The Effect of Different Doses of Extracorporeal Shock Waves on Experimental Model Mandibular Distraction

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Objective: Distraction osteogenesis (DO) is widely used treatment for the bone deformities. In addition extracorporeal shock wave therapy (ESWT) is a new perspective on noninvasive modalities of management of the bone regeneration. We examined the effects of 2 different single doses of ESWT on the consolidation period of DO of the rabbit mandible using stereological, radiological and immunohistochemical methods in the present study.

Methods: DO was performed unilaterally in the mandible of 18 New Zealand rabbits (6 months' old, weighing between 2.5 and 3 kg). The distraction zone of the mandible has received no treatment as controls. Group 2 (ESWT 500) received ESWT (single dose of 500 impulses 0.19 mJ/mm^2 energy flux intensity and 2155 mJ totally) were applied on the first day of the consolidation. Group 3 (ESWT 1000) treated with ESWT (single dose of 1000 impulses0.19 mJ/mm^2 energy flux intensity and 4310 mJ totally) were applied on the first day of the consolidation period. After the sacrification, radiologically bone mineral density, new bone formation, new fibrous tissue, and new vessel formation were analyzed using unbiased stereological methods.

Results: It was found a statistically significant difference between the study groups and control group in the bone mineral density measurements and the highest values were in the ESWT1000 group. In terms of stereological analysis, there was a significant difference between the study groups and control group ($P = 0.00$). The new capillary volume was highest in the E1000 group. Additionally, significant differences were found in point of the capillary volumes between the groups control and ESWT500 ($P = 0.001$), control and ESWT1000 ($P = 0.000$), ESWT500 and ESWT1000 ($P = 0.040$), respectively.

Conclusions: A total of 1000 impulses ESWT may induce the growth factors to enhance the newly formed bone regeneration.

Key Words: Cavalieri principle, distraction osteogenesis, extracorporeal shock waves, growth factors

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The treatment of bone deformities is a major challenging and alternative approaches can be applied in addition to several surgical methods. For the bone defects, common therapies are guided bone regeneration, bone grafts and distraction osteogenesis (DO).[1] The DO is a process in which tensile strength application to osteotomized bone segments and it results in bone regeneration.[1] In addition, the DO is commonly preferred in the treatment of congenital or acquired craniomaxillofacial defects.[2] The DO technique was used for the treatment of facial deformities in 1990 for the first time.[3] Additionally, it has been indicated that DO is considered as an effective approach in the treatment of craniosynostosis without any statistical disadvantage in terms of operative time, blood loss, need for transfusion or intensive care unit resources.[4] However, in this method, it takes a long time for the bone to dissipate and mature. This can be considered as a major disadvantage of the technique.[5] Several experimental studies have been conducted on shortening the duration of treatment and increasing osteogenesis, focusing on reducing or preventing these complications.[6–8] Various methods have been described that focus on promoting callus, such as low-intensity ultrasound, electrical stimulation, low-level laser, and growth factors.[9–16]

Extracorporeal shock wave treatment (ESWT) is considered as a one of the treatment approaches in the various disorders such as the musculoskeletal system fractures, lateral epicondylitis, Peyronie disease, calcified tendinitis, plantar fasciitis, and Achilles tendinopathy.[17–20] It was previously indicated that the expression of the growth factors related to the angiogenesis is boosted by ESWT. In the study of Williams[6] that is conducted on patients with musculoskeletal disorders like pseudoarthritis and tendinopathy, it was shown that the success rate for the treatment was high in patients subjected to ESWT. Also, in a previous study, it was shown that ESWT is effective in the treatment of the myofascial syndrome.[21] Similarly, Pfaff et al (2016) have investigated the increase in the mandibular bone regeneration. Also they have reported that growth factors induced the osteogenesis and angiogenesis and ESWT enhanced the bone healing.[22] However, there is not enough study about the use of ESWT in mandibular DO.

The studies have reported that ESWT application stimulates the new bone formation via leading the formation of microfractures. However, underlying mechanism of the regenerative effect of ESWT in the bone defects is not extensively understood. The recognized advantages of the ESWT over other methods are its property of being conservative, its effectiveness, its low cost, and its low rate of complication.[23] In addition, Van der Jagt et al (2009) have suggested that ESWT enhances the differentiation of bone marrow cells into osteoprogenitors and induces the growth factor releasing. They examined the bone architecture of ESWT—treated and untreated rat tibiae using microcomputed tomography (CT) scanning to evaluate the effects of ESWT on osteoporosis.[24]

The effect of the ESWT on the new bone formation in the distracted callus in the maxillofacial region has still been investigating. In the present study, we aim to research the effects exerted by different single doses of ESWT on mandibular DO with the help of histological, immunohistological, and unbiased stereological techniques as well as the radiological approach.

