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ORIGINAL ARTICLE

Evaluated periodontal tissues and oxidative stress in rats with neuropathic pain-like behavior

Ayşe Toraman[1] · Emine Toraman[2] · Mustafa Özkaraca[3] · Harun Budak[2]

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Abstract

Background Oxidative stress has a critical effect on both persistent pain states and periodontal disease. Voltage-gated sodium NaV1.7 (SCN9A), and transient receptor potential ankyrin 1 (TRPA1) are pain genes. The goal of this study was to investigate oxidative stress markers, periodontal status, SCN9A, and TRPA1 channel expression in periodontal tissues of rats with paclitaxel-induced neuropathic pain-like behavior (NPLB).

Methods and results Totally 16 male Sprague Dawley rats were used: control (n = 8) and paclitaxel-induced pain (PTX) (n = 8). The alveolar bone loss and 8-hydroxy-2-deoxyguanosine (8-OHdG) levels were analyzed histometrically and immunohistochemically. Gingival superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities (spectrophotometric assay) were measured. The relative TRPA1 and SCN9A genes expression levels were evaluated using quantitative real-time PCR (qPCR) in the tissues of gingiva and brain. The PTX group had significantly higher alveolar bone loss and 8-OHdG compared to the control. The PTX group had significantly lower gingival SOD, GPx and CAT activity than the control groups. The PTX group had significantly higher relative gene expression of SCN9A ($p = 0.0002$) and TRPA1 ($p = 0.0002$) than the control in gingival tissues. Increased nociceptive susceptibility may affect the increase in oxidative stress and periodontal destruction.

Conclusions Chronic pain conditions may increase TRPA1 and SCN9A gene expression in the periodontium. The data of the current study may help develop novel approaches both to maintain periodontal health and alleviate pain in patients suffering from orofacial pain.

Keywords Chronic pain · Oxidative stress · Nociceptive sensitivity · Periodontal disease · SCN9A · TRPA1

Abbreviations

SCN9A	Voltage-gated sodium channels, NaV1.7
TRPA1	Transient receptor potential ankyrin 1 channel
8-OHdG	8-hydroxy-2-deoxyguanosine
SOD	Superoxide dismutase
CAT	Catalase
GPx	Glutathione peroxidase
ROS	Reactive oxygen
RNS	Nitrogen species
NIH	National Institute of Health
PTX group	Paclitaxel induced group
i.p	Intraperitoneal
CEJ	Cementoenamel junction
qPCR	quantitative real-time PCR

✉ Ayşe Toraman
draysetoraman@gmail.com

[1] Hamidiye Faculty of Dentistry, Department of Periodontology, Health Sciences University, İstanbul 34668, Turkey

[2] Science Faculty, Department of Molecular Biology and Genetics, Atatürk University, Erzurum, Turkey

[3] Faculty of Veterinary Medicine, Department of Preclinical Sciences, Department of Veterinary Pathology, Cumhuriyet University, Sivas, Turkey

Introduction

Chronic pain is a common health condition problem that substantially reduces the quality of life; individuals often suffer from anxiety, depression, and restrictions in daily activities [1]. The pain exceeding the normal healing period, and a reduced neuronal threshold is considered a natural

feature of chronic pain [2, 3]. Chronic pain can occur as a result of cancer, myofascial pain syndrome, trigeminal neuralgia, diabetes, infections, temporomandibular disorders, and nerve-related diseases [4–7]. Neuropathic pain was shown to produce thermal hyperalgesia (increased pain sensitivity) and allodynia (pain resulting from non-painful stimuli) that contribute to the generation of reactive oxygen and nitrogen species (ROS; RNS) and cytokines. Oxidative stress (excessively increased ROS and, RNS) has a critical effect on persistent pain states such as inflammatory and neurogenic pain, neuropathic pain, and chemotherapy-induced pain [8]. It was shown that the amounts of ROS are increased in the dorsal horn neurons and spinal cord during neuropathic pain and inflammatory pain models [9, 10]. Excessively increased ROS formation causes damage to important compounds of cells such as lipids, protein, DNA, and carbohydrates. 8-hydroxy-2-deoxyguanosine (8-OHdG) is an oxidized nucleoside formed during DNA repair and used as a biomarker for oxidative DNA damage. In normal physiology, the antioxidant defense system maintains ROS activity and antioxidant balance by neutralizing excess free radicals, thus preventing damage. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are important enzymatic antioxidants [11, 12].

