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ORIGINAL ARTICLE

The long-term effects of extracorporeal shock waves on the epiphysis of the adolescent rat

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Abstract
Background Extracorporeal shock waves (ESW) have been successfully used to treat musculoskeletal injuries, tendinopathy, and plantar fasciitis. Different side effects of high-energy ESWT on bones, tendons, nerves, epiphysis, and cartilage have been discussed. Although the effects of ESW on the epiphysis in animal models have been described, no studies have investigated the long-term effect of ESW on the immature epiphysis in an animal model. The purpose of this study was to investigate the long-term effects of ESW on the immature epiphysis in rats.
Methods Sixteen 4-week old Wistar albino rats, average weight 116.3 grams (109.6–120.2 g) were used for these experiments. The rats were randomly divided into two groups, the ESW 1500 pulses (ESW1500p) group ($n = 8$) and the ESW 3000 pulses (ESW3000p) group ($n = 8$). In the ESW1500p group, 1500 pulses, at 4 bar, of 1-Hz shockwaves were applied, *once*, to the left knees of the rats. In the ESW3000p group, 3000 pulses, at 4 bar, of 1-Hz shockwaves were applied, *once*, to the left knees of the rats. The right knees ($n = 16$) of the rats in the two groups were used as the controls. The animals were sacrificed after an 8-month follow-up period. Femoral epiphyses were assessed by use of histology. The femoral length (FL), tibial length (TL), and femoral supracondylar mediolateral width (MLW) were measured.
Results There was no statistically significant difference between FL, TL, and MLW for the three groups ($P > 0.05$). The average histological scores were 8.8 (7–10), 17.8 (15–22), and 2.7 (0–4) in the ESW1500p, the ESW3000p groups, and the controls, respectively. The average histological score for the ESW3000p group was significantly higher than those for the ESW1500p group and the controls ($P < 0.001$). The average histological score for the ESW1500p group was significantly higher than that for the controls ($P < 0.001$).
Conclusions The histological findings of this study indicate that ESW increased the cellularity and basophilia of the extracellular matrix (ECM) in the adolescent rat epiphysis and there were no negative effects on extremity measurements in the long term.

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Introduction

The original treatment by extracorporeal shockwave (ESW) therapy was in lithotripsy. It was first applied to patients in 1980, to break kidney stones in urology [1]. Extracorporal shock waves (ESW) have been successfully used for treatment

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of musculoskeletal injuries [2], for example pseudoarthrosis [3, 4] patellar tendinopathy [5, 6], epicondylitis [3, 7, 8], plantar fasciitis [3], and osteonecrosis [9–11].

The exact mechanisms of action of ESW have remained controversial. Previous studies have demonstrated that ESW treatment increased the number of neovessels at the normal tendon–bone junction in dogs [12, 13]. The underlying mechanisms of ESW were reported to be effective inducement of tissue micro-damage, triggering angiogenesis via upregulation of vascular endothelial growth factor (VEGF), transforming growth factor-beta 1 (TGF β-1) [12], nitric oxide (NO) [3], and insulin-like growth factor [12, 14, 15].

Although overuse injuries of tendons and apophyses are seen with increasing frequency in children and adolescents as they increase their participation in organised sports, fitness, and dance activity [16], use of ESW for treatment is limited, because the effects of ESW on the epiphyseal growth plate are not well known. Although information on short and medium-term application of ESW on the epiphysis is documented, there is little information about the long-term effects of ESW on the epiphysis [17–20]. The research question of this study was whether application of ESW to the epiphysis will effect extremity length and histology of the epiphysis of adolescent rats in the long-term.

Methods

Study design

A randomised controlled study was designed. The independent variable was groups and the dependent variables were macroscopy and histology.

Animals

The experimental procedures used in this study were conducted in accordance with the National Institute of Health (NIH) Guide for the care and use of Laboratory Animals (NIH Publications no. 80-23, revised 1996). The experimental procedures were approved by the Institutional Review and Animal Ethics Use Committee of Cumhuriyet University Faculty of Medicine, and the study was conducted in accordance with accepted guidelines for the care and use of laboratory animals for research. Sixteen 4-week old Wistar albino rats, average weight 116.3 g (109.6–120.2 g) were used for these experiments. During the study, the animals were kept under standardised conditions; they were caged individually, and had free access to water and a standard pellet diet. Care for all rats was provided in accordance with animal care facility guidelines, and they were all handled carefully.