MATERIALS AND METHODS

In this study, 18 New Zealand rabbits, that are 6 months of age and with weights ranging between 2.6 and 3.2 kg, were used. The animals were obtained from Experimental Animals Research and Applying Center of Ondokuz Mayis University and were divided in a random manner into 3 groups, which are involved the control group and 2 experimental groups. The study was supported by the foundation of scientific research projects of the Ondokuz Mayis University.

All surgical procedures and experimental design including distraction processes were performed according to the study of Bereket et al.[25] The submandibular extraoral skin incision was performed along the inferior border of the mandibular body. A straight mandibular body osteotomy immediately anterior to the first premolar root was made with a surgical saw. Custom made bone-borne distractors were adapted along a plane perpendicular to the osteotomy (Fig. 1A). The periosteum, muscle, and skin were sutured in layers. After the operation, additional antibiotics (30 mg/kg of long-acting oxytetracycline) were administered intramuscularly, twice per week for 2 weeks. For pain relief, buprenorphine (0.03 mg/kg) was administered subcutaneously, twice daily for a week.

ESWT Applications

Following the 5 days latency period, the animal's mandibles were distracted at 0.35 mm twice daily (0.7 mm/day) for 10 days. The animals were divided into 3 groups, which are involved 2 experimental, and the control groups. Each group has 6 animals. The first group was designed as the control group (control, n = 6) and ESWT was not applied. In the 2nd group (ESWT500, n = 6) the single dose of 500 impulses ESWs at 0.19 mJ/mm^2 (energy flux intensity) and 5 Hz (2155 mJ totally) were applied using an ESWT device (OE050 focused applicator, Orthogold 100, MTS, Konstanz, Germany). In the 3rd group (ESWT1000, n = 6), the single dose of 1000 impulses was applied (4310 mJ totally) using the same device. Extracorporeal Shock Waves (ESW) were performed after the application of surgical lubricant gel to the skin on the 1st day of the consolidation period to the distraction zone. The dosage and the timing of ESWT were selected on the basis of literature.[26]

At the end of the 28 days of a consolidation period, the rabbits were sacrificed with high-dose sodium pentobarbitone (Pental; IE Ulagay, Istanbul, Turkey).

Histological and Stereological Analyses

Stereological evaluation of the samples was carried out at OndokuzMayis University Faculty of Medicine, Department of Histology and Embryology. Firstly, the tissues were decalcified in formic acid (%5) for 21 days. Following routine histological processing, 7-μm-thickness serial sections were taken according to systematic random sampling strategies using a rotary microtome (Leica RM 2135RT; Leica Instruments, Nussloch, Germany). According to the systematic random sampling manner, one of every 10 sections was selected. Then sections were stained using hematoxylin-eosin (HE) staining procedure and photographed by using light microscopy (Leica M 4000 B, Germany), with the color digital camera (Microbrightfield, Williston, VT) in stereological analysis system (Stereoinvestigator 9.0, Microbrightfield). The Cavalieri method, which is an accurate and fast stereological approach to volume calculation, was applied to estimate the total volume of new bone, connective tissue infiltration, and capillaries using light microscopy images.[26,27] Point-counting grids were applied to light microscopic images in the determination of area. The point density was determined considering acceptable coefficient of error (CE) after performing the pilot study. A grid space of 0.5 cm was used to cover the regions of interest. The CE was determined to be in the acceptable ranges (CE < 0.05) for volume estimation. The CE and the coefficient of variation were determined in accordance with the following formula:

Volume = t x a/p x $\sum$p ("t," section thickness; "a/p," the area representing each point on the point-counting grid (Dark blue

