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Assessment of IL-1β and VEGF concentration in a rat model during orthodontic tooth movement and extracorporeal shock wave therapy

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ARTICLE INFO

Article history:
Accepted 20 September 2012

Keywords:
Orthodontic movement
Shock wave
Cytokine
Periodontal ligament (PDL)

ABSTRACT

Objective: This study aimed to investigate PDL's cytokine concentration fluctuations after induction of orthodontic force with and without extracorporeal shock wave therapy in a rat model.

Materials and methods: An orthodontic appliance was fabricated and applied between the molars and the incisors of rats. The rats were treated by a single episode of 1000 shock waves and gingival crevicular fluid was collected for 3 days. The expression and concentration of IL-1β and VEGF were evaluated by ELISA assay. On day 3 all rats were sacrificed and histologic and immunohistochemical assays were applied.

Results: IL-1β concentration rose in both the treated and non treated shockwave groups on the first day, however it was statistically significantly higher in the treated group on day 2. No statistically significant difference was detected between the groups on day 3. The number/area of TRAP positive cells was higher in the non shockwave group than in the treated group. The percentage of cells expressing VEGF displayed the opposite trend. The findings regarding the immunohistochemical assay for IL-1β corresponded with those of the ELISA assay on day 3.

Conclusion: The application of shockwaves during orthodontic tooth movement influences the expression of IL-1β and VEGF and may alternate the periodontal remodelling expected rate.

1. Introduction

Extracorporeal generated shock waves were introduced for medical therapy approximately 20 years ago to disintegrate kidney stones. However, urology is not the only medical field which uses shock waves as a therapeutic method. Over the last 10 years, they have been used in different fields of medicine (orthopaedics, traumatology, veterinary medicine, treatment of impaired wound healing and burn injuries) to stimulate healing processes.[1–3] Shock wave therapy in all of these fields serves as a stimulation of healing processes by inducing neovascularisation and differentiation of stem cells into cells of the injured tissue to allow proper healing and

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0003–9969/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.archoralbio.2012.09.012

regeneration.[3–6] This is a completely different approach compared to urology where shock waves have been used for kidney stone disintegration.

Orthodontic tooth movement is achieved by the remodelling of periodontal ligament (PDL) and alveolar bone in response to mechanical loading.[7,8] The transduction of mechanical forces to the cells triggers a biologic response, which has been described as an aseptic inflammation because it is mediated by a variety of inflammatory cytokines and does not represent a pathological condition.[9,10] In contrast to chronic inflammatory responses, in which persistent stimuli sustain a long-lasting inflammatory response and result in tissue damage, the expression of inflammatory mediators after orthodontic force application is transitory and essential for orthodontic movement, as anti-inflammatory drugs are capable of blocking tooth movement.[11]

This tissue response initially involves vascular changes, followed by the synthesis of prostaglandins, cytokines and growth factors. Finally, such mediators are believed to activate tissue remodelling, characterized by selective bone resorption or deposition in the compression and tension regions of the PDL, respectively.[7,8,12] Both soft and mineralized connective tissue metabolism can be modulated by cytokines and growth factors, pointing to the possible involvement of such factors in tissue remodelling during orthodontic tooth movement.[13]

Rygh and Reitan[14] divided this biologic response to 3 phases of orthodontic tooth movement after the application of mechanical forces: initial tipping (few hours–~2 days after force application), lag phase (3–5 days after force application), and then tooth movement (7–14 days after force application). Rat models of tooth movement have provided in vivo evidence that interleukin-1β (IL-1β), tumour necrosis factor-alpha (TNF-α) and IL-6 are up-regulated in PDL cells and osteoblasts[15,16] and are important regulators in the bone remodelling process upon mechanical stimulation.[17–19] Vascular endothelial growth factor (VEGF) is the primary mediator of angiogenesis and serves various biological functions, such as increasing vascular permeability, promoting chemotaxis in human monocytes and involvement in bone resorption and formation.[20] As a result VEGF also plays an important role in orthodontic tooth movement.[21] All those cytokines are elevated in the gingival crevicular fluid of patients during orthodontic treatment within a few days after force application,[19,22,23] i.e. during the tipping and lag phase periods.

