Effect of Electrohydraulic Extracorporeal Shockwave Therapy on the Repair of Bone Defects Grafted With Particulate Allografts

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Abstract: This study determined the effect of electrohydraulic extracorporeal shockwave therapy (ESWT) on the healing of mandible defects repaired using particulate allogenic bone grafts. This study included 20 male Wistar rats aged 12 weeks. In all the animals, a critical-sized defect of 4-mm diameter was created in the mandible and the defect area was filled with particulate allograft. Next, the rats were divided into 2 groups, allograft (G) (n = 10) and allograft + ESWT (GE) (n = 10). On days 3, 5, and 7 after the grafting, rats in the GE group received ESWT involving 200 pulses with an energy flux density of 0.19 mJ/mm^2. Five rats in each group were sacrificed at the end of week 4 and at the end of week 8. Defect areas were examined radiologically by performing high-resolution computed tomography and stereologically by using the Cavalieri method. Obtained data were compared by performing statistical analysis. Radiological evaluation showed that bone density was higher in rats in the G group than in those in the GE group at week 4. In contrast, bone density was higher in rats in the GE group than in those in the G group at week 8. Stereological examination showed that new bone, connective tissue, and capillary volumes were higher in rats in the GE group than in those in the G group at both weeks 4 and 8. The authors' results indicate that repeated doses of ESWT accelerate the healing of bone defects repaired using allogenic bone grafts.

Key Words: Allograft, bone healing, electrohydraulic, shockwave

(*J Craniofac Surg* 2019;30: 1298–1302)

Bone defects in the maxillofacial region may occur congenitally or after surgical intervention and trauma. These defects heal spontaneously if their size does not exceed the critical size.[1] However, large bone defect healing may occur with fibrous tissue formation. Over the last 30 years, various bone grafts and substitutes[2] and biostimulative modalities (low-dose laser, low-density ultrasonography,[3] electrical and mechanical stimulation,[4,5] and extracorporeal shockwave therapy [ESWT][6]) have been examined for inducing bone regeneration in these defects.

Although autografts are the gold standard for treating bone defects, they are associated with disadvantages such as limited availability of materials and morbidity in donor site.[7] To prevent these complications, particulate allografts are used in maxillofacial and periodontal surgeries.[8–12] However, some procedures used for developing particulate allografts result in the loss of approximately 50% osteoinductivity, osteoconductivity, and mechanical properties.[13] Moreover, incorporation of particulate allografts in defect areas requires a long time (approximately 4–12 months).[8,12,14] Complete bone regeneration in the grafted region depends on several factors, including level of neovascularization; stimulation of growth factors; and proliferation, migration, and differentiation of osteoprogenitor cells.[15]

Extracorporeal shockwave therapy is a noninvasive and safe treatment modality that is recognized worldwide. Although shockwaves were first used to disintegrate renal stones and gallstones, they have been recently used for treating various orthopedic disease such as delayed and nonunion fractures, osteonecrosis, osteoarthritis, plantar fasciitis, calcific tendinitis, and lateral epicondylitis and have provided positive results.[16] Moreover, shockwave therapy increases the proliferation and differentiation of mesenchymal cells and stimulates the release of growth factors, thus inducing angiogenesis and osteogenesis.[16–18]

However, it is unclear how ESWT exerts osteostimulative effect. Experimental studies have shown that shockwave therapy induces osteogenesis and angiogenesis through various mechanisms as stated below. Moreover, ESWT induces bone turnover and type 1 collagen and osteocalcin production.[19] Furthermore, ESWT stimulates neovascularization and osteogenesis by increasing the release of important mediators such as bone morphogenetic protein-2, proliferating cell nuclear antigen, endothelial isoform of nitric oxide synthase, vascular endothelial growth factor [17,20,21] for bone healing and by increasing blood perfusion.[22] In addition, ESWT triggers a cascade of osteogenic and angiogenic transcription factors (CBFAL/RUNX2, HIF-1a, and vascular endothelial growth factor) in osteoblast cells.[23] One study showed that shockwaves increase the differentiation and proliferation of osteoblasts by inducing nitric oxide synthesis.[24] Another study reported that shockwave-induced osteoprotegerin release enhances ossification.[25]

Several recent studies have reported the positive effects of ESWT on bone healing. However, no study has reported the effect of the combination of ESWT with bone grafts on the healing of bone defects. The present study examined the effects of repeated doses of ESWT on the healing of bone defects repaired using allogenic grafts.

