Original article

Effect of low-energy shock waves in microfracture holes in the repair of articular cartilage defects in a rabbit model

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Keywords: articular; cartilage repair; marrow stimulation; shock waves

Background  Microfracture is a type of bone marrow stimulation in arthroscopic cartilage repair. However, the overall concentration of the mesenchymal stem cells is quite low and declines with age, and in the end the lesion is filled by fibrocartilage. The aim of this research was to investigate a novel method of enhancing microfracture by determining whether low-energy shock waves in microfracture holes would facilitate cartilage repair in a rabbit model.

Methods  Full-thickness cartilage defects were created at the medial femoral condyle of 36 mature New Zealand white rabbits without penetrating subchondral bone. The rabbits were randomly divided into three groups. In experimental group A, low-energy shock-wave therapy was performed in microfracture holes (diameter, 1 mm) at an energy flux density (EFD) of 0.095 mJ/mm² and 200 impulses by DolorClast Master (Electro Medical Systems SA, Switzerland) microprobe (diameter, 0.8 mm). In experimental group B, microfracture was performed alone. The untreated rabbits served as a control group. At 4, 8, and 12 weeks after the operations, repair tissues at the defects were analyzed stereologically, histologically, and immunohistochemically.

Results  The defects were filled gradually with repair tissues in experimental groups A and B, and no repair tissues had formed in the control group at 12 weeks. Repair tissues in experimental group A contained more chondrocytes, proteoglycans, and collagen type II than those in experimental group B. In experimental group B, fibrous tissues had formed at the defects at 8 and 12 weeks. Histological analysis of experimental group A showed a better Wakitani score \((P < 0.05)\) than in experimental group B at 8 and 12 weeks after the operation.

Conclusions  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. This model demonstrates a new method of improving microfracture and applying shock waves in vivo. However, longer-term outcomes require further study.

A rticular cartilage is a type of highly organized tissue with complex biomechanical properties and substantial durability.\(^1\) However, it has a poor intrinsic capacity for healing, and damage from trauma or degeneration can result in morbidity and functional impairment.\(^2\) Untreated lesions can lead to debilitating joint pain, dysfunction, and degenerative arthritis. Therefore, the management of articular cartilage defects continues to be one of the most challenging clinical problems for orthopaedic surgeons.\(^3,4\)

To date, cartilage repair strategies include debridement; bone marrow stimulation; and cell-based, cell plus scaffold-based, and whole-tissue transplantation techniques.\(^5\) Surgical procedures such as mosaicplasty and microfracture have shown stable clinical results in many cases. Additionally, autologous chondrocyte transplantation has been developed as a novel, improved treatment for cartilage defects.\(^6-8\) However, these modalities have some unresolved drawbacks, including unsatisfactory results despite the complexity of the surgical procedures, high cost, and adverse events associated with autologous chondrocyte transplantation.\(^9\)

Microfracture is a type of bone marrow stimulation in arthroscopic cartilage repair. It is commonly used as the first-line treatment for osteochondral lesions because of its minimal invasiveness, low associated morbidity, relatively short postoperative recovery time, and cost-effectiveness.\(^10-13\) Microfracture involves perforation of the subchondral plate in order to recruit mesenchymal stem cells from the bone marrow space into the lesion.\(^14,15\) However, the overall concentration of the mesenchymal stem cells is quite low and declines with age.\(^16\) Moreover, in the end the lesion is filled by fibrocartilage, which has different biomechanical properties from the native hyaline cartilage.\(^15\)

Shock waves have been employed for the management of uroliths in humans since 1977,\(^17,18\) and in the 1990s the application of shock waves was extended to musculoskeletal diseases. The effectiveness of treatment with extracorporeal

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Figure 1. Rabbit articular cartilage defect model and treatments with microfracture and shock waves. A: Corneal trephine (external diameter, 4.5 mm). B: Weight-bearing area of medial condyle. C: Cartilage removed from weight-bearing area of medial condyle by corneal trephine and flat surgical blade. D: Approximately full-thickness cartilage defect at the medial condyle. E: Cartilage defect after microfracture and low-energy shock waves in the microfracture holes (diameter, 1.0 mm). F: Handle of Swiss DolorClast Master (Electro Medical Systems SA, Switzerland) with micro-applicator (diameter, 0.8 mm).

