

# Expression of Substance P, Calcitonin Gene-Related Peptide and Vascular Endothelial Growth Factor in human dental pulp under different clinical stimuli

Javier Caviedes-Bucheli (✉ [javiercaviedes@gmail.com](mailto:javiercaviedes@gmail.com))

Pontificia Universidad Javeriana <https://orcid.org/0000-0003-0407-9847>

**Luis Fernando Lopez-Moncayo**

Universidad cooperativa de Colombia

**Hernan Dario Munoz-Alvear**

Universidad Cooperativa de Colombia

**Jose Francisco Gomez-Sosa**

Postgraduate Endodontic Department, Universidad Central de Venezuela

**Luis Eduardo Diaz-Barrera**

Universidad de la Sabana

**Hernando Curtidor**

Universidad Del Rosario

**Hugo Roberto Munoz**

Universidad de San Carlos de Guatemala

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## Research article

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# Abstract

*Background* To quantify the expression of SP, CGRP, and VEGF in human dental pulps as a response to occlusal trauma, moderate orthodontic forces, and occlusal trauma plus moderate orthodontic forces simultaneously.

*Methods* Forty human dental pulp samples were collected from healthy premolars indicated for extraction for orthodontic reasons in 20 patients. Patients were divided into four groups with 10 samples each (5 lower and 5 upper premolars from each patient): Healthy pulp control group, occlusal trauma group, moderate orthodontic forces group; and occlusal trauma plus moderate orthodontic forces group. Stimuli were applied for 24 h before tooth extraction in all experimental groups. All samples were processed, and SP, CGRP, and VEGF were measured by radioimmunoassay. The ANOVA test was performed to establish significant differences between groups and Tukey's HSD post hoc comparisons were also performed.

*Results* The highest increase in SP, CGRP, and VEGF expressions was found in the occlusal trauma plus orthodontic forces group, followed by the moderate orthodontic forces, the occlusal trauma and the control groups, with statistically significant differences between all groups (ANOVA  $p < 0.001$ ). All pairwise post-hoc comparisons were also significant (Tukey HSD  $p < 0.001$ ).

*Conclusion* SP, CGRP, and VEGF expressions significantly increase in human dental pulps when stimulated by occlusal trauma combined with moderate orthodontic forces, as compared with these two stimuli applied independently.

*Name of the registry:* Importance of Neurogenic Inflammation in the Angiogenic Response of the Dental Pulp as a Defensive Response

*Trial registration number:* NCT03804034

*Date of registration:* 01/15/2019 Retrospectively registered

*URL of trial registry record:* <https://clinicaltrials.gov/ct2/show/NCT03804034?term=NCT03804034&draw=2&rank=1>

## Background

When stimulated, the dental pulp can physiologically change its vascular, neural, and cellular components in order to adapt to masticatory function or events such as occlusal trauma and orthodontic forces. These stimuli may trigger a neurogenic inflammation through neuropeptides, such as Substance P (SP) and Calcitonin gene-related peptide (CGRP) [1], which may initiate a defense response and angiogenesis to prevent functional changes that could lead to necrosis [2-4].

Each neuropeptide must bind to a specific receptor in order to exert a biological function: for SP, the NK1 receptor is found in mast cells, macrophages, mesenchymal cells, fibroblasts, endothelial cells, and odontoblast-like cells [1]. This SP/NK1 complex had vascular effects, such as blood flow regulation and vasodilation, as well as inflammatory effects, promoting inflammatory cells chemotaxis and collagen formation as a defense mechanism [5].

SP/NK1 complex stimulates angiogenesis using direct mechanisms that modulate the endothelial cells and fibroblasts action by activating growth factors such as vascular endothelial growth factor (VEGF) and stimulating cell migration and proliferation to form mineralized tissues as a defense mechanism [2, 5, 6]. This complex also uses indirect mechanisms such as binding to granulocytes or macrophages, attracting cells with angiogenic potential [4, 7].

The CGRP exerts its biological activity by binding to CGRP receptors 1 or 2 (CGRPR1-2), with the former being the most sensitive protein to CGRP. CGRPR1 is a potent vasodilator that increases intrapulpal blood flow [8]; it is found on endothelial cells, mast cells, macrophages, lymphocytes, undifferentiated mesenchymal cells, fibroblasts, and odontoblast-like cells; and controls neurogenic inflammation and reparation processes combined with SP due to its angiogenic potential [1, 9].

Angiogenesis is mediated by several growth factors, including VEGF [10, 11] that is released in the pulp tissue as a response to a harmful stimulus to counteract emerging hypoxic areas by regulating oxygen and nutrient supply to cell populations that form new blood vessels [12, 13]. VEGF is also found in endothelial cells, mast cells, macrophages, lymphocytes, undifferentiated mesenchymal cells, fibroblasts, and odontoblast-like cells, controlling neurogenic inflammation and reparation processes combined with SP and CGRP due to its angiogenic potential [11, 14].

Occlusal trauma and orthodontic forces are clinical situations observed in patients undergoing orthodontic treatment that affect the human dental pulp, modifying its metabolism and causing vascular changes leading to hypoxia [4, 15]. The dental pulp response depends on the intensity, magnitude and duration of the stimulus. If the stimulus become constant, the resulting hypoxia will inevitably induce a defense response from the dental pulp via neurogenic inflammation and angiogenic mechanisms that could induce the formation of mineralized tissues [11, 13, 15-17].

Thus, the purpose of this study is to measure SP, CGRP, and VEGF expression in human dental pulp under occlusal trauma or moderate orthodontic forces, as well as both stimuli combined.