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Received April 2, 2018.

Accepted for publication October 31, 2018.

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This experimental study was supported by Project Management Office Coordinatorship of Ondokuz Mayis University with PYO.-DIS.1904.12.006 project code.

The authors report no conflicts of interest.

ISSN: 1049-2275

DOI: 10.1097/SCS.0000000000005213

METHODS

This study included 20 twelve-week-old male Wistar albino rats weighing 280 ± 15 g. The rats were obtained from the Laboratory Animal Center of Ondokuz Mayis University. During the study period, the rats were kept in cages maintained in rooms with a 12-/12-hour light/dark cycle, temperature of 22 ± 2°C, and average relative humidity of 50% and were given free access to standard food and drinking water. Moreover, the rats were examined daily by a veterinary doctor during the study period. This study was approved by the Animal Experiment Local Ethics Committee of the Ondokuz Mayis University (2011/64) and was supported by the Project Management Office of the Ondokuz Mayis University (PYO.DIS.1904.12.006).

Surgical Procedure

To induce general anesthesia, each rat received an intraperitoneal injection of 10 mg/kg ketamine hydrochloride (alphamine 10%; Alfasan International BV, Woerden, The Netherlands) and 3 mg/kg xylazine hydrochloride (Alfazyne 2% injectable; Alfasan International BV) before the surgery. The region for the surgery was shaved and sterilized using an antiseptic solution (povidone iodine). The animals were covered with a sterile cloth, with only the surgical area being exposed. Local anesthesia was induced by treating the surgical area with 0.5 mL articaine infiltration (Ultracain-DS; Hoechst Marion Roussel, Istanbul, Turkey). Next, the animals were stabilized by making them lay on their side, and a 1-cm incision was made in a posterior–anterior direction at the base of the right angulus. A full-thickness flap was designed showing the lateral surface of the ramus of the right mandible. Next, a critical-sized bone defect of 4-mm diameter was created using a low-speed trephine drill under saline irrigation. The defect area was standardized by leaving a 1-mm bone margin posterior and inferior to the defect. Allogenic particulate graft material (600–1250 μm; Mineross, Osteotech, Eatontown, NJ) was mixed with a sterile saline solution and was placed into the defect area by using a special vehicle (Fig. 1). The incision area in the subcutaneous tissue layers was closed using an absorbable suture (4/0 Vicryl; Ethicon, Brussels, Belgium) and a 4/0 silk skin suture (Doğsan, Trabzon, Turkey).

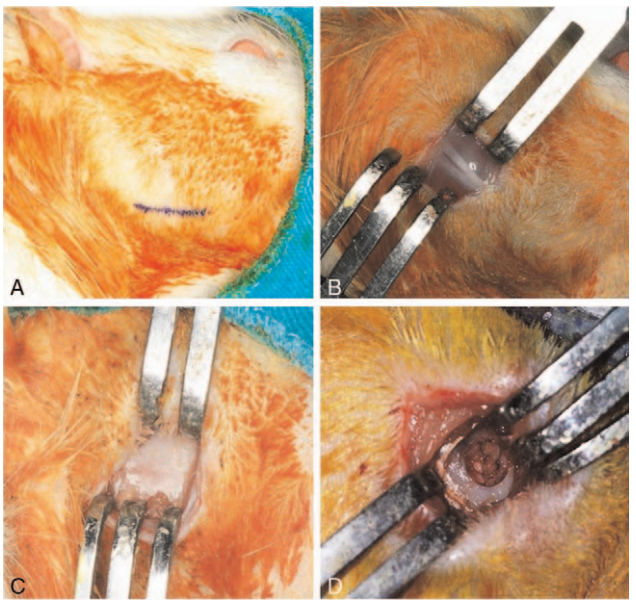

FIGURE 1. Planning of an incision in mandibular basis (A), preservation of the facial nerve branches after skin incision (B), achieving the lateral surface of the ramus by elevating the full thickness flap (C), application of particulate allograft to the critical size defect area (D).