shock waves has been shown in a range of diseases, including nonunion and chronic tendinopathies, by an increasing number of studies.19-24 Shock waves are single high-amplitude sound waves that propagate in tissue with a sudden rise from ambient pressure to maximum pressure at the wave front, followed by lower tensile amplitude.25 Although knowledge of the exact mechanism of the effect of shock waves in musculoskeletal diseases remains insufficient,26-30 two major mechanisms seem to be involved in the translation of mechanical shock-wave energy into its biological effects: membrane hyperpolarization and the formation of free radicals.31,32 It has been found that proliferation of bone marrow mesenchymal cells and production of growth factors are increased under the influence of shock-wave therapy.38,39 Recently, with the help of the endoscope, the micro-applicator of shock-wave equipment (Swiss LithoClast, Electro Medical Systems SA, Switzerland) has been used to manage uroliths directly in the ureter, urethra, and bladder. The question has thus arisen whether low-energy shock waves can be applied in microfracture holes to improve the results of microfracture in the treatment of cartilage defects under arthroscopy. The purpose of this study was to determine whether the repair results of microfracture in cartilage defects can be improved by the application of low-energy shock waves in microfracture holes.

METHODS

Animals and groups
Thirty-six skeletally mature and healthy New Zealand White rabbits (male or female, 6–8 months old, 2.5–3.0 kg body weight) were provided by the Experimental Animals Center of Chinese People’s Liberation Army (PLA) General Hospital, and were kept in a controlled environment with access to food and water. All the experimental protocols were in compliance with the Animal Welfare Act and were approved by the Institutional Animal Care and Use Committee of the Laboratory Animal Research Centre at Chinese PLA General Hospital.

All the rabbits were randomly divided into three groups. In experimental group A (n=24 knees), the rabbits were treated with shock waves in the microfracture holes at the cartilage defect. In experimental group B (n=24 knees), microfracture alone was performed. The rabbits with untreated defects served as the control group (n=24 knees).

Animal experiment
The rabbits were anesthetized by intramuscular injection of ketamine/xylazine/buprenorphine. The rear limb was shaved and then disinfected. The stifle joint of the hind leg was opened using sterile technique by a 3-cm-long medial parapatellar incision followed by lateral patellar luxation. The knee was flexed 120° to 140° to expose the weight-bearing area of the medial femoral condyle. A round chondral defect (4.5 mm in diameter) was created in the weight-bearing area of the medial femoral condyle. A round chondral defect (4.5 mm in diameter) was created in the weight-bearing area of the medial femoral condyle. A round chondral defect (4.5 mm in diameter) was created in the weight-bearing area of the medial femoral condyle. A round chondral defect (4.5 mm in diameter) was created in the weight-bearing area of the medial femoral condyle.

Intraoperatively, no bleeding from the subchondral bone was observed. Joint surfaces were kept moist with sterile saline irrigation every 30 to 60 seconds to maintain the viability of cells in the process of exposing articular and subchondral bone. In experimental group A, four microfracture holes (1 mm in diameter) were created in the cartilage defect using a 1-mm-diameter Kirschner needle to penetrate the subchondral bone to a depth of approximately 2 to 3 mm until bleeding was observed. Then shock-wave therapy was performed at an energy flux density (EFD) of 0.095 mJ/mm² and 200 impulses by placing the micro-applicator (diameter, 0.8 mm) of the Swiss DolorClast Master (Electro Medical Systems SA, Switzerland) in the microfracture holes (1 mm in diameter) (Figure 1E, 1F). In experimental group B, microfracture was performed alone at the defect without shock-wave therapy. In the control group, the defects were untreated. After the operation, the patella was repositioned and the knee capsule was closed by precise
and chondrocyte counts of tissue slices were determined. Chondrocyte count and histological scoring with hematoxylin solution. Collagen type II was stained tetrahydrochloride (DAB) solution, and counterstained brown. For immunohistochemistry, the unstained 5-µm sections and stains for histomorphometric and immunohistochemical assessment of reparative tissue types.

