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Original Research

Dentin hypersensitivity: expression of neuron/odontoblast receptors and release of neuropeptide in dental pulp

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Aim: This study assessed the activity of neuron/odontoblast receptors and neuropeptide release, under an animal model of dentin hypersensitivity (DH). **Methods:** Wistar rats were allocated in two groups (n=10): in test group DH was induced by a validated protocol in which a sports drink (pH 3.08) was ingested for 45d, while in control group, the animals ingested filtered water instead. Animals were euthanized and blood samples were collected to measure plasma corticosterone levels. Dental pulp samples (n=6) were processed for Western Blot and ELISA analysis of TRPV1 (mechano-, thermo- and chemoreceptor), P2X7 (adenosine triphosphate (ATP)-mechanosensitive receptor), TRPM8 (cold-sensitive receptor) and substance P (neurogenic peptide released by neuron activation and due to TRPV1 expression). Data were analyzed using Student's t tests (α=0.05). **Results:** DH significantly increased expression of TRPV1 ($p=0.002$), P2X7 ($p=0.007$) and substance P ($p<0.001$) but did not significantly affect the activity of TRPM8 ($p=0.079$). **Conclusion:** Under DH condition, neurons and odontoblasts expressed TRPV1 and P2X7 receptors as well as increased substance P release, demonstrating cellular and molecular mechanisms underlying DH.

Keywords: Dentin sensitivity. Neurons. Odontoblasts. Neurogenic Inflammation.

Introduction

Dentin hypersensitivity (DH) is a prevalent finding occurring approximately in 33.5% of the population according to a metanalytic study[1]. DH is described as a short, sharp pain, in response to thermal, evaporative, tactile, osmotic or chemical stimuli after exclusion of other pathologies[2,3]. For such, it is necessary that dentine becomes exposed to the oral environment, which occurs with gingival recession or loss of enamel due to wear processes, mainly erosion and abrasion[4].

The main hypotheses that explain DH are threefold: hydrodynamic, dentine innervation and odontoblastic theories[5-7]. The first, and most accepted attributes pain to the fluid flow within dentinal tubules, stimulating, by pressure difference, nerve endings. On the other hand, the innervation theory states that DH is due to the direct stimulation of nerve endings that extend from the pulp and are present in dentine. The odontoblastic theory suggests that odontoblasts act as receptor cells detecting diverse stimuli.

Dental pulp is innervated by two types of nerve fibers, A-fibers (myelinated) and C-fibers (non-myelinated)[8]. Acute pain, which characterizes DH, is related to the activation of the intradental A-fibers[5,9]. Functionally, A-fibers respond to several stimuli that induce intradentinal fluid flow, being associated to DH by the hydrodynamic theory[10]. This is due to the fact that increased intratubular fluid flow causes pressure change that consequently activates and stimulates A-fibers at the pulp-dentine interface by means of mechanoreceptors that activate pulp nerves[11].

Although over time of dentine exposure A-fibers may become less sensitive to hydrodynamic stimulation, nerve sensitization may persist triggered by inflammatory reactions (activation of C-fibers)[12]. That is the reason why DH has been considered a chronic state of pain by some researchers[10,12]. Although literature suggests that patients with DH can have severe pain after initial stimulation followed by lingering pain[9], the neurochemical mechanisms that involve potential pulp changes of teeth with DH remain unclear[5,13].

C-fibers do not respond directly to fluid flow stimuli, but their function in DH may be important because such fibers contain neuropeptides such as substance P and CGRP (calcitonin gene-related peptide), which cause local vasodilation, vascular permeabilization and edema formation, known as neurogenic inflammation[14]. In fact, while evaluating the photobiomodulation by low-level laser as a treatment for DH, a paper showed increased expression of substance P in thermal-sensitive dentine[15].