Since extracorporeal shock wave therapy (ESWT) can modulate healing processes, it might have an effect on orthodontic tooth movement and its cytokine profile in particular.

Our preliminary report[24] discussed the concept of applying shock wave therapy in dentistry in general and specifically in the orthodontic field. It also included some preliminary results that we attained from a pilot cytokines concentration study. The aim of the current paper is to present a complete array of the study in which we exclusively investigate the expression of IL-1β and VEGF in the periodonal tissues after induction of orthodontic force with and without ESWT in a rat model, during the first three days, i.e. the initiation stage of the biologic response to mechanical force.

2. Materials and methods

2.1. Research animals

Twenty female Sprague-Dawley rats (aged 3–4 months, body weight 260–280 g) were maintained under micro-isolator conditions, fed standard powdered chow and H_2O ad libitum, and kept at 25 °C while alternating 12-h periods of light and dark. All procedures were performed in accordance with the approved guidelines set forth by the Institutional Animal Care and Use Committee at the Technion – Israel Institute of Technology. Following acclimation (1 week), rats were randomized into 4 groups: without an orthodontic appliance or ESWT (negative control: $n = 5$); ESWT without an orthodontic appliance (positive control: $n = 5$); with an orthodontic appliance and without ESWT (ESWT/−: $n = 5$); and with an orthodontic appliance and ESWT (ESWT/+: $n = 5$). A general anaesthesia was induced using 75 mg/kg of ketamine and 10 mg/kg of xylazine, administered by an intramuscular injection.

2.2. The orthodontic system

Stainless steel ligature wires were bent and inserted beneath the contact point of the second and third maxillary molars to enclose the first and second maxillary molars as one unit on each experimental side, which was randomly chosen and shaved. The contralateral side was not used as control due to the possible effect of the ESWT. A Sentalloy® closed coil spring (20 cN, GAC, New York, USA)[25,26] was attached to this ligature wire and tightened to the teeth (Fig. 1). A transverse hole was drilled through both maxillary incisors at the apical third of the-crown using a drilling bur and the stainless steel ligature wire was inserted through the hole as described in the literature.[26] Most likely in case of pulp exposure occurred,

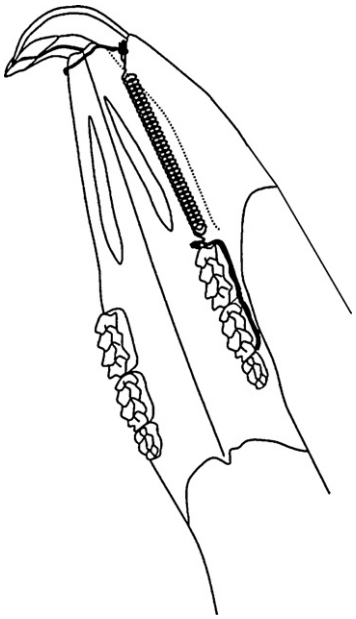

Fig. 1 – Schematic drawing of the rat model.

dentine continued to be build up thus forming a dentine bridge over the exposure site and sealed it during the next 3 days of the study.[27] The Sentalloy® spring was activated and subsequently attached to the ligature wire through the snout and the incisors. The delivered force of the spring was tested and confirmed to produce a force of 20 ± 2 cN after each measurement. There was no reactivation during the experimental period.

2.3. ESWT application

Based on literature reports[3,4] the characteristics of the ESWT were determined; on the day of the surgery, the rats were treated with a single application of unfocused 1000 impulses at EFD 0.1 mJ/mm^2, with a pulse rate of 5 pulses per second by DermaGold® (MTS, Konstanz, Germany) to the area of the maxillary tuberosity, i.e. the anatomical location of the 3 maxillary molars.

A prophylactic antibiotic, colicillin (0.1 mL/kg), was administered once to the rats from the ESWT/+ and ESWT/− groups in order to prevent infection that can be caused by trauma during the drilling or the application of the spring.

2.4. Gingival crevicular fluid (GCF) collection

GCF was collected under general anaesthesia each of the 3 days of the experiment. The small size of a rat molar cannot facilitate GCF collection separately from the tension and the pressure sides. Laminar flow while collecting rat GCF cannot be specified as arriving from either the tension or the pressure side as revealed by previous studies.[28–30] Therefore, GCF was considered as a whole for its fluctuations in IL-1β and VEGF's concentration.