For immunohistochemistry, the unstained 5-µm sections were deparaffinized and rehydrated, with endogenous peroxidase activity quenched by immersion of the sections in 30 ml/L H2O2 in methanol. The sections were then washed with phosphate-buffered saline (PBS) for 5 minutes, repeated 2 times, and the slides were incubated at 4°C overnight with monoclonal anti-mouse/human IgG (Santa Cruz Biotechnology, USA). Subsequently, the sections were rinsed in PBS for 5 minutes, repeated 2 times, and incubated with anti-mouse antibody (MaxVision, Maixin, Fujian, China) for 10 to 15 minutes at room temperature. Then the sections were washed again in PBS for 3 times, incubated in 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution, and counterstained with hematoxylin solution. Collagen type II was stained brown.

**Chondrocyte count and histological scoring**

Cartilage cell morphology in repaired tissue was analyzed and chondrocyte counts of tissue slices were determined using Image-Pro Plus 5.0 (Media Cybernetics, USA). Cartilage cells were counted in five different visual fields of a high-power lens (×40) in every H&E staining slice. The degree and the quality of healing in the microfracture holes and defects were assessed and scored blindly by the senior author (LI) and the first author (WANG), using the repair scoring systems of Wakitani et al40 for histological evaluation. The graded parameters included cellular morphology, matrix staining, surface regularity, bonding to adjacent cartilage, and height of reparative tissue. The maximum possible score in the Wakitani scoring system was 14 points (minimum, 0 points).

**Statistical analysis**

Results are reported as mean ± standard deviation. The average chondrocyte counts of the specimens were compared, and differences between groups were analyzed by analysis of variance (ANOVA). Differences in histological scoring between groups were analyzed using ANOVA. Statistical analyses were performed with SPSS 17.0 (SPSS Inc., IL, USA). The level of statistical significance was set at P <0.05.

**RESULTS**

**Gross observation**

All operative incisions at rear limbs underwent primary healing, and all rabbits survived the follow-up period of 4, 8, or 12 weeks without infection or synovitis in the operated knees. At 4 weeks after the operation, white hyaline tissue filled the microfracture holes and did not fill the defective area between the holes in both experimental groups A and B, while the defects in the control group were virtually unchanged (Figure 2A–2C). At 8-week follow-up, in experimental group A, the defects were partly filled by white hyaline tissue (Figure 2D). In experimental group B, the defects were filled by some white hyaline tissue and some fiber-like tissue (Figure 2E). In the control group, no regenerated tissues filled the defects (Figure 2F). At 12 weeks after the operation, in experimental group A, the defects were partly covered by smooth, glistening white hyaline tissue that was almost indistinguishable from the surrounding normal cartilage, although the defects were not filled completely (Figure 2G). In experimental group B, the defects were partly filled by rough fibrous tissues (Figure 2H). In the control group, the defects were still not obviously changed (Figure 2I).

**Cellular morphological, histological, and immunohistochemical evaluation of the articular cartilage repair**

**Four weeks after operation**

At 4 weeks after operation, in experimental group A, abundant new chondrocytes in microfracture holes were shown by H&E staining. At the inferior part and the bottom of the repair tissue, the chondrocytes were large and round or elliptical. At the superior part, the new chondrocytes were small but more intense. At the bottom of reparative tissue, abundant vascularization was seen, which was not observed in experimental group B (Figure 3A). Toluidine blue staining and safranin O staining showed a considerable amount of proteoglycan in newly formed cartilage tissue in experimental group A. The proteoglycan was more obviously at the inferior part and the bottom of the reparative tissue (Figure 3B, 3C). In type II collagen immunohistochemical staining, type II collagen was concentrated in the superior part of the
**Figure 2.** Gross observation at 4, 8, and 12 weeks after operation. A, D, G: In experimental group A, microfracture holes and defects were gradually and partly filled by white hyaline tissue at 4, 8, and 12 weeks after operation. B: In experimental group B, microfracture holes were filled by white hyaline tissue as in group A at 4 weeks after operation. E: In experimental group B, the defects were filled by some white hyaline tissue and some fiber-like tissue at 8 weeks after operation. H: In experimental group B, the defects were partly filled by rough fibrous tissues. C, F, I: The defects in the control group were virtually unchanged at 4, 8, and 12 weeks after operation. Bar=4 mm.