Periodontitis is an inflammatory condition that affects tooth-supporting tissues [13]. An imbalance between oxidant/antioxidant activities contributes to inflammation affecting the periodontium [14–16]. It has been suggested that the hydroxyl radical and superoxide anion are involved in bone reabsorption [17]. Oxidative stress causes periodontal bone loss through direct damage to the extracellular connective tissue and also indirectly by leading to proinflammatory cytokines/chemokines production [13].

Calcium influx through voltage-dependent calcium channels is an important pathway for maintaining intracellular calcium homeostasis [18]. It was suggested that increased extracellular calcium levels might support the osteogenic differentiation of human periodontal ligament stem/progenitor cells. It was reported that elevated extracellular and intracellular calcium levels inhibit bone resorption [18, 19]. TRPA1 is a member of the transient receptor potential (TRP) channel family. TRPA1 which has a much higher permeability to calcium compared to other TRPs allows an inward cation current that can modulate intracellular calcium-dependent pathways [20]. TRPA1 is often involved in pain, thermal, and chemical sensation is predominantly expressed in nociceptive sensory neurons [21]. TRPA1 can also be activated by various endogenous reagents involved in oxidative stress and inflammation [22, 23]. Nociceptive receptors on primary sensory fibers in periodontal tissues are involved in nociceptive mechanisms. Pulpitis and oral mucosal trauma were detected to trigger the expression of TRPA1 in dental tissues and oral mucosa [24, 25]. The SCN9A gene is involved in the formation of a part (alpha subunit) of the sodium channel called NaV1.7. These channels are found in small nociceptive fibers that transmit pain signals [26].

The present study intended to examine the impact of the paclitaxel-induced NPLB model on the oxidative stress level and status of periodontal tissues. The alteration in TRPA1 and SCN9A gene expressions in the tissues of the gingival and brain were investigated.

Material and method

In this study, the whole experiment procedure was performed in accordance with University of Atatürk Animal Experiments Local Ethics Committee protocol (HADYEK protocol number 2019 – 193). All experiments were consistent with the National Institute of Health (NIH) Guide for the care and use of animals.

The experiments were carried out on weighing 220–295 g male Sprague Dawley rats (n = 16). The sample size was determined by accepting an 80% power and a 95% confidence interval ($\alpha = 0.05$) [27]. All rats were housed in cages with constant temperature and light cycles (23 ± 1 °C, 12:12 h dark/light cycles). Rodent chow and water were given ad libitum.

Experimental design

Two groups (n = 8) were created. Control and PTX: intraperitoneal (i.p.) paclitaxel (2 mg/kg) injection was performed to create a NPLB model every two days and four times [28]. The pain-like behavior of rats was assessed by Randall–Selitto Analgesiometry Test at two points of the experiment (on days 7 and 21 of the first paclitaxel application). The rats were sacrificed under anesthetized at the end of the 22nd day. The brain tissues and mandible along with the periodontal tissue were removed.

Randall–Selitto analgesiometry test

The mechanical hyperalgesia in the rats was evaluated by Randall-Selitto test. A linearly-increasing pressure (a certain gram per second) was exerted on the plantar face of hind paw with Randall Selitto analgesiometer (Ugo Basile, Italy) [29]. While the mechanical force was applied, the pressure value at which the rat pulled its paw was accepted as the nociceptive. This threshold level was determined in all animals. A contraction or escape response observed in the animal was considered the presence of pain-like behavior.

