



ORIGINAL ARTICLE

Expression of NaV-1.7, TNF- α and HSP-70 in experimental flare-up post-extirpated dental pulp tissue through a neuroimmunological approach

Galih Sampoerno^a, Jenny Sunariani^{b,*}, Kuntaman^c

^a Department of Conservative Dentistry, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia

^b Department of Oral Biology, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia

^c Department of Microbiology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

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Abstract *Background:* Dental caries continue to represent a major problem which, if left untreated, will cause irreversible pulpitis. Root canal treatment constitutes one potential treatment intended to preserve teeth afflicted with irreversible pulpitis. During root canal treatment, pain or swelling, referred to as flare-ups, can occur at any point in the process.

Aim: To analyze the molecular aspect of the phenomenon of flare-up in vital dental pulp tissue following mechanical and bacterial trauma (extirpation and lipopolysaccharide [LPS] induction respectively) through a neurological approach, based on the expression of NaV-1.7 in neuron cells, and HSP-70, TNF- α in macrophage cells.

Method: This laboratory experimental study was conducted using 15 Sprague Dawley rats as subjects which were divided into three groups of five subjects: a control group, a pulp tissue extirpation group and an LPS induction followed by extirpation of pulp tissue group. Test samples were collected from the apical field of the mandibular incisor and subsequently examined using immunohistochemical methods.

Results: There were significant differences in Nav1.7, HSP70 and TNF α expression between the treatment groups. While a marked increase in the expression of HSP70 occurred, both Nav1.7, and TNF α expression decreased significantly.

Conclusion: Extirpating the dental pulp tissue will induce a more pronounced flare-up response from the molecules of the pulp tissue in vital teeth than those in inflamed vital pulp tissue.

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* Corresponding author at: Department of Oral Biology, Faculty of Dental Medicine, Universitas Airlangga, Jl. Prof. Moestopo 47, Surabaya, Indonesia.

E-mail addresses: sampoernogalih@yahoo.com (G. Sampoerno), jenny-s@fk.unair.ac.id (J. Sunariani), kuntaman@fk.unair.ac.id (Kuntaman).
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1. Introduction

A popular form of treatment for pulp perforated caries is that of root canal treatment featuring sequential stages such as root canal preparation, sterilization and obturation (Chandra and Gopikrishna, 2014). The term 'flare-up' refers to the intense pain and/or abscess that occurs during root canal treatment and constitute serious problems leading to an unscheduled consultation with a dentist (Torabinejad et al., 2014).

The pain associated with root canal treatment has multifactorial causes, including: the patient's general state of health, the condition of the pulp and periapical and, finally, the treatment procedure. The clinical conditions potentially resulting in flare-up comprise: apical periodontitis secondary to the treatment, incomplete cleaning of the root canal, recrudescence of chronic apical periodontitis and recurrent periapical abscesses. Meanwhile, the external factors that may contribute to the incidence of flare-up encompass over-instrumentation, incomplete root canal cleaning, debris contamination of the periapical tissue, overfilling, periapical lesions, retreatment and the general health of the host. Microbiological and immunological factors also play a role in flare-up due to possible changes in a range of factors including: local adaptation, periodontal tissue pressure, microbes, chemical mediators, cyclic nucleotides, in addition to immunological and psychological responses (Gark et al., 2010).

A study conducted in Denmark in 2002 found that 3.17% of unscheduled dental visits was due to severe pain during the root canal treatment period. (Walton, 2002). The incidence of flare-up being low may, however, have a considerable impact on patient, dentist and the dental clinic staff (Alamassi, 2017). A prospective study conducted in Israel over a period of eight months showed that 63.8% of 274 patients experienced post-endodontic pain (PEP) (Gotler et al., 2012). Another comparative study in Hyderabad reported 58% of 60 patients to have experienced mild to severe pain on the day after root canal treatment (Memon and Ali, 2013). Meanwhile, a cross-sectional study carried out in Pakistan between January and December 2009 indicating that 42.9% of 140 patients experienced post-operative pain for 24 h after obturation (Sadaf and Ahmad, 2014). This combined data showed that the pain experienced during or after treatment which should not exist, in reality, persists.