The upper second molar, on the experimental side, was isolated with cotton rolls and gently dried with an air syringe. Paper filter strips (Periopaper – gingival fluid collection strips; Pro Flow, Amityville, NY, USA) were carefully inserted into the gingival crevice between the upper first and second molars on the mesiopalatal aspect, until mild resistance was felt and allowed to remain there for 30 s. Then on removal, paper strips were covered with aluminium foil, placed in an Eppendorf tube and stored at −20 °C. Paper filter stripes contaminated with saliva or blood were disregarded and reapplied to the gingival crevice after 1 h. This method has been used to collect and analyze small volumes of biological fluids, i.e. GCF.[30] Lamster et al.[28,29] developed an approach for GCF sampling that standardizes the time of collection and reports the data as a total amount (or activity) in the timed sample.

2.5. IL-1β and VEGF's quantification – ELISA assay study

A twofold dilution series of standard solution from the kit (Quantikine® Rat IL-1β/VEGF, R&D Systems, Minneapolis, MN, USA) was prepared according to the manufacturer's instructions. One dry paper strip was inserted into each tubes of the standard solution and allowed to remain there for 30 s. Standard solution and sample paper strips were inserted into an individual test tube containing 100 μl of PBS. Tubes were allowed to stand at room temperature for 30 min, while manually shaking them every 5 min to facilitate extraction of the sample from the filter papers. After eluting the standard solution and the GCF from the paper strips by centrifugation at $14{,}000 \times g$ for 5 min, aliquots of the extracted sample were diluted twice with the Calibrator Diluent from the kit and used in the ELISA assay, performed according to the manufacturer's instructions. This assay uses a quantitative sandwich enzyme immunoassay technique. Affinity purified polyclonal antibody specific for mouse IL-1β/VEGF was pre-coated onto a microplate. Standards, controls and samples were pipetted into the wells and any rat IL-1β/VEGF present in the tested sample was bound by the immobilized antibody. An enzyme-linked polyclonal antibody specific for rat IL-1β/VEGF was added to the wells after washing away any unbound substances. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The intensity of the colour measured was in proportion to the amount of rat IL-1β/VEGF bound in the initial step. Sample values were read from the standard curve set to 450 nm with a wavelength correction between 540 and 570 nm, and measurement units expressed as pg/ml. All samples were assayed simultaneously.

Animals were euthanized after 3 days from appliance insertion.

2.6. Immunohistochemical staining

Maxillae were dissected en bloc, fixed in 10% formalin overnight at 4 °C, and placed in 4% ethylenediaminetetraacetic acid (EDTA) at 25 °C for 4–6 weeks of decalcification; the EDTA solution was changed every other day. Fully decalcified samples were dehydrated and paraffin embedded, and 7-μm-thick horizontal sections were prepared.

Sections obtained from the mesiopalatal root of the maxillary first molar on the experimental side underwent immunohistochemical staining for IL-1β and VEGF. Briefly, the sections were dewaxed, endogenous hydrogen peroxidase blocked with 0.3% hydrogen peroxide in methanol for 10 min at 25 °C and incubated with IL-1β (1:50, Rabbit, Santa Cruz Biotechnology Inc., Santa Cruz, CA, #sc 7884) and VEGF (1:50, Mouse, Thermo Fisher Scientific, CA, #jh 121) for 1 h at 25 °C. HRP polymer (SuperPicTure polymer, Invitrogen, Camarillo, CA) was added for 10 min followed by AEC chromagen (SuperPicTure polymer, Invitrogen, Camarillo, CA) for 10 min (both at 25 °C) to visualize the staining. Sections were counterstained with haematoxylin.

To identify osteoclasts, three slides per animal were stained for tartrate-resistant acid phosphatase (TRAP), as described previously.[31] The slides were evaluated at a magnification of 400× with a fixed measurement frame (450 mm × 450 mm). TRAP-positive cells were counted as multinucleate odontoclasts, which were observed on the surface of the alveolar bone.

