Is the reiteration of extracorporeal shock wave therapy beneficial to enhance the bone integrity in type 1 diabetic rats?

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Abstract

Aim: Type 1 Diabetes Mellitus (DM) is an important endocrine disease that causes disorders in the musculoskeletal system. Extracorporeal shock wave therapy (ESWT) may be effective in the improvement of these disorders due to its reported anabolic effects.

Materials and Methods: The effects of unfocused shock waves on the mandible in healthy and diabetic rats were investigated. 36 wistar albino rats were used and randomly divided into 4 groups: non-diabetic control (n-cont), non-diabetic eswt (n-eswt), diabetic control (d-cont), diabetic eswt (d-eswt). Geometric structure, bone mineral density (BMD) and biomechanical properties of the mandibles were analyzed. Datas were statistically analyzed by one-way variance analysis and Tukey post-hoc test.

Results: According to the results, BMD was lower in the diabetic groups. There was no significant increase in ESWT groups. The surface area of the mandible was significantly lower in the diabetic groups than in the n-cont group. However, it was significantly higher in the d-eswt group than in the d-cont group. Ultimate load values were found to be significantly lower in the d-eswt group than in the d-cont group. While there was no significant difference between the groups in terms of stiffness, ultimate strain and young modulus, lower ultimate stress values were observed in the d-eswt group compared to the d-cont group.

Conclusion: As a result, Type 1 Diabetes Mellitus caused a decrease in bone dimensions and bone mineral density, but did not affect the biomechanical properties. Interestingly, ESWT application caused the bone to be more fragile in diabetics.

Introduction

Type 1 Diabetes Mellitus (DM) is a multi-organ disease characterized by chronic hyperglycemia. It is due to insulin deficiency and has serious negative effects on the musculoskeletal system. In Type 1 DM, deterioration of the macroscopic structure of the bone and molecular and cellular changes occur in cortical and cancelous components and organic/inorganic substances. As a result, bone quality is impaired, and bone mineral density is low [1]; this condition is called diabetic osteopenia [2]. Although no specific pathogenesis has been indicated for this condition, studies have emphasized mechanical, hormonal, and vascular factors [3]. The changes that occur in bone tissue as a result of Type 1 DM are as follows:

1. Changes in bone physiology as a result of the disruption of microvascular structures: Decrease in the volume of cortical and trabecular bones [4] and alterations of bone's geometric structure [5].

2. Direct effects of hyperglycemia on cells and tissues: Decrease in bone mineral density, decrease in osteocalcin and osteoprotegerin [6], decrease in osteoblast differentiation and function, decrease in bone turnover [7], advanced glycation end products [8], increase in osteoclastogenesis, and decrease in osteoblastogenesis [2].

3. Hormonal conditions: Lack of insulin and insulin growth factors (lack of osteoblast stimulation and RUNX transcription), amylin deficiency (absence of osteoblast stimulation and osteoclast inhibition [2], oxidative stress (dysfunction of osteoblast and osteoclast and inhibition of osteoblastic differentiation), apoptotic cell death in osteoblasts [9], and abnormal cytokine and adipokine products [10].

As a result of all these effects, biomechanical properties decrease [11], fracture incidences increase, and bone healing

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is delayed [12]. To prevent these complications in diabetic individuals, new techniques are needed to increase bone density and strength and to regulate bone metabolism [3]. Due to its anabolic effect, extracorporeal shock wave therapy (ESWT) may be effective in improving bone formation and bone mineral density and in increasing the biomechanical properties of bone.

ESWT, which is known to induce angiogenesis and osteogenesis, is a non-invasive and effective treatment method for many musculoskeletal diseases (e.g., plantar fasciitis, calcific tendinitis of the shoulder, and delayed union or non-union of long bones) [13]. Shock waves have been shown to increase bone repair and regeneration by triggering the release of transcription factors, mediators, and growth factors (e.g., vascular endothelial growth factor, endothelial nitric oxide synthase, proliferating cell nuclear antigen, bone morphogenetic proteins, and osteocalcin) [14], stimulating the proliferation and differentiation of osteoprogenitor cells, and inducing osteoblastic activity [15]. These effects also induce mandibular fracture healing [16], shorten the consolidation time in mandibular distraction osteogenesis [17], increase bone mineral density and cortical and trabecular bone volume, and improve the biomechanical properties of bone [14]. As a result of these positive effects, shock waves are considered to have promising effects on the improvement of osteopenic bone tissue [18].

