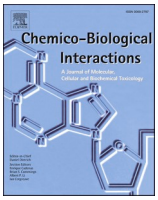
Chemico-Biological Interactions 366 (2022) 110128

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint



Research paper

Increased nociceptive sensitivity is associated with periodontal inflammation and expression of chronic pain genes in gingival tissues of male rats

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ARTICLE INFO

Keywords:
Periodontal disease
HCN channel
Kcns1
Chronic pain
Nociceptive sensitivity

ABSTRACT

Objective: This study aimed to evaluate the inflammatory response, hyperpolarization-activated cyclic nucleotide-gated 2 (HCN2), and voltage-gated potassium (Kv) 9.1 channel expression in rats with paclitaxel-induced neuropathic pain-like behavior.
Methods: Sixteen male Sprague Dawley rats were divided equally into two groups: control and paclitaxel-induced pain (PTX). The attachment loss and inflammatory cell infiltrate levels were analyzed histometrically and immunohistochemically. The gene expression of HCN2 and KCNS1 was analyzed by qPCR in the brain and gingival tissues.
Results: The attachment loss and prominent infiltration of inflammatory cells were significantly higher in the PTX group than in the control groups. In gingival tissues; the expression levels of HCN2 ($p = 0{,}0011$) were significantly higher and KCNS1 ($p = 0{,}0003$) were significantly lower in the PTX group than in the control groups.
Conclusion: Increased nociceptive sensitivity, may play a role in periodontal inflammation. KCNS1 may decrease and HCN2 expression may increase in periodontium in permanent chronic pain states. The results of the present study may be helpful in developing new approaches to alleviate pain and maintain periodontal health in patients suffering from orofacial pain.

1. Introduction

Persistent or recurrent pain that more than one-third of the world's population suffers from was recognized as an important public health problem by the World Health Organization [1,2]. Pain that continues beyond the normal healing period, typically persisting for more than 3–6 months, is defined as "chronic pain" [3]. Chronic pain can develop in conditions such as temporomandibular disorders, trigeminal neuralgia, migraine headaches, diabetic neuropathy, herpes zoster, arthritis, back injury, and cancer [2,4–6]. Chronic pain is classified as psychogenic, nociceptive, and neuropathic based on the effective mechanism [2]. Nociceptive pain can occur as a result of inflammation or tissue damage [7]. Neuropathic pain describes pain that results from damage to the nervous system or as a result of metabolic diseases [7,8].

Inflammation, an important defense mechanism, is a physiological response to damage, injury, or destruction caused by physical, chemical, or pathogens in tissues. But if the causative agent persists, it can become chronic and results in tissue damage [9]. Stimulation of peripheral bare nerve ending; leads to the release of neurotransmitters. Inflammation occurs if the local release of Substance P and other tachykinins is sufficient to initiate the inflammatory process [8,10]. Studies have suggested that neurogenic components contribute to inflammatory responses in various organs [10–12]. Since the nervous system is involved in the pathophysiology of various diseases, it has been suggested that neuropeptides may also play a role in the initiation and progression of oral diseases [11,13,14].

Periodontal disease is a chronic bacterial inflammatory process that mediates the destruction of periodontal tissues [15]. In periodontal

Abbreviations: Voltage-gated potassium (Kv) 9.1 channels, KCNS1; Hyperpolarization-activated cyclic nucleotide-gated 2 channel, HCN2; National Institute of Health, NIH; Paclitaxel induced group, PTX group; Intraperitoneal, i.p; Cementoenamel junction, CEJ; quantitative real-time PCR, qPCR.

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https://doi.org/10.1016/j.cbi.2022.110128
Received 9 August 2022; Received in revised form 16 August 2022; Accepted 19 August 2022
Available online 24 August 2022

tissues, pain sensation is primarily perceived by the free nerve endings of thin myelinated A-delta and unmyelinated C nerve fibers and carried to the spinal cord [16]. A delta fibers mainly carry thermal and mechanical stimuli; and C fibers carry mechanical, chemical, and thermal stimuli [17,18]. These sensory fibers in periodontal tissues contain a number of neuropeptides [19]. Few studies [10,20,21] have suggested a relationship between neuropeptides and neurogenic mechanisms in oral and periodontal inflammation.

