Advs Exp. Medicine, Biology - Neuroscience and Respiration (2016) 17: 57–65
DOI 10.1007/5584_2015_179
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Published online: 6 November 2015

The Influence of Shockwave Therapy on Orthodontic Tooth Movement Induced in the Rat

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Abstract

Shockwave therapy is used in medicine due to its ability to stimulate healing processes. The application of orthodontic force evokes an inflammatory reaction resulting in tooth movement. Shockwave therapy might have an effect on both inflammatory and periodonal ligament cytokine profiles. Our aim was to evaluate the fluctuations of different inflammatory cytokines after orthodontic force induction with and without shockwave therapy. An orthodontic appliance was applied between the rats' molars and incisors. In conjunction with the commencement of orthodontic force, the rats were treated with a single episode of 1000 shock waves and the gingival crevicular fluid was collected for 3 days. The expression and concentration of different cytokines was evaluated by a commercial 4-multiplex fluorescent bead-based immunoassay. The level of all cytokines displayed a similar trend in both shockwave-treated and untreated groups; the concentration peaked on the first day and declined thereafter. In all cases, however, the cytokine levels were smaller in the shockwave-treated than in untreated animals; a significant difference was found for sRANKL and borderline difference for IL-6 on Day 1. We conclude that shockwave therapy during the induction of orthodontic tooth movement influences the expression of inflammatory cytokines.

Keywords

Cytokines • Gingival crevicular fluid • Healing • Inflammation • Orthodontic tooth movement • Shockwave therapy • Teeth

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1 Introduction

Shock waves have been applied clinically for the first time in renal stone therapy (Graff et al. 1988), where they were casually noted to have an effect on the iliac bone with primary osteocyte damage, followed by osteoblastic stimulation. Consequently, shockwave therapy is widely applied in musculoskeletal disorder treatment (Tamma et al. 2009). Over the last 10 years, this therapy has been used in other fields of medicine, such as treatment of impaired wound healing, burn injuries (Novak et al. 2008; Sathishkumar et al. 2008; Haupt 1997), and erectile dysfunction (Vardi et al. 2012). The recent transformation in the application of shock waves for the stimulation of the healing process has become possible due to the understanding of the effects of shock waves on neovascularization, differentiation of stem cells into injured tissue cells, and the release of different growth factors (Sathishkumar et al. 2008; Nishida et al. 2004; Wang et al. 2003). Recently, the anti-inflammatory effect of shockwave therapy has also been reported (Mittermayr et al. 2012; Mariotto et al. 2009). Although the biochemical mechanisms underlying this effect are not fully understood, extracorporeal shockwave therapy (ESWT) may modulate endogenous nitric oxide (NO) production by positively affecting endothelial NO synthase activity and subsequently suppressing NF-κB activation (Mariotto et al. 2009). In addition, the level of macrophage-derived inflammatory proteins (MIP-1α and MIP-1β) and the oxidative burst of leukocytes were reduced after ESWT (Mittermayr et al. 2012).

Orthodontic tooth movement is an accepted model for inducing and resolving inflammation during a limited time period (Proffit 2006). The prolonged pressure applied by the orthodontic appliance on a tooth results in its movement due to a weakening of the periodontal ligament (PDL), a collagenous supporting structure, as the bone around the tooth remodels. Like any inflammatory reaction, it involves two phases. An acute phase (the first 1–3 days) is initiated by resident macrophages that undergo activation and release inflammatory mediators enabling propagation and maturation of the inflammatory response (Eugene Roberts 2005). When stimulation (i.e., the orthodontic pressure) is sustained, a shift occurs toward the chronic phase, which is characterized by tissue destruction and resolution due to persisting inflammatory process.

Rat models of tooth movement have provided *in vivo* evidence that the receptor activator of nuclear factor kappa-B ligand (RANKL), interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), and IL-6 are up-regulated in PDL cells and osteoblasts (Hazan-Molina et al. 2013; Kim et al. 2007; Bletsa et al. 2006; Alhashimi et al. 2001; Shiotani et al. 2001), and are important regulators of bone remodeling in response to mechanical stimulation (Yamaguchi 2009; Ren et al. 2007; Kanzaki et al. 2006; Uematsu et al. 1996; Le and Vilcek 1987). These cytokines are elevated in the gingival crevicular fluid of patients during orthodontic treatment within a few days after force application (Nishijima et al. 2006; Lowney et al. 1995; Grieve et al. 1994).