The primary aim of this study was biomechanically, geometrically, and radiologically evaluation the effect of ESWT on osteopenic bone tissue in type 1 diabetics. To analyze this effect, primarily the variables of bone mineral density and bone fracture resistance were investigated.

Materials and Methods

Subjects and sample size

The experimental study protocol was adopted by the Ondokuz Mayıs University's Local Ethics Committee on Experimental Animals. The guidelines for the use and care of laboratory animals were followed throughout the entire study. Power analysis was done for minimum sample size determination. In this analysis, type-I and type-II errors were accepted as 5% and 80% respectively. The standardized effect size was accepted as 0.8 according to literature searching. As a result, the minimum required sample size was calculated as a total of 24. This number was equally distributed to 4 groups and 6 rats were placed in each group according to the randomization principles. Due to

Figure 1. A. Eswt device, B. Unfocused applicator and application.

the risk of death of diabetic subjects, 36 three-month-old male Wistar rats weighing 280–300 g were used in this study. The rats to be included in the study were selected by a simple random sampling method. The subjects were randomly divided into four groups, with nine rats in each group. Randomization was carried out by choosing random numbers after the subject numbers were written on the cards.

1. Non-diabetic control (n-cont)
2. Non-diabetic ESWT (n-eswt)
3. Diabetic control (d-cont)
4. Diabetic ESWT (d-eswt)

Animals were kept in standard conditions (12-h day and night, temperature of 22 ± 2 ℃, relative humidity of 40%–60%, free feed and drinking water, and good ventilation). The polyuria and weight status of the subjects were checked by the veterinarian each day during the entire study.

Diabetic model design

The diabetic groups were administered streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.1 M citrate buffer with a pH of 4.5 as a single intraperitoneal dose of 50 mg/kg. The animals in the non-diabetic group were injected with an i.p. sodium citrate buffer solution. Three days after the injection, fasting blood levels were measured with a glucometer (Accu-Chek Pro, Roche Diagnostics, Germany) from blood samples obtained from the tail veins. The diabetic model was realized when the blood glucose level was 250 mg/dl. After the diabetic model was confirmed, five weeks were allowed to pass to see its effects on bone metabolism. Blood glucose levels were re-measured on the day of sacrification.

ESWT application and sacrification

The subjects in the ESWT groups were sedated with i.p. 20 mg/kg ketamine hydrochloride and 1 mg/kg xylazine hydrochloride injections. On the 35th, 37th, and 39th days of the experiment, shock wave therapy was applied in three sessions in the mandible corpus at the molar tooth level, with 200 impulses, 5 Hz, and 0.19 mJ/mm^2 energy flow density values in each session (Figure 1). After 13 weeks of STZ injections, all subjects were sacrificed by administering a high dose of thiopental (100 mg/kg). The mandible samples were cleaned of soft tissues, and a radiological examination was performed to evaluate bone density.

Bone mineral density (BMD)