**Figure 3.** Four weeks after operation. In group A, abundant new chondrocytes in microfracture holes were shown by H&E staining. At the bottom of reparative tissue, abundant vascularization was seen (A). Toluidine blue staining and safranin O staining showed a considerable amount of proteoglycan in newly formed cartilage tissue in group A (B, C). In type II collagen immunohistochemical staining, type II collagen was concentrated in the superior part of the new tissue (D). In group B, H&E staining showed that new chondrocytes were very intense in the whole repair tissue, and no vessels appeared at the bottom (E). Toluidine blue staining and safranin O staining indicated less proteoglycan in the regenerated tissue (F, G). Type II collagen immunohistochemical staining showed that type II collagen was rich in the whole regenerated tissue (H). In the control group, no tissue appeared in the defects (I–L). Bar=50 µm.

repair tissue, where small new chondrocytes were intense (Figure 3D). As compared with experimental group A, in experimental group B, H&E staining showed that new chondrocytes were very intense in the whole repair tissue,
Eight weeks after operation. In group A, H&E staining showed that microfracture holes were full of hyaline-like cartilage tissue, and there were rich blood vessels at the bottom. Chondrocytes with the typical structure of hyaline cartilage lacunae were arranged in layers at the superior part and in columns at the caudomedial part (A). In toluidine blue staining and safranin O staining, the proteoglycan was more obvious at the inferior part of the regenerated hyaline-like cartilage in the holes (B, C), and type II collagen immunohistochemical staining showed that type II collagen was concentrated in the superior part (D). In group B, H&E staining showed that the microfracture holes were filled by regenerated tissue, the cartilage cells were dense and disordered, and some fibrous tissues had formed (E). Toluidine blue staining and safranin O staining showed little proteoglycan formed in the tissue (F, G). Type II collagen was at the surface layer of the reparative tissue with type II collagen immunohistochemical staining (H). In the control group, there was still no newly formed tissue appearing in the defects (I–L). Bar=50 µm.

Eight weeks after operation

At 8 weeks after operation, in experimental group A, H&E staining showed that microfracture holes were full of hyaline-like cartilage tissue, and there were rich blood vessels at the bottom. Chondrocytes with the typical structure of hyaline cartilage lacunae were arranged in layers at the superior part and in columns at the caudomedial part (Figure 4A). In toluidine blue staining and safranin O staining, the proteoglycan was more obvious at the inferior part of the regenerated hyaline-like cartilage in the holes (Figure 4B, 4C), and type II collagen immunohistochemical staining showed that type II collagen was concentrated in the superior part (Figure 4D). In experimental group B, H&E staining showed that the microfracture holes were filled by regenerated tissue, the cartilage cells were dense and disordered, and some fibrous tissues had formed (Figure 4E). Toluidine blue staining and safranin O staining showed little proteoglycan formed in the tissue (Figure 4F, 4G). Type II collagen was at the surface layer of the reparative tissue in type II collagen immunohistochemical staining (Figure 4H). In the control group, there was still no newly formed tissue appearing in the defects (Figure 4I–4L).

