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Periodontal cytokines profile under orthodontic force and extracorporeal shock wave stimuli in a rat model

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Background and Objective: Extracorporeal shock wave therapy has been used in various clinical conditions as a result of its ability to stimulate healing processes in acute and chronic inflammatory states. Orthodontic force application triggers an inflammatory reaction in the periodontal tissue surrounding the involved teeth, resulting in tooth movement. Preliminary work revealed that extracorporeal shock wave therapy increased the expression of the inflammatory cytokines involved. Our aim was to investigate the expression of inflammatory cytokines in the periodontal tissues following orthodontic force induction, with and without shock wave therapy, in experimental rats.

Material and Methods: An orthodontic appliance was fabricated and applied between the molars and the incisors of adult Wistar rats. In conjunction with orthodontic force commencement, the rats were treated with a single episode of 1000 shock waves. Every day, during the 3 d of the study, rats were killed and the immunolocalization of RANKL, interleukin (IL)-1β, IL-6 and tumor necrosis factor-alpha was evaluated.

Results: The percentage of the area staining positively for all inflammatory cytokines during the first 2 d decreased statistically significantly more in the shock wave-treated group compared with the nontreated control group. On the first day, the percentage of the area staining positively for IL-1β and RANKL on the compression side peaked in both groups, with a sequential rise in the number of TRAP-positive cells.

Conclusion: The induction of shock wave therapy during orthodontic tooth movement influences the expression of different inflammatory cytokines in the tissue and might alter the expected periodontal remodeling rate.

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Extracorporeal shock waves are acoustic waves that can induce a mechanical wave which passes through the cell with a cavitational effect. High-energy shock waves have been used mainly for the treatment of kidney, gall bladder or salivary gland stones, while many researchers have applied lower energy shock waves as a nonsurgical treatment method in the field of orthopedics (1–6). The use of shock waves has been extended to several other fields of medicine, such as traumatology and veterinary medicine, and shock waves have been used for the treatment of

impaired wound healing, burn injuries and erectile dysfunction (7–10). This wider use is a result of the ability of shock waves to stimulate healing processes (9,11–13) and to reduce inflammatory reactions (14,15). In addition, extracorporeal shock wave therapy (ESWT) has been applied in the field of dentistry, although reports are still very scarce [(8,9,16) H. Hazan-Molina, A. Reznick, H. Kaufman, D. Aizenbud, submitted].

Orthodontic tooth movement is a model for inducing and resolving periodontal inflammation during a limited time period (17); it involves a short, acute phase lasting 1–3 d and prolonged chronic phases lasting 3–4 weeks (18). Experimental tooth-movement models in rats have provided *in-vivo* evidence that RANKL, interleukin (IL)-1β, tumor necrosis factor-alpha (TNF-α) and IL-6 are up-regulated in periodontal ligament cells and osteoblasts [H. Hazan-Molina, A. Reznick, H. Kaufman, D. Aizenbud, submitted (19,20)]. In addition, these cytokines are important regulators in the bone-remodeling process upon mechanical stimulation (21–23). Recently, an increase in the expression of those cytokines in the gingival crevicular fluid was described after application of ESWT during orthodontic tooth movement in rats (H. Hazan-Molina, A. Reznick, H. Kaufman, D. Aizenbud, submitted).

The aim of this study was to investigate, in greater detail, the expression of cytokines in the periodontal tissues of a rat model in two separate sets of experiments: (i) the use of ESWT alone vs. a sham control (no orthodontic force); and (ii) induction of orthodontic force, with and without ESWT.

Material and methods

All procedures were approved by the Institutional Animal Care and Use Committee at the Technion – Israel Institute of Technology (IL-0005-01-11).

Research animals

Fifty-one male Wistar rats, 3–4 mo of age and weighing 260–280 g, were used in this study. The animals were fed a standard pellet diet with water *ad libitum*, and were kept at 25 ± 2°C in alternating 12-h periods of light and dark. Following acclimation (1 week), the rats were randomly categorized into four groups: control (without an orthodontic appliance or ESWT; $n = 6$); ESWT (ESWT without an orthodontic appliance; $n = 15$); Spring (with an orthodontic appliance and without ESWT; $n = 15$); and Spring + ESWT (with an orthodontic appliance and ESWT; $n = 15$). General anesthesia, comprising 75 mg/kg of ketamine (Rotexmedica, Trittau, Germany) and 10 mg/kg of xylazine (Eurovet Animal Health B.V, Bladel, the Netherlands), administered by an intramuscular injection in the hind-limb, was induced for application of the orthodontic appliance and/or the shock wave therapy.