The incidence of flare-up in root canal treatment among non-vital teeth is higher than that of their vital counterparts (Alves, 2010). Nevertheless, another theory regarding post-endodontic discomfort proved that root canal treatment conducted on teeth with irreversible pulpitis is more painful (Segura-Egea et al., 2009). Root canal treatment of vital pulp-induced post-endodontic pain confirmed a significantly higher incidence and intensity compared to that resulting from the treatment of non-vital tooth or that following endodontic retreatment (Gotler et al., 2012). Post-operative pain occurs with higher intensity more frequently (53% of cases) following root canal treatment compared to other dental procedures (Krasniqi and Daci, 2017). The correlation between flare-up frequency and intensity following endodontic treatment of pulp conditions remains controversial (Sipavičiūtė and Maneliėnė, 2014). Since flare-up may occur during root canal treatment, this study involved the use of animal subjects with pulp extirpation. The research sought to analyze the molecular

aspects of flare-up, specifically the neuroimmunological response involving expression of NaV-1.7 in neuron cells and HSP-70, TNF- α in both macrophage cells and in vital dental pulp tissue following extirpation compared to the inflamed dental pulp tissue subjected to lipopolysaccharide (LPS) induction and extirpation.

2. Materials and method

2.1. Experimental design

This research was laboratory-based and experimental in nature, involving post-test only control group design. All reported procedures had been reviewed and approved by the Universitas Airlangga Faculty of Dental Medicine Health Research Ethical Clearance Commission (Certificate number 272/HRECCFODM/X/2018). The level of pain experienced by the subjects of this study was measured by observing the expression of NaV-1.7 due to pulp extirpation.

2.2. Experimental procedures

This study featured 15 male Sprague Dawley rats as subjects that satisfied the inclusion criteria, specifically; in good health, weighing 450 g, aged 20 weeks and possessing completely erupted incisors. The subjects were randomly divided into three groups, a control group receiving no treatment, a pulp-extirpated group and an LPS-induced followed by pulp extirpation group.

The control group received no treatment and its members were sacrificed at the same time as those of the treatment group. Prior to treatment, all treatment group members were anaesthetized by means of an intraperitoneal injection of ketamine (80 mg/kg) and xylaxine (10 mg/kg) diluted in sterile Phosphate Buffer Saline (PBS). The lower incisor of treatment group I (pulp extirpation group) was decapitated as far as the level of the interdental papilla (3 mm) in order to create a flat surface and open the pulp chamber by means of a high-speed handpiece pana-max NSK OM-T0307E Japan) and fissure bur (Dia-Burs TC-21 ISO 160/014 FG LOT D14G007800 MANI Inc 8-3 Kiyohara Industrial Park Utsunomia, Tochigi, Japan). The tooth was extirpated with a barbed broach (VDW GmbH – Bayerwaldstr. 15-81737 Munich-Germany) to a depth of ± 21 mm and initial fit of the apices root canal. The barbed broach was inserted into the root canal before being rotated 360 degrees and the pulp tissue was extracted. The tooth was subsequently sealed with glass ionomer cement and terminated after 24 h, as the highest incidence of flare-up was considered to occur 0–24 h after root canal treatment before declining. For the second treatment group (LPS + extirpation), an access opening was made on the lower incisor, prior to the administering of an intrapulpal injection of lipopolysaccharide (LPS) stock isolated from *Porphyromonas gingivalis* (Ultrapure lipopolysaccharide from *Porphyromonas gingivalis* – TLR4 ligand, Catalog # tlr1-pplps. Version #14F18-MM. Invivo-Gen. 3950 Sorrento Valley Blvd. Suite 100 San Diego, CA 92121 – USA). After 48 h, the time required for acute inflammation to result from LPS induction, the pulp tissue was extirpated. The teeth were then sealed with glass ionomer cement and terminated after 24 h.

The subjects were sacrificed by means of an injected overdose of ketamine and xylazine, with the mandible being isolated. The mandibles were fixated in 10% buffered formaline for 24 h and subsequently decalcified using 4% Ethylene Diamine Tetraacetic Acid (EDTA) for 30 days before being made into a paraffin block.