Histopathological analyzes

The right mandibles of rats were removed and fixed in 10% neutral formaldehyde solution for 48 h. The samples were decalcified with EDTA. After, 10 sections of 20 µm thickness were obtained from the samples embedded in paraffin blocks. Then, sections of 5 µm were taken and stained with Hematoxylin-Eosin. All sections were taken along the 1st and 2nd molars in the buccolingual plane. The equally distant sections of each tooth were selected for histometric analysis. The alveolar bone level of the first molars has measured the distance (µm) between the cementoenamel junction (CEJ) and the alveolar bone crest (CEJ-ABC).

Immunohistochemical analyzes

5 µm sections taken on polylysine slides were passed by xylol and alcohol series, after washing with PBS, and treated with 3% H2O2 for 10 min. Antigen retrieval was performed using antigen retrieval solution for 2 × 5 min at 500 watts. After the tissues were washed with PBS were incubated with 8-OhDG primary antibody (Santa cruz, Cat no. sc-66,036) at a dilution rate of 1/200 at room temperature for 45 min. Secondarily; Large Volume Detection System: anti-Polyvalent, HRP (Thermofischer, Catalog no: TP-125-HL) was used as recommended by the manufacturer. DAB (3,3′-Diaminobenzidine) was used as chromogen. After counterstaining with Mayer's Hematoxylin, it was covered with entellan and examined at a magnification of 40x under a light microscope. A semi-quantitative evaluation was done. The immunopositive in all epithelium of the mandibular keratinized mucosa cells was evaluated as 50%> severe (+++), 20–50% moderate (++), 20% < mild (+), no immunopositive = absent (-).

Enzyme activity analyzes

Rat gingival homogenates were prepared. Quantitative protein determination was performed spectrophotometrically at 595 nm according to the Bradford method [30] using bovine serum albumin as a standard. SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), and GPx (EC 1.11.1.9) activities were spectrophotometrically measured according to Sun [31], Aebi [32], and Beutler [33], respectively.

Real-time PCR (qPCR)

RNA was extracted from the gingival tissues including the sulcular epithelium around molar teeth (Invitrogen 12,183,025 RNA Mini Kit). cDNA synthesis was performed from the isolated RNA using the cDNA synthesis kit (Biolabs, E6300S). qPCR was used to determine the levels of SCN9A and TRPA1 genes mRNA expression. Quantitative gene expression was detected by SYBR Green (Biorad-Cat no: 10,000,076,382) [34]. Table 1 presented the primers used. The ΔCT method was used to analyze relative gene expression results.

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

Gene Symbols	Accession Number	Primer	Sequence (5'→3')
Trpa1	NM_207608.1	Forward	TCCAAACCTCC-GAAATAG
		Reverse	ATGTTAGTGGCCTTGTGC
Scn9a	NM_133289.2	Forward	TCTCCCTTCAGTCCTC-TAA
		Reverse	AACAAAGTCCAGC-CAGTT
Gapdh	NM_017008.4	Forward	CCTTCATTGACCTCAAC-TAC
		Reverse	TCGCTCCTGGAAGATG-GTGAT

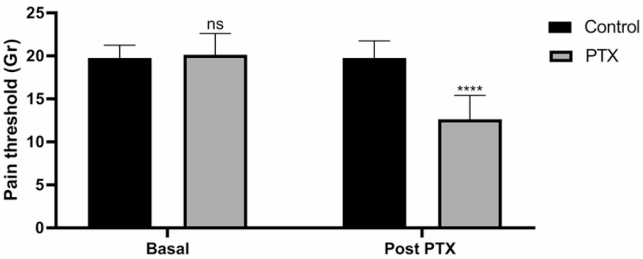

Fig. 1 Randall- selitto analgesiometer measurement values. Data shown are mean ± SD (n = 8/each group). ****$p < 0.0001$ significantly different from the Control group

Statistical analysis

Results were evaluated by GraphPad Prism Software version 8.0 (GraphPad Software, San Diego, CA). The values were presented as mean ± standard deviation. Student's t-test was used to evaluate the difference between groups ($p < 0.05$).

