Aim: To compare the efficiency of extracorporeal shock wave (ESW) treatment with hyaluronic acid (HA) viscosupplementation in an experimental rabbit cartilage defect model.

Materials and methods: A total of 24 New Zealand rabbits were randomly divided into 4 groups: HA, ESW, ESW + HA, and control. Chondral defects were created in the left knees of the rabbits. HA viscosupplementation was performed on the HA and ESW + HA groups, and after 24 h, 0.16 mJ/mm² ESW was performed on the ESW and ESW + HA groups. After an 8-week follow-up, the rabbits were sacrificed and histopathological examination of the defects was carried out. In addition, immunohistochemistry was performed by the avidin-biotin peroxidase method using vascular endothelial growth factor (VEGF), transforming growth factor beta 1 (TGF-β1), and type II collagen antibodies, and the results were evaluated semiquantitatively.

Results: There was a significant difference between the control group and the ESW group in terms of Pineda score and type II collagen expression; between the control group and the HA group in terms of Pineda score, VEGF expression, type II collagen expression, and TGF-β1 expression; and between the control group and the ESW + HA group in terms of Pineda score, VEGF expression, type II collagen expression, and TGF-β1 expression.

Conclusion: The results show that both treatment methods have positive therapeutic effects on the articular cartilage defect model in terms of the parameters studied.

Key words: ESW, HA, cartilage defect, VEGF, TGF-β1, type II collagen

1. Introduction

The physical properties of extracorporeal shock wave (ESW) treatment were first described in 1959 by Eisenmenger. Shock waves are mainly sound waves. Approximately 30 years ago, shock waves began to be applied clinically in various centers in Germany in order to break up renal stones. In 1980, Chaussy first used ESW in a human for the purpose of breaking up a kidney stone at the University of Munich (1). This nonoperative method has since replaced previous treatments as the gold standard in the treatment of urinary system stones (1–8).

In the past 20 years, ESW has been used to safely and effectively to treat various medical conditions (9). ESW is expanding its applications from urinary calculi treatment to orthopedic settings such as pseudoarthrosis (10,11), patellar tendinopathy (12,13), epicondylitis (10,14,15), plantar fasciitis (10,16), and osteonecrosis (17,18).

Studies conducted in recent times have shown that the mechanism of ESW can be performed with osteoblastic and/or angiogenesis-stimulating agents such as transforming growth factor beta 1 (TGF-β1) and vascular endothelial growth factor (VEGF). In another study, a significant increase was identified in colony forming unit-osteoprogenitor cells (colony forming unit, CFU-O) and TGF-β1 quantities as a result of performing ESW in rats (19–22).

Hyaluronic acid (HA) is an extracellular high-molecular-weight polysaccharide that is a constituent of many tissues, especially loose connective tissues (23). HA is the most important glycosaminoglycan of the extracellular matrix in articular cartilage. It is also responsible for the viscoelastic properties of synovial fluid. The average molecular weight in normal healthy people is 4–5 million daltons. Animal studies have revealed that HA injection
reduces joint pain and retards the degenerative process. It is also reported that HA has modulated inflammation, neutrophile chemotaxis, macrophage proliferation, phagocytosis, and angiogenesis (24).

The purpose of the present study is to compare the effects of HA and/or ESW treatment on articular cartilage defects.

2. Materials and methods

2.1. Animals
The experimental procedures used in this study were carried out in accordance with guidelines of the National Institutes of Health regarding the care and use of laboratory animals. The experimental procedures were approved by the Institutional Review and Animal Ethics Committee of Cumhuriyet University Faculty of Medicine, and the study was conducted according to accepted guidelines on the care and use of laboratory animals. A total of 24 adult New Zealand rabbits (Cumhuriyet University Animal Center, Sivas, Turkey) with an average weight of 2.4 kg were used in these experiments. Rabbits were randomly divided into 4 groups: control, ESW, HA, and ESW + HA. During the study, the animals were kept under standardized conditions and caged individually. They had free access to water, and a standard pellet diet. Care of all rabbits was provided in accordance with Animal Care Facility guidelines.

2.2. Preparation of cartilage defects
The animals were induced using ketamine HCl 50 mg/kg (Ketalar®, Pfizer, Turkey) plus xylazine 6 mg/kg IM (Rompun®, Bayer, Turkey). During the study, a standard medial parapatellar approach was used to expose the left knee. A defect was created in the weight-bearing area of the medial femoral condyle using a 3-mm drill and incised until the subchondral layer was seen (Figure 1). Careful hemostasis and a layered closure were performed to ensure a watertight seal, and the rabbits were allowed to move freely in the postoperative period; 4 mg/kg Carprofen (Rimadyl, Pfizer Inc., UK) was given as postoperative analgesia for 3 days.