All radiological examinations were performed by a blinded radiologist. High-resolution computed tomography images were obtained to measure the bone density of the mandibles. For these measurements, we used a multislice tomography device with 16 sequential detectors (Aquilion 16 system, Toshiba Medical Systems Corporation, Tokyo, Japan), 250 mAS, 120 kV, 512 × 512 matrix, and 0.5 mm reconstruction thickness scan data.
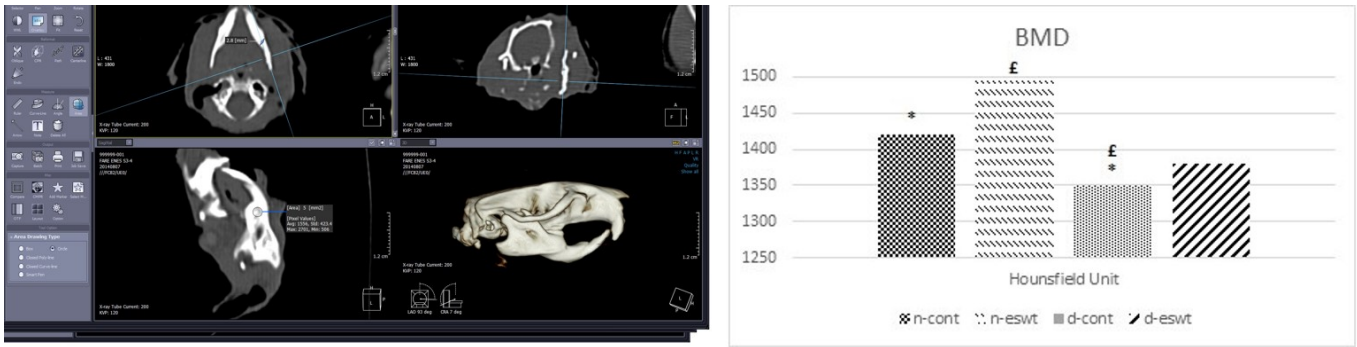
Figure 2. A. 3D reconstruction and sections of rats cranial bones, measurement of bone mineral density from tomography sections as Hounsfield Unit, B. Mean bone mineral density values. (The same symbol above the columns indicates statistical significance).
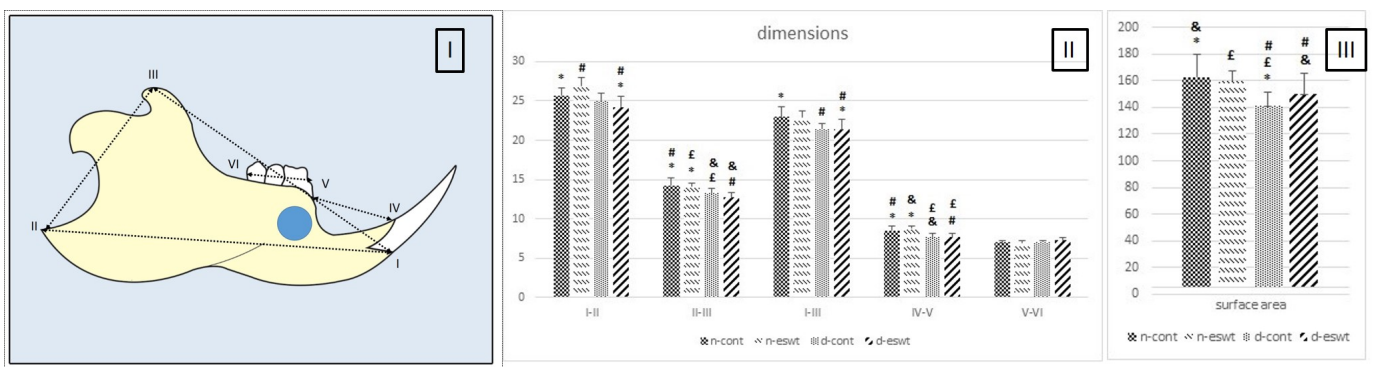

Figure 3. A. Dimensional analysis of mandible, reference points, determination of surface area, B. Geometric dimensions of the mandible mm^2, C. Mandible surface area mm^2. (The blue circle indicates where the bone mineral density was measured. Same symbols in chart columns indicate statistical significance).