2.3. Immunohistological staining

The paraffin blocks were cut into 4 μm slices using a microtome, placed in polysine slides and heated to 56–58 °C overnight. The activity of endogen peroxide was eliminated by incubating the slides in 3% hydrogen peroxide for 30 min at room temperature. The slides were deparaffinated in xylol for five minutes and washed with tap water four times, rehydrated in 95% alcohol for five minutes, 70% alcohol for five minutes and, finally, washed with tap water for five minutes. The slides were immunohistologically stained using monoclonal antibody, anti-rat anti-Nav1.7 (CST, USA), anti-HSP70 (SantaCruz Biotech. USA) and anti-TNF α (SantaCruz Biotech. USA), before being counterstained with Haematoxylin Meyer (Pizem and Coem, 2003; Soini et al., 1998). The expression of Nav1.7 in the pulp neuron cell and that of HSP70 and TNF α in the macrophage cells were observed under a light microscope (Nikon, Japan) at 1000 \times magnification, with pictures taken using a digital camera (Sony, USA).

2.4. Data analysis

The data obtained was statistically analyzed using One-way ANOVA with post-hoc Tukey HSD (SPSS for windows) in order to identify differences between groups at a significance level of 0.05.

3. Results

The mean and standard deviation of each expression has been tabulated and presented in Tables 1, 2 and 3. The Kolmogorov Smirnov test result for each variable of each group confirmed the data to be normally distributed, as did that of the Levene's test which confirmed the data to be homogeneous. As can be seen from the contents of Table 1, the expression of Nav1.7 among the groups differed considerably, with the highest recorded expression of Nav1.7 occurring in the pulp extirpation group. This was significantly higher than that of the LPS + pulp extirpation group ($p = 0.001$) and the control group ($p = 0.001$). The level of expression in the LPS + pulp extirpation group was significantly higher than that in the control group ($p = 0.001$). The results are contained in Fig. 1.

Table 1 Mean and Standard Deviation of Nav1.7 Expression. The different superscript letters are statistically significant (Tukey HSD, $P < 0.05$).

No	Group	Mean \pm SD	P Value
1	Control	0.8 \pm 0.8367 ^a	0.001
2	Pulp Extirpation	10.6 \pm 1.1402 ^b	
3	LPS + Pulp extirpation	5.4 \pm 1.1402 ^c	

Table 2 Mean and Standard Deviation of TNF α Expression. The different superscript letters are statistically significant (Tukey HSD, $P < 0.05$).

No	Group	Mean \pm SD	P Value
1	Control	0.8 \pm 0.8367 ^a	0.001
2	Pulp Extirpation	10.4 \pm 1.5166 ^b	
3	LPS + Pulp extirpation	6.4 \pm 2.3022 ^c	

Table 3 Mean and Standard Deviation of HSP70 Expression. The different superscript letters are statistically significant (Games-Howell, $P < 0.05$).

No.	Group	Mean \pm SD	P Value
1	Control	0.6 \pm 0.8944 ^a	0.001
2	Pulp Extirpation	5.2 \pm 2.8636 ^b	
3	LPS + Pulp extirpation	13.4 \pm 1.3416 ^c	

As can be seen from Table 2, a significant difference in TNF α expression was also revealed. The highest expression was recorded in the pulp extirpation group and was significantly higher than that of both the LPS + pulp extirpation group ($p = 0.007$) and the control group ($p = 0.001$). The level of expression in the LPS + pulp extirpation group was significantly higher than that of the control group ($p = 0.001$). The results are presented in Fig. 2.

As can be seen in Table 3, a significant difference of HSP70 expression was also identified. The highest expression of TNF α recorded occurred in the LPS + pulp extirpation group and was significantly higher than that of the pulp extirpation group ($p = 0.003$) and the control group ($p = 0.001$). Meanwhile, the level of expression in the pulp extirpation group was considerably higher than that of the control group ($p = 0.044$). The results are contained in Fig. 2. The histological imaging of Nav1.7, TNF α , and HSP70 can be seen in Fig. 3.

4. Discussion

This study revealed a level of expression of HSP-70, TNF- α and Nav1.7 in pulp-extirpated group to be higher than in the LPS + pulp extirpation group. The increase in HSP-70 is caused by trauma resulting from pulp extirpation which compromises the cell walls of pulp tissue. The damaged cells release HSP-70, recognized by TLR-4 present on the surface of macrophage cells, which may activate Myd88 and initiate intracellular signal transduction that activate IRAK and recruit TRAF-6 that phosphorylate the inhibitor of IKK. This may, in turn, activate I- κ B kinase, inhibit I- κ B kinase and activate NF- κ B. The inflammation signal within the cell may release HSP-70 from macrophages to inhibit IKK and NF- κ B in order to prevent transcription of proinflammatory cytokines. HSF, as the transcription factor that unbinds the HSP-70, will enter the nucleus and express HSP-70.