## Methods

This experimental study was conducted following the recommendations of the Colombian Ministry of Health regarding ethical issues of scientific research involving human tissues. It was approved by the Bioethics Committee of the Faculty of Dentistry at the Universidad Cooperativa de Colombia in Pasto (CBE07). Written informed consent was obtained from all patients participating in this study. This study was registered in clinicaltrials.gov under the title: "Importance of Neurogenic Inflammation in the

Angiogenic Response of the Dental Pulp as a Defensive Response”, trial registration number NCT03804034, dated 01/15/2019, URL: <https://clinicaltrials.gov/ct2/show/NCT03804034?term=NCT03804034&draw=2&rank=1>

Human dental pulp was obtained from 20 freshly extracted maxillary and 20 mandibular premolars with orthodontic indications from 20 healthy patients, 8 men and 12 women, aged between 18 and 30 years. Patients taking medications for pain, smokers, and pregnant women were excluded. All the teeth included in this study had a normal masticatory function with a clinically and radiographically complete root development, without caries, restorations, orthodontic forces, periodontal disorders, or parafunctional habits.

The sample size was calculated with the TAMAMU 1.1® program (Tokio, Japan) based on the mean and standard deviation of the variables measured in previous studies [18, 19], using a 95% confidence interval and a sample power of 80%, to obtain a total sample size of 20 patients (5 per group). Patients were randomly assigned (Random.org App, Randomness and Integrity Services Ltd, Dublin, Ireland) to each group for a total of 10 premolars (5 upper and 5 lower from the same side of each patient) per group: a) Control group (5 upper and 5 lower healthy premolars from the same side); b) Occlusal trauma (5 upper and the 5 lower premolars that were in contact under occlusal trauma); c) Orthodontic force (5 upper and 5 lower premolars); d) Occlusal trauma and orthodontic force (5 upper and 5 lower premolars that were in contact under occlusal trauma and orthodontic force). Contralateral teeth were also extracted for orthodontic reasons but were not used in the study. (Fig. 1).

In the occlusal trauma group (Fig. 1b), an occlusal interference was placed on the mandibular premolars. An articulating paper was used to mark the contact area between the upper and mandibular premolars indicated for extraction, following the study model reported by Caviedes Bucheli et al. [18]. Lower premolar was acid-etched with 37% phosphoric acid (SuperEtch; SDI Ltd, Bayswater, Victoria, Australia) for 15 seconds. After washing and drying, a bonding agent (STAE; SDI Ltd) was applied and light-cured for 15 seconds, and finally a 2mm resin block (Filtek Z350; 3M Espe, Seefeld, Germany) was placed and light-cured (40 sec) over the contact area of the upper premolar. An articulating paper was used to verify that only the premolars indicated for extraction had contact during normal occlusion as well as in lateral movements. Patients were given chewing gums and instructed to repeat 20 masticatory cycles for 30 sec, followed by a 30 sec rest interval, and repeat the sequence for 30 min. This chewing cycle was repeated three times every 8 h for the first 24 h and it was confirmed by each patient in a format with the established schedules.

For the moderate orthodontic force group (Fig. 1c), the occlusal surface of the first mandibular molar was raised using a block of resin (Filtek Z350; 3M Espe, Seefeld, Germany) until the premolars were out of occlusion before applying an orthodontic force, following the study model reported by Caviedes-Bucheli et al. [19]. Convertible standard buccal tubes (Orthorganizer, Carlsbad, CA) were bonded on the buccal face of the first upper and lower molar with resin (Light Bond; Reliance Orthodontic Products Inc, Itasca, IL). A McLaughlin, Bennett, and Trevisi (MBT) slot size 0.022 bracket (Ref. 702-393 MC, Orthoorganizer)

was bonded over the buccal face of the upper and lower first premolars. A 0.0017/0.025 titanium molybdenum alloy (TMA) wire cantilever was inserted into each first molar tube and the wire was bent buccally to form a helix with an activation angle of 45°. The cantilever was clinched to the distal end of the tube, and the orthodontic force was measured with an orthodontic dynamometer to 56g exerting a tipping and extrusive force on the premolar for 24 h before being extracted.

In the occlusal trauma and moderate orthodontic force group (Fig. 1d), occlusal interferences combined with orthodontic force were performed as previously described; this force was applied to the teeth for 24 h before being extracted, without placing the resin bumpers in the posterior molars.

## Sample Collection

All the teeth included in this study were anesthetized with 1.8 ml of 4% prilocaine without a vasoconstrictor (Pricanest, Ropsohn Therapeutics Ltd.) by infiltrative injection for maxillary premolars and inferior alveolar nerve block injection for mandibular premolars. The teeth were extracted 5 min after administering the anesthetics with conventional methods. Immediately after the extraction, the teeth were sectioned using a Zekrya bur (Dentsply, Tulsa, OK) in a high-speed handpiece irrigated with saline solution. The pulp tissue was obtained using a sterile endodontic excavator, placed on an Eppendorf tube, snap frozen in liquid nitrogen, and kept at -70 °C until the radioimmunoassay test was performed.

## Radioimmunoassay (RIA)

Tissue samples were defrosted without thermal shock, dried on a filter, and weighed on an analytic balance. Peptides were extracted by adding 150 µL of 0.5 M acetic acid and double boiled in a thermostat bath for 30 min.