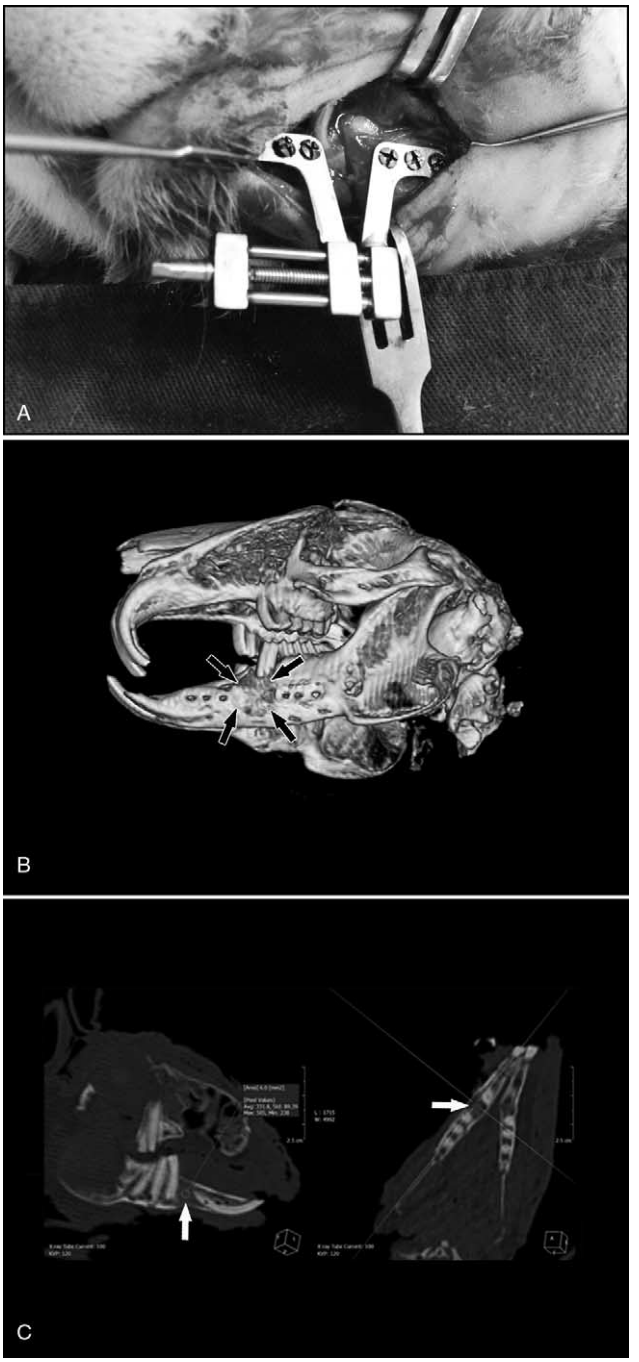

FIGURE 1. (A) Intraoperative view after the placement of the custom-made distractor device. (B and C) Computerized tomographic images of the distracted area (arrows).

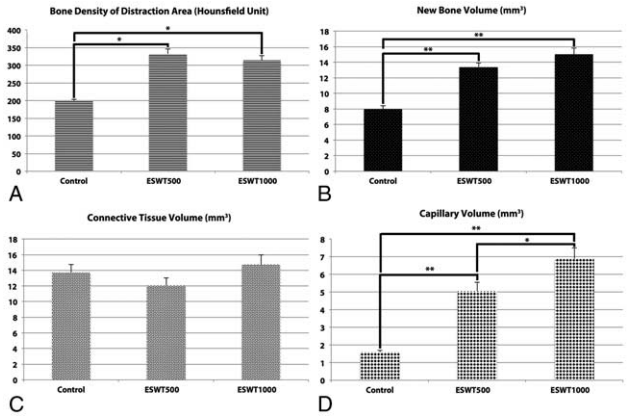

FIGURE 2. (A) The graphic showing bone density values of the control and laser groups. (B) The new bone volumes of the groups. (C) The connective tissue volumes of the groups. (D) The capillary volumes of the groups.

square); "$\sum$p," the total number of points that hit to the interested area (red points).

Immunostaining Procedures

The immunohistochemical staining of sections also was performed on sections using anti-bone morphogenetic protein (BMP)-7, anti-transforming growth factor β1 (anti-TGFβ1), anti- vascular endothelial growth factor (VEGF), and anti-collagen antibody (Abcam, Cambridge, MA). In the immunohistochemical analyses, a streptavidin-biotin complex (Abcam) was used. HRP/DAB chromogen kit (Abcam) was used as chromogen and the slides were counterstained with Mayer hematoxylin (Sigma, Saint Louis, MO).

Radiologic Evaluation

CT images were obtained from all groups after the consolidation period (Fig. 1 B and C). Therefore, the positions of all rabbits were arranged with the occlusal plane perpendicular to the horizontal plane. Images were obtained using HR-CT (Aquilion 16 system, Toshiba Medical System Corporation, Tochigi-ken, Japan) in accordance with the standard protocol. Axial slices were obtained from the mandible samples including all distracted area. Density measurements using Hounsfield Units (HU) were performed, in the determined area, located between the distracted bone segments.[27] The measurements of the bone density values were carried out twice with the same examiner.