2.7. Histomorphometry

Three non sequential sections of each animal were randomly selected. In those sections, two regions from the mesiopalatal root of the maxillary first molar were randomly selected for examination under a Zeiss Axioskop 2 plus microscope (Carl Zeiss, Gottingen, Germany). All images of each specimen were

captured using a CCD camera. The obtained images were analyzed using an ImageJ, an image-analysis software (ImageJ 1.440, National Institute of Health, USA). The percentages of cells expressing IL-1β and VEGF were calculated from all sections at 200× magnification both for the tension and compression sides by a pathologist blinded to the nature of the studies.

2.8. Statistical analysis

The data was evaluated using SPSS software, version 17 (SPSS Inc., Chicago, IL, USA). All results are expressed as mean ± SD (SD). The differences in IL-1β/VEGF levels between the groups in the ELISA assay were assessed by ANOVA and Scheffe *post* hoc multiple-comparisons test. The differences in the percentages of cells expressing IL-1β and VEGF and TRAP-positive cells between the groups were assessed by Kruskal–Wallis ANOVA on ranks, and multiple comparisons were adjusted by Mann–Whitney *U* test with Holm's sequential Bonferroni correction. Statistical significance was assumed to be $p < 0.05$.

3. Results

3.1. ELISA assay study

Of the two tested cytokines, IL-1β was the only detected cytokine along the study timeframe.

Fig. 2 presents IL-1β concentrations during the days of the study. No statistical significant difference was found in IL-1β concentrations between all groups except on day 2. IL-1β concentration rose in both ESWT/+ and ESWT/− groups on the first day, but was higher in the ESWT/+ group (257.45 pg/ml and 166 pg/ml, respectively). In both groups this rise was found to be statistically significant in comparison to the level on day 0 ($p < 0.05$). However, on day 2 while IL-1β concentration kept rising in the ESWT/+ group, it decreased in the ESWT/− group (277.63 pg/ml and 136.36 pg/ml, respectively). The increased IL-1β concentration in the ESWT/+ group was found to be statistically significant in comparison to all three other groups and its level on day 0 ($p < 0.05$). On day 3, IL-1β concentrations in both groups decreased and reached a lower level in the ESWT/+ group (75.09 pg/ml and 94.72 pg/ml, respectively), revealing a statistically significant difference than its level on the previous day ($p = 0.005$).

IL-1β concentrations in the positive and negative control groups did not change throughout the timeframe of the study in a statistically significant manner and were not found to be statistically different between the groups.

3.2. TRAP Staining

The number/area of TRAP positive cells was statistically significantly higher in the ESWT/− group than in the ESWT/+ group, on the compression side ($p < 0.0001$). No difference was found between all other groups (Fig. 3).

3.3. Histomorphometry of IL-1β

The immunohistochemical staining for IL-1β in the different groups is presented in Fig. 4. The expression of this cytokine is mainly in the alveolar bone with quite smilar levels in both the ESWT/+ and the ESWT/− groups. No statistically significant difference was detected between the control groups and between the ESWT/+ and ESWT/− groups (Fig. 5). However, a statistically significant difference in the percentage of cells expressing IL-1β was found between the negative control group and both the ESWT/+ and ESWT/− groups (on both sides) ($p < 0.05$). The same finding was revealed for the positive control group.

3.4. Histomorphometry of VEGF

The immunohistochemical staining for VEGF in different groups is presented in Fig. 6. The expression of this cytokine is both in the PDL and alveolar bone with an apparent difference between the ESWT/+ and ESWT/− groups. Fig. 7 describes the percentage of cells expressing VEGF. A statistically significant difference was found between the positive control and both the negative control and the ESWT/− groups, on both of its sides ($p < 0.05$). In addition, the percentage of cells expressing VEGF was statistically significantly higher in the ESWT/+ group compared to the ESWT/− group (on both the tension and pressure sides) ($p < 0.05$).