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 a control because of the short distance of this area from the experimental side and hence the possibility of some effect of the ESWT taking place. A Sentalloy® (20cN; GAC, New York, NY, USA) closed-coil spring was attached to this ligature wire and tightened to the teeth. A transverse hole (with a diameter of 0.3 mm) 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 previously (24). When pulp exposure occurred, dentine continued to build up, thus forming a dentine bridge over the exposure site and sealing it during the following 3 d of the study (25). The Sentalloy® spring was activated and subsequently attached to the ligature wire through the incisors. The force of the spring delivered was confirmed to be 20 ± 2 cN. There was no reactivation during the experimental period.

ESWT application

Based on reports in the literature (9,11), on the day of fixing the orthodontic device, a single application of 1000 unfocused impulses at an energy flux density of 0.1 mJ/mm^2 and a pulse rate of five pulses per second was delivered, by DermaGold® (MTS, Konstanz, Germany), to the area of the maxillary tuberosity (i.e. the anatomical location of the three maxillary molars) of rats in the ESWT and Spring + ESWT groups.

A prophylactic antibiotic, colicillin (0.1 mL/kg) (100 mg/mL of ampicillin + 250,000 IU/mL of colistin sulphate; Egevet, Izmir, Turkey) was administered once to the rats of the Spring and Spring + ESWT groups in order to prevent infection that can be caused by trauma during drilling or the application of the spring.

Five rats from each of the Spring, Spring + ESWT and ESWT groups and two rats from the control group were killed each day by CO_2 inhalation.

Immunohistochemical staining

Maxillae were dissected *en bloc*, fixed in 10% neutral buffered formalin (pH 6.8) 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 to obtain a horizontal planar surface cut through the teeth.

Sections obtained from the mesio-palatal root of the maxillary first molar on the experimental side underwent immunohistochemical staining for IL-1β, IL-6, RANKL and TNF-α. Briefly, the sections were dewaxed, endogenous hydrogen peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 10 min at 25°C and the sections were incubated with IL-1β (#sc 7884, 1 : 50, Rabbit; Santa

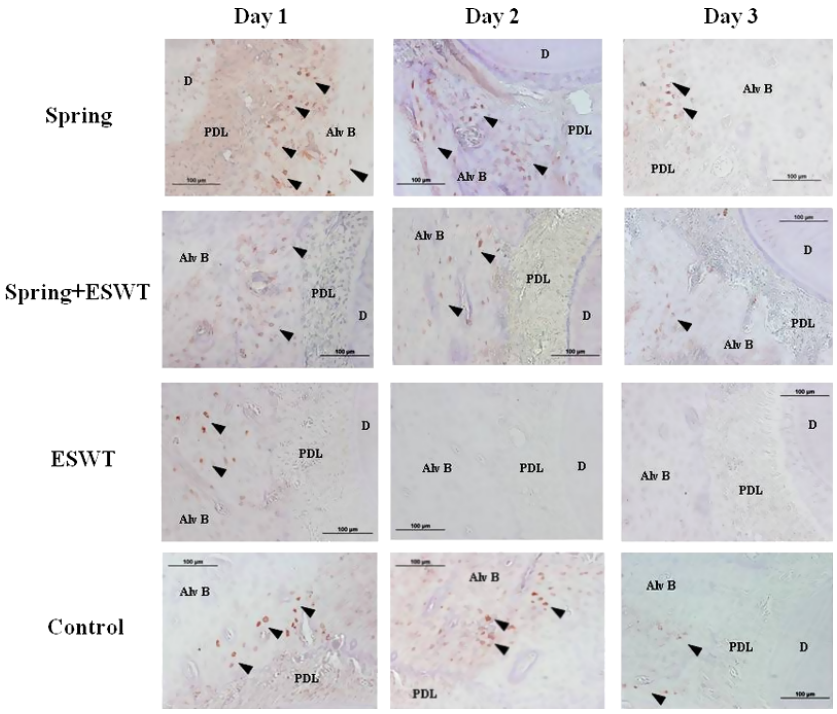

Fig. 1. Immunohistochemical staining for RANKL on the pressure side during the 3 d of the study. Alv B, alveolar bone; Control, without an orthodontic appliance or extracorporeal shock wave therapy (ESWT); D, dentin; ESWT, ESWT without an orthodontic appliance; PDL, periodontal ligament; Spring, with an orthodontic appliance and without ESWT; Spring + ESWT, with an orthodontic appliance and ESWT.