Besides the release of substance P and CGRP by C-fibers, such neuropeptides can also be secreted following nerve sensitization of inflammatory receptors expressed in odontoblasts[10]. These ion channels include TRPV1, a member of the transient receptor potential (TRP) family of cation channels[10,16,17], that acts as mechano-, thermo- and chemosensors[5].

Neurons and odontoblasts can express indeed TRPM8, a cold-sensing ion channel receptor[10,16,17]. TRPM8 expression was detected in 58% of sensory neurons innervating the dental pulp of rats[18], and in cultured odontoblasts from rat and human dental pulp[19].

P2X7 is another odontoblast receptor[20], which participates of the sensory function of teeth by releasing extracellular adenosine triphosphate (ATP) in response to physical stimuli[21,22]. Once activated by P2X7, inflammatory cells release pro-inflammatory cytokines that initiate and maintain a persistent inflammatory process[23]. However, to best authors' knowledge, the expression of P2X7 from the DH perspective remains unexplored.

Owing to the putative expression of neuron and odontoblast receptors and the still debate around the release of neuropeptides in the context of DH and ultimately the importance of deepening the understanding on its physiopathology under a validated animal model of DH, this study assessed the activity of mechano-, thermo- and/or chemoreceptors as well as of mechanoreceptive ion channel in neurons and odontoblasts. The null hypothesis tested was that DH would not be associated with activity of receptors in neurons and odontoblasts and neuropeptide release.

Materials & Methods

Ethical aspects, experimental design and sample size

All procedures performed in this study were in accordance with the ethical standards in compliance with the guidelines of the National Council for Animal Experimentation Control (CONCEA) and Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines for the care and welfare of animals. This study was reviewed and approved by the local Ethics Committee on Animal Experimentation (protocol #2017/029).

Twenty male Wistar rats (ANILAB) were housed in groups of two, in polypropylene cages containing pine shavings in an open system, ventilated shelves with temperature ranging between 22 and 24 °C in the animal facility, in an environment with luminosity control (12h light/dark cycles). The animals were randomly assigned to two groups (test and control), as follows: test group: ten rats received a sports drink (Gatorade®, pH 3.08) *ad libitum*, for 45 days, to induce HD as described elsewhere[24,25]; control group: ten rats received filtered water (pH 6.30) *ad libitum*, for 45 days. Both groups received the same feed (Presence Ratos e Camundongos, Presence Nutrição Animal) *ad libitum*. All animals were weighed before the commencement and at the end of the experiment, for control and certification of welfare.

Sample size was calculated (G*Power 3.1.9.4) from the data collected in pilot study and determined that 6 rats per group would provide 90% power to detect an effect size of 2.317 with a 2-sided α of 0.05. Four animals were added in order to provide material for scanning electron microscopy (SEM) analysis and to compensate for dropouts/losses.

Sample collection and processing

At the end of the 45-day period of ingestion of the beverages, the rats were euthanized by decapitation without previous anesthesia to avoid anesthetic-induced increase in plasma corticosterone concentrations[26]. Blood aliquot was collected in heparin-coated tubes and centrifuged (10 min/1000 ×g). Plasma was used to determine corticosterone level by enzyme-linked immunosorbent assay (ELISA).

Subsequently, the teeth (lower molars) were carefully extracted to prevent dental pulp exposure. Teeth were gently cleaned with sterile gauze and physiological solution, aiming to remove any retained periodontal tissue. They were then stored at -80°C until further analysis. Each sample was composed of a pool of all lower molars (6 teeth). Dental pulp was extracted by triturating the samples in a mortar and pestle. During the extraction process, samples were kept in nitrogen solution avoiding protein denaturation. Samples were then resuspended in RIPA Lysis Buffer (Santa Cruz Biotechnology) containing a protease inhibitor cocktail (Sigma, USA). The homogenized samples were centrifuged (10,000 rpm/10 min/4°C), and the supernatant was collected. Homogenized samples were stored at -20°C.