SP expressions (Phoenix Peptide Pharmaceutical RK-061-05) and CGRP (Phoenix Peptide Pharmaceutical RK-015-02) were determined by competitive binding assays using the corresponding kits for each substance following previously published protocols [18-20]. The VEGF expression was equally determined by competition binding assays using a human, recombinant VEGF165 (G143AB; Genentech, Inc., Los Angeles, CA, USA), primary antibody, polyclonal rabbit antiserum to VEGF165 (27906-17, Genentech, Inc., Los Angeles, CA, USA), and human recombinant <sup>125</sup>I-VEGF tracer (NEX328, NEN Life Science Products, Inc. Boston, MA, USA). Each sample solution (50 µL) was incubated in tubes at 18 °C for 20 h with 100 µL of primary antibody, and 100 µL of different peptide concentrations (SP:  $7.42 \times 10^{-3}$ -0.89 pmol/mL; CGRP:  $2.64 \times 10^{-3}$ -0.32 pmol/mL; VEGF:  $2.5 \times 10^{-4}$ - $3.2 \times 10^{-2}$  pmol/mL) was added with 50 µL of radiolabeled factors and incubated for 24 h. Bound fractions were precipitated with secondary antibody (100 µL), 100 µL of fetal bovine serum, and 500 µL of RIA buffer containing 1% polyethylene glycol 8000. After 2-h incubation at 20 °C, the tubes were centrifuged at 3,000 rpm for 45 min at 4 °C. The supernatants were decanted, and the pellet radioactivity was read in a gamma counter. The peptide concentration was determined according to calibration curves of the radioactivity in each mixture. A blinded operator analyzed the treatment effects applied to the teeth in the pulp tissue samples. The

samples were coded so blinded operator could not know what stimuli was applied on the tissue being analyzed.

## Statistical Analysis

Results are presented as SP, CGRP, and VEGF expressions in pmol/mg of pulp tissue. Means and standard deviations, as well as minimum and maximum values for each neuropeptide and VEGF, were calculated. A Kolmogorov-Smirnov (KS) test was used to determine if the results obtained for each peptide adjust to normality, in order to perform an analysis of variance (ANOVA) test to determine statistically significant differences, followed by Tukey's HSD post-hoc and Bonferroni correction tests at a significance level of  $p < 0.05$ .

## Results

SP, CGRP, and VEGF expressions were found in all 40 analyzed pulp tissue samples. KS test showed a normal distribution for the 3 peptides measured. The mean SP expression in the control group was  $0.3665 \pm 0.0198$  pmol/mg of pulp tissue. For the occlusal trauma group, the mean SP expression increased to  $0.7432 \pm 0.028$  pmol/mg; in the orthodontic force group, the mean expression was  $1.0163 \pm 0.0025$  pmol/mg. The highest SP expression was found in the occlusal trauma plus orthodontic force group, with a mean expression of  $1.5021 \pm 0.0104$  pmol/mg (Fig 2). ANOVA ( $p < 0.001$ ) test found statistically significant differences between groups and Tukey's HSD and Bonferroni tests showed statistically significant differences in all possible pairwise comparisons ( $p < 0.001$ ).

The mean CGRP expression in the control group was  $0.0367 \pm 0.0019$  pmol/mg of pulp tissue. In the occlusal trauma group, the mean CGRP expression increased to  $0.0555 \pm 0.0020$  pmol/mg; in the orthodontic force group, the mean expression was  $0.0749 \pm 0.0032$  pmol/mg. The highest CGRP expression was found in the occlusal trauma plus orthodontic force group, with a mean expression of  $0.0958 \pm 0.0028$  pmol/mg (Fig. 3). ANOVA ( $p < 0.001$ ) test found statistically significant differences between groups and Tukey's HSD and Bonferroni tests also showed statistically significant differences in all possible pairwise comparisons ( $p < 0.001$ ).

Mean VEGF expression in the control group was  $0.0004 \pm 0.0001$  pmol/mg of pulp tissue. For the occlusal trauma group, the mean VEGF expression increased to  $0.0010 \pm 0.0001$  pmol/mg, followed by the moderate orthodontic force group with a mean expression of  $0.0014 \pm 0.0001$  pmol/mg. The highest VEGF expression was found in the combined occlusal trauma and moderate orthodontic force group with a mean expression of  $0.0025 \pm 0.0003$  pmol/mg (Fig. 4). ANOVA ( $p < 0.001$ ) test found statistically significant differences between groups and Tukey's HSD and Bonferroni test also showed statistically significant differences in all possible pairwise comparisons ( $p < 0.001$ ).

## Discussion

SP, CGRP, and VEGF play a crucial role in the defense mechanisms of pulp tissue against any harmful stimuli by activating angiogenesis and neurogenic inflammation that induce reparation through mineralized tissue formation [11, 16, 21].

Control group healthy premolars, that were not subjected to any stimulus, showed SP and CGRP basal levels that allow pulp tissue homeostasis with appropriate oxygen and nutrient supply, which contributes to the proper functioning and maintenance of a healthy pulp. These findings were consistent with the baseline levels in healthy human dental pulps reported in previous studies [18, 19].

VEGF expression in the control group shows the physiological levels in the human dental pulp that are required for tissue response to masticatory function in order to provide oxygen supply for different cell populations based on angiogenic mechanisms [22].

The statistically significant increase in SP, CGRP, and VEGF expressions between the control and experimental groups can be explained similarly, taking into consideration that increased expression of these proteins generates biological defense events that are proportional to the type, nature, and magnitude of the stimulus.

Dental pulp cellular activity increases when subjected to harmful stimuli, thereby producing SP and CGRP [21]. SP binds to the NK1 receptor of endothelial cells, inducing mitotic activity to form new blood vessels. Moreover, SP induces VEGF release, modulating the action of endothelial cells and fibroblasts, allowing cell migration and proliferation, either directly or indirectly, and attracting cells with angiogenic potential [10, 22].