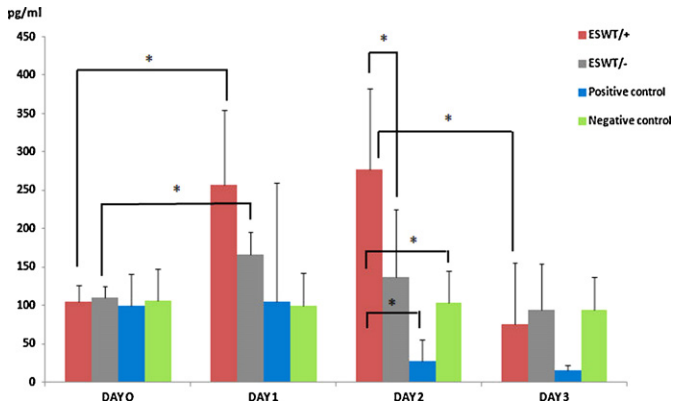

Fig. 2 – (A) IL-β levels (pg/ml) in the GCF of the four groups. *$p < 0.05$.

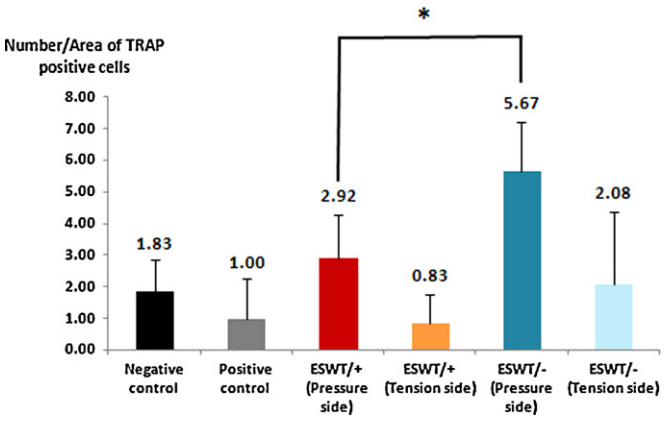

Fig. 3 – The number/area of TRAP positive cells. $^{*}p < 0.001$.

4. Discussion

The results of the ELISA assay in this study show increased levels of IL-1β concentration in response to the induction of an orthodontic force following ESWT. While many studies have emphasized the beneficial effects of ESWT in musculoskeletal tissue and bone regeneration, to date, few have determined its effect on oral bacteria, periodontal tissue healing, and alveolar bone resorption resulting from chronic inflammation of the periodontium.[2,3] No report to date has examined its influence on different inflammation mediators and growth factors in the periodontium, in the presence of a sterile inflammatory process as induced by the orthodontic force presented in this study. In order to conduct a preliminary assessment of the effect of ESWT on the inflammatory process as whole and specifically on its initiation, we chose 2 cytokines that are known to present increased concentrations following activation of orthodontic force and the application of ESWT, i.e. during the initial tipping and lag phase periods. However, orthodontic tooth movement is a complex biological and biochemical process mediated by a large array of cytokines leading to different cell activity. Since no report to date has described orthodontic tooth movement modification under