2.3. HA injection
In the HA and ESW + HA groups, 0.3 mL (Orthovisc 15 mg 2 mL ampoule, Anika Therapeutics, Inc., USA) was injected into the joint after the cartilage defect was created and the skin was closed. Passive flexion-extension movements were applied to the knee in order to distribute the HA homogeneously in the joint.

2.4. ESW procedure
ESW was performed 24 h after the preparation of cartilage defects using a Storz Masterpuls MP200 device (KARL STORZ GmbH & Co. KG T, Tuttingen, Germany). A 1-Hz shockwave at an energy flux density (EFD) of 0.16 mJ/mm², and 500 impulses, was applied to the left knees of the rabbits in the ESW and ESW + HA groups. The rabbits were anesthetized using injections of ketamine HCl 50 mg/kg (Ketalar®, Pfizer, Turkey) and xylazine 10 mg/kg IM (Rompun®, Bayer, Turkey).

2.5. Histological evaluation
After the tissues taken were fixed in 10% formaldehyde solution for 24 h, they were decalcified in 10% formic acid solution. On macroscopic examination following decalcification (Figure 2), the sagittal sections taken passing through the center of the defective regions after the tissue follow-up procedure were embedded in paraffin. For morphological examination, 3-μm-thick sections were taken from the paraffin blocks obtained. Sections stained with hematoxylin–eosin (H–E), safranine-0, and toluidine blue were examined using a light microscope. The safranine-0 and toluidine blue used in the determination of the quantities of proteoglycan and cartilage were evaluated semiquantitatively. In the evaluation, the Modified Pineda (25) Histological Grading Scale was used (Table 1).

2.6. Immunohistochemical method and evaluation
The sections were placed on positively charged slides and deparaffinized in xylene, and then put into distilled water after the hydration process. In order to eliminate endogenous peroxidase activity, 1% hydrogen peroxide was applied on the sections for 10 min, and then they were washed with phosphate-buffered saline (PBS). Antigen retrieval was performed in a microwave for 35 min at high frequency in EDTA (pH 8.4) solution. For the purpose of reducing nonspecific staining, the sections were incubated with UV block for 20 min and then with
mouse VEGF (Thermo Scientific, UK), rabbit polyclonal type II collagen (Novocastra, UK) antibody, and rabbit polyclonal TGF-β1 (Gene Tex, USA), and for 1.5 h in a humid environment at 32 °C. Antibody determination was performed using AEC chromogen (Scytek, USA), and sections were counterstained with Mayer’s hematoxylin. After dehydration, they were closed with mounting medium (Scytek, USA). The sections were examined under light microscope. In the regenerated tissue, the expression of VEGF, type II collagen, and TGF-β1 was evaluated semiquantitatively as no staining (-), weak staining (+), moderate staining (++), and strong staining (+++), according to the intensity and extensity of staining.

2.7. Statistical analysis
SPSS 15.0 (SPSS Inc., Chicago, IL, USA) was used to record and evaluate the results. The distribution and type of data suggested the use of nonparametric test procedures; thus, the Kruskal–Wallis test and Mann–Whitney U-test were used. In the first step, the Kruskal–Wallis test was used to check the null hypothesis. The null hypothesis was rejected at a P value of <0.05.

3. Results
During the follow-up, 1 of the rabbits in the HA group died. The remaining 23 rabbits were included in the evaluation. Among all of the groups, there was a significant difference in terms of VEGF, type II collagen staining, and Pineda scores (P < 0.05). When the groups were compared in pairs, a significant difference was found between the control group and the ESW group in terms of Pineda score and type II collagen expression (P < 0.05), and no significant difference was found between them in terms of VEGF and TGF-β1 expression (P > 0.05). When the control group and the HA group were compared, a significant difference was found in terms of Pineda score, VEGF expression, type II collagen expression, and TGF-β1 expression (P < 0.05). When the control group and ESW + HA group were compared, there was a significant difference in terms of VEGF and TGF-β1 expression (P < 0.05), but there was no significant difference in terms of Pineda score and type II collagen expression (P > 0.05). When the ESW group and the HA group were compared, a significant difference was found in terms of VEGF expression and TGF-β1 expression (P < 0.05), but there was no significant difference in terms of and type II collagen expression and Pineda score (P > 0.05). When the HA and the ESW + HA group were compared, there was

Figure 2. White arrows indicate cartilage defects of control and study groups. a. control group, b. HA group, c. ESW group, d. ESW + HA group.
no significant difference in terms of Pineda score, VEGF expression, type II collagen expression, and TGF-β1 expression (Figure 3–7). The modified Pineda scores, VEGF, type II collagen, and TGF-β1 expression of the control and study groups are presented in Table 2.