Shockwave exposure

Shockwave exposure was performed by using a Storz Masterpuls MP200 device (Karl Storz, Tuttlingen, Germany). Animals were randomly divided into two groups, the ESW1500p ($n = 8$) group and the ESW3000p ($n = 8$) group. 1500 pulses, at 4 bar, of 1 Hz (EFD = 0.38 mJ/mm^2) shockwaves were applied once to the adductor tubercle of the left knees of the rats in the ESW1500p group; 3000 pulses, at 4 bar, of 1 Hz (EFD = 0.38 mJ/mm^2) shockwaves were applied once to the adductor tubercle of the left knees of the rats in the ESW3000p group. The right knees of the rats ($n = 16$) were used as the controls. For all shockwave exposure, the rats were anaesthetised by injection with ketamine HCl 75 mg/kg (Ketalar®, Pfizer, Turkey) plus xylazine 6 mg/kg IM (Rompun®, Bayer, Turkey).

Macroscopic measurements and histological examination

The animals were sacrificed after an 8-month follow up period. Femoral epiphyses were assessed by use of histology. A single histologist, unaware of the identity of the samples, performed the histological analysis. The epiphyses were decalcified by use of buffered formic acid (pH 2) for 48 h [21]. All soft tissue was removed. The femoral length (FL), tibial length (TL), and femoral supracondylar medio-lateral width (MLW) were measured by use of a 0–150 mm digital calliper (Mitutoyo, UK; code no: 500-181-21).

Bones were fixed in 10 % neutral formalin, dehydrated through increasing concentrations of ethanol, then embedded in paraffin. We obtained paraffin sections 4–6 μm thick and stained these by use of the conventional haematoxylin–eosin (H&E) technique. Sections of stained tissues were evaluated under an Olympus (Tokyo, Japan) BX51TF light microscope, and histological assessment was performed in accordance with a modified version of guidelines for the growth plate as used by Quintana al. [22] (Table 1).

Statistical analysis

SPSS 17.0 (SPSS, Chicago, IL, USA) was used to record and evaluate the results. Results were analysed by use of Kruskal–Wallis and ANOVA tests, with the Tukey test for post-hoc comparisons. A P value <0.05 was regarded as statistically significant.

Macroscopic measurements

Table 2 illustrates the measurements of the femoral length (FL), tibial length (TL), and distal femoral medio-lateral width (MLW). No statistically significant difference was determined between the three groups ($P > 0.05$).

Table 1 Histological score of epiphyseal plaque changes, modified from guidelines used by Quintana et al. [22]

	Score
Structure	
Normal	0
Partial increase	1
Total increase	2
Hypercellularity of reserve zone	
Normal	0
Slight	1
Moderate	2
High	3
Hypercellularity of proliferative zone	
Normal	0
Slight	1
Moderate	2
High intense	3
Hypercellularity of hypertrophic zone	
Normal	0
Slight	1
Moderate	2
Intense	3
Basophilia of extracellular matrix H&E staining	
Normal	0
Slight	1
Moderate	2
Intense	3
Hypertrophy of perichondral ring	
Normal	0
Slight	1
Moderate	2
Severe	3
Epiphyseal interface	
Normal	0
Partial increasing	1
Total increasing	2
Metaphyseal interface	
Normal	0
Partial increasing	1
Total increasing	2
Total	21

Histological findings

ESW1500p

Epiphyseal plaque was observed to be limited to a narrow area, within normal limits (Fig. 1a). Cartilage cells, forming columns in the epiphyseal–diaphyseal direction, had typical cell stratigraphy. The extracellular matrix (ECM) of cells had yet to reach the calcification and ossification zones; very little basophilia was observed. Almost no basophilia was observed in the ECM of calcification or in the ossification zones (Fig. 1b, c).