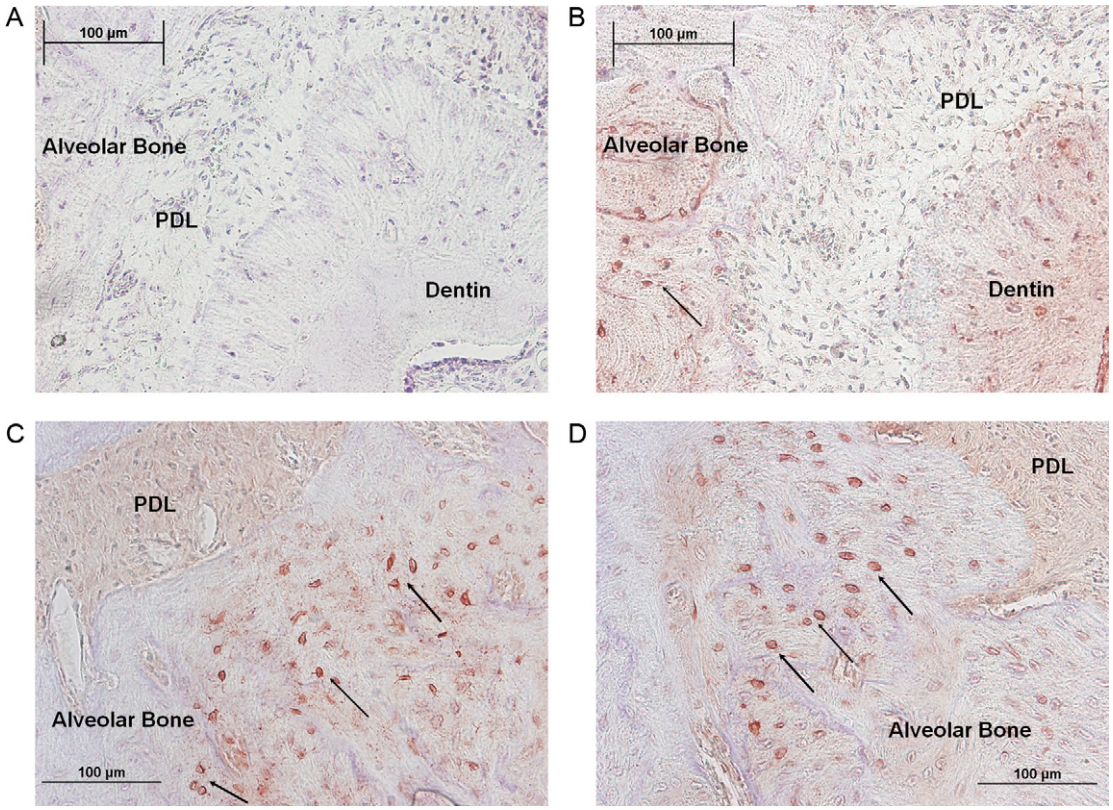

Fig. 4 – Immunohistochemical staining for IL-1β on the tension side: (A) negative control, (B) positive control, (C) ESWT/+ group and (D) ESWT/− group. PDL, periodontal ligament. Arrows indicating cells expressing IL-1β.

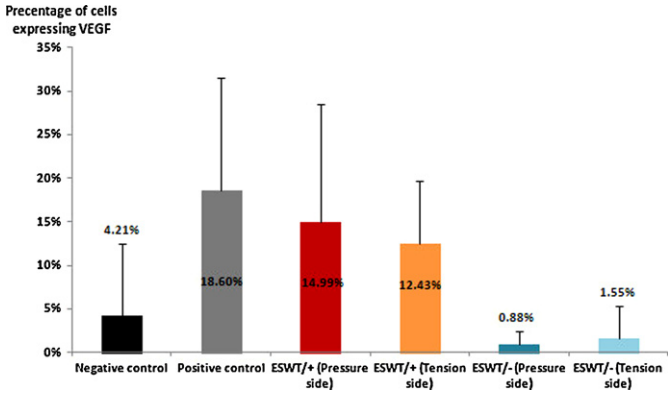

Fig. 5 – The percentage of cells expressing IL-1β in the different study groups.

ESWT, either from the clinical aspect or from a histochemical and histological one, we may assume different types of influences both additive and contractive over the time of force application, as noted in this study. Most studies that have described IL-1β levels in the GCF during orthodontic tooth movement[32,33] have reported up-regulation with a concentration peak achieved 24 h after application of force. Our findings suggest that in both ESWT/+ and ESWT/− groups IL-1β levels confirm this data, though they rose at a higher pace in the ESWT/+ group, indicated by the steeper incline between the bars in Fig. 2. However, while IL-1β concentration reached its peak in the ESWT/− group on the first day, it kept rising in the ESWT/+ group and reached its peak only one day later. This finding corresponds with a recent report of Han et al.[34] who described an increased level of IL-1 in cultured tenocytes after the induction of ESWT and the report of Stojadinovic et al.[35] describing a significant increased expression of IL-1β in skin grafts in a murine model. Furthermore, between days 2 and 3 its level declined at a faster pace than in the ESWT/− group, as depicted in the steeper decline between the bars in the graph in Fig. 2. The kinetics of IL-1β levels indicates that ESWT may induce up-regulation of IL-1β secretion and faster resolution of this acute phase inflammation cytokine during tooth movement.