In our previous preliminary study, the application of shock waves during orthodontic tooth movement increased acutely the expression of IL-1β in the gingival crevicular fluid on the compressed side of the PDL on Day 2, along with a reduction in the number of TRAP (tartrate resistant acid phosphatase) positive cells, characteristic of osteoclasts on Day 3. These findings contradicted each other, since IL-1β is known to promote osteoclastogenesis by inducing RANKL (receptor activator of nuclear factor κβ ligand) expression on stromal cells and synergizes with RANKL to promote osteoclast differentiation (Bloemen et al. 2011; Lee et al. 2010; Takayanagi 2007; Teitelbaum 2006). In an attempt to resolve the controversy, in the present study we set out to further investigated the expression of acute phase cytokines in the rat periodontal tissue using an alternative method of cytokine determination. The study was conducted in two separate sets of experiments. In the first set, ESWT was compared to a sham control (no orthodontic force). In the second set, orthodontic force was applied with and without ESWT. Both sets of experiments lasted for 3 days; the time that covers the acute phase of the orthodontic inflammatory reaction.

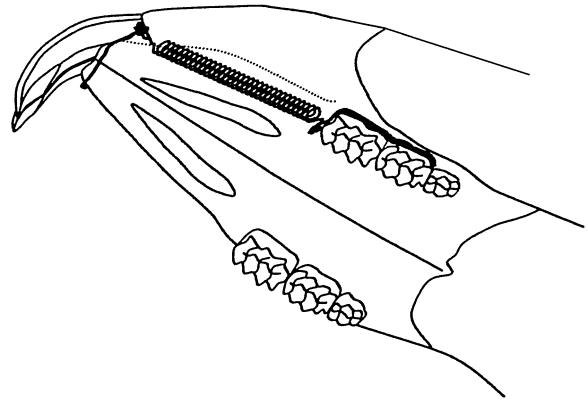

Fig. 1 Scheme of the orthodontic rat model

2 Methods

All procedures were approved by the Institutional Animal Care and Use Committee at the Technion – Israel Institute of Technology (IL-0005-01-11). Twenty one, 3–4 months old, Wistar male rats, weighing 260–280 g were used in this study. The animals were fed a standard pellet diet and water ad libitum, and kept at 25 °C (±2 °C) while alternating 12-h periods of light and dark. Following acclimation (1 week), rats were randomly categorized into 4 groups: without an orthodontic appliance or ESWT (negative control: n = 3); ESWT without an orthodontic appliance (ESWT: n = 6); with an orthodontic appliance and without ESWT (Spring: n = 6); and with an orthodontic appliance and ESWT (Spring + ESWT: n = 6). General anesthesia using 75 mg/kg of ketamine (Rotexmedica; Trittau, Germany) and 10 mg/kg of xylazine (Eurovet Animal Health B.V.; Bladel, the Netherlands), administered by intramuscular injection in a hindlimb, was induced for the application of the orthodontic appliance and for the shock wave therapy.

2.1 The Orthodontic System

In each rat, the experimental side was randomly chosen and the skin above it was shaved. A stainless steel ligature wire (0.012″) (SIA; Orthodontic Manufacturer, Caserta, Italy) was bent and inserted beneath the contact point of the second and third maxillary molars, thus enclosing the first and second maxillary molars as a single unit on each experimental side. The contralateral side was not used as control due to short distance from the experimental side and the possible effect of the ESWT. A 20 cN sentalloy closed coil spring (GAC; Central Islip, NY) 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 previously described (Ren et al. 2004). When the pulp exposure occurred, dentine continued to build up thus forming a bridge over the exposure site and sealing it during the following 3 days of the study (Inoue and Shimono 1992). The Sentalloy® spring was activated and subsequently attached to the ligature wire through the incisors. The spring's delivered force was tested and confirmed to produce a force of 20 ± 2 cN. There was no reactivation during the experimental period.

2.2 ESWT Application

Based on the literature reports (Sathishkumar et al. 2008; Nishida et al. 2004), right after surgical application of the orthodontic appliance, the rats from the ESWT and Spring + ESWT groups were treated with a single application of 1000 unfocused impulses at EFD 0.1 mJ/mm^2, with a

pulse rate of 5 pulses per sec by DermaGold (MTS; Konstanz, Germany) to the area of the maxillary tuberosity, i.e., the anatomical location of the 3 maxillary molars.