Voltage-gated potassium (Kv) channels have received considerable attention as regulators of pain due to their essential role in shaping nociceptive signals. It has been suggested that inflammatory agents can be directly modulated some Kv channels. Inflammatory mediators directly inhibit K^+ conductivity [22] KCNS1, encoding the Kv9.1 subunit, is the first Kv gene associated with development of chronic pain in humans [23].

Hyperpolarization-activated cyclic nucleotide-gated 2 (HCN2) channel mediates the rhythmic electrical activity of cardiac cells and plays an important role in modulating membrane potentials, dendritic integration, and action potential thresholding in neurons [24]. HCN2 channel activity has been reported to be strongly associated with neuropathic and inflammatory pain [25].

The present study aimed to investigate the status of the periodontal tissues in rats with paclitaxel-induced neuropathic pain-like behavior. Gene-level expressions of HCN2 and KCNS1 in the brain and gingival tissues were evaluated.

2. Material and method

2.1. Ethics statement

This study was performed according to the local ethics committee protocol of Atatürk University for Animal Experiments (HADYEK protocol number 2021-4). All experimental procedures were carried out in accordance with the National Institute of Health (NIH) Guide for the care and use of animals.

2.2. Animals

We used sixteen male Sprague Dawley rats (220–295 g) in this study. The sample size was calculated by assuming 80% power and 95% confidence interval ($\alpha = 0.05$); eight animals per group were needed [26]. The rats were maintained on a 12-h light/dark cycle with temperatures at 21 ± 2 °C. The rats had access to water and rat chow ad libitum.

2.3. Experimental groups and period

Rats were randomly divided into two groups (n = 8). Control and PTX: Paclitaxel (2 mg/kg) was administrated to induce neuropathic pain-like behavior by intraperitoneal (i.p.) injection every two days and four times [27]. The pain-like behavioral assessment was conducted with Randall–Selitto Analgesiometry Test on days 7 and 21 of the first injection of paclitaxel [27,28]. At the end of the 22nd day, the mandible along with the neighboring tissue surrounding the first molar teeth and brain tissues were removed from rats following sacrification under anesthesia with xylazine hydrochloride (10 mg/kg i.p. Rompun, Bayer, Istanbul, Turkey) and ketamine hydrochloride (40 mg/kg kg i.p. Ketalar, Pfizer, Istanbul, Turkey).

2.4. Randall–Selitto Analgesiometry Test

We used the Randall-Selitto test to evaluate mechanical hyperalgesia in the rats. The device is applied to the base of the animal's hind paw with a constant increasing force (a certain gram per second). At the start of the application, the pressure (grams) with which the animal pulls its paw is recorded as the pain threshold value. The pain-like behavior is considered present if the animal begins to exhibit an escape or convulsion response. The pain-like behavior threshold of each rat was determined by applying pressure to the hind paws of all animals on the Plantar Randall Selitto analgesiometer testing equipment (Ugo Basile, Italy) [28,29].

2.5. Histopathological analyzes

The right mandibles surrounding the neighboring tissues were removed from sacrificed rats and fixed in 10% neutral formalin solution for 48 h. The samples were decalcified in EDTA solution, embedded in paraffin blocks. Then, 10 sections of 20 μm thickness were taken from each block and shaved. Afterward, samples were cut into 5 μm-thick sections along the molars in a buccolingual plan. All sections were stained with Hematoxylin-Eosin. Attachment loss of the first molar of the mandibulary was assessed by measuring the distance (μm) between the cementoenamel junction (CEJ) and the coronal position of the junctional epithelium attached to the root surface [30]. The number of inflammatory cells in connective tissue adjacent to the junctional epithelium was counted at a magnification of x 40. Inflammatory cell infiltrates were evaluated as absent (0), mild (1), moderate (2), severe (3), and very severe (4) [31].

