression. The increase in CGRP expression in this study is in line with previous studies stating that CGRP expression was significantly higher in pulps with irreversible pulpitis compared to normal pulps.²¹ Orthodontic forces can trigger the release of neuropeptides e.g., CGRP from nociceptive fibers in periodontal tissues.²² The response of the pulp sensitivity test showed a higher mean of CGRP expression in the group with irreversible pulpitis.²¹

Extirpation of the pulp tissue increased the expression of HSP70 and then caused damage to the pulp tissue, thereby HSP70 leaving the cells. The release of HSP70 from the cell was recognized by TLR4 on the macrophage cells' surface. It further activated MYD88 which triggered intracellular signal transduction, resulting in IRAK activation. The activation of IRAK caused TRAF6 to phosphorylate the IKK inhibitors, which triggered and inhibited I- κ B. The I- κ B was then inactivated, and thus NF- κ B could be activated. The presence of inflammatory signals in these cells caused the HSP70 bound with HSP to separate because the HSP70 tried to inhibit TRAF6. Thus, the transcription process did not occur, and proinflammatory cytokines were not expressed. HSF as a transcription factor that unbinds with HSP70 entered the cell nucleus and caused the expression of HSP70. This increased HSP70 expressing cells. This study is in line with several previous studies which also suggested that the dental pulp experienced an increase in HSP70 expression after exposure to mild trauma. Meanwhile, HSP70 expression increased significantly within 24 hours after tooth separation.¹⁹ Then, it gradually decreased one week after wedge placement. Evidence shows HSPs act as molecular chaperones to maintain homeostasis during injury and are expressed physiologically to respond mechanical stress

and/or stimulation. HSP expression in fibroblasts increases during traumatic occlusion.²⁴ Heat stress increases the expression of the HSP70 protein, which is very important in tissue repair and regeneration.⁶

The increase in TNF α expression resulted in the increase of NaV1.7 expression. Meanwhile, the increase in TNF α expression was due to physical damage during extirpation which was responsible for the increase in HSP70 expression. TNF α via TNFR could have activated TRAF-2, which triggered MEK. MEK subsequently activated MAPK which then caused NaV1.7 to be expressed. This is in line with several previous studies which also suggested that by accelerating channel activation and increasing VGSC expression in NF κ B-dependent mechanisms as well as p38 MAPK signaling pathways in the CNS neurons, TNF α led to the increasing Na⁺ current.²⁵ As VGSC got affected by TNFR, it then promotes excitation in primary afferent neurons, which possibly explain the mechanism of sensitization associated with neuropathic pain and inflammation.²⁶ TNF α up-regulated NaV1.7 and mRNA in both DRG neurons and adrenal chromaffin cells.²⁷

The increase in CGRP and HSP70 after the administration of LPS *Porphyromonas gingivalis* followed by pulp tissue extirpation

In this study, there was an increase in the expression of CGRP after extirpation of the dental pulp tissue preceded by the administration of LPS *Porphyromonas gingivalis*. The increase in CGRP was caused by two main factors. The first factor is the administration of LPS *Porphyromonas gingivalis*, a compound of gram-negative bacteria which can activate innate immune receptors e.g., TLR4. LPS via the TLR4 pathway in the early phase of infection could have activated MYD88 that triggered IRAK signal transduction. The activation of IRAK caused TRAF6 to phosphorylate the IKK inhibitors, which triggered and inhibited I- κ B. The I- κ B was then inactivated, and thus NF- κ B could be activated. The expression of NF- κ B caused the increase in TNF- α expressing cells. TNF- α then bound to the TNFR receptor to activate TRAF2, which triggered MEK. The MEK subsequently activated MAPK adapters that could increase the CGRP expressing cells.

Meanwhile, the second factor is the extirpation of the pulp tissue which damages sensory nerve cells and thus releases CGRP.

CGRP could be recognized by CALCRL on the surface of GPCR. The activation of GPCR via CGRP could have stimulated AC that produced cAMP. After that, this cAMP could have activated PKA as an adapter, which subsequently activated transcription factors that caused protein synthesis and increased CGRP expression.