2.3 Gingival Crevicular Fluid (GCF) Collection

GCF was collected under general anesthesia during the 3 days of the experiment. 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) were longitudinally cut, carefully inserted into the gingival crevice until mild resistance was felt and remained there for 30 s. Upon removal, the paper strips were covered with aluminum foil, placed in an Eppendorf tube and stored at −20 °C. Paper filter strips contaminated with saliva or blood were placed aside and reapplied to the gingival crevice after 1 h. This method has been used to collect and analyze small volumes of biological fluids (Perinetti 2004). Sampling of GCF often collects the entire volume of fluid at the sampled site, and this volume varies from tooth site to tooth site. As a result, an approach to GCF sampling was developed, which standardizes the time of collection and reports the data as a total amount (or activity) in the timed sample (Lamster et al. 1998; Lamster 1997).

2.4 Determination of Cytokine Concentrations

Cytokine concentrations (pg/ml) were determined using a commercial 4-multiplex fluorescent bead-based immunoassay (Procarta Cytokine Assay Kit; Affymetrix, Santa Clara, CA) and the Luminex 100 IS Instrument (Luminex; Austin, TX). The multiplex kit used was capable of detecting pro-inflammatory cytokines: IL-1β, IL-6, soluble receptor activator of nuclear factor kappa-B ligand (sRANKL) and tumor necrosis factor (TNF)-α.

For the assay, each paper strip was inserted into an individual test tube containing 75 μL of PBS. Tubes were kept at room temperature for 30 min, and were shaken every 5 min to facilitate extraction of the sample from the filter papers. After eluting the gingival crevicular fluid from the paper strips by centrifugation at 14,000 × g for 5 min, 50 μL aliquots/well of the gingival crevicular fluid samples were incubated with anti-rat multi-cytokine beads at room temperature for 1 h in the dark. The unbound material was removed by filtration. Twenty-five microliters/well of detection antibodies were added, and reactions were incubated at room temperature for 30 min in the dark. Twenty-five microliters/well of streptavidin–phycoerythrin were then added, and the plates were incubated at room temperature for an additional 30 min in the dark. One hundred and twenty microliters/well of reading buffer were added and the plate was read in a plate reader. Concentrations of cytokines in each sample were extrapolated from standards by means of Luminex 100 Integrated System 2.3 software (Austin, TX). All samples were run in duplicates.

2.5 Statistical Analysis

Results were expressed as means ± SE. The inter-group differences in IL-1β/IL-6/sRANKL/TNF-α levels in the multiplex fluorescent bead-based immunoassay were assessed with the Kruskal-Wallis test on ranks, and multiple comparisons were adjusted by the Mann-Whitney U test with Holm's sequential Bonferroni correction. The differences in the cytokine levels between the 3 days of the experiment were assessed by the Wilcoxon signed-rank test. Statistical significance was assumed to be at $p < 0.05$. Data were evaluated using SPSS software, ver. 17 (SPSS, IBM, Chicago, IL).

3 Results

All cytokines displayed a similar trend in both the Spring + ESWT and Spring groups, whereby