Cruz Biotechnology, Santa Cruz, CA, USA), IL-6 (#sc 1265,1 : 50, Rabbit; Santa Cruz), RANKL (#sc 7628,1 : 50, Rabbit; Santa Cruz) and TNF-α (#LC-C42300,1 : 400, Rabbit; LifeSpan BioSciences, Seattle, WA, USA) antibodies. Horseradish peroxidase polymer (SuperPicTure polymer; Invitrogen, Camarillo, CA, USA) was added for 10 min followed by AEC chromogen (SuperPicTure polymer; Invitrogen) for 10 min (both at 25°C) to visualize the staining. Sections were counterstained with hematoxylin.

To identify osteoclasts, three nonsequential slides per rat were randomly chosen and stained for TRAP, as described previously (26). The slides were evaluated with a × 40 objective in a fixed field of view (432 μm × 325 μm). TRAP-positive cells were counted as multinucleate odontoclasts, which were observed on the surface of the alveolar bone.

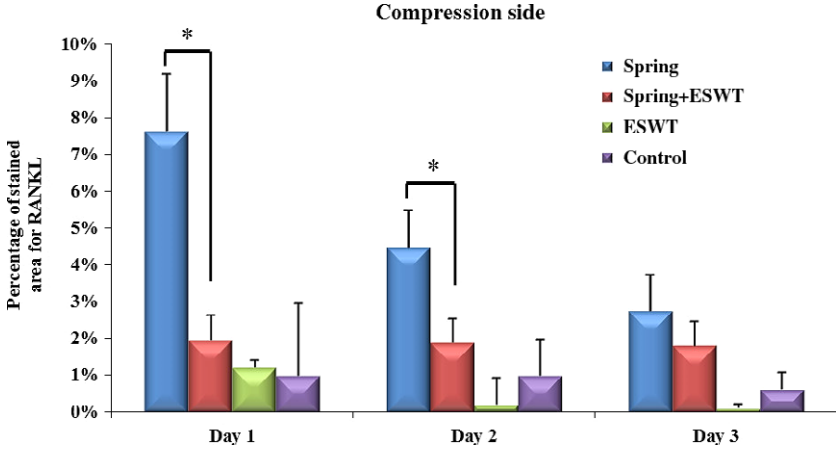

Fig. 2. Percentage of the area staining positively for RANKL on the compression side during the 3 d of the study. Control, without an orthodontic appliance or extracorporeal shock wave therapy (ESWT) (control); ESWT, ESWT without an orthodontic appliance; Spring, with an orthodontic appliance and without ESWT; Spring + ESWT, with an orthodontic appliance and ESWT. Data represent mean + standard error of the mean. *$p < 0.05$.

Histomorphometry

Three nonsequential sections of each embedded maxilla 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, Göttingen, Germany). All images of each specimen were captured using a Charge-Coupled Device camera. The images obtained were analyzed using ImageJ image-analysis software (IMAGEJ 1.440; National Institutes of Health, Bethesda, MD, USA). The area staining positively for IL-1β, IL-6, RANKL and TNF-α was measured under a × 20 objective and calculated as a percentage of the total area from all sections of both the tension and compression sides.

Statistical analysis

The data were evaluated using SPSS software, version 17 (SPSS Inc., IBM, Chicago, IL, USA). All results are expressed as mean ± standard error of the mean. The differences between the groups in the percentage of area staining positively for IL-1β, IL-6, RANKL and TNF-α, and in the number of TRAP-positive cells, were assessed using one-way ANOVA with Scheffé's *post-hoc* test for normal distributed data or by the Kruskal–Wallis test with Holm's sequential Bonferroni correction. Statistical significance was assumed to be $p < 0.05$.