Western Blot

Total extracted proteins were evaluated using colorimetric BCA protein dosing kit (Thermo Scientific). Protein samples (80 µg) from lower molars were separated by electrophoresis in polyacrylamide gel SDS-PAGE 10% and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4 °C with a blocking buffer [PBS 5% (p/v) of skim milk and 0,1% Tween 20 and rinsed three times with PBS 0,1% Tween 20. Afterwards, they were incubated in a PBS solution containing 5% skim milk and 0,1% Tween 20 containing primary antibody for anti-rat TRPV1 (Alomone Labs, CAT# ACC029, LOT# ACC030AN2302, 1:1000 overnight), anti-TRPM8 (Alomone Labs, CAT# ACC-049, LOT# ACC049AN1402, 1:500 overnight), and anti-P2X7 (Alomone Labs, CAT# APR-004, LOT# APR004AN2302, 1:500 overnight). After washing, the membranes were incubated with specific HRP conjugated secondary antibodies and rinsed again. Membranes were then developed with a chemiluminescent kit as described in the instruction manual. Image J software was used to measure the bands by optical density to quantify the protein level of the TRPV1, TRPM8 and P2X7 receptors in dental pulp. To compensate for any differences in the amount of loaded protein, the intensity of the test band was divided by the intensity of GAPDH (Cell Signaling Technology®, Denver, MA, USA) band for each sample.

Enzyme-linked immunosorbent assay (ELISA)

Protein levels of substance P (SP) in dental pulp (Phoenix Pharmaceuticals®, Inc., Burlingame, California, EUA – range 0.07 - 2.24 ng/ml) and corticosterone dosage in plasma (Cayman Chemical® – Ann Arbor, MI, USA – range 8.2 – 5,000 pg/ml) were evaluated by ELISA according to manufacturer's specifications of the kits used.

SEM

The hemimandibles of the remaining four animals of each group were extracted and stored in formaldehyde. Subsequently, the soft tissue was removed and the hemimandibles underwent dehydration with ethyl alcohol, in increasing concentrations: 25% (20 minutes), 50% (20 minutes), 75% (20 minutes), 95% (30 minutes) and absolute alcohol (100%) for 1 hour. Then, hexamethyldisilane (HMDS) was used for 10 minutes to dry the samples and a layer of gold (200 Aº) was deposited on them (Sputter Coater EMITECH, Model: K450. Kent, United Kingdom) to be analyzed in a scanning electron

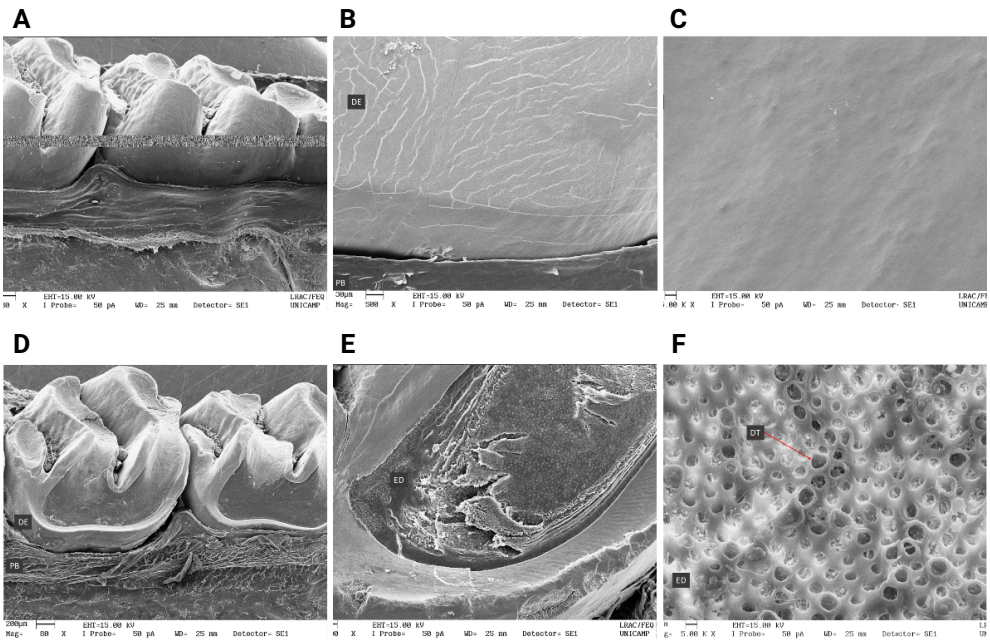
microscope (Leo 440i, LEO Electron Microscopy/Oxford. Cambridge, England). Photomicrographs were obtained at 80x, 500x and 5,000x magnification.