4. Discussion

HA is a glycosaminoglycan constituent of synovial fluid and cartilage matrix, and plays an important physiological role in synovial joints (26–27). Intra-articular hyaluronans have been approved by the US Food and Drug Administration (FDA) since 1997 for treating osteoarthritis knee pain, and there are a number of different hyaluronic acid preparations for the treatment of knee osteoarthritis (28–30). Positive effects of HA on repair tissue have been reported in studies related to chondral defects (31).

Dorotka et al. (32) evaluated the influence of shock waves on the proliferation of human chondrocytes and ovine bone marrow stromal cells at EFDs of 0, 0.02, and 0.06 mJ/mm² and 0, 500, and 1000 impulses. They concluded that the proliferation of BMSC increased under the effect of shock-wave therapy, which provides an additional explanation for its effectiveness in the treatment of pseudarthrosis. Such proliferation, however, is not characteristic of chondrocytes. Chen et al. (22) found that the expression of TGF-β1 and VEGF-A significantly increased in femoral defects of rabbits after ESW (EFD of 0.16 mJ/mm² and 500 impulses). Nishida et al. reported (33) that a low-level shock wave enhanced the expression of VEGF and its receptor. Wang et al. (34) reported that in the repair of full-thickness articular cartilage defects in rabbits, low-energy shock waves in microfracture holes facilitated the production of hyaline-like cartilage repair tissues more than microfracture alone. A clinical study conducted on long-bone nonunions revealed that shockwave-promoted bone healing was associated with systemic elevations of serum NO levels and osteogenic growth factors, including TGF-β1, VEGF, and bone morphogenetic protein 2 (BMP-2) (35). Another study concluded that ESW has a chondroprotective effect in subchondral bone remodeling in the initiation of an osteoarthritis model of the anterior cruciate ligament of transected knees in rats (36).

Articular cartilage shows only a limited capacity for repair due to the lack of inherent mechanisms of repair in mature articular cartilage. Options for the repair or replacement of focal cartilage lesions include abrasive chondroplasty, subchondral drilling, and microfracture. In these procedures, bone marrow-derived stem cells

Table 1. Histological grading scale for the defects of cartilage (Pineda et al. (14)).

<table>
<thead>
<tr>
<th>Category</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell morphology</strong></td>
<td></td>
</tr>
<tr>
<td>Hyaline cartilage</td>
<td>0</td>
</tr>
<tr>
<td>Mostly hyaline cartilage</td>
<td>1</td>
</tr>
<tr>
<td>Mostly fibrocartilage</td>
<td>2</td>
</tr>
<tr>
<td>Mostly noncartilage</td>
<td>3</td>
</tr>
<tr>
<td>Noncartilage only</td>
<td>4</td>
</tr>
<tr>
<td><strong>Matrix-staining (metachromasia)</strong></td>
<td></td>
</tr>
<tr>
<td>Normal (compared with host adjacent cartilage)</td>
<td>0</td>
</tr>
<tr>
<td>Slightly reduced</td>
<td>1</td>
</tr>
<tr>
<td>Markedly reduced</td>
<td>2</td>
</tr>
<tr>
<td>No metachromatic stain 3</td>
<td>3</td>
</tr>
<tr>
<td><strong>Surface regularity</strong></td>
<td></td>
</tr>
<tr>
<td>Smooth (&gt;3/4)</td>
<td>0</td>
</tr>
<tr>
<td>Moderate (&gt;1/2–3/4)</td>
<td>1</td>
</tr>
<tr>
<td>Irregular (1/4–1/2)</td>
<td>2</td>
</tr>
<tr>
<td>Severely irregular (&lt;1/4)</td>
<td>3</td>
</tr>
<tr>
<td><strong>Thickness of cartilage</strong></td>
<td></td>
</tr>
<tr>
<td>&gt;2/3</td>
<td>0</td>
</tr>
<tr>
<td>1/3–2/3</td>
<td>1</td>
</tr>
<tr>
<td>&lt; 1/3</td>
<td>2</td>
</tr>
<tr>
<td><strong>Integration of donor with host adjacent cartilage</strong></td>
<td>0</td>
</tr>
<tr>
<td>Both edges integrated</td>
<td>0</td>
</tr>
<tr>
<td>One edge integrated</td>
<td>1</td>
</tr>
<tr>
<td>Neither edge integrated</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total maximum</strong></td>
<td>14</td>
</tr>
</tbody>
</table>

* Total smooth area of the reparative cartilage compared with the entire area of the cartilage defect.