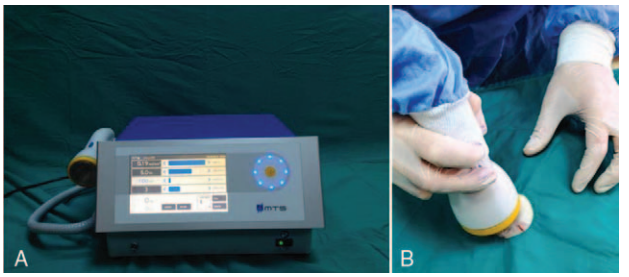

FIGURE 2. Extracorporeal shockwave therapy device and applicator (A), application of extracorporeal shockwave therapy to subjects (B).

For infection prophylaxis and pain control, the rats were postoperatively treated with 50 mg/kg cefazolin sodium intramuscularly (Sefazol; Mustafa Nevzat Co, Istanbul, Turkey) and 5 mg/kg tramadol HCL (Contramal; Abdi Ibrahim Ltd, Istanbul, Turkey) once per day for 3 days. Moreover, the wound area was dressed with an antiseptic solution (Neo-Caf Spray Intervet, MSD, Aprilia, Italy) for 5 days.

Protocol for Extracorporeal Shockwave Therapy Application

On day 3 after the grafting, the rats were randomly divided into graft (G) (n = 10) or graft + ESWT (GE) (n = 10) groups. On days 3, 5, and 7 after grafting, rats in the GE group were administered electrohydraulic-based ESWT (Orthogold 100; MTS, Konstanz, Germany) involving 200 pulses at a frequency of 5 Hz and energy flux density of 0.19 mJ/mm^2 per session. In all the rats, ESWT was administered by the same researcher. Before administering ESWT, the rats were sedated by administering an intraperitoneal injection of 20 mg/kg ketamine hydrochloride (alphamine 10%) and 1 mg/kg xylazine hydrochloride (Alfazyne 2% injectable). The skin of the rats was covered with an ultrasonography gel, and ESWT was administered by positioning an unfocused applicator of OP155 device at a right angle to the defect area (Fig. 2). As a placebo effect, the same procedure was performed with the device being turned off in rats in the G group.

Five rats in each group were sacrificed at the end of week 4 (4G and 4GE groups) and the remaining 5 rats in each group were sacrificed at the end of week 8 (8G and 8GE groups) by intraperitoneally injecting high-dose ketamine hydrochloride (alphamine 10%) and xylazine hydrochloride (Alfazyne 2% injectable).

EVALUATION METHODS

Radiological Evaluation

Bone density (BD) was measured by obtaining a high-resolution computer tomography image by using a multislice CT device with 16 detectors (Aquilion 16 system; Toshiba Medical Systems Corporation, Tokyo, Japan) by using a scanning protocol specified for small animals (tube current: 250 mAS, voltage: 120 kV, matrix: 512 × 512, algorithm: bone, and reconstruction thickness: 0.5 mm). DICOM files were transferred to OsiriX (Pixmeo SARL, Geneva, Switzerland) for analysis. In sagittal section images, BD was measured by superimposing circles with a surface of 13 mm^2 on the defect area and by calculating Hounsfield unit values (Fig. 3).

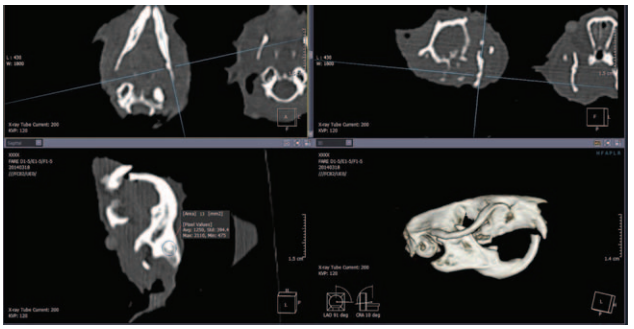

FIGURE 3. Measuring the bone density value of the defect area in images transferred to software program.