Statistical Analysis

The data were compared with 1-way analysis of variance using SPSS (Ver: 21, IBM Corporation for Mac) statistics program. Post-hoc Tukey test was used in the multiple comparisons. All comparisons were considered significant at $P < 0.05$.

RESULTS

Radiological Assesment

The data obtained from the measurements of the bone density (Hounsfield Unit) of the distracted area in Computed Tomography, the bone density values belonging to the experimental groups were noted to be higher than the control group in comparison (Fig. 2A). The highest values were found in the T1000group. Although there was no significant difference between ESWT500 and ESWT1000 groups ($P > 0.05$), there were significant differences between the groups control and ESWT500 ($P = 0.003$), Control and ESWT1000 respectively ($P = 0.013$).

Volumetric Assessment

In terms of newly formed bone volume, there were significant differences in the ESWT500 and ESWT1000 groups compared to control group ($P = 0.000$ and $P = 0.000$, respectively). However, no significant difference was found between the experimental groups ($P > 0.05$)(Fig. 2B).

When compared the groups in terms of the connective tissue volume, there were no significant differences between the all groups ($P > 0.05$) (Fig. 2C).

Additionally, significant differences were found in point of the capillary volumes between the groups Control and ESWT500 ($P = 0.001$), Control and ESWT1000 ($P = 0.000$), ESWT500 and ESWT1000 ($P = 0.040$), respectively (Fig. 2D). The highest capillary volume value was found in the ESWT1000 group.

Immunohistochemical Findings

When examined the immunohistochemically stained slides, the density of positively stained cells have supported the data obtained from stereological analyses. Especially, the most intense staining with anti-VEGF, anti-collagen antibody, and anti-BMP-7 was observed in the ESWT1000 group in immunohistochemical staining (Fig. 3).

DISCUSSION

DO is a current approach in the treatment of maxillofacial deformities but some major postoperative problems may occur during treatment because of long periods of consolidation. Therefore researchers have performed a variety of studies, which aimed at reduce the consolidation time via increasing new bone formation and maturation in the distracted area.

It has been shown that several biostimulative treatment modalities such as low-level laser therapy, ultrasound, hyperbaric oxygen, electrophysiological and electromagnetic field applications have also accelerated new bone formation in some studies.[8,13,28–32]

ESWT is a noninvasive method for treatment of bone fractures.[17,18,28–32] It has been reported that ESWT accelerates healing in long bones by increasing cell differentiation and neovascularization in animal models.[33] The other study performed on rabbits has reported that ESWT enhances the callus amount and biomechanical properties.[34] However, there are a few studies of the effects of ESWT on new bone formation in mandibular DO.[35] In this context, in our study, possible positive effects of ESWT on regeneration were investigated in the DO model with different single doses (500 and 1000 doses).

It has been investigated how the new bone formation is affected by different doses of ESWT using stereological methods. It was reported that the low dose of ESWT (500 impulses) has a positive effect on the increase in the new bone volume. This possible effect is evidenced by the fact that volume of the new bone is at the peak level in ESWT1000 group. Also, it was evident that the mineral density of the bone (HU) was increased in ESWT1000 group.

Our results are consistent with the findings of Lai et al.[35] However, their results showed that the ESWT dose has a positive effect on the DO consolidation, which is different from our study (500 pulses at 14 kV and 500 pulses at 21 kV). In a study focusing on the influence of ESW, it was observed that ESW administration caused significant capillary formation and new trabecular production.[28] It was not possible to compare our results with others because of the differences in energy density of ESWT, shock wave production type, shot counts, and devices.[17,18,35,36]

