

Effects of fibroblast growth factors 2 and low intensity pulsed ultrasound on the repair of knee articular cartilage in rabbits

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Abstract. – OBJECTIVE: To investigate the effect of low intensity ultrasound irradiation combine with fibroblast growth factors (FGF2) on the repair of the knee articular cartilage and to explore its mechanism in rabbit.

MATERIALS AND METHODS: The model of the rabbit knee joint injury was established. 40 rabbits were divided into four groups, including control group, model group, FGF2 group and FGF2 + low intensity pulsed ultrasound group (FGF2 + LIPU). The knee joints of rabbits were taken at 4 and 8 weeks, respectively. Histopathological changes were detected by Hematoxylin and Eosin stain (HE) and evaluated by Wakitani score. The expression of FGF2 mRNA was detected by Real-time polymerase chain reaction (RT-PCR) and the levels of Collagen I and Collagen II protein were analyzed by Western blotting.

RESULTS: In FGF2 group and FGF2 + LIPU group, it was found that the tissues of knee joint were gradually repaired following the change of time. Further, the recovery was better in FGF2 + LIPU group. Cartilage defect areas were filled with cartilage-like cells and the repair surface was fused with surrounding cartilage in FGF2 and FGF2 + LIPU groups. Wakitani scores were consistent with HE results. The expressions of FGF2 mRNA were higher in FGF2 and FGF2 + LIPU group than the model group. Western blotting results showed that the levels of Collagen I and Collagen II protein in FGF2 and FGF2 + LIPU groups were significantly increased compared with that in model group.

CONCLUSIONS: FGF2 and LIPU combined application on the rabbit knees joint repair is better than FGF2 alone. FGF2 and LIPU combination can promote the synthesis and secretion of collagen in chondrocytes, promote the differentiation and maturation of chondrocytes during the repair of cartilage defects.

Key Words:

Low intensity ultrasound irradiation, Fibroblast growth factors (FGF2), Knee articular cartilage, Rabbit, Collagen I, Collagen II.

Introduction

Articular cartilage enables the bones of the knee joint to move over one other without rubbing. The most common cause of osteochondral lesions is trauma in the United States¹. Articular cartilage can wear away when osteoarthritis affects a joint, leaving the bone to rub on bone. Degenerative lesions can be of differing shapes and depths, and the stiffening of subchondral bone leads to cartilage matrix breakdown and less shock absorption².

It still remained a challenge to treat articular cartilage lesions about the knee. There was currently a wide range of options available, from various types of operations to more conservative measures. A recent use of growth factors have increased people's interest^{3,4}. Fibroblast growth factors (FGFs) are a family of growth factors, with members involved in wound-healing, angiogenesis, various endocrine signaling pathways, and embryonic development^{4,5}. FGFs are key regulators in the processes of proliferation and differentiation of the wide variety of cells and tissues⁶. FGF2 is also known as basic fibroblast growth factor. FGFs play important roles in wound healing as well as in stimulating blood vessel growth. FGF1 and FGF2 stimulated the proliferation of fibroblasts and angiogenesis by growing granulation tissue, early in the wound-healing process^{7,8}. Previous research⁹ suggested that FGF2 was sufficient to repair articular cartilage damage. Low-intensity pulsed ultrasound (LIPU) was a pressure or sound wave with the capability to transfer mechanical energy into biological tissues¹⁰. The LIPU application on treatment of articular cartilage injury has been drawn the attention. Previous investigation¹¹ indicated that LIPU had significant therapeutic potential in treating a severe articular cartilage injuries in our animal study. We inve-

stigated the effect of FGF-2 combines with low intensity ultrasound irradiation on the repair of knee articular cartilage in rabbits and the effects of the expression of Collagen I and II.

Materials and Methods

Animals

40 pure adult healthy adult New Zealand rabbits were obtained from Medical Experimental Animal Center of Guangdong Province, regardless of male and female, weight 2.0-2.5 kg, free drinking water and feeding. Ethical approval was approved by the Medical Ethics Committee of Animal Care and Use Committee of the China Medical University.

Animal Model Establishment

A cartilage damage model of the knee joint was established. Briefly, after hair from knee removing, rabbits were anesthetized with the 30 g/L sodium pentobarbital (Lukang Pharmaceutical Limited by Share Ltd., Shandong, China) by intravenous injection. Under the aseptic condition, the anterior incision of the knee was selected, and the skin was cut about 2 cm long. Holes of 3.0-mm diameter, evenly spaced, were drilled using a hand drill (Synthes, Mathys AG, Bettlach, Switzerland), until the articular surface of the intercondylar fossa of femur was exposed. After hemostasis, the wound was washed with isotonic saline. Penicillin sodium (Lukang Pharmaceutical Limited by Share Ltd., Shandong, China) was injected at a dose of 0.01 mg/kg for 3 days after the operation. Animals were kept individually in metal cages and fed with standard rabbit diet and water ad libitum.

Experimental Grouping

Rabbits were divided into control group, model group, FGF2 group, FGF2 + LIPU group. Rabbits in control group were treated with skin injury but no knee injury. The collagen membrane containing only PBS (Geistlich Bio-Gide, Wolhusen, Switzerland) was implanted for rabbits before suture in model group. 100 ng/mL FGF2 (Sigma-Aldrich, St. Louis, MO, USA) was also performed for rabbits in FGF2 group. In FGF2 + LIPU group, rabbits were irradiated with low intensity pulsed ultrasound (Smith & Nephew Inc., Memphis, TN, USA) at 72 h after injury during 100 ng/mL FGF2 treatment. The ultrasound signal was composed of a 200-microsecond burst of 1.5 MHz and 30.0 ± 5.0 mW/cm² spatially and temporally averaged

incident intensity. The ultrasound treatment protocol started 3 days after operation for 20 min/d continuously until the animals were euthanized.