Statistical analysis

After ascertaining data compliance to normal distribution and homoscedasticity, the groups were compared using Student's t tests (GraphPad Prism, GraphPad software Inc, California, USA), with the significance level set at 0.05.

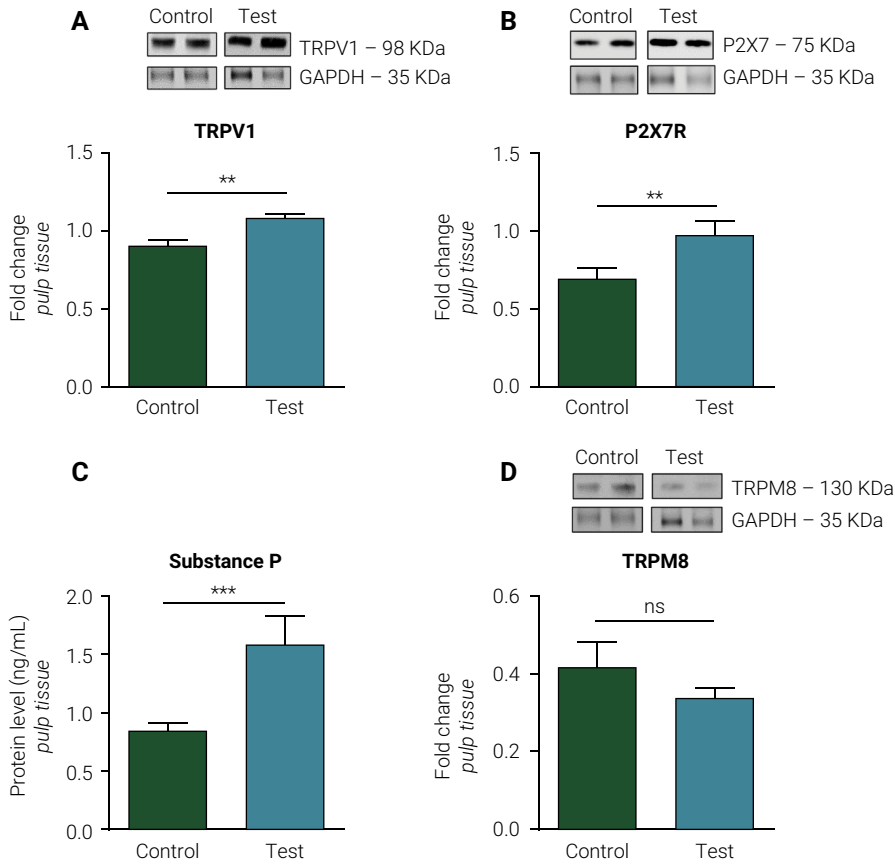
Results

SEM photomicrographs substantiated the exposure and patency of dentinal tubules in test animals, i.e., that received the sports drink (Figures 1a-f). The top row are images from the control group, and the bottom row images are from the test group. The first column (A and D) are zoomed-out images (50x magnification) that show the buccal and occlusal surfaces of the molars, it is possible to notice the amount of enamel loss and dentine exposure on SEM image D, while the dentine in the control group is not exposed. The second column (B and E) are a 500x magnification of the cervical area, again the control group has enamel, while the test group image shows the amount of enamel loss, dentinal exposure, and dentinal tubules are visible. The last column (C&F) are 5,000X magnification show enamel in the control group and exposed and open dentinal tubules in the test group.