Results

Histomorphometry analysis

Immunohistochemical staining for RANKL on the pressure side is presented, for the different groups, in Fig. 1. Positive immunostaining of this cytokine was found in the alveolar bone with an apparent difference between the Spring and Spring + ESWT groups. Figures 2 and 3A describe the percentage of the area that stained positively for RANKL and IL-1β, respectively. The percentage of the area that stained positively was statistically significantly higher in the Spring group compared with the Spring + ESWT group, on the compression side, on the first day for both cytokines

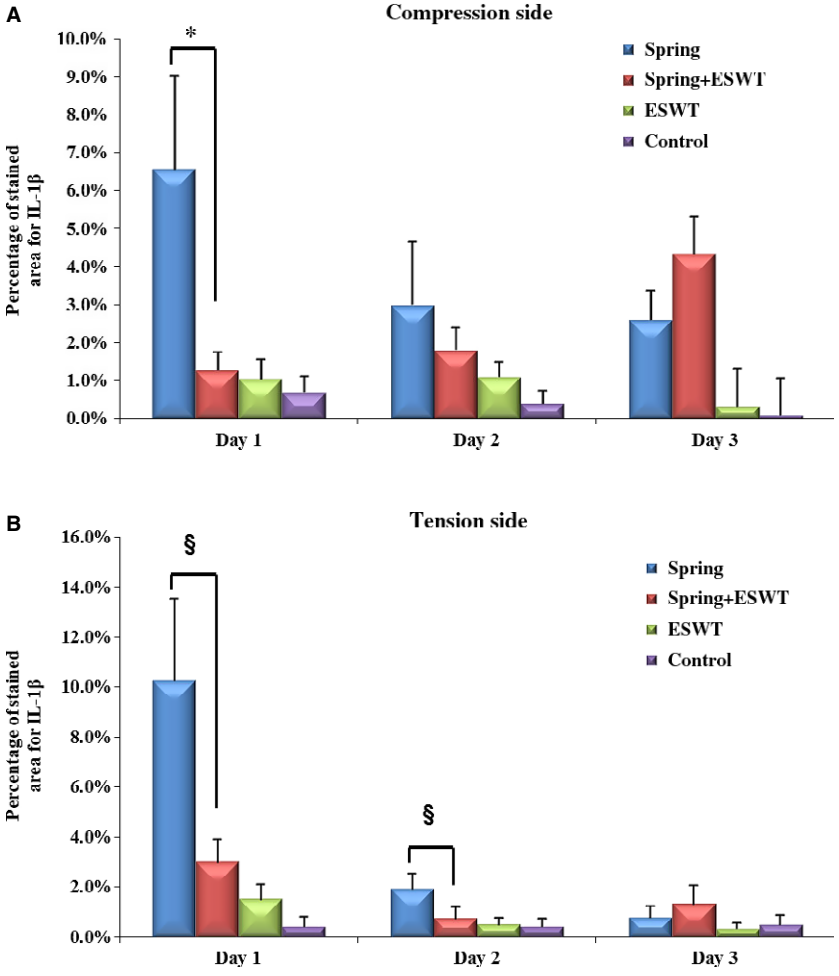

Fig. 3. Percentage of the area staining positively for interleukin-1β (IL-1β) during the 3 d of the study. (A) Compression side. (B) Tension side. Control, without an orthodontic appliance or extracorporeal shock wave therapy (ESWT) (control); ESWT, ESWT without an orthodontic appliance; Spring, with an orthodontic appliance and without ESWT; Spring + ESWT, with an orthodontic appliance and ESWT. Data represent mean + standard error of the mean. *$p < 0.05$, §$p < 0.1$.

($p = 0.008$ and $p = 0.036$, respectively) and on the second day only for RANKL ($p = 0.02$). Although, regarding RANKL, we did not find any difference between the groups on the tension side during all days of the study, the Spring + ESWT group showed a lower percentage of area staining positively for IL-1β, with a tendency towards statistical significance compared with the Spring group on both day 1 and day 2 ($p = 0.058$ and $p = 0.051$, respectively) (Fig. 3B).

Figures 4 and 5 describe the percentage of the area that stained positively for TNF-α and IL-6, respectively. The percentage of the area staining positively for TNF-α in the Spring group was statistically significantly higher compared with that of the Spring + ESWT group, on both sides, on most days ($p \leq 0.05$) (Fig. 4). However, regarding IL-6, this result remained only on the tension side and only for the first day ($p = 0.039$) (Fig. 5).