In addition, there was an increase in the expression of HSP70 after extirpation of the dental pulp tissue preceded by the administration of LPS *Porphyromonas gingivalis*. The increase in HSP70 was caused by two main factors. The first factor is the administration of LPS *Porphyromonas gingivalis*, a compound from gram-negative bacteria responsible for activating innate immune receptors e.g., TLR4. LPS via the TLR4 pathway in the early phase of infection could have activated MYD88, which then triggered IRAK signal transduction. The activation of IRAK caused TRAF6 to phosphorylate the IKK inhibitors, which triggered and inhibited I- κ B. The I- κ B was then inactivated, and thus NF- κ B could be activated. The presence of inflammatory signals in these cells caused HSP70 bound with HSP to separate because the HSP70 tried to inhibit IKK and NF- κ B. The transcription process did not occur, and proinflammatory cytokines were not expressed. HSF, a transcription factor that unbound with HSP70, entered the cell nucleus, thereby causing the expression of HSP70 to increase. This is in line with a previous study where periapical lesions increased macrophage infiltration and activated muscle tissue's inflammatory pathways as well as HSP70 and LPS's serum concentrations in rats.²⁸

Meanwhile, the second factor is the extirpation of the pulp tissue which damages pulp tissue and thus released HSP70 from the cell. The release of HSP70 activated MYD88, which then triggered IRAK signal transduction. The activation of IRAK caused TRAF6 to phosphorylate the IKK inhibitors, which triggered and inhibited I- κ B. The I- κ B was then inactivated, and NF- κ B could be activated. The presence of inflammatory signals in these cells caused HSP70 bound with HSP to separate as HSP70 tried to inhibit IKK and NF- κ B. Thus, the transcription process did not occur, and proinflammatory cytokines were not expressed. HSF, a transcription factor that unbound with HSP70, would enter the cell nucleus causing the expression of HSP70 to increase. This increased

HSP70 expression.

The decrease in Nav-1.7 after the administration of LPS *Porphyromonas gingivalis* followed by pulp tissue extirpation

In this study, there was an decrease in the expression of Nav1.7 after extirpation of the dental pulp tissue preceded by the administration of LPS *Porphyromonas gingivalis*. The decrease in Nav1.7 expression was due to the decrease in TNF α expression caused by the overexpression of HSP-70. Overexpression of HSP-70 caused inhibition of TRAF-6 which phosphorylates the IKK inhibitors and prevented NF- κ B activation. The decrease in TNF expression is in line with the previous study which also suggested that extracellular HSP functioned as a dangerous signal to stimulate the immune response, while intracellular HSP functioned as a negative regulator to control inflammation.²⁹

Extracellular or exosome-bound HSP70 could bind to TLR2 or TLR4, activate NF- κ B, and produce TNF α , IL-1 β , and IL-6. Besides that, through interaction with TRAF6, HSP70 can act as an anti-inflammatory because of inhibiting LPS-mediated activation of NF- κ B.³⁰ The overexpression of HSP70 will block NF- κ B activation and nuclear translocation of p50/p65 via IKK-mediated inhibition of I κ B (NF- κ B inhibitor) phosphorylation.³¹ HSPA plays a significant role in defending the oral cavity in either pathological and healthy conditions. Meanwhile, Janus-faced HSPA can be pro-inflammatory and anti-inflammatory.³² Decreased TNF α activated TNFR which then triggered TRAF2. TRAF2 then activated MEK and declined the Nav1.7 expression. Similarly, a previous study mentioned that TNF α could be responsible for the up-regulation of the NF- κ B signaling pathway involved in this process, as well as Nav1.7 in DRG neurons of mice with DN.³³ An increase in sodium current (VGSC) in formerly damaged DRG neurons may be umpired by the overproduction of TNF α . Meanwhile, increased TNF α may be responsible for upregulation of Nav1.3 and Nav1.8.²⁶

Extirpation of the pulp tissue increased the expressions of CGRP, HSP70, and Nav1.7 in the pulp tissue. Increased CGRP in nerve cells and HSP70 in macrophage cells inclined TNF α . Consequently, the increased TNF α triggered an increase in Nav1.7 which caused flare-ups. Increased CGRP could directly uplifted Nav1.7 through AC and PLC pathways in nerve cells.

The administration of LPS followed by the pulp tissue's extirpation caused overexpression of HSP70 which would inhibit TRAF6. As a result, it decreased TNF α and NaV1.7. The administration of LPS caused cells to undergo apoptosis, which could cause a decrease in the pulp tissue and flare-ups as well. Based on its molecular aspect, LPS induction followed by pulp extirpation (inflamed vital pulp) showed a less pronounced flare-up compared to that of the extirpated dental pulp (vital dental pulp).

Conclusions

The extirpation of the pulp tissue increased the expressions of CGRP, HSP70, and NaV1.7 in the pulp tissue. Increased CGRP in nerve cells and HSP70 in macrophage cells could enrich TNF, thereby causing NaV1.7 to increase. An increase in CGRP can directly incline NaV1.7 through the AC and PLC pathways. The administration of LPS followed by the pulp tissue's extirpation causes over-expression of HSP70, which will inhibit TRAF6. Consequently, it decreases TNF, which then decline NaV1.7.

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Declaration of Interest

The authors declare that there are no conflicts of interest.

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