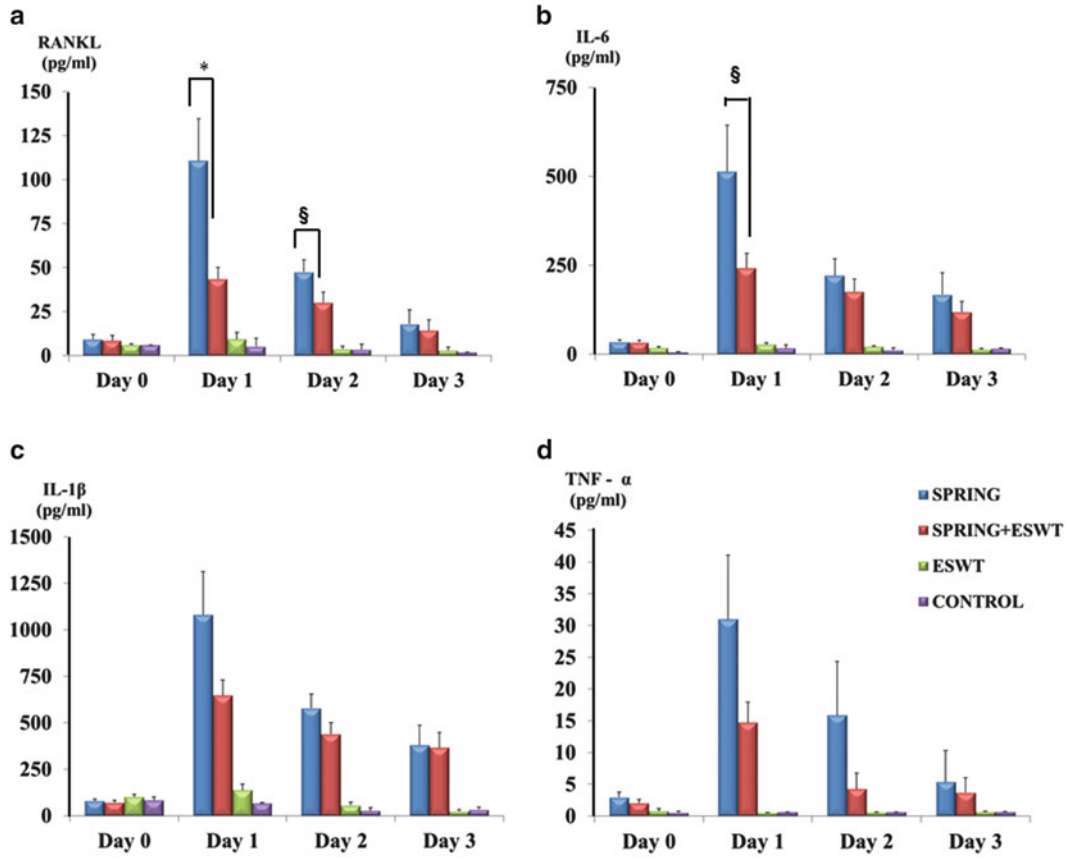

Fig. 2 Concentration of different cytokines in the four study groups during a 3-day study. (**a**) – sRANKL; (**b**) – IL-6; (**c**) – IL-1β; (**d**) – TNF-α. Data are means ± SE. *p = 0.01; §p = 0.07

the concentration peaked on Day 1 and decreased thereafter (Fig. 2). In all cases, the level of cytokines was smaller in the Spring + ESWT than that in the Spring group. However, a statistical significance was only revealed in sRANKL concentration ($p = 0.01$) and a borderline significance in the IL-6 concentration ($p = 0.07$). On Day 2, the difference in cytokine concentrations between the two groups decreased, with sRANKL in the Spring + ESWT group maintaining a borderline lower level compared with the Spring group ($p = 0.07$) (Fig. 2). On Day 3, all differences in cytokine concentrations between the two groups levelled off.

The ESWT and negative control groups also displayed a similar trend with no statistically significant difference found between the two in regard to all cytokines during the 3 days of the study (Fig. 2).

We also examined the change in each cytokine concentration during the 3 days in each group. In the Spring + ESWT and Spring groups, increases in all cytokines were significant between Days 0 and 1 ($p \leq 0.01$). Then, on Day 2 the level of cytokines reverted in both groups, but the reversion was significant only regarding sRANKL and IL-1β ($p \leq 0.05$).

In the negative control group, all cytokines remained quite stable and their change was largely insignificant between the days of the experiment (Fig. 2). Nevertheless, in the ESWT group, sRANKL displayed a significant increase from Day 0 to Day 1 ($p = 0.04$), and IL-1β and IL-6 demonstrated a significant decrease from Day 1 to Day 2 ($p < 0.05$).

4 Discussion

In the present study we determined the effects of extracorporeal (ESWT) shockwave therapy on the expression of cytokines in the periodontal tissues after induction of orthodontic force. The force of 20 cN was distributed over the two first maxillary molars. Since the human molar is approximately 20 times larger than the rat molar (Sato et al. 1984), the effect of force could be estimated to be the same as the force of 200 cN on a human molar, which is considered a substantial orthodontic force (Lee et al. 2013).