TRAP staining

The number/area of TRAP-positive cells was statistically significantly higher in the Spring group than in the Spring + ESWT group after the first and second days, on the compression side ($p = 0.035$ and $p = 0.029$, respectively). However, no difference was found between these groups after the third day (Fig. 6).

Discussion

This study presents the effects of ESWT on the histological immunostaining of several different cytokines in periodontal tissues after induction of orthodontic force. A force of 20 cN was distributed over the two first-maxillary molars. As a human molar is approximately 20 times larger than a rat molar (27), the effect of the force could be estimated to be the same as a force of 200 cN (20/2*20) on a human molar, which is considered a reasonable orthodontic force (28).

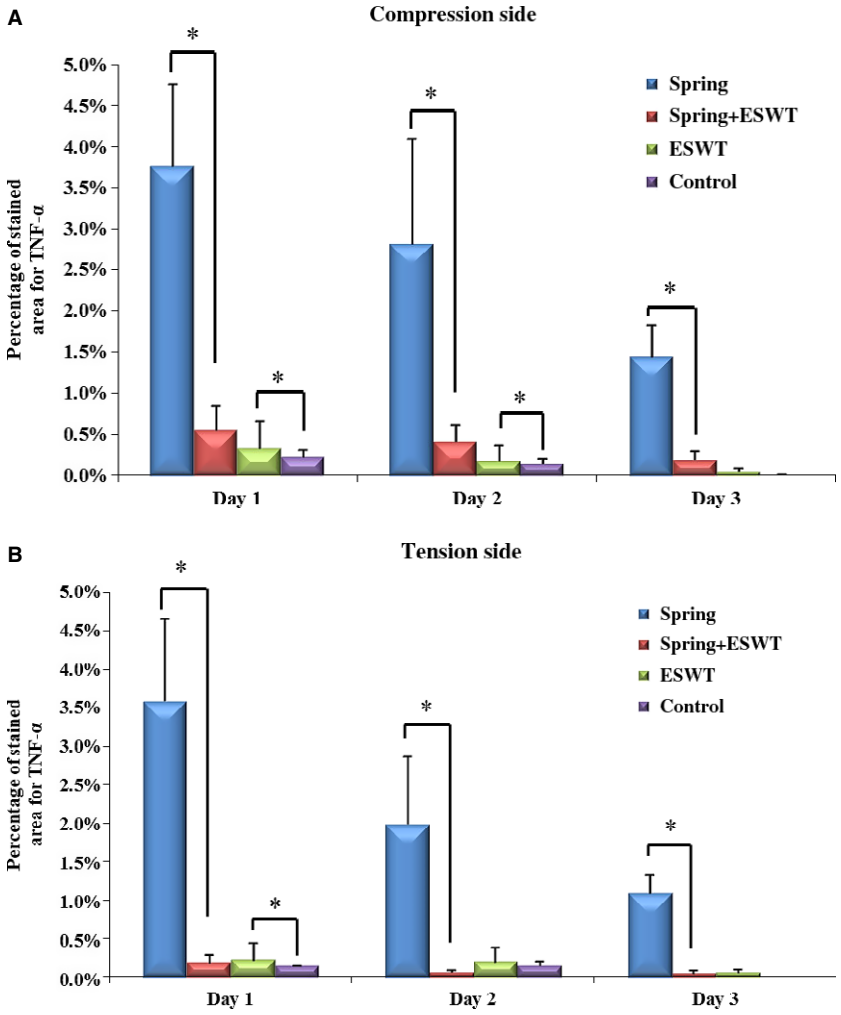

Fig. 4. Percentage of the area staining positively for tumor necrosis factor-α (TNF-α) during the 3 d of the study. (A) Compression side. (B) Tension side. Control, without an orthodontic appliance or extracorporeal shock wave therapy (ESWT) (control); ESWT, ESWT without an orthodontic appliance; Spring, with an orthodontic appliance and without ESWT; Spring + ESWT, with an orthodontic appliance and ESWT. Data represent mean + standard error of the mean. *$p < 0.05$.