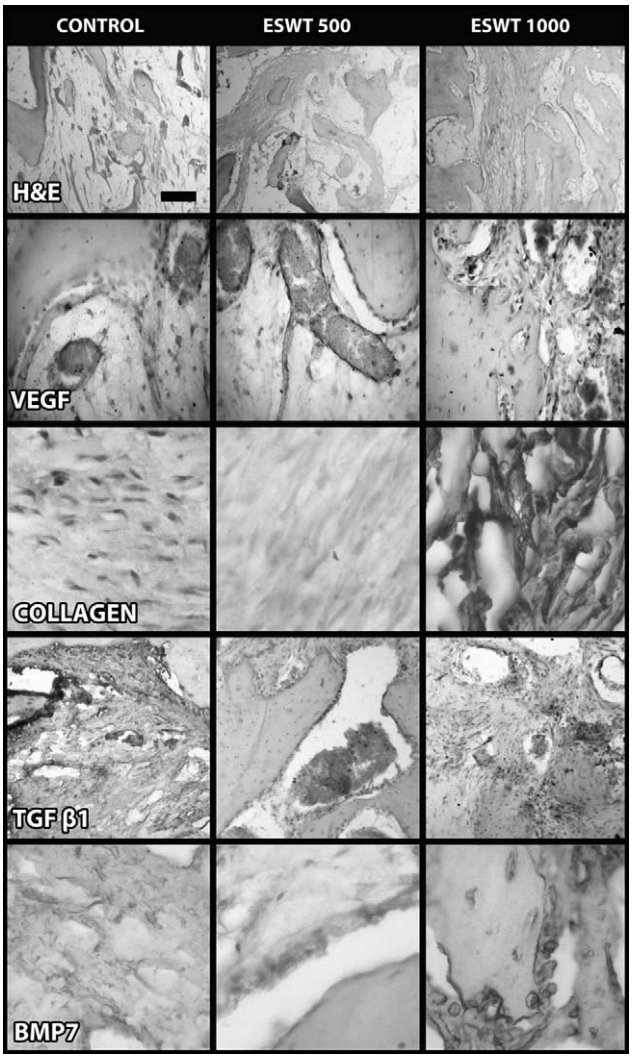

FIGURE 3. Representative histological and immunohistochemical images of samples. The density of positively stained cells was supported the data obtained from stereological analyses. Especially, the most intense staining with anti-VEGF, anti-collagen antibody, anti-BMP-7 was observed in the ESWT1000 group. (Bar 250 μm). ESWT, extracorporeal shock wave therapy; TGF-β1, transforming growth factor; VEGF, vascular endothelial growth factor.

Biochemical and catabolic reactions in fractured bone tissue are caused by stimulations of biophysical type, by the alteration of various growth factors. ESWT causes to alterations in the expressions of the angiogenic and osteogenic growth factors that function in boosting the bone regeneration. Wang et al investigated that ESWT possesses biological effects on bone healing, using a model of fracture in rabbits.[18] They suggested that there was an increase in the bone durability, there was evidence of more cortical bone formation, and neovascularization and that angiogenic growth factors like VEGF, endothelial nitric oxide synthase (eNOS), BMP-2, and proliferating cell nuclear antigen have higher secretion levels in the experimental group. Furthermore, Sathiskumar et al investigated the effects of ESWT on alveolar bone regeneration in rats and they have found higher levels in rats subjected to 300 and 1000 pulse ESWT.[35] Lai et al[26] have reported that ESWT has a high ability to increase angiogenesis and bone regeneration and reduce the time needed to complete the consolidation process. Data obtained from this study support the above-mentioned literature.

Our study indicated that newly formed bone and capillary volumes in the ESWT1000 group are significantly increased in comparison to other groups. The stereological data in this study were supported with increased BMP-7 and VEGF expressions according to immunostaining analyses. Although osteogenesis emerged in all groups, the higher rate of bone formation in ESWT1000group was considered to be related to the increased contribution of BMP-7 to bone healing at a late stage. During the natural course of bone healing, BMP-7 levels rise after 2 weeks. It was indicated that BMP-7, BMP-2, and BMP-4 play a similar role in DO. Likewise, an increased level of VEGF leads to a higher rate of neovascularization. The presence of markedly voluminous neovascular structures, bone, and increased collagen deposition in the ESWT1000 group suggests that even at late stages the bone regeneration is maintained in this group. Additionally, this finding suggests that there is a tight coordination between areas of neovascularization and new bone formation.

In this study, ESWT's different treatment protocols on DO were analyzed. The increase in the regeneration of the distracted bone, induced by ESWT, may be related to the expression of growth factors. Our immunohistochemical findings, especially enhanced VEGF expression, supported this statement. In another approach, in vitro and in vivo studies have suggested that ESW treatment enhances the proliferation and differentiation of fibroblast. In this context, ESW may activate the gene expression for TGF-β1 and collagen types III and I. In addition, it was reported that an increase in nitric oxide release is also present in the early phase of treatment. Following this, activation of eNOS and VEGF may be related to TGF-β1.[36]

In this regard, further studies are required in this area to the determine the optimum dose of ESWT, which is a new treatment. Additionally, new experimental studies are required for a better understanding. The prevention of the potential side effects that might arise in the period of long DO treatment may be facilitated by the positive contribution of ESWT to quality and mineralization of bone. However, it is required to shed light on this subject with experimental studies.

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