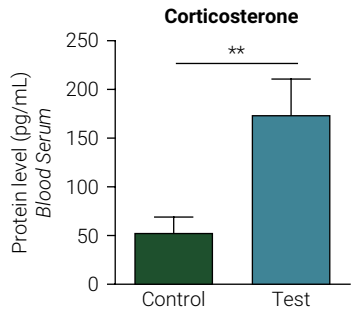
Legend: Top row images a, b and c from the control group; Bottom row images d, e and f from the test group; DE- Dental enamel, PB- Periodontal bone, ED- Exposed dentine in areas of enamel loss, DT- Dentinal tubule.
Figure 1. Scanning electron microscopy (SEM) photomicrographs of rat teeth from control and test groups.

The test group demonstrated significantly higher levels of TRPV1 (p = 0.002; Figure 2A) and P2X7 receptors (p = 0.007; Figure 2B) and substance P (p < 0.001; Figure 2C). However, there was no difference in the level of TRPM8 between groups (p = 0.079; Figure 2D).



Legend: The symbol (***) indicates p < 0.001 and (**) indicates p < 0.01.
Figure 2. Column diagram of the mean expression of TRPV1 (A), P2X7 (B), Substance P (C), and TRPM8 (D) in dental pulp of the rats of control and test groups.

Corticosterone level was significantly higher in the test group (p = 0.050; Figure 3), while there was no difference in the body weight of the rats in the two groups (p = 0.756; Table 1).



Legend: The symbol (**) indicates p = 0.05, Student's t test.
Figure 3. Column diagram of the mean plasma corticosterone level of the control and test groups.

Table 1. Average and standard deviation of the initial and final weight (g) of the control and test animals.

Time	Control	Test
Initial	332.8 (12.8)	331.6 (10.4)
Final	399.3 (21.6)a	402.6 (13.2)a

Legend: Final weight followed by the same lowercases did not differ from each other.

Discussion

The rat DH model used in this study has proved to cause substantial surface loss of dental hard tissues, exposure and patency of dentinal tubules (Figure 1), in alignment with papers that validated this method of inducing hypersensitive dentine[24,25]. With respect to this model, it is worth noting that in a previous paper by our group[27], despite the severity of the erosion lesions, no histological changes such as inflammation and reparative dentin formation have been noticed in the underlying pulp tissue.

In addition to structural and morphological changes on the dental hard tissues, it is worth noting that our results showed a more than threefold increase in plasma corticosterone levels in the group with DH. This allows inferring that there was pain associated with the wear lesions, a finding consistent with the fact that most of the animals subjected to the same DH model presented high scores of pain in previous investigations[24,25]. Although one can argue that corticosterone levels was increased as a consequence of handling, all animals was habituated to daily manipulation and fast handled for similar time prior to euthanasia, which was performed by decapitation without anesthesia for data quality. Despite the severity of surface loss, dentine exposure and pain in the test group, which could imply limitations in feeding, animals of both groups had no weight differences.

Based on the findings of this paper, the null hypothesis that HD would not be associated with activity of receptors in neurons and odontoblasts and neuropeptide release was rejected as DH significantly increased expression of TRPV1, P2X7 and substance P under the DH animal model adopted herein.