Twelve weeks after operation

At 12 weeks after operation, in experimental group A, the regenerated tissue from microfracture appeared as hyaline-like cartilage with H&E staining. Chondrocytes with hyaline cartilage lacunae were arranged in columns at the superior part and in columns at the caudomedial part (Figure 5A). Toluidine blue staining and safranin O staining showed that type II collagen was intense at the caudomedial part of newly formed cartilage, and some vessels appeared at the bottom (Figure 5A). Toluidine blue staining and safranin O staining showed that proteoglycan was intense at the caudomedial part of newly formed cartilage.
Figure 5. Twelve weeks after operation. In group A, the regenerated tissue appeared as hyaline-like cartilage with H&E staining. Chondrocytes with hyaline cartilage lacunae were arranged in columns at the caudomedial part of newly formed cartilage, and some vessels appeared at the bottom (A). Toluidine blue staining and safranin O staining showed that proteoglycan was intense at the caudomedial part (B, C). Type II collagen was obvious at the superior part and surface layer of the new cartilage with immunohistochemical staining (D). In group B, H&E staining showed that the superior part of the regenerated tissue was loose fibrous tissue and fibrocartilage, and the inferior part contained some vessels and chondrocytes with disordered distribution (E). Toluidine blue staining and safranin O staining showed little proteoglycan formed in the tissue (F, G). Immunohistochemical staining indicated little type II collagen (H). In the control group, a small amount of fibrous tissue appeared at the defects with H&E staining (I–L). No regenerated tissue was observed in the defect areas with toluidine blue staining, safranin O staining, and type II collagen immunohistochemical staining (I–L). Bar=50 µm.

Chondrocyte count and histological scoring

In experimental group A, the mean chondrocyte counts were 2315±114 (4 weeks after operation), 2184±156 (8 weeks after operation), and 1867±93 (12 weeks after operation). In experimental group B, they were 2383±117 (4 weeks after operation), 2294±82 (8 weeks after operation), and 1431±106 (12 weeks after operation). In the control group, no repair tissue appeared in the defects at 12 weeks after operation. No statistically significant difference in chondrocyte count was noted between experimental groups A and B at 4 weeks and 8 weeks (P>0.05), but at 12 weeks after operation the average chondrocyte count of experimental group A was greater than that of experimental group B, with the difference being statistically significant (P<0.05).

The mean histological scores were 6.13±0.83 (4 weeks after operation), 4.00±1.06 (8 weeks after operation), and 2.75±1.04 (12 weeks after operation) in experimental group A; and 6.25±0.71 (4 weeks after operation), 6.75±0.71 (8 weeks after operation), and 7.75±0.71 (12 weeks after operation) in experimental group B. Because in the control group no repair tissue appeared in the
defects at 12 weeks after operation, the mean histological scores were 14±0 at all three time points. No statistically significant difference was found between the scores of experimental groups A and B at 4 weeks (P >0.05). The scores of experimental group A were significantly different from those of experimental group B at 8 and 12 weeks and from those of the control group at 4, 8, and 12 weeks (P <0.05) (Figure 6). These scores showed that the repair results of the application of low-energy shock waves in microfracture holes were significantly better than those of microfracture alone at 8 and 12 weeks after the operation.

**Figure 6.** Bar chart showing significant differences between the Histological scores of experimental groups A and B and the control group at 8 and 12 weeks after operation. The histological result of newly formed cartilage tissue in experimental group A was better than that in the other two groups at 8 and 12 weeks after operation.

**DISCUSSION**

Although knowledge of the exact mechanism of the effect of shock waves remains insufficient, the effectiveness of shock-wave treatment in musculoskeletal diseases has been demonstrated by a growing number of studies. Many studies have indicated that shock-wave therapy can affect the cellular ultrastructure and the proliferation of marrow stem cells, as well as enhancing the expression of some biological factors.37-41