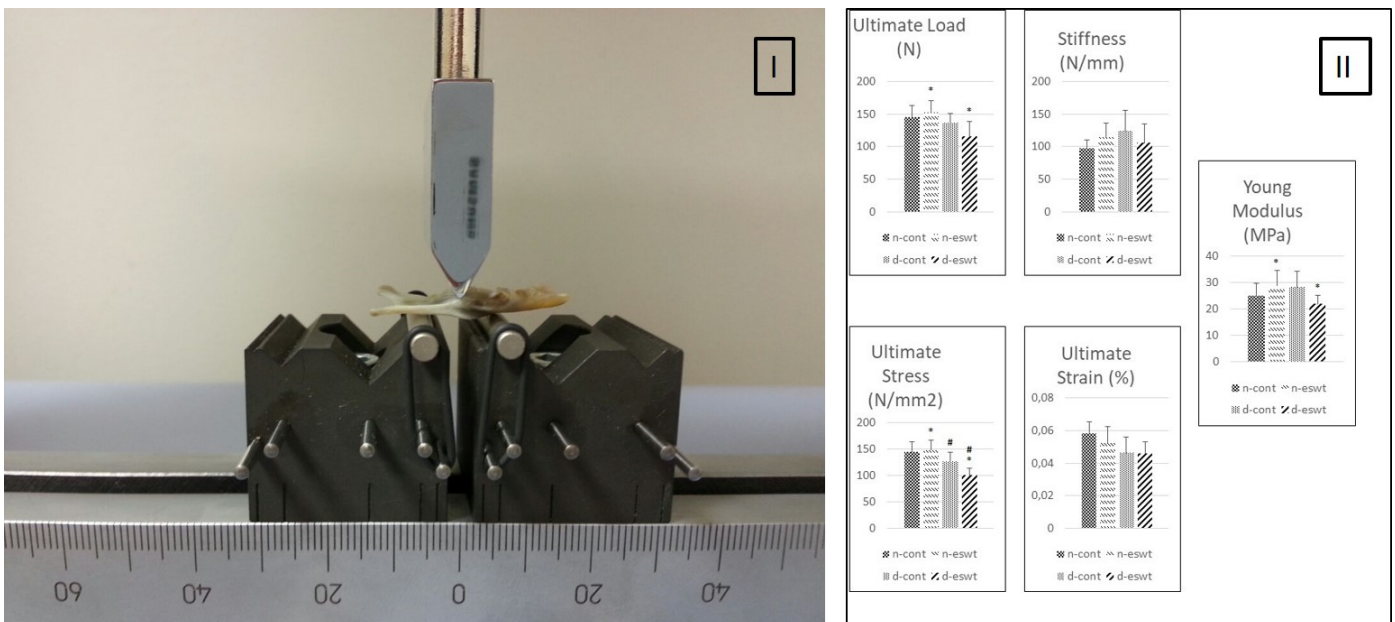**Figure 4.** Biomechanical testing method and results. (Same signs indicate statistical significance).

The resulting DICOM files were transferred to OsiriX (Pixmeo Sarl, Switzerland), and analyses were performed on a small animal scanning interface (Figure 2). For bone mineral density measurements, Hounsfield unit (HU) values were identified using a 5 mm^2 circular area at the anterior level of the roots of the mandibular

first molar (Figure 3).

Geometric and biomechanical evaluation

All geometric and biomechanical testing were performed by a blinded researcher. The samples subjected to radiological evaluations were stored at −20 ℃. Dimensional measurements of the mandible were performed with a digital caliper. The guide points for the measurements were identified as follows: I–mandible symphysis, II–the most posterior of the angle, III–the highest point of the coronoid process, IV–incisor tooth, V–the most anterior of the molar teeth, and VI–the most posterior of the molar teeth [19]. The triangular area between points I–II–III represents the surface area of the mandible (Figure 3).

Prior to biomechanical testing, the samples were slowly warmed up at room temperature and kept moist in saline-impregnated gauze for testing. Biomechanical analyses were performed using a three-point bending test with a servo-hydraulic high-precision universal test machine (Shimadzu AGS-X 10 kN, Shimadzu Corp., Tokyo, Japan). It was placed horizontally with the lateral of the mandible pointing downward, centered on the supports placed at 11-mm intervals. The force-applying end of the device was directed to the inferior third molar tooth (Figure 4). To minimize variability, all samples were placed exactly the same in terms of location and direction. The displacement of the force applicator was set to 5 mm/min. We continued to apply force until there was a complete vertical fracture of the mandible. We automatically obtained the load/deformation graph and recorded the maximum load (Newton, N) values at the time of fracture. The following data were calculated: stiffness (N/mm), ultimate stress (N/mm^2), ultimate strain (%), and Young's modulus (MPa).

In the load/deformation curve, there is a linear area where the bone shows elastic properties and a plastic area where it is irreversibly damaged, and the yielding point separates these two areas. While the elastic region is related to the mineral component of the bone, the plastic region is related to the collagen component [20]. Stiffness is the slope of the linear section. Strain is the degree of displacement in the transverse direction at a given load point. This indicates the flexibility of the bone and is independent of geometry. Ultimate stress is the ratio of the force to the cortical area, indicating the bone's resistance to fracture. Young's modulus is defined as the stress occurring per unit elongation and indicates the flexibility of the bone [1].

Statistical evaluation

Descriptive values of the variables were computed as mean and standard deviation. The goodness of fit of the observations to the normal distribution was checked by Shapiro-Wilk test. Because the data were normally distributed, the one-way ANOVA model was used for comparing the groups. When differences were detected between the groups, pairwise evaluations were analyzed using Tukey's test. If P-value was less than < 0.05, it was considered statistically significant.