Table 2. All quantitative data from control and study groups are presented: modified Pineda scores, VEGF expression, type II collagen expression, and TGF-β1 expression. Data are expressed as mean and standard error of mean.

<table>
<thead>
<tr>
<th></th>
<th>Control group (n = 6)</th>
<th>ESW group (n = 6)</th>
<th>HA group (n = 5)</th>
<th>ESW + HA group (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified Pineda score</td>
<td>3.33 ± 0.52</td>
<td>1.50 ± 0.55 *</td>
<td>2.00 ± 0.71 *</td>
<td>1.33 ± 0.52 *</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.33 ± 0.52</td>
<td>0.33 ± 0.52</td>
<td>1.80 ± 0.84 b</td>
<td>1.17 ± 0.41 b</td>
</tr>
<tr>
<td>Type II collagen</td>
<td>0.50 ± 0.55</td>
<td>1.50 ± 0.55 *</td>
<td>2.20 ± 1.10 *</td>
<td>1.83 ± 0.75 *</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>0.50 ± 0.84</td>
<td>0.83 ± 0.41</td>
<td>1.80 ± 0.45 b</td>
<td>1.50 ± 0.55 b</td>
</tr>
</tbody>
</table>

* vs control group; b vs ESW group
Figure 3. Histological findings using hematoxylin–eosin (a–d) and toluidine blue (e–h) stains. The defect was untreated (control group) (a–e), treated with ESW (b–f), treated with HA (c–g), and treated with ESW + HA (d–h) (Original magnification ×100).

Figure 4. Immunohistochemical staining for VEGF (a–d). The defect was untreated (control group) (a), treated with ESW (b), treated with HA (c), and treated with ESW + HA (d) (Original magnification ×100).
(BMSC) are stimulated to migrate from the subchondral bone to the site of cartilage defect. However, this results in the formation of fibrocartilage rather than hyaline cartilage, which contains more fibrous tissue, and has significantly less proteoglycan and more type I collagen in terms of biochemistry (37).

The purpose of this study was to determine the nonsurgical efficiency of HA and ESW in cartilage defects by comparing the efficiency of ESW and HA without developing a microfracture in the experimental cartilage defect model. Among the evaluation parameters of our study, we used the quantity of type II collagen as well as tVEGF, TGF-β1, and Pineda score, and we tried to demonstrate how newly developed cartilage resembles hyaline cartilage. As a result, it was observed that in both treatment methods more type II collagen was synthesized than in the control group. There was no difference in the quantities of TGF-β1 and VEGF in the ESW group as compared to the control group. Hausdorf et al. (38) did not find any significant increase with ESW in TGF-β1 quantity;
however, Wang (21) applied ESW and reported significant increases depending on dose in samples taken from the femur distal after bone marrow culture. Although we applied 500 impulses at 0.16 mJ/mm² dosage as specified by Wang, we found no increase in TGF-β1. We think that this result may be associated with the fact that TGF-β1 evaluation was performed 8 weeks after ESW was carried out. Furthermore, since we did not apply microfracture in the subchondral bone, it may be that the migration of the growth factors, which are thought to be stimulated in the bone marrow, to the cartilage defect area became difficult. The advantage of the model used is that ESW and HA viscosupplementation processes are nonoperative techniques. Thus, we think that both methods may be used in treating cartilage defects, and that they may yield better results if applied in combination with surgical techniques. In the patient group where HA and ESW techniques were combined, an increase was determined in VEGF and type II collagen expression. The most important limitations of the study are a lack of information about when or in which sessions ESW needs to be applied, and about EFD and impulse quantity.

In conclusion, we think that both treatment methods have positive therapeutic effects on articular cartilage in terms of the parameters studied. Due to its effect on VEGF and TGF-β1, HA is a better treatment than ESW in EFD and the impulses studied. In order to evaluate the effect of ESW on type II collagen synthesis in particular, new studies should be conducted at different EFD and impulse quantities.

Acknowledgment
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References