Table 2 Measurements of the femoral length (FL), tibial length (TL), and supracondylar femoral medio-lateral width (MLW)

	ESW1500p ($n = 8$)	ESW3000p ($n = 8$)	Controls ($n = 16$)	Results
FL (mm)	37.0 ± 0.7	37.6 ± 1.0	36.8 ± 1.2	$P = 0.411$
TL (mm)	39.9 ± 1.5	41.1 ± 1.6	40.8 ± 1.5	$P = 0.620$
MLW (mm)	7.0 ± 0.3	7.9 ± 1.6	6.9 ± 0.3	$P = 0.283$
Histological score	8.8 ± 1.0[a]	17.8 ± 2.7[a,b]	2.7 ± 1.0	$P < 0.001$

Data are presented as mean ± SD

[a] $P < 0.001$ versus controls

[b] $P < 0.001$ versus ESW1500

ESW3000p

In this group, the most striking characteristic finding was that the basophilic staining in the ECM of the epiphyseal cartilage was significantly greater (Fig. 1d–f) than in the other groups. Epiphyseal plate boundaries were irregular. Even the ECM surrounding the cartilage cells during ossification of epiphyseal plate was stained basophilic. In this group, most of the epiphyseal plate cartilage was composed of differentiating cartilage cells close to epiphyseal side. Observation of the blood vessels between the highly basophilic cartilage cells was also interesting (Fig. 1e). Cartilage cells sometimes spread to the trabecular region of the diaphysis and they were basophilic ECM (Fig. 1f).

Controls

In this group, the epiphyseal plaque and the thickness of the epiphyseal plaque were normal (Fig. 1g, h), the cartilage ECM basophilia was very low, and acidophilic areas were rare. Calcification and ossification layers were wider than for the other groups.

The average histological score was 8.8 (7–10), 17.8 (15–22), and 2.7 (0–4) for the ESW1500p group, the ESW3000p group, and the controls, respectively. The average histological score for the ESW3000p group was significantly higher than those for the ESW1500p group and the controls ($P < 0.001$). The average histological score for the ESW1500p group was significantly higher than that for the controls ($P < 0.001$) (Table 2; Fig. 2).

Discussion

We evaluated the long-term effect of ESW on the immature epiphysis of rat. Histological assessment and semi-