Results

Among the 36 subjects, two from the d-cont group died during anesthesia, and two from the d-eswt group died because they were unable to tolerate the diabetic condition. The remaining subjects tolerated the diabetic model and ESWT applications well. Whereas normal weight gain and body development were observed in all non-diabetic subjects, polyuria and weight loss were observed in rats in the diabetic groups (Table 1). In addition, edema due to mild inflammation was observed in the first three days after ESWT applications.

A significantly higher blood glucose level was found in the diabetic groups than in the non-diabetic groups (p <0.05) in the blood glucose level measurements made after the diabetic model was created and on the day the experiment was terminated. The weight of the subjects was significantly lower in the diabetic groups than in the non-diabetic groups (Table 1). The bone mineral density results are demonstrated in Figure 2. The all geometric parameters of mandible are stated in Figure 3. The biomechanical parameters are shown in Figure 4.

Discussion

This experimental study is the first to examine the effects of shock waves on the biomechanical properties of bone and bone mineral density in diabetics. This study was conducted because it is thought that ESWT could be effective in preventing/ameliorating the complications that occur in bone tissue in diabetic individuals due to its reported anabolic effects on bone tissue.

During the experiment, as expected, an excessive increase in blood glucose levels and significant weight loss were observed in diabetic subjects. One of the important points is the timing of complications in bone caused by chronic blood glucose levels. Studies have reported an approximately 50% reduction in the trabecular bone in the tibia, femur, and vertebrae four weeks after STZ application [21], decreased bone mass characterized by low bone formation, and a significantly decreased BMD after 8–12 weeks [18]. For this reason, in our study, ESWT was applied five weeks after the STZ application, and the duration of the experiment was determined to be 13 weeks.

Geometric structure (size and shape), architecture (cortical and cancelous bone amount), and bone content (organic and inorganic components) are affected because of cellular and molecular changes that occur as a result of Type 1 DM [1]. Spontaneous/provoked fractures [1,2,20] and delayed bone healing are considered a result of decreased BMD [6] and the mechanical strength of bone tissue [22]. In this study, a significant decrease in BMD was found in the d-cont group compared to the n-cont group. However, evaluating BMD alone is not sufficient to determine the risk of fractures in diabetics. It is also necessary to define bone quality. Bone quality is related to bone content and geometric structure [8]. Bone content consists of organic and inorganic components (i.e., collagen network, elastin, proteoglycan, bone turnover, and bone mineralization) [20], and it is the main factor determining biomechanical property [1]. The geometric structure of bone is also considered a significant factor in determining

Table 1. Average weight and blood glucose levels of subjects in groups.

	3 days after STZ injection				At time of sacrification (13 weeks after STZ injection)			
	n-cont	n-eswt	d-cont	d-eswt	n-cont	n-eswt	d-cont	d-eswt
Weight (g)	284.9	293.4	280.3	295.6	389.2	385.6	255.1	248.7
Blood glucose level (mg/dL)	105.4	110.2	296.4	302.5	108.9	105.7	421.1	443.8

bone strength [23]. In this study, the surface area of the mandible was significantly lower in the d-cont group than in the n-cont group. In some studies with human subjects, there is evidence that smaller bones form in people with diabetes. Saha et al. found smaller bone areas in adolescents with Type 1 DM [24]. Nyman et al. found a significantly reduced femoral diaphysis length in rats with Type 1 DM compared to the control group [4]. In our study, there was also a significant decrease in the weight of the subjects in the diabetic groups. These results confirm that bone mineral density decreases and that the geometric structure of the bone is affected in diabetic subjects.