Stereological Evaluation

Tissue samples were separated from soft tissue and were immediately fixed in 10% formaldehyde. After fixation, the tissues were decalcified in 5% formic acid solution for 21 days and were embedded in a paraffin block. Next, 5-μm serial sections were obtained in a coronal plane. Next, 1/6th of these sections were selected by performing systematic random sampling, and the selected sections were incubated at 60°C for 1 night. After deparaffinization, the sections were stained with hematoxylin–eosin, defect areas were identified using an optical microscope (Leica DM4000 B; Leica, Tokyo, Japan) under ×10 magnification, and photographs were obtained using a color digital camera (MicroBrightField, Williston, VT) (Fig. 4). The obtained photos were transferred to a stereological analysis system (Stereo Investigator 9.0; MicroBrightField), and volumes of new bone (NB) tissue, connective tissue (CT), and capillaries (CA) were determined using Cavalieri method. The Cavalieri method was performed using point-counting grids with 50-μm intervals. The intervals between the points were determined using a method described by Onger et al.[26]

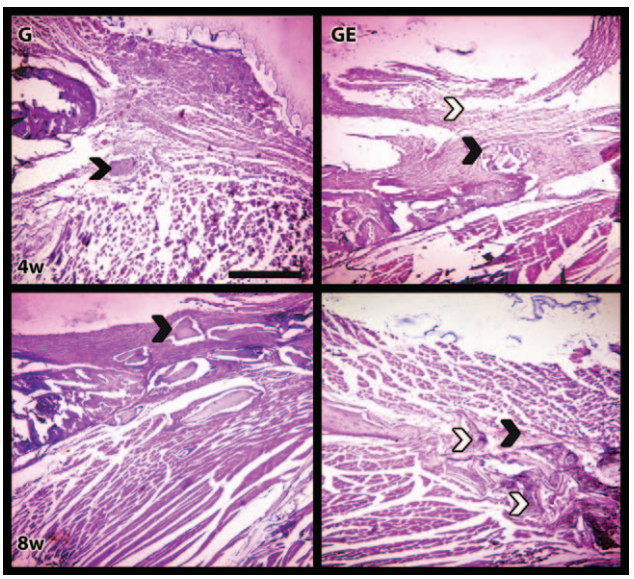

FIGURE 4. Histological appearance of sections obtained from the 4th and 8th weeks of samples. White arrows refer to new bone areas. Black arrows refer to particulate allograft. (Bar: 250 μm, Stain: hematoxilen-eosine).

Statistical Evaluation

Data obtained by performing radiological and stereological evaluations were assessed using SPSS statistical software package (ver. 13.0; Chicago, IL). Comparison between the groups was performed using ANOVA, and pairwise comparison was performed using Tukey post-hoc test. P value of <0.05 was considered statistically significant.

RESULTS

General Findings

We observed that the study protocol was well tolerated by the rats included in the study. However, rats in the GE group (1 rat in 4GE and 1 rat in 8GE) showed swelling for 3 days after the administration of ESWT that did not cause any complication and that was resolved spontaneously.

Bone Density

Bone density values of the defect areas are shown in Figure 5. The highest BD value was obtained for rats in the 8GE group, and the lowest BD value was obtained for rats in the 4GE group. Moreover, rats in these 2 groups showed statistically significant difference in BD values ($P < 0.05$). The mean BD value of rats in the 8GE group was higher than that of rats in the 8G group; however, this difference was not statistically significant ($P > 0.05$). Moreover, no significant difference was observed in the mean BD values of rats in the 4GE and 4G groups.

New Tissue Volume

Rats in both the 4GE and 8GE groups showed higher NB, CT, and CA volumes than those in the 4G and 8G groups. Moreover, rats in the 4GE group showed significantly higher NB, CT, and CA volumes than rats in the 4G group ($P < 0.05$). However, the difference between these parameters was not statistically significant for rats in the 8GE and 8G groups. Moreover, rats in the 8G group showed higher NB, CT, and CA volumes than rats in the 4G group ($P > 0.05$). However, rats in the 8GE group showed lower NB, CT, and C volumes than rats in the 4G group, with a significant difference being observed only with respect to CA volume (Fig. 6).