Results

Behavioral tests

Figure 1 shows the nociceptive threshold values. In this study, the pain threshold was significantly lower in the PTX group (12.63 ± 2.77) compared to the control group (19.75 ± 1.49; $p < 0.0001$). These results indicate that NPLB occurs in PTX-treated rats.

Alterations in TRPA1 and SCN9A relative mRNA expression in the brain and gingival tissues

The PTX group showed significantly higher SCN9A mRNA expression in the brain (0.15 ± 0.03 fold; p = 0.0002) and gingiva (0.20 ± 0.04 fold; p = 0.0002) than in the control group (Fig. 2). The PTX group had significantly higher relative mRNA expression levels of TRPA1 in both brain (0.27 ± 0.04 fold; p < 0.0003) and gingiva (0.17 ± 0.01 fold; p = 0.0002) than the control (Fig. 3).

Gingival SOD, GPx, and CAT activity

Table 2 shows levels of SOD, GPx, and CAT activity in the gingival tissues. The PTX group (59.14 ± 16.3; $p = 0.013$) showed a significantly lower gingival SOD activity level than the control group (141 ± 29.27). There was a significant decrease in the level of CAT activity in the PTX group (16.82 ± 3.08; $p = 0.03$) compared to the control group (25.08 ± 3.43). The gingival GPx activity level of the PTX group (0.58 ± 0.14; $p = 0.0002$) was significantly lower than the control group (1.17 ± 0.06).

Histopathological and immunohistochemical findings

Our results showed that there was a significant increase in distance measurements for CEJ–ABC in the PTX group (255.33 ± 3.88) than in the control (114.50 ± 7.23; p < 0.001) (Table 2; Fig. 4). Detected immunopositivity was in gingival epithelial tissue (Table 2; Fig. 4). No significant 8-OhDG immunopositivity was observed in the control group (0.16 ± 0.40). A moderate level of 8-OhDG immunopositivity was detected in the PTX group (1.83 ± 0.40; $p < 0.05$).

Discussion

Our results showed that increased nociceptive sensitivity causes lower SOD, CAT, and GPx activity, higher 8-OHdG immunostaining in gingival tissue, and alveolar bone loss. Furthermore, the results of this study showed high mRNA expression of SCN9A and TRPA1 genes in gingival tissues in a chronic pain-like behavior model. To the best of our knowledge, this is the first research to assess the relationship between NPLB, oxidative stress, SCN9A, TRPA1, and changes in the periodontium.

Chronic pain activates multiple intracellular pathways including ROS formation such as protein folding leading to ROS generation [9, 35]. For example, increased afferent inputs might increase the release of proteins such as glutamate and inflammatory mediators, and excess glutamate can lead to excessive ROS production in neurons. Studies indicate that ROS levels are elevated in the spinal cord, dorsal root ganglion (DRG), and dorsal horn neurons during neuropathic pain [9, 10, 36]. Komirishetty et al. [37] reported increased SOD activity accompanying increased oxidative damage in the spinal cord after the neuropathic

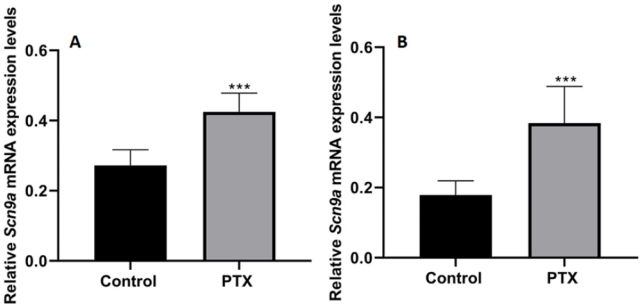

Fig. 2 The increase in gene expression of SCN9A in paclitaxel-induced chronic neuropathic pain-like behavior. (**A**) q-PCR analysis of SCN9A in brain tissues of groups. (**B**) q-PCR analysis of SCN9A in periodontal soft tissues of groups. Data shown are mean ± SD (n = 3/each group). ***$p < 0.001$ significantly different from the Control group