2.6. RNA isolation, cDNA synthesis, and quantitative real-time PCR (qPCR)

The brain (cerebral cortex) samples and the gingival tissues around the left mandibular molar teeth were collected including the sulcular epithelium from the animals in all groups. Then and RNA isolation was performed using the RNA isolation kit (Invitrogen 12183025 RNA Mini Kit). The mRNA expression levels of the KCNS1 and HCN2 genes were detected with qPCR. Quantitative gene expression analysis was determined using the SYBR Green (Biorad-Cat no: 10000076382) method. The primers used are described in Table 1. The PCR reaction mix was prepared for template DNA containing 25 μl and amplification reactions were performed as follows: 50 °C for 2 min, 95 °C for 10 min, 45 cycles at 95 °C for 10 s, and anneal/extension at 60 °C for 1 min [32]. Relative gene expression data were analyzed using the ΔCT method [33].

2.7. Statistical analysis

Statistical analyses were performed with GraphPad Prism Software version 8.0 for Windows (GraphPad Software, San Diego, CA). The significance level was set at $p < 0.05$. The values are expressed as mean ± standard deviation. Immunohistochemical data were analyzed with the Mann–Whitney *U* test. Between-group differences were analyzed using Student's t-test for other results.

3. Results

3.1. Behavioral tests

Mechanical nociceptive responses threshold test results are presented in Fig. 1. Our results showed that the response to pain-like behavior decreased significantly in the PTX group (8.87 ± 3.7)

Table 1
Gene-specific primers showing the names, gene symbols, and GenBank accession numbers.

Gene Symbols	Accession Number	Primer	Sequence (5'→3')
Hcn2	NM_053684.2	Forward	GGACCATCGGGAAGAAGATGTA
		Reverse	GCTGAGATCATGCTGAACCTTG
Kcns1	NM_053954.2	Forward	ATCGCCGCCATGTGCATCCAC
		Reverse	AGGCGCGACGACACCTCGAAG
Gapdh	NM_017008.4	Forward	CCTTCATTGACCTCAACTAC
		Reverse	TCGCTCCTGGAAGATGGTGAT

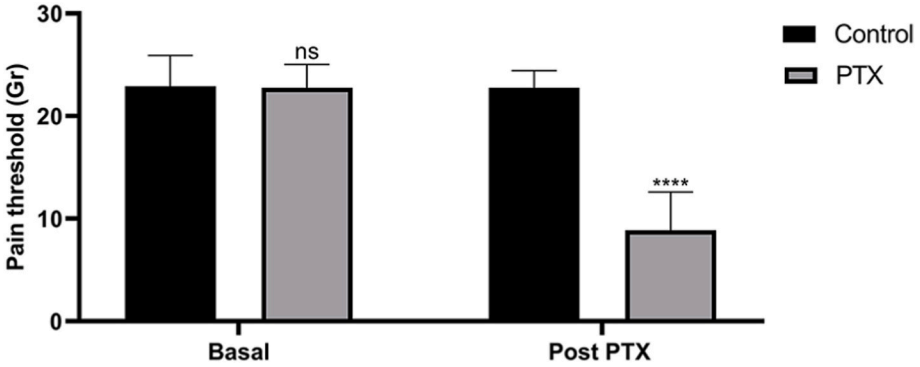

Fig. 1. Randall-selitto analgesiometer measurement values. Data shown are mean ± SD (n = 8/each group). ****p < 0.0001 significantly different from Control group.

compared to the control group (22.75 ± 1.6; p < 0.0001). This is an indication that PTX administration causes neuropathic pain-like behavior in rats.

3.2. Changes in Hcn2 and Kcns1 mRNA expression in the brain and gingival tissues

There was significantly less mRNA expression of KCNS1 in both brains (−0.53 ± 0.12 fold; p = 0.001) and gingival tissues (−0.03 ± 0.005 fold; p = 0.0003) of the PTX group than in the control group (Fig. 2). Fig. 3 shows the relative mRNA levels for HCN2 in the brain and gingival tissues. The mRNA expression of HCN2 in both brain (0.23 ± 0.03 fold; p < 0.0001) and gingival tissues (0.03 ± 0.003 fold; p = 0.0011) of the PTX group was significantly higher than in the control group.