DISCUSSION

This study is the second one in which the effect of ESWT on mandibular bone defect healing was examined by us. In our previous study that we used same experiment protocol, both sham group and test group were used. According to the results of the

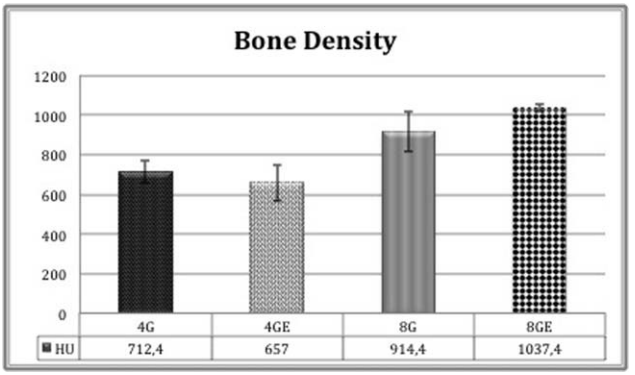

FIGURE 5. Mean Hounsfield Unit from the defect area as a result of high-resolution computer tomography examination.

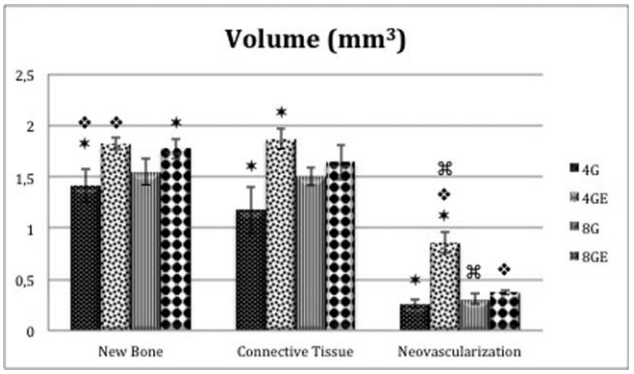

FIGURE 6. Average tissue volumes obtained as a result of stereological analysis. (The same asterisk expresses statistical significance between groups).

study, it was concluded that ESWT was not effective in bone defect healing in which no graft materials were applied.[27] For this reason, only the subjects who had graft applied were included in this study.

Graft materials are used for achieving acceptable level of healing of bone defects from the perspective of structural integrity and functionality. Particulate grafts are often used for guided tissue regeneration (alveolar augmentation, extraction socket protection, and sinus wall augmentation) and maxillary/mandibular reconstruction.[28,29] Several factors, including type of graft, number of living cells in the defect region, growth factors, or vascularity of the defect region, affect graft incorporation.[15,30] Optimum NB tissue formation requires the presence of sufficient number of osteoprogenitor cells in defect region. These cells need to be induced by growth factors and need to undergo revascularization to generate a bone phenotype.[31] Experimental studies have shown that the use of platelet-rich fibrin, which contains growth factors, along with graft materials increases bone healing.[32] Another factor affecting graft incorporation is graft resorption. Rapid resorption of graft material negatively affects regeneration. In contrast, slow resorption of graft material and presence of residual graft material after the completion of bone healing results in the formation of a composite tissue and in incomplete regeneration.[8] Biostimulation methods promote graft resorption and NB tissue formation in the defect region. Although rapid bone regeneration in the grafted region is clinically desirable, no completely successful treatment strategy is available for allograft incorporation at present.[12] The present study assessed whether the biostimulation effect of ESWT because of the formation of new bone and induction of angiogenesis increased graft incorporation.

However, optimum parameters for the induction of osteogenesis by ESWT have not yet been established. High energy density values suppress the osteogenic effect of ESWT, and low energy density values exert an osteostimulative effect.[16] Studies have shown that an average of 500 pulses with low energy flux density (0.16–0.22 mJ/mm^2) are effective for bone regeneration.[33,34] In the present study, we applied 600 pulses of low energy flux density by using an unfocused applicator. We used unfocused shockwaves in the present study to induce a regional effect and thus to increase angiogenesis, cell migration, and growth factor release.