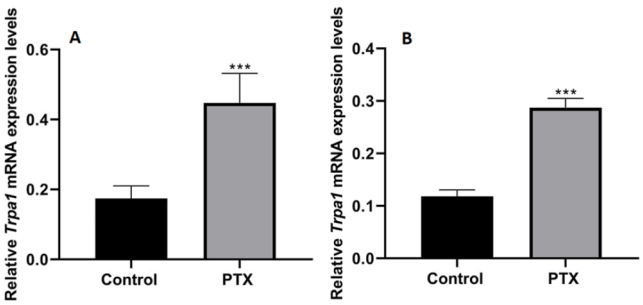

Fig. 3 The increase in gene expression of TRPA1 in paclitaxel-induced chronic neuropathic pain. (**A**) q-PCR analysis of TRPA1 in brain tissues of groups. (**B**) q-PCR analysis of TRPA1 in periodontal soft tissues of groups. Data shown are mean ± SD (n = 3/each group). ***$p < 0.001$ significantly different from the Control group

Table 2 Comparison of distance for the CEJ–ABC around teeth and SOD, GPx, and CAT activity levels and semi-quantitative analyses of 8-OHdG in the gingival tissues between the groups

Groups	Control	PTX
SOD (U/mg-protein)	141 ± 29.27	59.14 ± 16.3*
GPx (U/mg-protein)	1.17 ± 0.06	0.58 ± 0.14***
CAT (U/mg-protein)	25.08 ± 3.43	16.82 ± 3.08*
Distance for the CEJ–ABC (µm)	114.50 ± 7.23	255.33 ± 3.88***
8-OhDG immunopositivity	0.16 ± 0.40	1.83 ± 0.40*

Comparison of distance for the CEJ–ABC around teeth and SOD, GPx, and CAT activity levels and semi-quantitative analyses of 8-OHdG in the gingival tissues between the groups (mean ± SD). Data shown are mean ± SD (n = 3/each group for SOD, GPx, and CAT analyses; n = 8/each group for analyses of 8-OHdG and distance for the CEJ–ABC). *$p < 0.05$, ***$p < 0.001$ significantly different from Control group