We selected the cytokines for the study which are known to respond with increases following orthodontic force activation (Uematsu et al. 1996; Lowney et al. 1995; Grieve et al. 1994) to assess the effect of ESWT on the inflammatory process in general and on its initiation in particular. The evidence of similar pattern of biologic reactions in the presence of ESWT indicates that this treatment leads to a modification of the inflammatory process rather than to its elimination. Spring activation, as a means of achieving orthodontic tooth movement, created an inflammatory response expressed by increases in the concentration of all cytokines. The increases peaked 24 h after the application of force application, which is in accord with previous reports (Karacay et al. 2007; Ren et al. 2007; Dudic et al. 2006; Nishijima et al. 2006; Tian et al. 2006). The anti-inflammatory effect of ESWT, previously reported by Mariotto et al. (2009), was validated in this study as all cytokines were reduced in the GCF after shockwave therapy. Moreover, a significant decrease in the concentration of sRANKL after ESWT may indicate a positive effect exerted by ESWT on bone formation. That is also supported by Tamma et al. (2009) who have reported a decrease in the ratio of sRANKL to osteoprotegerin after ESWT, suggesting the inhibition of osteoclastogenesis.

Several studies that have described a specific effect of ESWT in different cell cultures consisting of an elevation of different cytokines (Nishida et al. 2004; Han et al. 2009; Hofmann et al. 2008). In the present *in vivo* study, concentration of all cytokines in both ESWT and negative control groups did not differ significantly on all study days. Application of ESWT in *in vivo* model should be followed by induction of inflammatory processes, such as flap creation (Yan et al. 2008), ischemia-induced myocardial dysfunction (Nishida et al. 2004), periodontal disease induction (Sathishkumar et al. 2008), or spring activation during orthodontic force application to detect changes in cytokine concentration. ESWT alone is probably insufficient to cause an appreciable change in the tissue cytokine profile.

In our previous study (Hazan-Molina et al. 2013), we have observed a significantly higher concentration of IL-1β in the GCF on Day 2 in the Spring + ESWT group, along with a reduced number of TRAP positive cells, a constituent of osteoclasts, on the compressed side of PDL on Day 3, compared with the Spring alone group. IL-1β promotes osteoclastogenesis by inducing RANKL expression in stromal cells and synergizes with RANKL to promote osteoclast differentiation later on (Bloemen et al. 2011; Lee et al. 2010; Takayanagi 2007; Teitelbaum 2006). IL-6 is also known as a potential osteoporotic factor due to its effect on osteoblast lineage cells (Chung et al. 2003; Ota et al. 2001). Thus, we could expect a similar pattern of expression of IL-1β, IL-6, and RANKL in the GCF during the study days. The present findings regarding IL-1β, IL-6, and RANKL in GCF were generally in line with the previous reports above mentioned and with our expectations, although only a trend was noted in some cases. Furthermore, Han et al. (2009) detected a non-statistically significant decrease in IL-1β concentration, along with a statistically significant decrease in IL-6. This divergent effect, compared with our previous studies (Hazan-Molina et al. 2013; Hazan-Molina et al. 2011), might be explained by the use of different cytokine detection methods, i.e., ELISA and 4-multiplex fluorescent bead-based immunoassay in the previous and in this study, respectively. The inability to detect other than IL-1β cytokines in the GCF in the previous study and

the contradiction in the expression pattern between IL-1β and TRAP positive cells led us to search for a different detection method more suitable for small volumes. Multiplex fluorescent bead-based immunoassay has been known as a very sensitive and specific method for the detection and quantitation of different proteins in human sera and other body fluids of small volumes (Martins et al. 2004; Kellar et al. 2001). The ability to consistently repeat the pattern of expression of all cytokines in the present study may indicate that the 4-multiplex fluorescent bead-based immunoassay proved superior to ELISA in cytokine detection in GCF.

It is likely that despite some anti-inflammatory effect of ESWT, the initial inflammatory reaction may be strong enough to propagate into an orthodontic tooth movement. Furthermore, since a faster healing process is expected, due to a smaller inflammatory reaction, paradoxically an even larger orthodontic tooth movement could be anticipated with ESWT for the same orthodontic force applied during the same time frame.

The surprising results presented in this study raise a question of whether the anti-inflammatory effect of ESWT may have a clinical potential to be implemented in enhancing orthodontic tooth movement. This study focused on the first 3 days of the inflammatory reaction. During this time frame the tooth is only minimally displaced in the alveolar socket. Further research should be conducted through a longer time frame (at least 3 weeks), comparing the amount of orthodontic tooth movement with and without the effect of ESWT. In addition, ESWT may be also implemented in the future for the treatment of different lesions, involving acute inflammatory reactions in the oral cavity with damage to the alveolar bone, due to the ability of ESWT to reduce the inflammatory process and inhibit osteoclastogenesis. One example is periodontal diseases that often leads to osseous defect and teeth loss or periodontal inflammation around implants (peri-implantitis).

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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