The results of the histomorphometry regarding IL-1β expression correspond well with the results of the ELISA

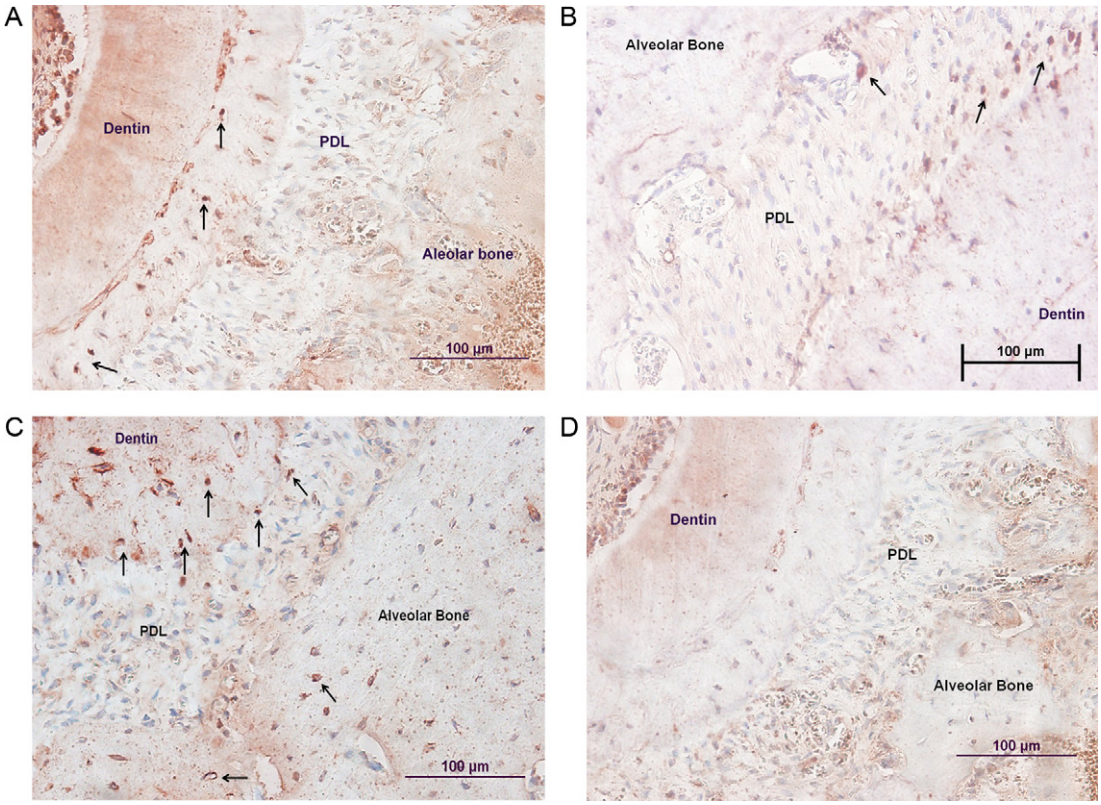

Fig. 6 – Immunohistochemical staining for VEGF on the pressure side: (A) negative control, (B) positive control, (C) ESWT/+ group and (D). ESWT/− group. PDL, periodontal ligament. Arrows indicating cells expressing VEGF.

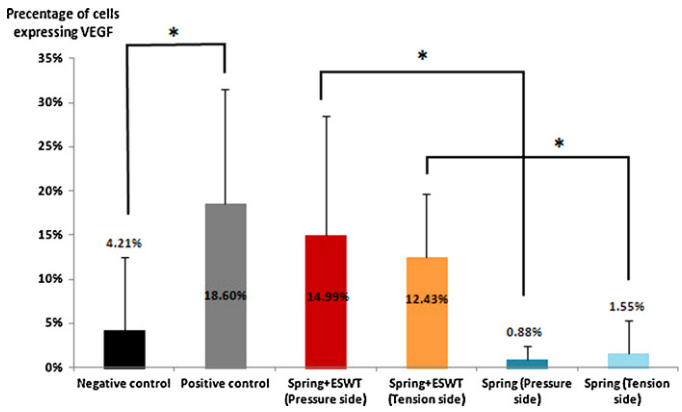

Fig. 7 – The percentage of cells expressing VEGF in the different study groups. $^{*}p < 0.05$.

study on day 3 of the experiment for the ESWT/+ and ESWT/− group as there was no difference between the groups in both sets of tests. Similarly, the positive and negative control groups displayed the same trend as their concentration/percentage of expressing IL-1β was much smaller than the study groups.