Furthermore, CGRP acts synergistically with SP by increasing inflammatory mediators, compromising the pulp blood flow and collateral circulation due to hypoxia caused by occlusal trauma, orthodontic forces, or a combination of both stimuli, initiating a compensating mechanism to correct the resulting damage, avoiding the vascular collapse that will be higher according to the intensity of the stimulus [19, 23, 24]. CGRP intervenes in the formation of new vessels via the cAMP–PKA pathway activation, which is related to endothelial cell stimulation through its receptor CGRPR1. It also boosts the action of VEGF [25] and fibroblast and odontoblast stimulation to form a mineralized tissue as a defense mechanism [4, 26].

SP and CGRP induce VEGF expression to initiate angiogenesis as a defense mechanism of the human dental pulp [7, 9, 23]. This may support other studies' hypothesis that occlusal trauma and orthodontic forces cause hypoxic areas due to increased cellular activity intended for mineralized tissue formation as a defense mechanism [4].

Angiogenesis is initiated as the VEGF expression level increases, creating vascular destabilization through increased hypoxia-inducible factor 1 [13]. The binding of VEGF to VEGF-R2 is responsible for the differentiation, proliferation, migration, and tubulogenesis of endothelial cells. Subsequently, VEGF bound to VEGF-R1 acts on the stalk cells and is involved in the guidance and control of cell formation in the new vasculature until its stabilization in order to reach normoxia and reduce its expression [12, 14].

Finally, in the occlusal trauma plus the moderate orthodontic forces group, SP, CGRP, and VEGF expressions were statistically significantly higher than those in other groups. It could be inferred from these results that the synergy between the two stimuli affects the pulp microcirculation even more because it compromises blood flow and collateral circulation and creates hypoxia in the pulp tissue, caused by vascular congestion that if not controlled, could lead to necrosis [4, 13, 27, 28].

Therefore, this investigation is relevant because its scope is closer to the patients' clinical condition under orthodontic treatment, and because patients under this treatment suffer permanent occlusal interferences during teeth movement, this could lead to clinical situations such as altered response to sensitivity tests and reduced lumen and canal size due to dentin formation as a defense mechanism [29, 30].

Due to the in vivo nature of the samples, an immediate stimulus model was performed. This prevented the evaluation of SP, CGRP, and VEGF expressions over a more extended period, which is considered as a study limitation. However, neuropeptides and VEGF are released immediately after stimuli, and therefore their effects are also immediate and sustained as well [4]. Moreover, there is evidence on the literature that supports that a 24h period is enough to provide a stimuli/effect response [19]. Another limitation that could affect the results is the non-compliance of the patients to follow home-instructions, although clear and concise directions were given to every patient participating in the study and they signed a commitment form to faithfully follow the instructions given.

## Conclusions

Within the limitations of this study, it can be concluded that SP, CGRP, and VEGF expressions significantly increase in human dental pulps when stimulated by occlusal trauma combined with moderate orthodontic forces, as compared with these two stimuli applied independently.

## Abbreviations

**SP:** Substance P

**NK1:** Neurokinin 1

**CGRP:** Calcitonin Gene-Related Peptide

**CGRPR1:** Calcitonin Gene-Related Peptide Receptor 1

**CGRPR2:** Calcitonin Gene-Related Peptide Receptor 2

**VEGF:** Vascular Endothelial Growth Factor

**VEGF-R1:** Vascular Endothelial Growth Factor Receptor 1

**VEGF-R2:** Vascular Endothelial Growth Factor Receptor 2

# Declarations

**Ethics approval and consent to participate:** This study was approved by the ethics committee of the Faculty of Dentistry at the Universidad Cooperativa de Colombia in Pasto (CBE07)

**Consent for publication:** Not applicable

**Availability of data and materials:** The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests:** The authors declare that they have no competing interests

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## Authors' contributions:

All authors have read and approved the manuscript

**JCB.** Conceptualization, Methodology, Supervision, Project Administration, Writing Original Draft.

**LFLM.** Validation, Investigation, Resources.

**HDMA.** Validation, Investigation, Resources.

**JFGS.** Conceptualization, Data Curation, Visualization.

**LED.** Methodology, Validation.

Methodology, Validation

**HRM.** Formal Analysis, Writing Review & Editing.

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## Figures



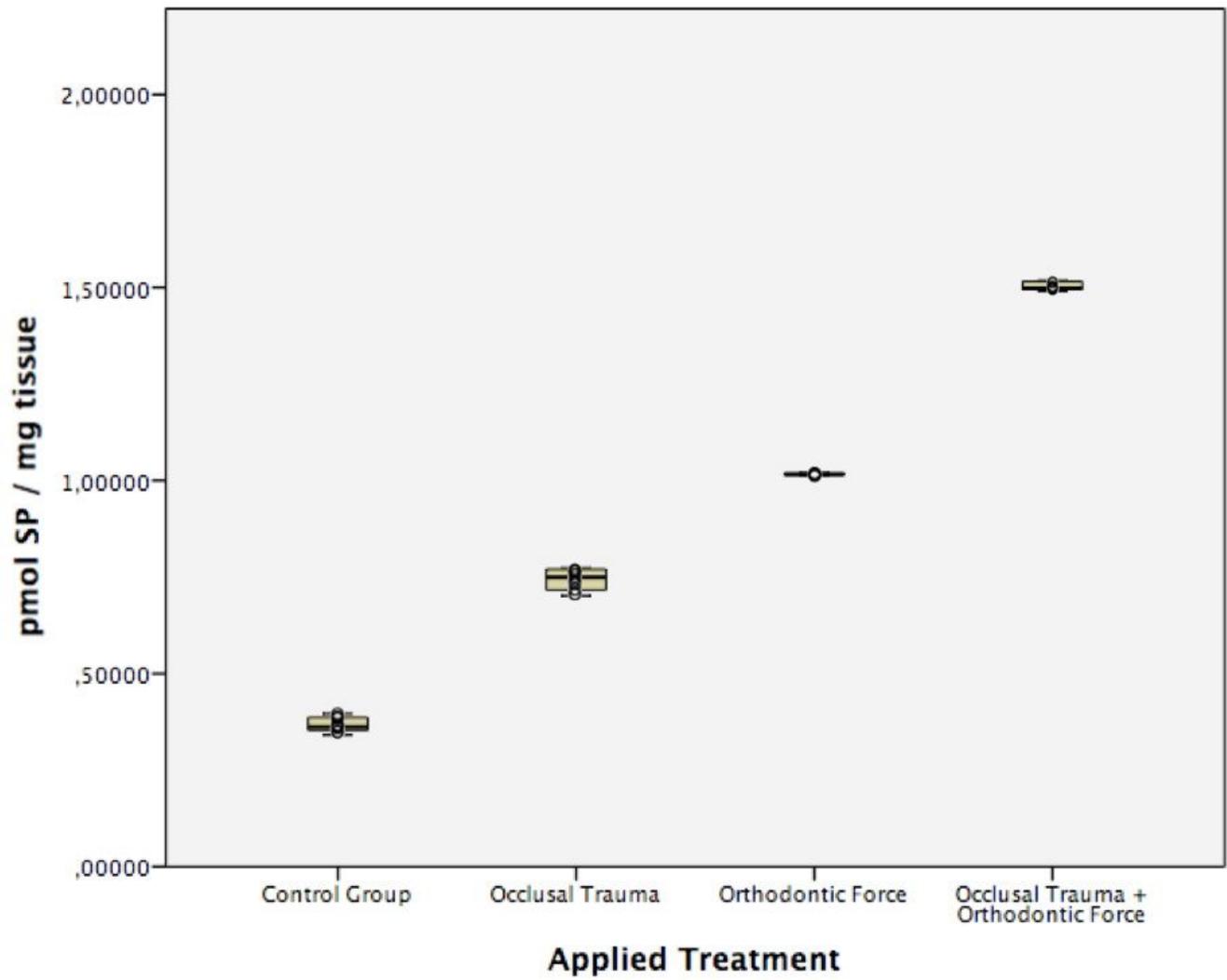
**Figure 1**

Clinical images of the different stimuli applied to the patients in each group. A) Control Group (without occlusal trauma nor orthodontic force); B) Occlusal trauma group; C) Moderate orthodontic force group; D) Occlusal trauma plus moderate orthodontic force group.