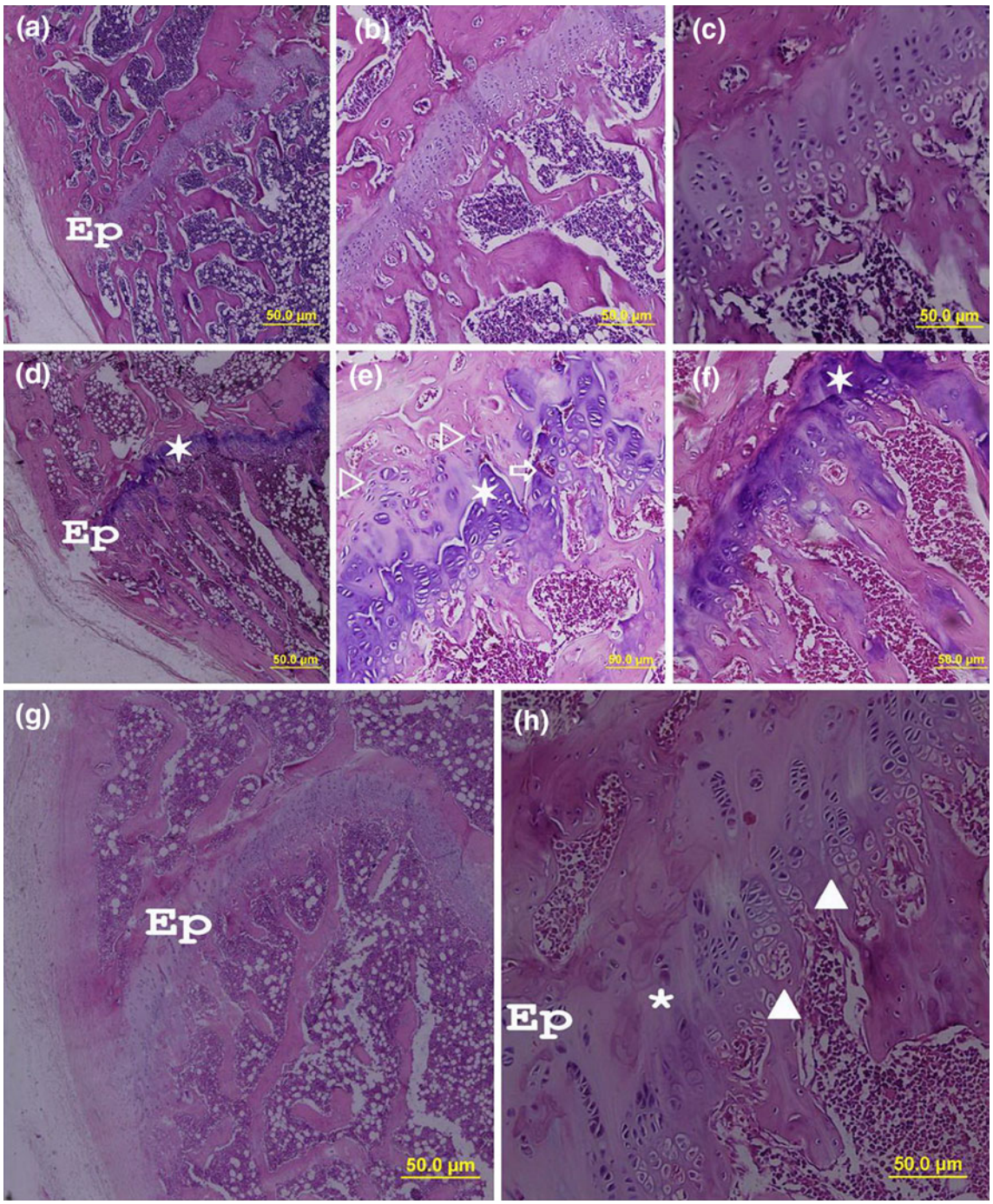

Fig. 1 Morphological structure of the epiphyseal plaque of the experimental groups. In the ESWT1500 group (**a–c**) epiphyseal plaque was observed in a limited area (**a**) and the cartilage ECM was slightly basophilic (**b–c**). In the ESWT 3000p group (**d–f**), a highly basophilic matrix (*star*), rich blood vessels (*arrow*), and the abundance of the differentiating chondrocytes (*arrowhead*) were remarkable (**d**, **e**). In some areas, the cartilage cells had spread to the bone trabecular surfaces and had begun to synthesise a basophilic (*blue areas*) matrix in this area (**f**). In the control group (**g**, **h**), most of the epiphyseal plaque (Ep) was composed of the calcification and ossification layers (*filled arrowhead*), and pale basophilic staining of the cartilage extracellular matrix (*asterisk*) was observed. Staining H&E

quantitative scoring were different for the ESW1500p and ESW 3000p groups. In the ESW3000p group, especially, prominent basophilia was observed in the ECM of newly differentiated cartilage cells and in the epiphyseal–perichondral area. This may be indicative of significant regeneration of cartilage tissue and may help to explain the mechanism of tissue recovery after ESW. Although the histological scores were different in this study, FL, TL, and

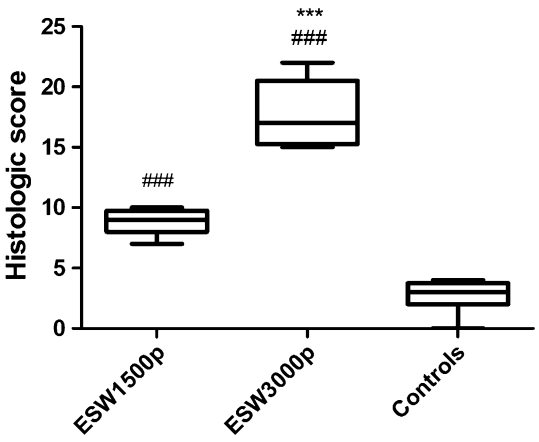

Fig. 2 Histological scores for the study groups. $^{****}P < 0.001$ versus ESW1500p, $^{\#\#\#}P < 0.001$ versus controls

MLW parameters were not statistically significantly different.

Histological assessment in this study was conducted on the basis of guidelines for the growth plate, using the grading used by Quintana et al. [22]. The purpose of the study by Quintana et al. was to evaluate cell loss in all zones of the epiphyseal plate. In our study, however, because ESW had positive effect (i.e. increasing ECM and cellularity) in the epiphyseal area, it was modified by maintaining basic conditions.

Hutchinson and Ireland [23] reported that over 25 million children participate in school-sponsored sports, and an additional 20 million participate in extracurricular organised sports. Over the past decade, the increased intensity in training, more pressure for success, new opportunities for structured play, and more organised advanced leagues and travelling teams have led to a corresponding increase in overuse injuries in skeletally immature athletes. Over-use injuries in young athletes include diagnoses such as little leaguer's shoulder, little leaguer's elbow, osteochondritis dissecans of the elbow, tennis elbow, and distal radial epiphysitis. ESW is a useful method of treatment for most over-use injuries in adults, especially tendinitis.