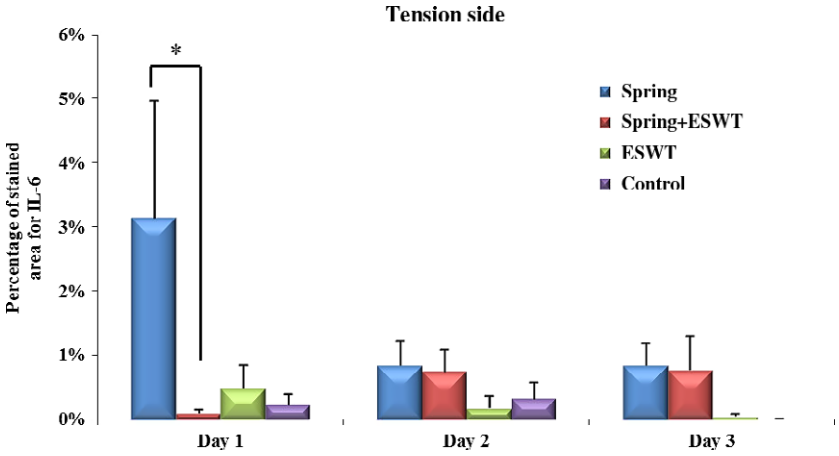

Fig. 5. Percentage of the area staining positively for interleukin-6 (IL-6) on the tension side during the 3 d of the study. Control, without an orthodontic appliance or extracorporeal shock wave therapy (ESWT) (control); ESWT, ESWT without an orthodontic appliance; Spring, with an orthodontic appliance and without ESWT; Spring + ESWT, with an orthodontic appliance and ESWT. Data represent mean + standard error of the mean. *$p < 0.05$.

Comparision between ESWT (positive) and sham (negative) control groups

Several studies have reported that the application of ESWT on cells obtained from different tissues such as bone, tendon etc. caused a marked elevation in the expression of several cytokines (29–33). However, in this *in-vivo* study, no statistically significant difference was found, on any of the study days, in the percentage of the periodontal area of both the positive and negative control groups that stained positively for any of the cytokines analyzed (except for TNF-α), on both compression and tension sides. This finding was also evident in the gingival crevicular fluid of rats, as recently reported (H. Hazan-Molina, A. Reznick, H. Kaufman, D. Aizenbud, submitted). In contrast to the *in-vitro* studies, the application of ESWT should always follow an inflammatory induction process, such as raising a skin flap (34), ischemia-induced myocardial dysfunction (11), periodontal disease (9) or activation of a spring during orthodontic force application. ESWT application alone might not be sufficiently effective to cause marked changes in the expression of several cytokines in the periodontal tissue.

Furthermore, TNF-α is a principal cytokine involved in mediating acute inflammation and apoptotic cell death. It is possible that the shock waves themselves caused physical trauma to the periodontium, leading to the induction of acute inflammation and apoptosis, thus creating an increase in the percentage of the area staining positively for TNF-α in the ESWT group compared with the negative-control group. Tamma *et al.* (33) reported that the application of

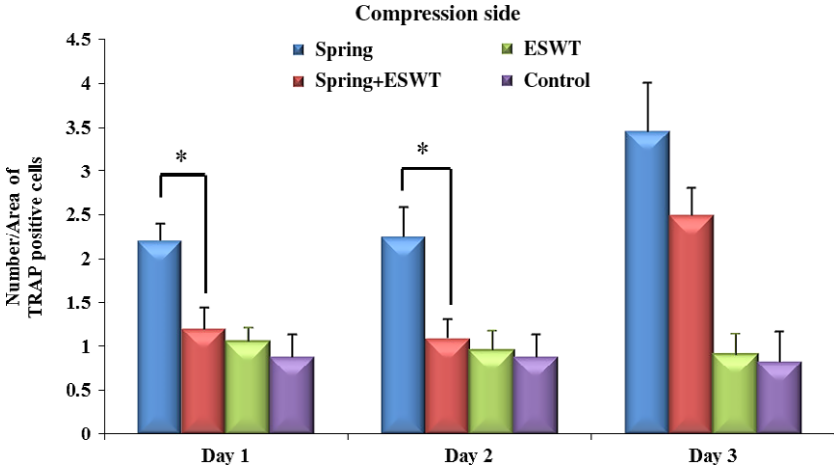

Fig. 6. Number/area of TRAP-positive cells on the compression side during the 3 d of the study. Control, without an orthodontic appliance or extracorporeal shock wave therapy (ESWT) (control); ESWT, ESWT without an orthodontic appliance; Spring, with an orthodontic appliance and without ESWT; Spring + ESWT, with an orthodontic appliance and ESWT. Data represent mean + standard error of the mean. *$p < 0.05$.