The effect of shock waves on cells differs according to energy density, from the development of intercellular gaps to frank detachment of endothelial cells and basement membrane damage.31,42 Steinbach et al42 found that the cell membrane can be altered by shock waves of 0.12 mJ/mm² and is the most sensitive part of the cell. In research on the application of extracorporeal shock-wave therapy (ESWT) (1500 shock waves of 0.5 mJ/mm²) to femoral heads of adult Sprague-Dawley rats, Mayer-Wagner et al43 found that extracorporeal shock waves caused alterations of hyaline cartilage on a molecular and ultrastructural level, and that the alterations on the ultrastructural level included expansion of the rough-surfaced endoplasmic reticulum, detachment of the cell membrane, and necrotic chondrocytes. Therefore, high-energy ESWT may cause degenerative changes in hyaline cartilage similar to those in initial osteoarthritis. Dorotka et al44 evaluated the influence of shock waves on the proliferation of human chondrocytes and ovine bone marrow stromal cells (BMSCs) at EFDs of 0, 0.02, and 0.06 mJ/mm² and 0, 500, and 1000 pulses. The research showed that the proliferation of BMSCs was increased under the influence of shock-wave therapy, and chondrocytes had less proliferative potential than untreated controls and were not positively affected. Chen et al45 found that expression of transforming growth factor-β1 (TGF-β1) and vascular endothelial growth factor-A (VEGF-A) was significantly increased in femoral defects of rabbits after ESWT (0.16 mJ/mm², 500 impulses). Nishida et al46 reported that a low level of shock waves enhances the expression of VEGF and its receptor, Flt-1, in cultured human umbilical vein endothelial cells in vitro, with a maximum effect noted at 0.09 mJ/mm².

In our early research using a rabbit model, we found that the microfracture holes were expanded and the subchondral bone around the holes was badly damaged when the EFD was higher than 0.095 mJ/mm² and the frequency was more than 200 impulses. According to the reports cited above, an EFD of 0.095 mJ/mm² can increase proliferation of marrow stromal cells and expression of TGF-β1 and VEGF. Therefore, in this research we chose low-energy shock waves at an EFD of 0.095 mJ/mm² and 200 impulses, and the shock-wave application was performed directly in the microdrill holes (1 mm in diameter) through a micro-applicator (0.8 mm in diameter). At 4, 8, and 12 weeks after the operation, histological and immunohistochemical staining showed proliferation of chondrocytes and blood vessels in microfracture holes in the shock-wave group, and more obvious increase of proteoglycan and type II collagen in the regenerated area than in the group treated with microfracture alone. At 8 and 12 weeks after surgery, chondrocytes with the typical structure of hyaline cartilage lacunae were arranged in columns and distributed in layers as in normal cartilage in the regenerated tissues of the shock-wave group. However, in the group treated with microfracture alone, the distribution of cells was dense and disordered, and fibrous tissue had formed in the repair tissues at 8 and 12 weeks. All these results demonstrated that microfracture with shock-wave therapy in the microfracture holes at an EFD of 0.095 mJ/mm² and 200 impulses produced better regenerated tissue than microfracture therapy alone.

Dorotka et al44 reported that the proliferation of ovine BMSCs could be increased under the influence of shock-wave therapy. Chen et al48 found that expression of TGF-β1 and VEGF-A was significantly increased in femoral defects of rabbits after ESWT. Nishida et al45 reported that a low level of shock waves enhances the expression of VEGF and its receptor, Flt-1, in cultured human umbilical vein endothelial cells in vitro at 0.09 mJ/mm². The results of these studies and the present
study indicate that the impact of low-energy shock waves on the bone marrow blood in microfracture holes enhanced the generation of hyaline cartilage cells and blood vessels in the regenerated tissues.

At 4, 8, and 12 weeks after the operation, the control group showed either no or minimal spontaneous repair tissue at defects, with the subchondral bone maintaining its integrity. This has also been observed in previous studies on chondral defects. Therefore, the results indicated that full-thickness chondral defects with a diameter of 4.5 mm were not prone to much spontaneous healing within 12 weeks after surgery.

In the present study, we did not find repair of subchondral bone in the microfracture holes, and we found that all the cartilage defects in both of the experimental groups were not completely filled. Therefore, the long-term results of shock-wave treatment in microfracture holes remain to be explored. Meanwhile, future study will focus on the results of use of different EFDs and impulses in large animals.

On the basis of the present study, we conclude that in the treatment of articular cartilage defects in adult New Zealand White rabbits, low-energy shock waves applied directly in the microfracture holes generated hyaline cartilage-like tissue in the holes and defects within 12 weeks after operation, a better result than the regenerated fibrous tissue observed after application of microfracture alone. Moreover, low-energy shock waves increased the generation of hyaline cartilage cells and blood vessels in the regenerated tissue. However, the long-term results in the rabbit model and the results of different EFDs and impulses in large animals require further study.

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