3.3. Histopathological and histometrical findings

The attachment loss was significantly higher in the PTX (103.00 ± 5.86) group than in the control groups (6.50 ± 2.58; p < 0.001) (Table 2, Fig. 4). As shown in Table 1 and Fig. 4, prominent infiltration of inflammatory cells was observed in periodontal tissues of the PTX group (2.66 ± 0.51) as compared with the control group (0.33 ± 0.40; p < 0.05).

4. Discussion

This study demonstrated that periodontal inflammation and connective tissue destruction occurred in rats with experimental chronic pain-like behavior. Furthermore, our study revealed increased mRNA expression of HCN2 and decreased mRNA expression of KCNS1, which play an important role in chronic pain, in gingival tissues. To our knowledge, this study is the first report in dentistry to evaluate the relationship between chronic pain-like behavior, HCN2, KCNS1, and periodontal inflammation.

In chronic pain, it has been shown that there is a change in the expression of chronic pain genes in both the central nervous system and peripheral tissues. Changes in neurotransmission directly influence the messages received and sent by neurons, while changes in ion channels can alter the transmission of received messages by decreasing or increasing neuronal excitability [34]. The studies [34,35] indicate that KCNS1 contributes to an increase in pain sensitivity and, in many cases, an increased occurrence of chronic pain conditions in both healthy and chronic pain individuals. KCNS1 expression in the rat dorsal root ganglion was shown to be downregulated in a model of peripheral neuropathic pain-like behavior [35]. Tsantoulas et al. demonstrated that mechanical nociceptive sensitivity in basal and experimental neuropathic pain-like behavior model elevated in peripheral neurons of mice lacking KCNS1 [23]. In our study, the expression of KCNS1 in brain tissue was significantly lower in the PTX group than in the control group. We found that KCNS1 expression was significantly lower in the gingival tissue of the PTX group than in the control group.

Studies [24,25] have shown that the expression of HCN channels involved in the modulation of pain signaling is increased in chronic pain. Up-regulation of HCN protein expression has been observed in neurons in the dorsal root ganglion [36] and spinal cord [24] of rats with neuropathic and inflammatory pain-like behavior. Ding et al. [25] reported that inhibition of HCN channel activity in the Gasserian ganglion

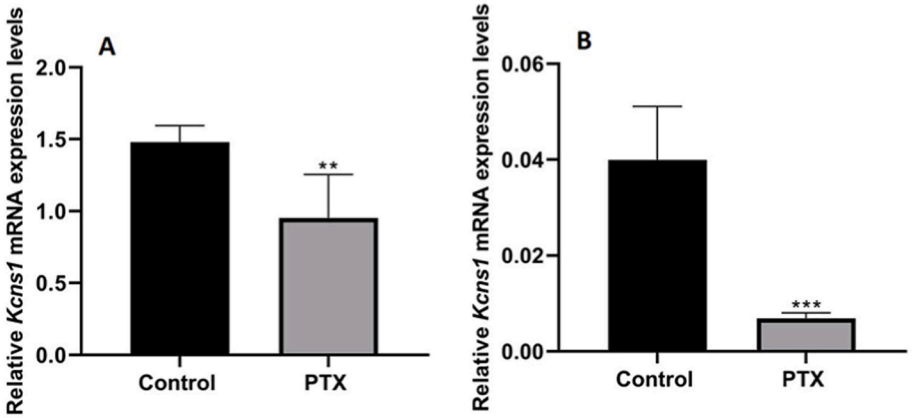

Fig. 2. The decrease in gene expression of KCNS1 in paclitaxel-induced chronic neuropathic pain. (A) q-PCR analysis of KCNS1 in brain tissues of groups. (B) q-PCR analysis of KCNS1 in periodontal soft tissues of groups. Data shown are mean ± SD (n = 3/each group). **p < 0.01, ***p < 0.001 significantly different from Control group.