ESWT on osteoblasts led to a fast pro-apoptotic effect on the osteoblasts directly reached by ESWT, in addition to the immediate lysis of some of the cells.

Comparison between the Spring and Spring + ESWT groups

The anti-inflammatory effect of ESWT is also validated in the current study, as the percentage of the area staining positively for all periodontal inflammatory cytokines during the first 2 d of the study on both compression and tension sides was reduced in a statistically significant manner in the Spring + ESWT group compared with the Spring group. This is complementary to a previous report in which only the concentration of RANKL in the gingival crevicular fluid among the same cytokines was statistically significantly reduced on the first day (H. Hazan-Molina, A. Reznick, H. Kaufman, D. Aizenbud, submitted).

Moreover, IL-1β has been known to promote osteoclastogenesis by inducing RANKL expression on stromal cells and synergizing with RANKL to promote later stages of osteoclast differentiation (35–38). This scenario might be supported by our study, as on the first day we observed a peak in the percentage of the area staining positively for IL-1β and RANKL on the compression side of the periodontium, both in Spring and in Spring + ESWT groups. Sequentially, on the second day following application of force, the number of TRAP-positive cells in those two groups increased. The statistically significant difference between the Spring and Spring + ESWT groups in the percentage of the area staining positively for RANKL and in the number of TRAP-positive cells, on the compression side, on the first and second days after application of force, supports the positive effect of ESWT on periodontal bone formation and suggests its inhibitory effect, as reported by Tamma *et al*. (33).

In a preliminary study (39), we observed a contrasting result regarding the IL-1β concentration in the gingival crevicular fluid: it was statistically significantly higher on the second day in the Spring + ESWT group compared with the Spring group. Yet, the number of TRAP-positive cells per area was reduced in the Spring + ESWT group compared with the Spring group, on the compressed side of the periodontal ligament, on day 3, as presented in this report.

This difference might be explained by the use of different cytokine detection methods: ELISA in the preliminary study (16,39) and immunohistochemical staining in this study. The inability to detect cytokines other than IL-1β in the gingival crevicular fluid in the preliminary study by ELISA (16,39) and the contrast in the expression pattern between IL-1β- and TRAP-positive cells led us to search for a different and more sensitive detection method in a different medium. Immunohistochemical staining is a very sensitive and specific method for the detection of different proteins in tissue. The fact that the inflammatory cytokine-expression patterns could be replicated in a consistent manner in all groups may indicate that immunohistochemical staining is a more sensitive and appropriate detection method for cytokines in tissue compared with determination of cytokine concentration in the gingival crevicular fluid by ELISA. If detection and quantitation of different proteins in body fluids of small volumes (like gingival crevicular fluid) is desired, a more sensitive and specific detection method should be considered, such as multiplex fluorescent bead-based immunoassay.

A somewhat constant percentage of area staining positively for RANKL was found in the Spring + ESWT group, in contrast to its decrease in the Spring group during the 3 d of the study. However, a similar rate of increase in TRAP-positive cells in both groups was indicated by the similar increases of the bar charts in Fig. 6. Thus, although there was a statistically significantly smaller percentage of area staining positively for RANKL and a statistically significantly smaller number of TRAP-positive cells in the Spring + ESWT group compared with the Spring group, the rate of increase in TRAP-positive cells was the same between the groups. This possibly indicates a similar rate of periodontal bone removal on the compression side, although with a smaller number of cells. These findings may indicate more efficient orthodontic tooth movement in the Spring + ESWT group.

In light of this current study and other studies [(9), H. Hazan-Molina, A. Reznick, H. Kaufman, D. Aizenbud, submitted, (33,40,41)], ESWT may be implemented to reduce the periodontal inflammatory process and inhibit osteoclastogenesis. The practical use of ESWT in the periodontal disease healing process, with or without orthodontic force stimuli, may include the treatment of different periodontal lesions, involving acute and chronic inflammatory reactions with damage to the alveolar bone. Its overall clinical effect on orthodontic tooth-movement biology and the velocity of the orthodontic process still needs to be determined.

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