The higher TRPV1 expression in the group having hypersensitive dentine gives evidence to previous conjectures that the experience of pain via exposed dentine may involve the activation of TRPs[17]. According to the quoted paper, such expression relies on receptors present in both nerve fibers and odontoblasts. As in the current study dental pulp was extracted from triturated lower molars, the origin of TRPV1 (if from nerve fibers or odontoblasts) cannot be differentiated. However, in the clinical setting probably the most important expression of TRPV1 would originate from odontoblasts, which extents up to inner dentine from pulp[28], and thereby represents the outermost cellular component that external stimuli would encounter in tooth structure. On the other hand, nerve fibers possibly would have a secondary role, as they are present in dentinal tubules only approximately 100 µm from the pulp[29]. This discussion is in line with a previous paper in which the authors mentioned that TRPV1 is detected mainly in odontoblasts[16].

Activation of TRPV1 has been shown to promote release of substance P[10], causing neurogenic inflammation through vasodilation, vascular permeabilization and edema formation[10]. It seems therefore reasonable to consider that the increased expression of TRPV1 and substance P release in DH context, as it occurs in pulp from carious teeth[30] and teeth diagnosed with irreversible pulpitis[31], has the potential to cause neurogenic inflammation and evoke pain. Worth mentioning is that once substance P is elevated, it is further increased during the inflammatory process, thereby sustaining, and exacerbating the neurogenic inflammation[14], which may explain DH as a lingering pain in some patients and even help to explain the eventual need of endodontic treatment for irreversible pulpitis of teeth having non-carious cervical lesions.

Under the current model, expression of the P2X7 receptor in the pulp was also higher in the test group, providing evidence on the sensory role of odontoblasts under a DH scenario. This role can be ascribed to the increased TRPV1 expression that induces ATP release to the extracellular space by stimulated odontoblasts[32]. In fact, membrane deformation caused by dentinal fluid movement activates mechanosensitive channels-TRP[33]. ATP is then released into the extracellular space, acting as a neurotransmitter, and activates P2X7 receptors, which trigger intracellular signaling pathways thereby activating the neuron to establish neurotransmission with neurons[33].

Despite the borderline p value suggests a trend toward pulp samples of rats having DH present increased TRMP8 activity, no statistically significant difference was detected among the test and control groups. One possible reason for this finding may be the fact that TRPM8 starts to be sensitive to cooling approximately below 25 °C and 22 °C in dental pulp nerves[34] and odontoblasts[35], respectively, and the sports drink was available and consumed at temperature close to this threshold (22-24 °C). One can speculate that lower temperatures, similar to the temperature that the drink is consumed in real life situations, neurons and odontoblasts would have increased response, thereby elevating calcium ions and TRPM8 activity[17].

Although there exist other neurogenic peptides and numerous classes of mechano- and thermosensitive channels as well as ligand- and voltage-gated ion channels, their roles when dentine is exposed remain to be unraveled. Yet, by assessing some

of the main neuron and odontoblast receptors (TRPV1, TRPM8 and P2X7) and neurogenic peptide (substance P) under an animal model of DH, this paper contributes to the comprehension on the physiopathology and advancing in strategies to prevent and treat DH. Despite further studies are warranted to perform immunohistochemical to deepen the comprehension of the cellular expression of the receptors, the present investigation opens perspectives to advance strategies to prevent and treat DH. This may include the incorporation of pharmacological antagonists of neuron and odontoblast receptors to dentine desensitizing formulations.

In conclusion, under DH condition, neurons and odontoblasts expressed TRPV1 and P2X7 receptors as well as increased substance P release, demonstrating cellular and molecular mechanisms underlying DH.

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Data Availability

Datasets related to this article will be available upon request to the corresponding author.

Author Contributions

Giovanna Correa Denucci: conceived the idea, designed the experiment, collected data, analyzed data, wrote and revised the manuscript. **Henrique Ballassini Abdalla**: collected data, analyzed data, revised the manuscript. **Juliana Trindade Clemente-Napimoga**: conceived the idea, designed the experiment, analyzed data, revised the manuscript. **Cecilia Pedroso Turssi**: conceived the idea, designed the experiment, analyzed data, revised the manuscript.

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