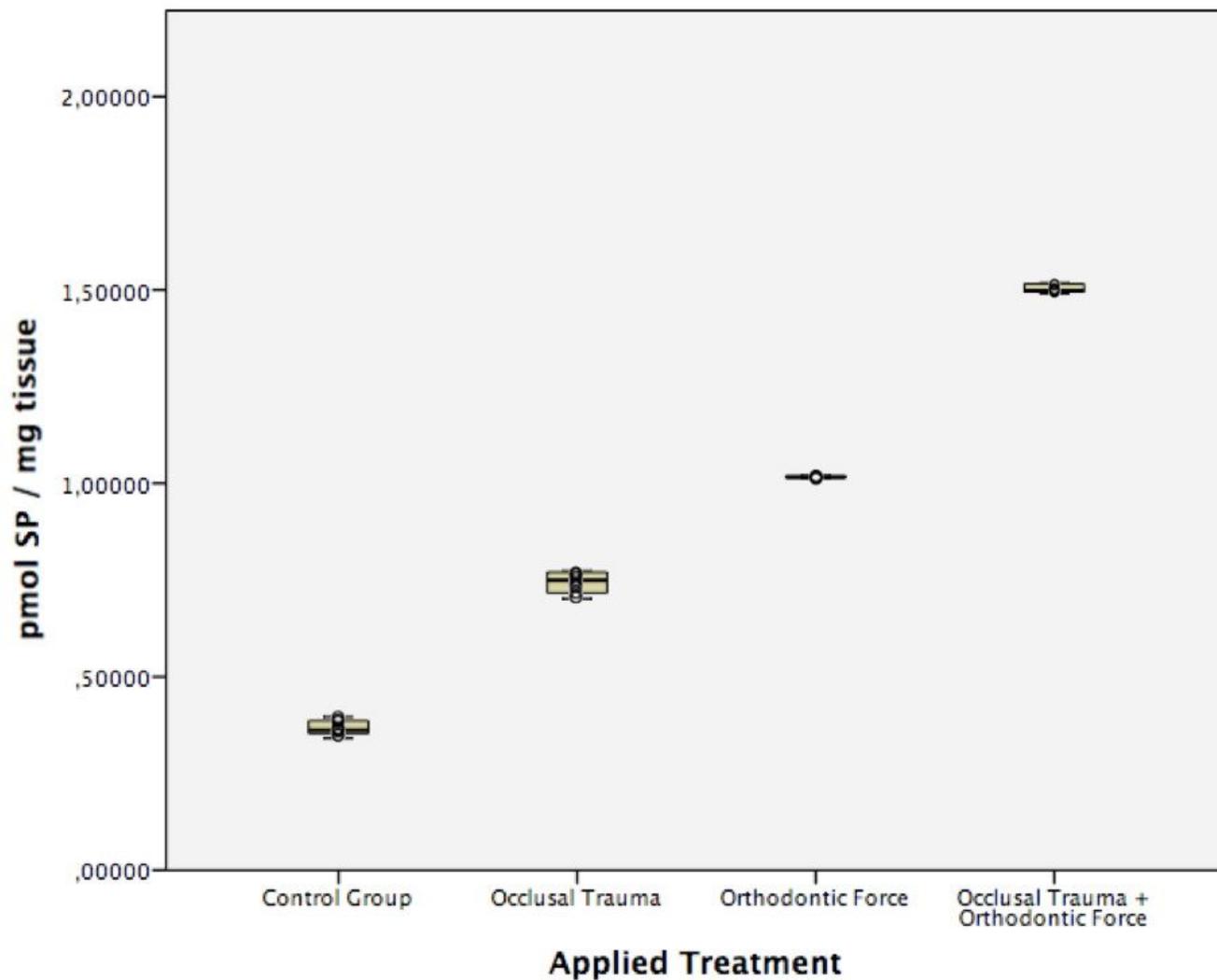
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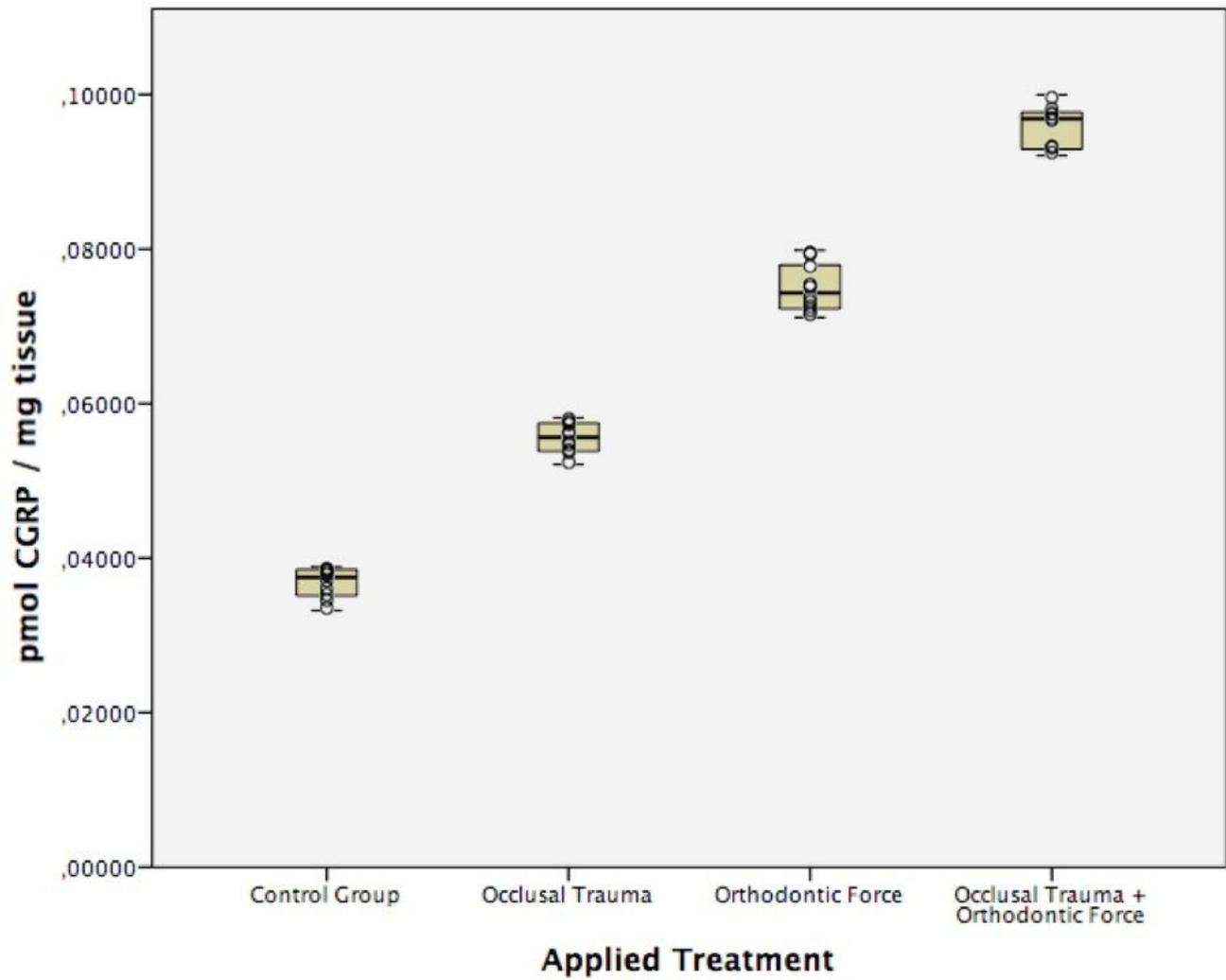
**Figure 2**

Substance P (SP) expression (pmol/mg) in human dental pulp from teeth under different clinical stimuli.