Deterioration of biomechanical properties, less resistance to tension forces, a significant decrease in the ultimate load values at break, and lower absorption energy, displacement values, and fracture resistance[4,20] have been reported in Type 1 DM. Thus, the bone becomes more fragile. Factors such as ultimate load and stiffness are used to assess bone strength associated with the mineral content of bone [3]. Low ultimate load and high stiffness values indicate that the bone is more fragile [20]. Although lower ultimate load and higher stiffness values were observed in the d-cont group in this study, these results were not statistically significant. The literature shows that bone stiffness increases [20] or decreases [3] due to diabetes. These contradictory findings are explained by several factors, such as the sex of the subjects, the duration of exposure to the diabetic condition, and the biomechanical test method [20]. Ultimate stress, ultimate strain, and Young's modulus are the other parameters used to evaluate bone fragility [3,23]. Ultimate stress also provides information about the collagen structure of the bone and its mineral content. Although studies have reported that ultimate stress decreased significantly in diabetics [3,20], no significant difference was observed between the d-cont and n-cont groups in terms of ultimate stress, ultimate strain, and Young's modulus in this study. When all biomechanical markers are considered together, the bone seems to be relatively less mineralized in diabetics. However, when we evaluate them with the BMD and geometric results, bone mineralization can be considered impaired due to diabetes, and thus bone strength decreases. Although the results show that diabetic bones are more fragile, weaker, and harder, new studies with more subjects may be useful in confirming these relative results.

The unfocused shock waves used in the treatment are short-term, high-energy acoustic waves that expand and spread between tissues. Although the penetration depth of unfocused sound waves is small, the area affected is wider [18]. ESWT has been successfully used clinically in many musculoskeletal diseases. However, a small number of studies have shown that shock waves have promising effects on diseases in which the bone microenvironment

is disrupted, such as diabetes or osteoporosis. ESWT has been reported to cause a significant increase in bone mineral density and bone strength, as well as a higher growth factor release rate, in the treatment of osteoarthritis among osteoporotics [25]. However, it seems that ESWT has been used in clinical and animal experiments to improve the complications occurring in soft tissues due to diabetes. Studies on ESWT have aimed to improve the healing of diabetic foot [26], diabetic polyneuropathy [27], acute and chronic wound healing in soft tissue [28], and chronic plantar fasciitis [29]. Studies have reported that ESWT can induce diabetic wound healing by stimulating revascularization and increasing tissue regeneration. In the current study, ESWT application had no significant positive effect on bone mineral density in either the diabetic or the non-diabetic group. However, there was a significant increase in the surface area of the mandible in the d-eswt group compared to the d-cont group. Regarding the biomechanical results, the ESWT application in the non-diabetic groups did not cause a significant change, while ESWT applied in the diabetic groups caused significantly lower ultimate load values. Ultimate stress values were significantly reduced in the d-eswt group compared to the n-eswt group and in the d-eswt group compared to the d-cont group. Similarly, there was a significant decrease in Young's modulus in the d-eswt group compared to the n-eswt group. Note that although these results have a positive effect on the increase in bone volumes in diabetics, shock waves cause a significant decrease in strength. In other words, unfocused shock waves locally deteriorate the biomechanical properties of the mandible, and the bone becomes more fragile in diabetics. Despite the positive effects of ESWT on healthy bones, researchers have also reported no positive effects. ESWT reduces mechanical endurance and causes delayed bone healing [16,30]. In two our previous studies, ESWT was negatively effective in the regeneration of critical-sized bone defects but was positively effective in allograft-applied defects [30,31]. The negative effect of ESWT on critical bone defect regeneration was also observed in the diabetic groups. However, there are differences in terms of shock wave parameters, number of sessions, application time, and applicator type in all these studies. The shock wave parameters and the number of sessions necessary for the biostimulating effect on bone tissue have not yet been determined. Therefore, there is a need for new study designs with different shock wave parameters.

In this study, dimensional analysis, bone density analysis, and biomechanical analysis were performed to reveal the power–structure relationship. Explaining the relationship between biomechanical changes and specific metabolic changes is not the purpose of this study. The duration of

exposure of the rats to an uncontrolled hyperglycemic state was 13 weeks. This is unlikely to fully represent a chronic diabetic condition in humans. Subjecting animals to a longer period of hyperglycemia can lead to further deterioration in bone's material properties. However, it is not possible for subjects with severe weight loss and polyuria to survive for a long period. Nevertheless, the lack of histomorphometric and immunohistochemical analysis can be considered among the other limitations of this study. In addition, although many recent experimental studies have examined the biostimulatory effect of shock waves, there is still no consensus on the most appropriate therapeutic shock wave parameters. Therefore, the effects of ESWT on the biomechanical properties of bone tissue and bone mineral density in diabetics are still unclear, and further studies are needed on this subject.

Ethics approval

The experimental study protocol was adopted by the Ondokuz Mayıs University's Local Ethics Committee on Experimental Animals (Approval no: 30.12.2011/124).

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