Biostimulative methods induce bone healing during angiogenesis and proliferation phases.[35] Osteoblast migration and proliferation, graft ingrowth, and matrix formation occur between the first 7 and 14 days of bone healing. Therefore, angiogenesis and growth factor stimulation during this period is important.[24] In the present study, we repeatedly applied shockwaves during the early healing period (3 sessions during the first week of wound healing) to induce biostimulation.

Radiopacity of allografts is sufficient enough to be recognized during bone healing.[8] In the present study, we analyzed BD in the defect area of each rat by measuring Hounsfield unit values on sagittal section images obtained by performing high-resolution computer tomography. Lower BD values were obtained for rats in the 4GE group than for rats in the 4G group, and higher BD values were obtained for rats in the 8GE group than for rats in the 8G group; however, these differences were not statistically significant. During the healing period, allografts present in the defect region initially undergo resorption because of increased osteoclast activity, which reduces radiopacity.[14] NB tissue is unorganized in week 4 of bone healing but replaces the graft tissue in a well-organized manner by week 8 of healing.[24] Therefore, BD values increase in week 8. BD values obtained in the present study suggest that ESWT reduces BD by increasing graft resorption and immature NB tissue volume in the early period. However, ESWT increases BD as a long-term effect in bone defects treated with allografts, suggesting that increase in mature bone volume at the end of week 8 plays a role in increasing BD value. Although BD values of rats in the 8GE group were significantly higher than those of rats in the 4GE group, no significant difference was observed in the BD values of rats in the 8G and 4G groups. This result suggests that shockwave therapy further increases BD in the bone defect treated with an allograft. In contrast, Barnes et al[34] reported that ESWT reduces BD as a long-term effect in osteotomy gaps treated with autogenous grafts. The difference between the results reported by Barnes et al and our result may be due to the use of different shock wave parameters. The optimum interval and the shock wave number are currently being determined. Another reason may be that autogenous cancellous bone grafts have been used in other study. [34] The effect of ESWT on different graft materials has not yet been determined. New studies should be planned to compare these results.

For a realistic analysis, we performed stereological evaluation that allows three-dimensional volumetric assessment of tissues. We used the Cavalieri method because it allows the most unbiased evaluation and determined NB, CT, and CA volumes. Rats in the 4GE and 8GE groups showed higher NB, CT, and CA volumes than rats in the 4G and 8G groups, indicating that ESWT stimulated healing in bone defects repaired using allografts. However, rats in the 4GE group showed a reduction in NB, CT, and CA volumes compared with rats in the 8GE group. In contrast, NB, CT, and CA volumes increased in rats in the 4G group compared with those in rats in the 8G group. These results suggest that ESWT exerts a promising effect on allograft-treated bone defects in the short term and that this effect does not remain stable in the long term.

A limitation of our study is that there are few subjects in each group and this may cause a type 2 error. To increase the power, this study should be done with a higher number of subjects. The mandible has a limited area to create a defect. In particular, the angulus area is composed of a very thin cortical bone. To avoid the risk of complications, thicker bone areas should be selected. Therefore, the study should be performed in a different bone or region and larger subjects. As another limitation of this study, the amount of remained graft material at the end of the healing period was not examined. Therefore, the effect of ESWT on the graft resorption process is not clear. It is useful to plan new experimental studies, in which the remaining graft material was determined by micro-CT and stereological methods.

CONCLUSION

Extracorporeal shockwave therapy is a noninvasive treatment method that has proven to be a osseostimulation effect. Therefore, many complications can be prevented when used in clinics to accelerate the healing process. Our results indicate that ESWT

accelerates the healing of bone defects repaired using allografts in the early period. This study was the first to investigate the efficacy of allograft together with ESWT on bone defect healing. However, additional studies should be performed to reach a precise conclusion because of the lack of studies on this topic.

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