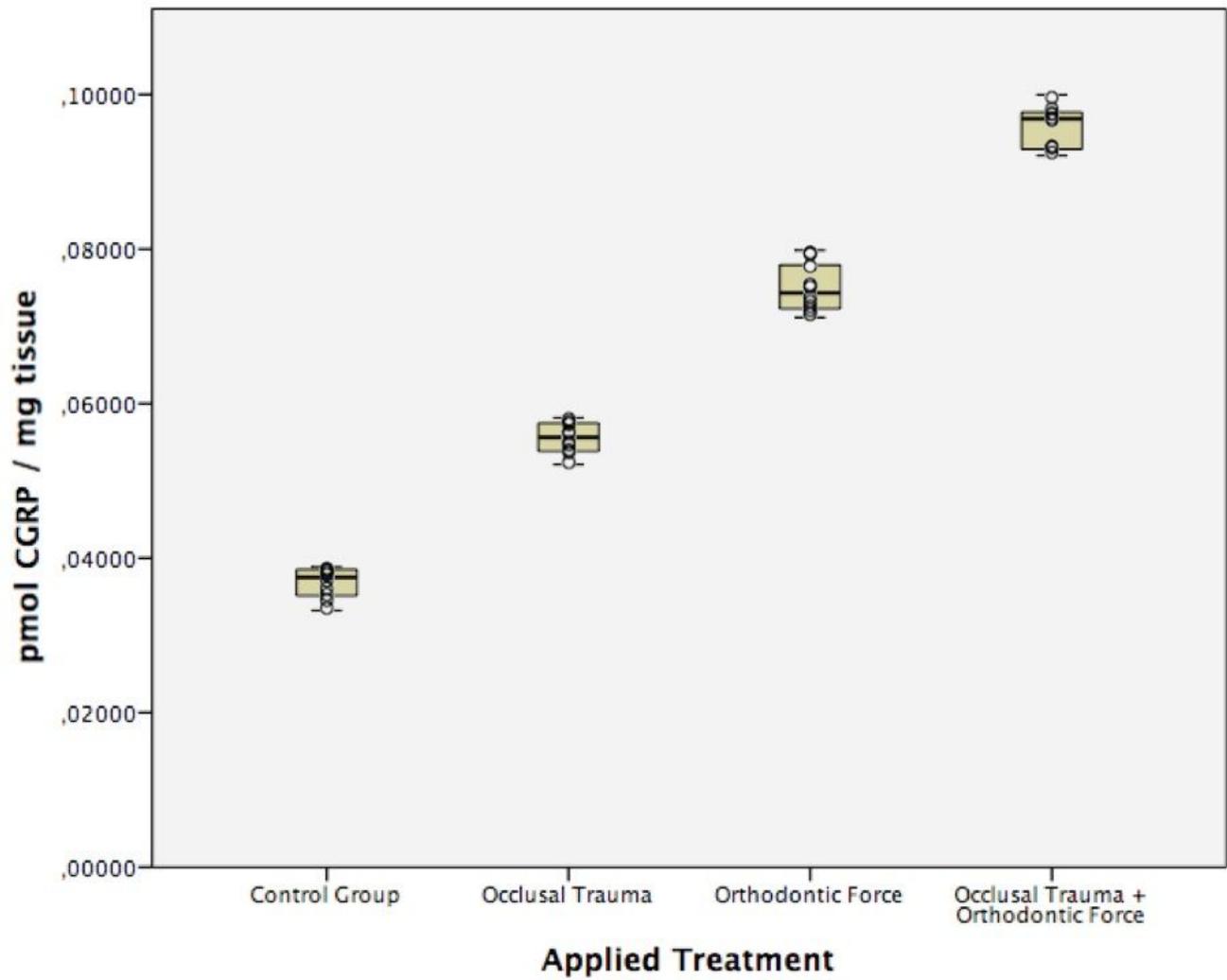
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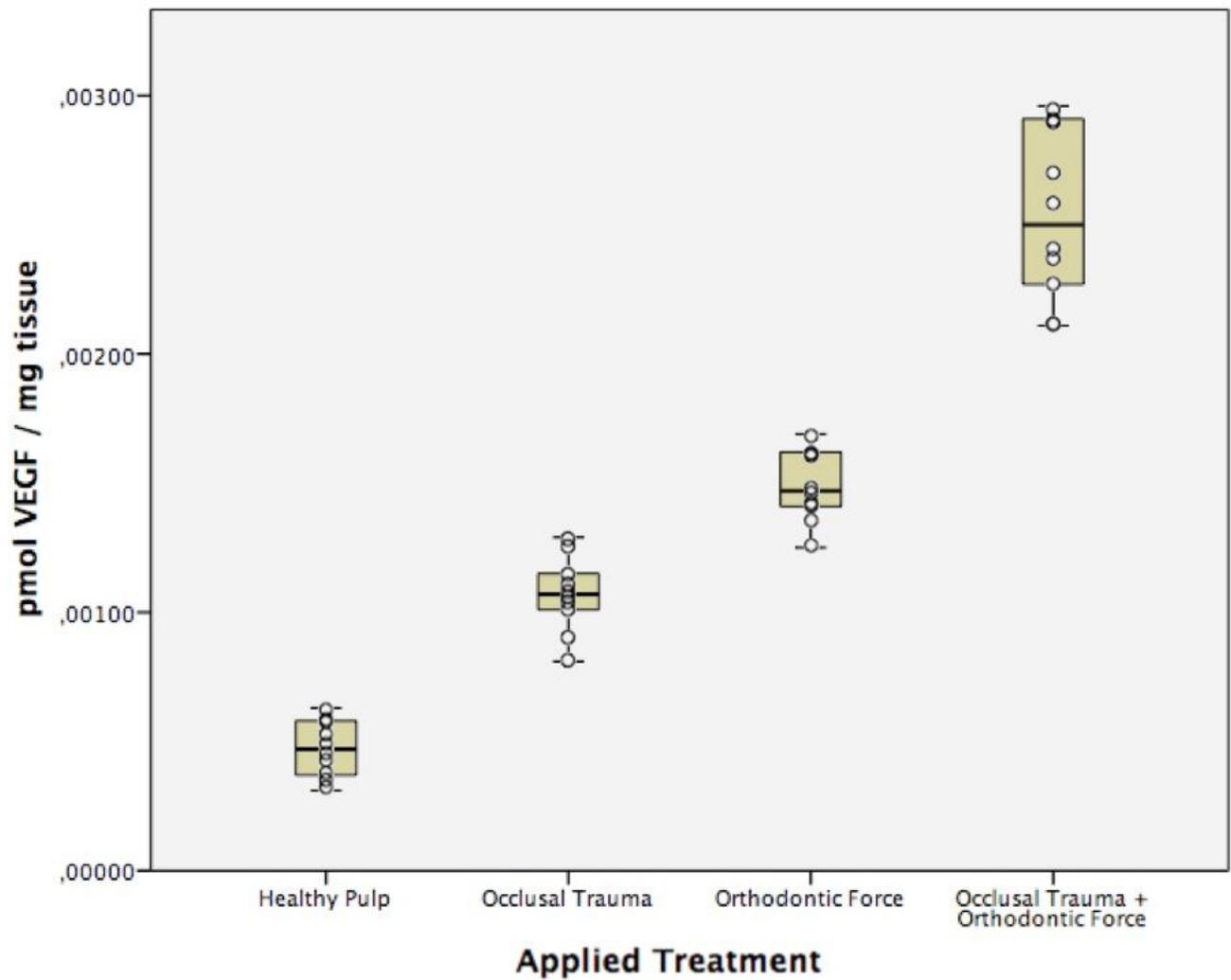
**Figure 3**