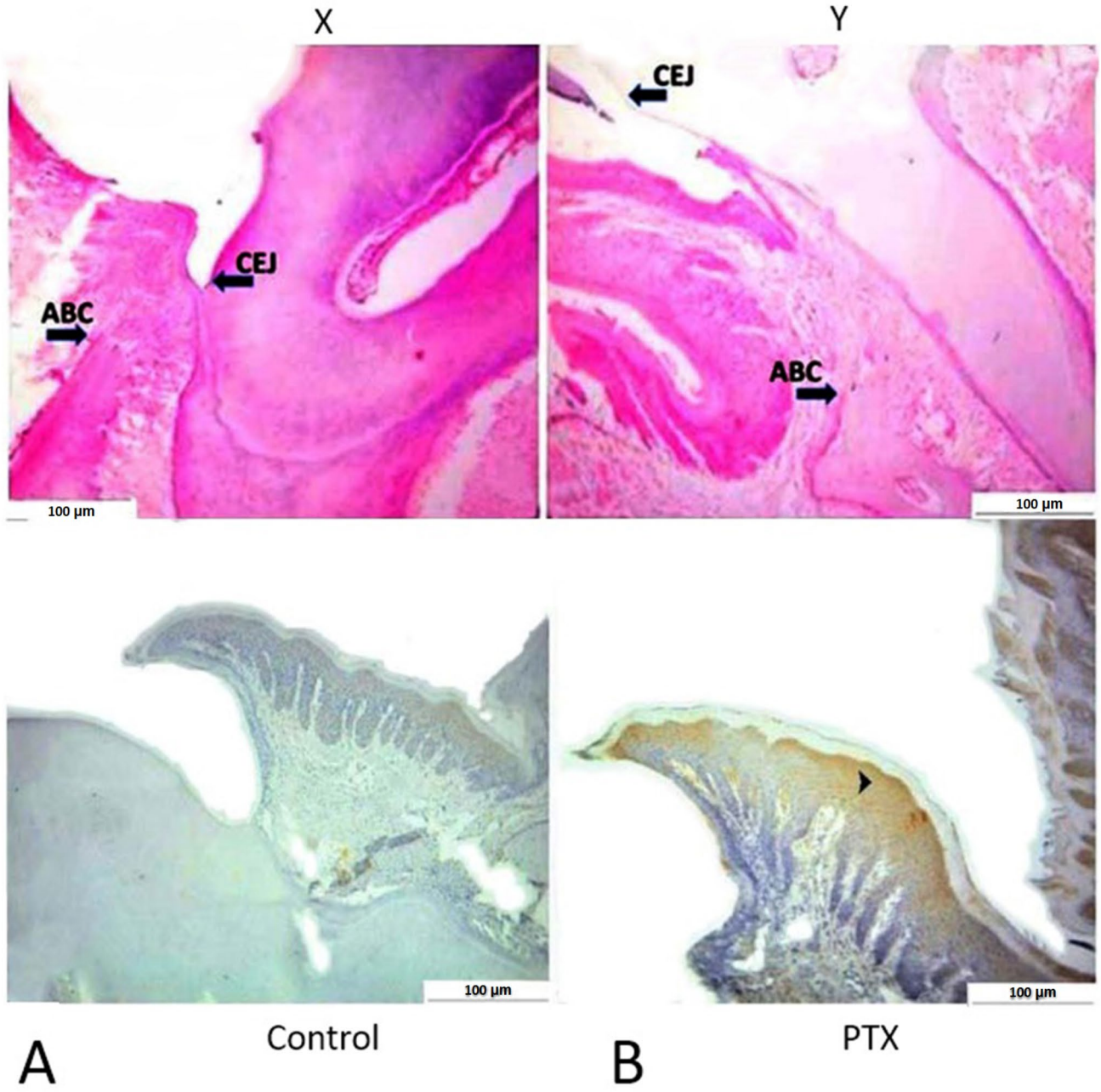

Fig. 4 **X** and **Y;** Histopathological findings of gingival mucosal tissues from all groups in the buccolingual sections of mandibular first molars (H&E staining). (X) Control group section, a normal histologic view. (Y) PTX group section. CEJ; cementoenamel junction, ABC; alveolar bone crest. **A** and **B;** Immunohistochemical staining of 8-OHdG in the gingiva, presented as the cell number/unit square (mm^2). (**A**) Control group section. (**B**) PTX group section. Arrow indicates positive staining

pain model. Guedes et al. [38] suggested that GPx activity was elevated in the spinal cord of rats with neuropathic pain. Zhao et al. [36] showed a decrease in SOD activity and an increase in 8-OHdG level in the spinal cord after experimental chronic neuropathic pain was induced. We did not find any study evaluating ROS levels in peripheral tissues in neuropathic pain conditions. Our results suggested that there was a significant decrease in SOD, CAT, and GPx levels in the gingival tissue of the PTX group. We also found an increase in the level of oxidative damage marker 8-OHdG in the gingival tissue.

Kerckhove et al. [39] show that oxidative stress in the case of paclitaxel-induced neuropathic pain causes damage to neuronal and non-neuronal cells, as well as macrophage activation and ultimately overproduction of proinflammatory cytokines. The increased ROS/RNS stimulates the channel activities of TRPA1, mainly expressed in peptidergic C fibres [21, 40]. Materazzi et al. [41] detected that