This result is in accordance with those of previous research undertaken which revealed that the highest increase of HSP70 expression occurred 24 h post-injury, returning to normal levels after 24 h (Pileggi et al., 2009). Another study of tooth

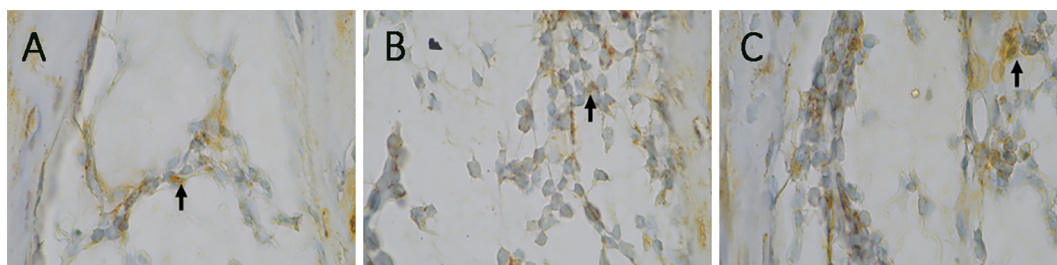


Fig. 1 NaV expressed by control group dental pulp neuron cells (A), by pulp extirpation group neuron cells (B) and by LPS + pulp extirpation group neuron cells (C) at 400 \times magnification (black arrows).

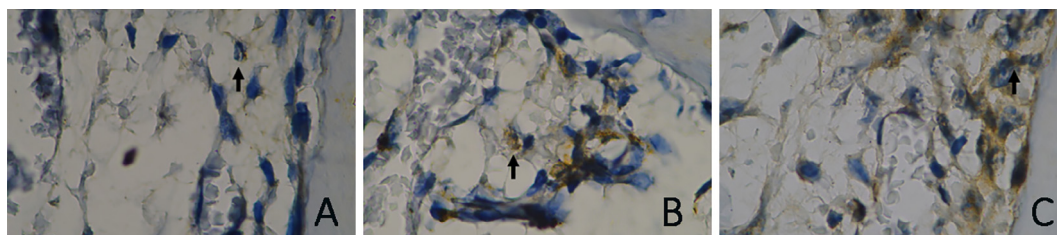


Fig. 2 HSP70 expressed by control group dental pulp neuron cells (A), by pulp extirpation group neuron cells (B) and LPS + pulp extirpation group neuron cells (C) at 400 \times magnification (black arrows).

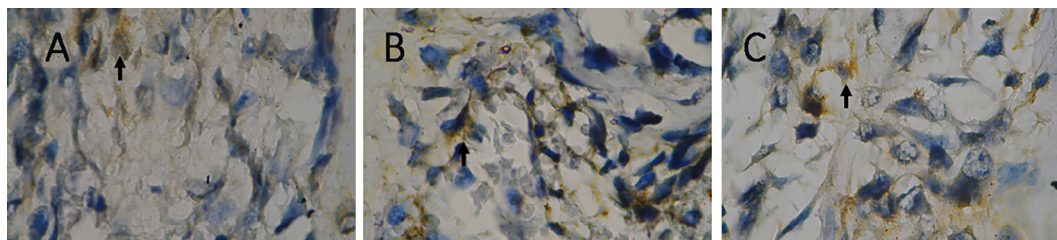


Fig. 3 TNF- α expressed by control group dental pulp neuron cells (A), by pulp extirpation group neuron cells (B) and LPS + pulp extirpation group neuron cells (C) at 400 \times magnification (black arrows).

separation also found an increase in HSP70 expression after 24 h, gradually decreasing over the course of a week (Nakano et al., 2013). HSPs constitute molecular chaperones which maintain homeostasis that will be physiologically expressed in response to mechanical stress and/or stimulus. HSP will be expressed in dental pulp tissue to defend against damage due to mechanical stress during orthodontic treatment (Nakano et al., 2013).