Calcitonin gene-related peptide (CGRP) expression (pmol/mg) in human dental pulp from teeth under different clinical stimuli.



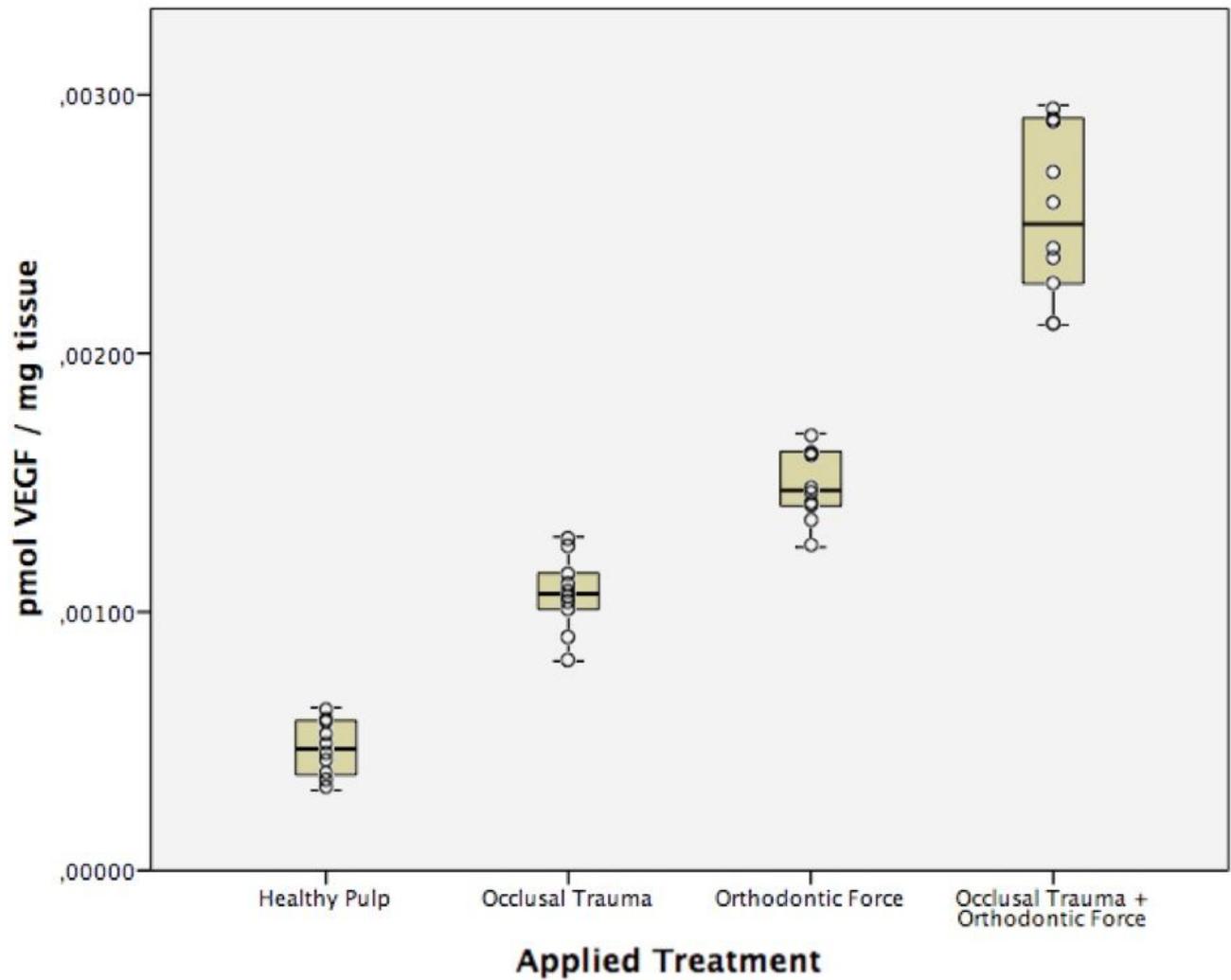
**Figure 3**

Calcitonin gene-related peptide (CGRP) expression (pmol/mg) in human dental pulp from teeth under different clinical stimuli.



**Figure 4**

Vascular endothelial growth factor (VEGF) expression (pmol/mg) in human dental pulp from teeth under different clinical stimuli.



**Figure 4**

Vascular endothelial growth factor (VEGF) expression (pmol/mg) in human dental pulp from teeth under different clinical stimuli.

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