IL-1β has been known to promote osteoclastogenesis by inducing the receptor activator of nuclear factor-kB ligand (RANKL) expression on stromal cells and synergizing with RANKL to promote later stages of osteoclast differentiation.[36–39] However, Lee et al.[37] suggested that IL-1β can inhibit RANK by induced proteolytic shedding of the macrophage colony-stimulating factor (M-CSF) receptor c-Fms that is required for RANK expression. This finding is supported in our study by the decrease in the number/area of TRAP positive cells in the ESWT/+ group on day 3, following the increase in IL-1β concentration in the same group, on day 2 as indicated in the ELISA results. Moreover, the statistically significant decrease in the number/area of TRAP positive cells after ESWT might indicate the positive effect of ESWT on bone formation. This is further supported by Tamma et al.[40] who reported a decrease in RANKL to Osteoprotegerin (RANKL/OPG) ratio after ESWT suggesting inhibition of osteoclastogenesis.

VEGF was not detected during the three days of the study by the ELISA assay. The reports regarding VEGF's detection in the GCF are inconsistent in the literature; Kaku et al.[41] reported its detection in the GCF of humans as early as one day after force application, however, Pinkerton et al.[42] demonstrated its down regulation 6–24 h from force application. Miyagawa et al.[21] reported VEGF's detection only seven days after force application. Despite the above, VEGF was detected by the immunohistochemistry assay on day 3. Many studies have described up regulation in VEGF's expression and the angiogenic effect of ESWT by immunohistological means in different surgical procedures.[43–48] This explains the statistically significant difference between the ESWT/+ and ESWT/− groups in the percentage of cells expressing VEGF. Furthermore, in the negative control group, we observed a lower level of expression of VEGF, compared to the other groups. This can be explained by a reported constitutive VEGF expression in periodontal tissues cells, which are subjected to intermittent chewing forces, regardless of tooth movement.[21]

Several studies have described the effect of ESWT on naïve cells of different tissue cultures and have reported a marked elevation in different cytokines.[4,34,49] Accordingly, the positive control group presented a statistically significant increase in the percentage of cells expressing VEGF compared to the negative control group. Nevertheless, in the ELISA assay, IL-1β concentrations of both the positive and negative control groups were not found to be statistically different on all the days and the concentration levels did not change in a statistically significant manner between the days. The same finding repeated in the histomorphomerty of IL-1β.

According to the pressure–tension theory, with the application of light constant force on the teeth, blood flow through the partially compressed PDL decreases as soon as fluids are expressed from the PDL space.[50] These alterations in blood flow quickly induce changes in oxygen levels, which stimulate the release of other biologically active agents, such as IL-1β and nitric oxide (NO).[51] Recent reports indicate that one of the possible molecular mechanisms of action of ESWT seems to be its capacity to keep local NO contents at a physiological level in the early phase of inflammatory response, and to enhance NO production either through a non-enzymatic or enzymatic pathway.[52] One of the suggested regulation pathways in the resolution of sterile PDL inflammation induced by orthodontic tooth movement is the reciprocity of NO – NF-κB concentrations. Further research is warranted to study these pathways for possible involvement of NF-κB expression induction. Studies under similar conditions should also evaluate the changes in the concentration of other cytokines and for a longer period, as well as the clinical rate of tooth movement in a prolonged follow up period in order to detect its significant acceleration in comparison to regular orthodontic force systems.

In conclusion the results of this study indicate that the application of ESWT during the induction of an orthodontic force influences the concentration of different inflammatory cytokines. This effect may result in attenuation and enhanced periodontal remodelling leading to shortening of the orthodontic induced PDL inflamatory process which may finally lead to a faster orthodontic tooth movement than expected.

Further investigation is warranted to advocate the ESWT clinical aspect of this complex PDL tissue activity and the large array of cytokine mediated cell behaviour, characterized by selective bone resorption or deposition in the compression and tension regions.

Funding

None.

Competing interest

None declared.

Ethical approval

None declared.

Acknowledgments

This study was conducted with the support of the LAZAROFF fund for research in maxillofacial surgery – Technion, Israel Institute of Technology and the Israel Orthodontic Society (IOS) research grant 2010.

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