The higher TNF α expression which occurred after pulp extirpation in this study was possibly due to an increase in HSP70 from macrophages in response to physical damage during extirpation. Such damage will be recognized by Toll-Like Receptors (TLR)-4 on macrophage cell surfaces and, subsequently, activate Myeloid differentiation primary response 88 (Myd88), inducing intracellular signal transduction to activate Interleukin-1 Receptor-Associated Kinase (IRAK) that recruits TNF receptor associated factor (TRAF-6). TRAF-6 will phosphorylate I- κ B Kinase (IKK) inhibitor, thus activating I- κ B kinase with the result that I- κ B becomes inactive. Nuclear Factor Kappa B (NF- κ B) may be activated and, in turn, activate TNF α . This result is in accordance with those of previous studies reporting an rise in TNF α expression due to increasing HSP70 in response to trauma. This is followed

by modulation of messenger Ribonucleic Acid (mRNA), TNF α and Receptor activator of nuclear factor kappa-B ligand (RANKL) expression in human periodontal ligament due to compression force (Mitsunashi et al., 2011). HSP70 exogenous acts as a cytokine in human monocyte by stimulating proinflammatory cascade signal transduction, followed by upregulation of interleukin (IL) -1 β , IL-6 and TNF α expression (Nair et al., 2015). The induction of HSP70 in vivo can regulate TNF α production after injury (Morimatsu et al., 2010). HSP70 binds plasma membrane with high affinity causing rapid intracellular calcium flux that activates NF κ β , thus increasing the expression of proinflammatory cytokine TNF α , IL-1 β and IL-6 in monocytes. The two pathways of signal transduction are activated by exogenous HSP70. One pathway depends on CD14 and intracellular calcium which initiate expression of TNF α , IL-1 β and IL-6, while the other is CD14-independent yet depends on intracellular calcium resulting in TNF α expression without IL-1 β or IL-6 (Nair et al., 2015).

This study also confirmed the increasing expression of NaV-1.7, possibly also due to a rise in TNF α expression caused by HSP70 produced by macrophages in response to physical damage during dental pulp extirpation. TNF α

activates TNF Receptor-Associated Receptor 2 (TRAF-2). The TRAF-2 triggers MAPK Kinase (MEK) that, in turn, activates Mitogen-activated Protein Kinase (MAPK). This may cause expression of sodium channel NaV-1.7 which plays a role in inflammation-induced dental pain (Beneng et al., 2010). The increased axonal expression and augmentation of NaV1.7 in dental pulp contributes to the increasing response of spontaneous pain which is characteristic of dental pain (Luo et al., 2008).

Dental pain constitutes a serious health problem, although some VGSC isoform as well as epithelial sodium channels have been identified in dental pulp tissue performing different function and with different locations. Nav1.7, Nav1.8 and Nav1.9 are crucial to inflammation of dental pulp. The isoform of sodium channels is increasingly the potential target of contemporary pulpitis treatment (Suwanchai et al., 2011). The expression of VGSC in the dorsal root ganglion (DRG) of a damaged neuron shows that these channels are, indeed, involved in the sensation of pain following nerve injury. The expression of NaV1.7 in DRG plays a role in mediating pain from the sciatic neuron after a compression injury and exposure to NP (Mukai et al., 2014). An increasing signal into VGSC denotes an initial event required for activating microglia post-injury (Jung et al., 2013). This confirms that NaV1.7, NaV1.8 and NaV1.9 are involved in the production of chronic inflammatory pain in the first few weeks after injury (Strickland et al., 2008). A nerve injury notably induces changes in the levels of NaV1.3, NaV1.5 and NaV1.7. In the spine, NaV1.7 is strongly expressed after nerve injury and is correlated to the pain experienced (Shao et al., 2016).

4.1. Post-LPS induction and pulp extirpation (mechanical trauma) increases in HSP-70

Observation of the LPS-induced group prior to pulp extirpation (mechanical irritation) showed an increase in HSP70 expression which may be due to two main reasons, namely; LPS from *Porphyromonas gingivalis* and dental pulp extirpation. LPS constitutes a substance from gram-negative bacteria that can trigger an innate immune response. The receptor that plays a role in this response is TLR4. LPS binds TLR4 and activates Myd88, inducing signal transduction of IRAK that recruits TRAF6 which, subsequently, activates HSF1 factor transcription, thus increasing the expression of HSP70. A similar result was explained by Pereira et al. (2017), who stated that periapical lesions cause an increase in macrophage infiltration, activation of inflammatory cascade in muscles and an intensification of HSP70 and LPS serum concentration in rats (Pereira et al., 2017).