paclitaxel application stimulated oxidative stress by products that ultimately activate TRPA1 in mice. It was reported that the activation of the TRPA1 increased on nerves carrying pain induced by cold after dental bleaching [42]. TRPA1 expression was observed to be upregulated in myelinated nerve fibers of teeth with pulpitis [43]. In the current study, TRPA1 mRNA expression was significantly increased in the gingival tissue of rats. This increase may have occurred in the nociceptive nerve endings in the periodontium. The human [44, 45] and rat studies [46] suggested that differential expression of SCN9A may be an important contributor to the development of trigeminal pain. Animal studies showed that SCN9A gene expression and function are increased in models of neuropathic pain, diabetic, inflammatory, and bone cancer [47–50]. Zhang et al. [51] revealed a significant increase in the expression of the SCN9A gene with the qPCR method in DRG of rats with paclitaxel-induced neuropathy. Li et al. [52] reported that the expression of the SCN9A gene in DRG was upregulated in paclitaxel-induced neuropathy in rats and humans with neuropathic pain. Our study demonstrated that the expression of the SCN9A gene was significantly increased in the gingival tissue of rats. Tumor necrosis factor-α and its downstream signal molecule nucleus factor-kappa B, and prostaglandin E2 were statemented to upregulate Nav1.7 mRNA expression [53, 54]. Inflammation associated with oxidative stress in gingival tissues may have contributed to the increase in SCN9A.

It was reported that there was an important relationship between oxidative stress biomarkers and gingival inflammation and alveolar bone resorption [55]. It was indicated that increased intracellular ROS levels contribute to bone resorption by directly promoting osteoclast differentiation and activity [56]. It was shown that the gingival SOD, GPx, and CAT activity decreases [57, 58] or increases [13, 14, 55] in gingival inflammation and periodontal disease. Akalın et al. [58] suggested that relations may exist between periodontal status and gingival SOD activity. Studies showed an important increase in 8-OHdG levels of gingiva in periodontal disease [56, 59]. In our study, alveolar bone loss was found in the PTX group with increased oxidative stress.

Study limitations: First, this present study presented only results in the paclitaxel-induced NPLB model. The present research does not estimate SCN9A and TRPA1 levels in periodontal tissues in other diseases such as diabetes, trigeminal neuralgia, which cause neuropathic pain, orthodontic treatment pain. Second, the levels of SCN9A and TRPA1 gene expression were determined using qPCR in tissues. It is known that transcript levels generally do not reflect protein levels or protein activation status. Further experiments such as western blotting analysis should be carried out. Third, the study was not include micro-CT analysis, which better assesses alveolar destruction, and TRAP staining. Studies reported that different sex-related immune cells and female hormones may increase pain sensitivity in women [60, 61]. In the current study, we studied male rats to exclude the action of estrogen on nociceptive behavior. But, the use of single-sex was another limitation of the study.

Conclusions

In this study, we found that oxidative stress and oxidative DNA damage increased in healthy periodontium with increased nociceptive sensitivity and alveolar bone loss occurred, probably with the contribution of increased oxidative stress. Our results indicated that SCN9A and TRPA1 expressions increased in gingival tissues in chronic pain-like behavior condition. Considering that ROS/RNS increases alveolar bone resorption, which occurs as a result of the inflammatory process and that the expression of SCN9A and TRPA1 may be associated with inflammatory mediators, investigating the effect of these genes in periodontal diseases may help develop new approaches both to maintain periodontal health and alleviate pain in patients suffering from orofacial pain. This study may be a guide in this regard, but further studies at the molecular level are needed.

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Author contributions Ayşe Toraman: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Final approval of the version to be submittedEmine Toraman: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - Original Draft, Visualization, Final approval of the version to be submittedMustafa Özkaraca: Methodology, Formal analysis, Investigation, Writing - Original Draft, Visualization, Final approval of the version to be submittedHarun Budak: Conceptualization, Methodology, Writing - Original Draft, Visualization, Final approval of the version to be submitted.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests The authors declare no competing interests.

Ethical approval This study was performed according to the University of Atatürk University Animal Experiments Local Ethics Committee protocol (HADYEK protocol number 2019 – 193). All experiments were consistent with the National Institute of Health (NIH) Guide for the care and use of animals.

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