Cuff et al. [24] followed athletes in years 9–12 for 1 year and reported that 1685 (51 %) of 3276 athletes meeting the study criteria had some type of injury. They also reported that 584 respondents (18 %) suffered an overuse injury, whereas 152 (5 %) overuse injuries reported were tendonitis (238), shin splints (214), and lower-extremity stress fracture (75). The most common acute injuries were muscle strain (467), joint sprain (432), and fracture (313). It is possible ESW can be used to treat most such injuries during high school. However, as mentioned before, the most important problem related to ESW is that possible effects of ESW on physis of children and adolescents is not well known.

The reason the distal femur was chosen for this study is that many diseases for which treatment is possible by ESW, for example overuse injuries related to both extensor mechanisms (quadriceps–patella–patellar tendon complex) and flexor mechanisms (hamstring muscles, pes anserinus, and biceps tendon), knee fractures, osteochondritis dissecans (OCD), and knee osteonecrosis, are associated with the knee, and as the femur is the longest bone, possible differences in extremity length because of epiphysis damage can be more distinctly determined.

Saisu et al. [18] investigated the effect of ESW on the bone mineral content (BMC) of the long bones of immature rabbits. They showed that ESW induces overgrowth and a local increase in BMC. They administered 1000 and 5000 pulses, and 100 MPa shock waves, to the femoral shaft. Six weeks after exposure, the length, width, and the BMC of femoral bones were higher than those of controls. In contrast, the femoral length, tibial length, and distal femoral width in our study was not statistically significantly different between the experimental and the control groups after an 8-month exposure period (1500 pulses, 3000 pulses).

The effect of ESW on immature physis is controversial. Yeaman [17], investigated the proximal tibia of eighteen 5-week-old male rats. They were exposed to 1500 shock waves at 20 kV in a Dornier XL-1, Six of these rats and six age-matched controls and sacrificed 2, 4, and 10 weeks later. They reported that eight of the 18 (44 %) treated animals had focal growth plate dysplastic lesions attributable to the treatment. In the group sacrificed at 10 weeks, two of the six (33 %) treated animals had extensive dysplastic lesions which were associated with marked shortening of the shocked limb. In our study, FL, TL, and MLW were not statistically significantly different between ESW groups and controls. This result can be explained in two ways. First, Yeaman's study had a maximum 10-week follow-up period whereas in our study the follow up period was 8 months. Second, and more importantly, Yeaman used high-energy ESW with a lithotripsy device whereas we used a portable ESW device specific to orthopaedic applications in this study.

Nassenstein et al. [19] investigated the effects of high-energy shock waves on the structure of the immature epiphysis in rats. They applied 800 ESW (0.32 mJ/mm^2) to the proximal tibia epiphysis of rats. They reported that under the conditions used in human shock wave therapy, no damage occurred to the rabbit epiphysis. Ozturk et al. [20] reported that ESW stimulated growth of the immature tibial epiphysis in rabbits. In Ozturk's study, high-energy ESW (14 kW, 0.6 mJ/mm^2, 1500 and 3000 shots three times with an interval of 7 days) was administered to the tibial epiphysis instead of the human dose (0.32 mJ/mm^2, 800); after 6 weeks they found, histologically, that the thickness

of the epiphyseal plaque had increased after exposure to 1500 and 3000 ESW. We evaluated the long-term effect of ESW on the immature epiphysis of rats. Histological assessments and semi-quantitative scoring were different for the ESW1500p, and ESW3000p groups. For the ESW3000p group, especially, prominent basophilia was observed in the ECM of new differentiated cartilage cells and the epiphyseal–perichondral area. This may be indicative of significant regeneration of cartilage tissue and may help to explain the mechanism of tissue recovery after ESW.

This study had a few limitations:

1 The opposite extremity was used as a control and the systemic effect of ESW on the opposite extremity was not known.
2 Standardised doses of ESW have not previously been described.
3 Extremity alignment is very important for evaluation of the long-term effect of ESW on epiphysis. ESW may cause partial injury of the epiphyseal plate related to varus and valgus deformity. Radiographic evaluation was not used to measure extremity alignment in this study.

The histological findings of this study showed that ESW increased the cellularity and basophilia of ECM in the adolescent rat epiphysis and there were no long term negative effects on extremity measurements. Therefore, further studies both on use of different doses of shock waves and on measurement of extremity alignment are needed to enable safe use of ESW in childhood and adolescent.

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Conflict of interest The authors declare they have no conflict of interest.

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