After exposure to LPS, the level of HSP70 rises (with details of the initial increase available in an hour), reaching a maximum level in two hours and returning to normal in six hours (Zhang et al., 2013). Pulp extirpation may damage the pulp cells and release HSP-70 that will be recognized by the TLR-4 present on the surface of macrophage cells. This may activate Myd88, and the intracellular signal transduction that initiates IRAK and recruits TRAF-6 that phosphorylates the inhibitor of IKK. These may activate I- κ B kinase and NF- κ B, while inhibiting I- κ B kinase. The inflammation signal inside the cell may release HSP-70 from macrophages to inhibit IKK and NF- κ B, thereby preventing transcription of proinflammatory

cytokine. HSF, as the factor transcription that unbinds HSP-70, will infiltrate the nucleus and synthesize HSP-70.

4.2. The decrease in TNF- α and NaV-1.7 after exposure to LPS and pulp extirpation (mechanical trauma).

The results of this study indicated a decrease in TNF- α after pulp extirpation of LPS-induced pulp. The low level of TNF- α expression was possibly caused by the overexpression of HSP-70 that prevents TRAF-6 phosphorylating the IKK inhibitor. Therefore, NF- κ B will not be activated which may reduce the expression of TNF- α . This finding concurs with that of a previous study which concluded that overexpressed HSP70 can inhibit the increase in cytokine production induced by LPS which controls the fever response (Dokladny et al., 2010).

This study also proved that HSP70 is able to control animal cytokine expression through inhibition of NF- κ B pathways. Induction of HSP inhibits proinflammatory cytokine production and blocks the activation of NF- κ B in respiratory epithelial cells. This might be correlated to the stabilization of I κ B α by inhibiting IKK activation (Sheppard et al., 2014). The increase in the level of HSP70 inhibits proinflammatory cytokine production induced by LPS through a mechanism involving NF- κ B inactivation and binding TRAF6, thereby preventing its ubiquitination (Chang et al., 2017). HSP70 protects dental pulp cells and inhibits the early stages of inflammation. HSPA plays an important role in the oral defense system, both under normal and pathologic conditions. However, this role is "Janus-faced" in character since it can be both that of immune stimulator and regulator. The upregulation of HSP70 may suppress cytokine expression induced by LPS which correlates to the inhibition of I κ B α and NF- κ B degradation. Thus, overexpression of HSP70 may regulate the expression of cytokine induced by LPS through NF- κ B pathways (Dokladny et al., 2010).

This study also confirmed decreasing NaV-1.7 expression in the LPS + pulp extirpation group. The low level of TNF- α expression was possibly caused by the overexpression of HSP-70 that prevents TRAF-6 from phosphorylating the IKK inhibitor. Therefore, NF- κ B will not be activated which, in turn, reduces the expression of TNF- α . The low level of TNF α can also reduce TRAF2 by TNFR activation, further decreasing MEK activation by TRAF2 and, by extension, NaV-1.7 expression. This result supports that of a previous study by (Huang et al., 2014) which stated that increasing TNF α expression is suspected of upregulating NaV1.7 in rodent DRG neuron with DN involving the NF- κ B signaling pathway. The increase in sodium flow (VGSC) in injured DRG might be mediated by over-production of TNF α (Chen et al., 2011). The increasing TNF α is supposed to correlate to increase the regulation of NaV1.3 and NaV1.8 after DRG nerve injury (He et al., 2010). The effect of TNFR activation on VGSC increases primary afferent neuron excitation which may explain the mechanism of sensitization that correlates to neuropathic and inflammatory pain (Leo et al., 2015). TNF α increases the flow of Na⁺ by accelerating channel activation and augmenting the VGSCs expression that depends on the signaling pathway of NF- κ B and p38 MAPK in central nervous system neurons (Chen et al., 2015).

5. Conclusion

Based on its molecular aspect, the extirpated dental pulp (vital dental pulp) demonstrated a more pronounced flare-up response compared to that of LPS induction followed by pulp extirpation (inflamed vital pulp).

Declaration of Competing Interest

There is no conflict of interest

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