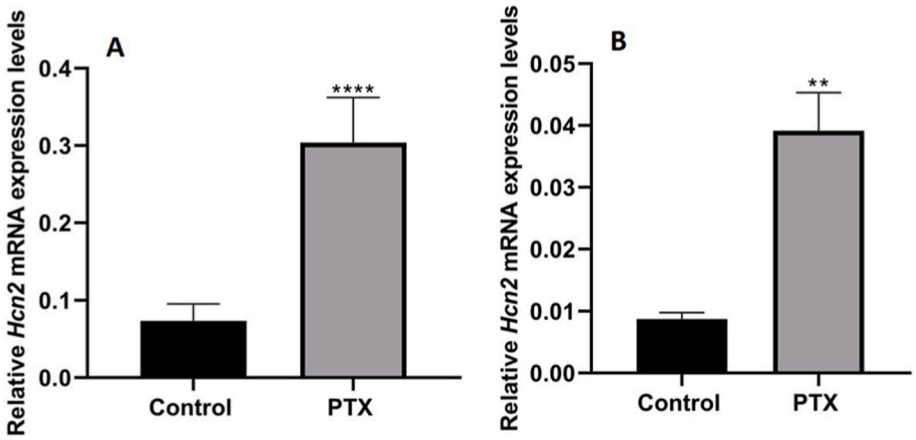

Fig. 3. The increase in gene expression of HCN2 in paclitaxel-induced chronic neuropathic pain. (A) q-PCR analysis of HCN2 in brain tissues of groups. (B) q-PCR analysis of HCN2 in periodontal soft tissues of groups. Data shown are mean ± SD (n = 3/each group). **p < 0.01, ****p < 0.0001 significantly different from Control group.

Table 2
Comparison of Attachment loss around teeth and Inflammatory cell infiltrates between the groups.

Groups	Attachment loss (μm)	Inflammatory cell infiltrates
Control	6.50 ± 2.58[a]	0.33 ± 0.40[a]
PTX	103.00 ± 5.86[b]	2.66 ± 0.51[b]

Comparison of Attachment loss around teeth ($p < 0.001$) and Inflammatory cell infiltrates ($p < 0.05$) between the groups (mean ± SD). Data shown are mean ± SD (n = 8/each group). Different letters ([a,b]) in the same column indicate significant differences among groups.

reduced trigeminal neuropathic pain. Wells et al. [37] detected that HCN2 gene expression increased in trigeminal ganglia neurons hours after pulp injury. Ebadian et al. [11] suggested that HCN2 polymorphism may affect the progression of chronic periodontitis and inflammatory gum disease due to its role in inflammatory mechanisms. Our results showed that the level of HCN2 expression was significantly higher in the brain tissues of the PTX group as compared with the control group. In addition, the level of HCN2 expression was significantly higher in the gingival tissues of the PTX group than in the control group in this study.

In periodontal inflammation, the number of inflammatory cells in the gingival connective tissue and junctional epithelium increases by the infiltration of inflammatory cells [38]. Subsequent to the initial inflammatory response, connective tissue destruction begins. As the inflammatory process develops, the destruction also occurs in the periodontal ligament and alveolar bone [19] and alterations in attachment levels on periodontium [39]. In the current study, prominent inflammatory cell infiltration was observed in the periodontal tissues of

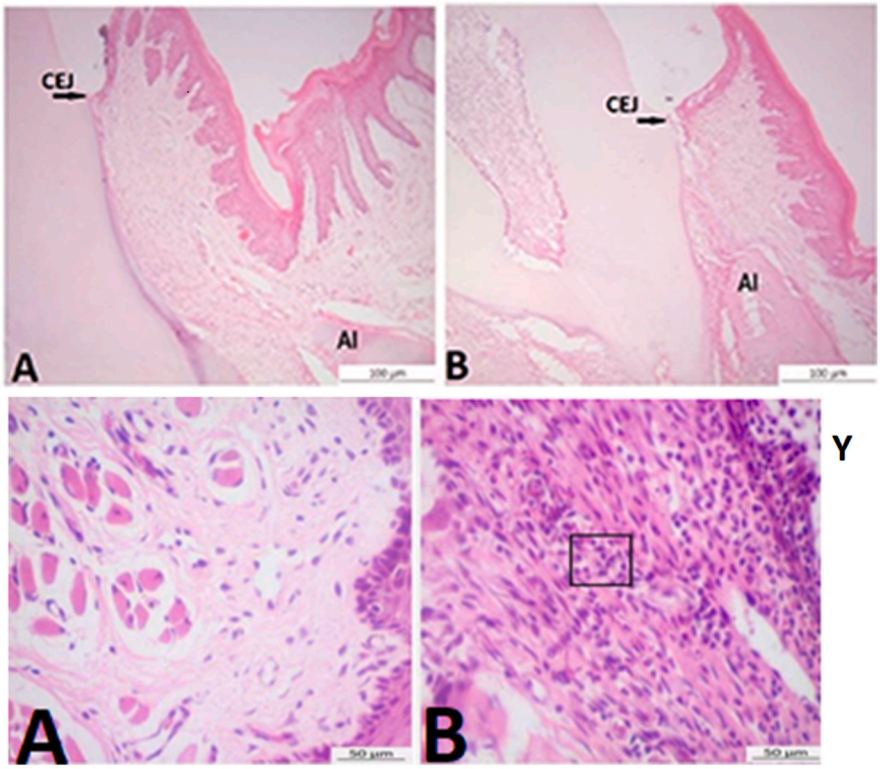

Fig. 4. X; Histopathological findings of gingival mucosal tissues from all groups in the buccolingual sections of mandibular first molars (H&E staining). (A) Control group section, a normal histologic view. (B) PTX group section.Arrows; cementoenamel junction (CEJ), Al; alveolar bone crest. **Y;** Number of inflammatory cells in the gingiva, presented as the cell number/unit square (mm2). The number of inflammatory cells in the connective tissue adjacent to the junctional epithelium. (A) Control group section. (B) PTX group section.

the PTX group. And, we observed increased attachment loss in the PTX group as compared with the control group. These results revealed that experimental chronic pain can induce inflammation in periodontal tissues and destruction in tooth support tissues.

Study limitations: First, our results were obtained only in the paclitaxel-induced chronic pain-like behavior model. Our study does not evaluate the levels of HCN2 and KCNS1 in pain in orthodontic treatment or chronic diseases in dental support tissues. Second, the gene expression of HCN2 and KCNS1was analyzed by qPCR in tissues. Transcript levels often do not necessarily reflect protein levels or protein activation status. The lack of western blotting analysis that better evaluates the level of protein expression are additional limitation. It has been suggested that sex may affect pain sensitivity, tolerance, and analgesia. Studies show that female hormones (especially estrogen) and different sex-related immune cells play a role in increased pain sensitivity in women [40,41]. In this study, male rats were preferred to rule out the effect of estrogen on nociceptive behaviors. However, another limitation of this study is that it is a single-sex animal study.

5. Conclusions

We observed that increased nociceptive sensitivity can cause inflammation and tissue damage in the healthy periodontium. Our results revealed that HCN2 expressions increased [36,42] and KCNS1 expressions decreased [43,44] in periodontal tissues in presence of chronic pain-like behavior. Because studies suggest that KCNS1 and HCN2 can be directly modulated by inflammatory mediators, it would be interesting to explore the function of these genes in inflammatory periodontal diseases. In addition, the results of the present study may be helpful in developing new approaches to alleviate pain and maintain periodontal health in patients suffering from orofacial pain. Further studies are needed to investigate the role of these genes in pains occurring in the periodontium.

The author's contribution statement

Ayşe Toraman: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Final approval of the version to be submitted. Emine Toraman: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - Original Draft, Visualization, Final approval of the version to be submitted. Mustafa Özkaraca: Methodology, Formal analysis, Investigation, Writing - Original Draft, Visualization, Final approval of the version to be submitted. Harun Budak: Conceptualization, Methodology, Resources, Writing - Original Draft, Visualization, Final approval of the version to be submitted.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

Acknowledgement

We are thankful to Professor Dr. Serhat KÖSEOĞLU from Department of Periodontology, Faculty of Dentistry, İstanbul Medeniyet University, Istanbul, Turkey for many valuable opinions during the study.

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