Rabbits in each group were sacrificed at 4 and 8 weeks, respectively. Five rabbits in each group were sacrificed, and the tissues of the knee joints were taken out. Some of them were stored in liquid nitrogen and partially in 4% paraformaldehyde (SolaiBao Biotechnology, Beijing, China). The color, cartilage surface and defect area of joint were observed in different group.

Histopathological Changes

A tissue slide was randomly selected from each group and stained with hematoxylin and eosin (HE, Bolait Chemical Co., Ltd., Wuhan, China). The repair of articular cartilage was observed under light microscope (Fuji Photo Film Co., Ltd., Tokyo, Japan). Wakitani method was used to evaluate the histological scoring of the specimens by Blind method (Table I). The method was based on cell morphology, matrix staining, surface regularity, thickness of the body and the surrounding cartilage. Aspects of the organization to repair the situation, the highest 14 points were the poor repair, the lowest 0 points were repair closed to normal cartilage.

RT-PCR Analysis

Total RNA was extracted using TRIzol Kit (Cat. no. 74104, Qiagen, Duesseldorf, Germany) following the manufacturer's instructions strictly. The quality and quantity of RNA were detected. Then, 500 ng of total RNA were obtained to generate cDNA by a reverse transcription kit (TaKaRa, Dalian, China). Quantitative polymerase chain reaction (qPCR) was carried out to measure the mRNA levels. The relative levels of target mRNA were standardized through GAPDH gene as reference. The relative expression of FGF2 mRNA in each group was calculated by 2^{-Ct} method.

Western Blotting

Collagen I and II protein levels were checked by BCA method (Sigma-Aldrich, St. Louis, MO, USA). Transmembrane was performed to transfer proteins to polyvinylidene difluoride (PVDF) (Thermo Fisher Scientific, Waltham, MA, USA). Blocking was performed using 5% skimmed milk. After washing, membranes were incubated with corresponding antibodies including rabbit anti-Collagen I antibody (1:1000, Thermo Fisher Scientific, Waltham, MA, USA), rabbit anti-Collagen II antibody (1:2000, Thermo Fisher Scientific, Waltham, MA, USA) and anti- β -actin

(1:1000, ab9845, Abcam, Cambridge, MA, USA) overnight at 4°C. The membranes were washed and incubated with anti-rabbit IgG-horseradish peroxidase (HRP) secondary antibody (1:1000, MBS435036, MyBioSource, San Diego, CA, USA) at room temperature for 1 h. Signals detection was performed using ECL method (Sigma-Aldrich, St. Louis, MO, USA). Relative expression levels of each protein were normalized to endogenous control β-actin using ImageJ software (Informer Technologies, Inc., Shingle Springs, CA, USA).

Statistical Analysis

The data were expressed as mean ± standard deviation (mean ± SD) and were analyzed by SPSS19.0 statistical software (Armonk, NY, USA). Comparisons between two groups were performed using *t*-test. Abnormal distribution data were compared between groups using one-way ANOVA. *p*<0.05 was considered to be statistically significant.

Results

General Observations

Joint fluid volume was colorless, transparent and viscous, while the appearance of articular cartilage was milky and shiny in control group (Figure 1A, E). After 4 weeks, there was a little adhesion in the knee joint, and the articular fluid

was turbid in model group. The cartilage defect area was lower than the surrounding cartilage, and the surrounding cartilage surface was rough (Figure 1B). In the FGF2 group, the synovial fluid was clear, and the cartilage defect area was filled with repair tissue, partly higher than the surrounding cartilage surface. But the surrounding tissue color was bright with smooth surface (Figure 1C). In the FGF2 + LIPU group, the repair tissue was filled with milky white transparent cartilage-like tissue, and neonatal cartilage surface was smooth. The outline of the restoration area was blurred (Figure 1D). After 8 weeks, compared with the control group, the color was light yellow, and the depression was obvious in model group (Figure 1F). In the FGF2 group, the joint was no adhesions and contracture, and cartilage defect repair tissue was filled completely (Figure 1G). In the FGF2+LIPU group, defect zone repair tissue was close to normal hyaline cartilage with surface gloss. The surface was smooth, and the surrounding cartilage tissue boundary was not obvious (Figure 1H). The results indicated that the repairing effect of FGF2 + LIPU were better than that of FGF2 from general appearance observation.

Histopathological Changes

In control group, articular cartilage surface was smooth, and four-layer structure was clearly visible. (Figure 2A, E). After 4 weeks, in model group,

Table I. Standard of Wakitani.

Index	Tissue expression	Scores
Cell morphology	Hyaline cartilage	0
	Hyaline cartilage is the main content	1
	Fibrocartilage is the main content	2
	Contains a small amount of cartilage	3
Matrix staining	No cartilage	4
	Normal	0
	Stain light	1
	Stain decrease significantly	2
Cartilage surface1)	No stain	3
	Smooth (>3/4)	0
	Coarser (1/2-3/4)	1
	Irregular (1/4-1/2)	2
Cartilage thickness2)	Especially rough (<1/4)	3
	>2/3	0
	1/3-2/3	1
The degree of binding between the filling and the surrounding cartilage	<1/3	2
	Totally combination	0
	Partial combination	1
Total scores	No combination	2
	---	14

Note: 1) The ratio of smooth surface to total defect area of cartilage; 2) The ratio of the average thickness of the packed cartilage to the thickness of the surrounding cartilage.

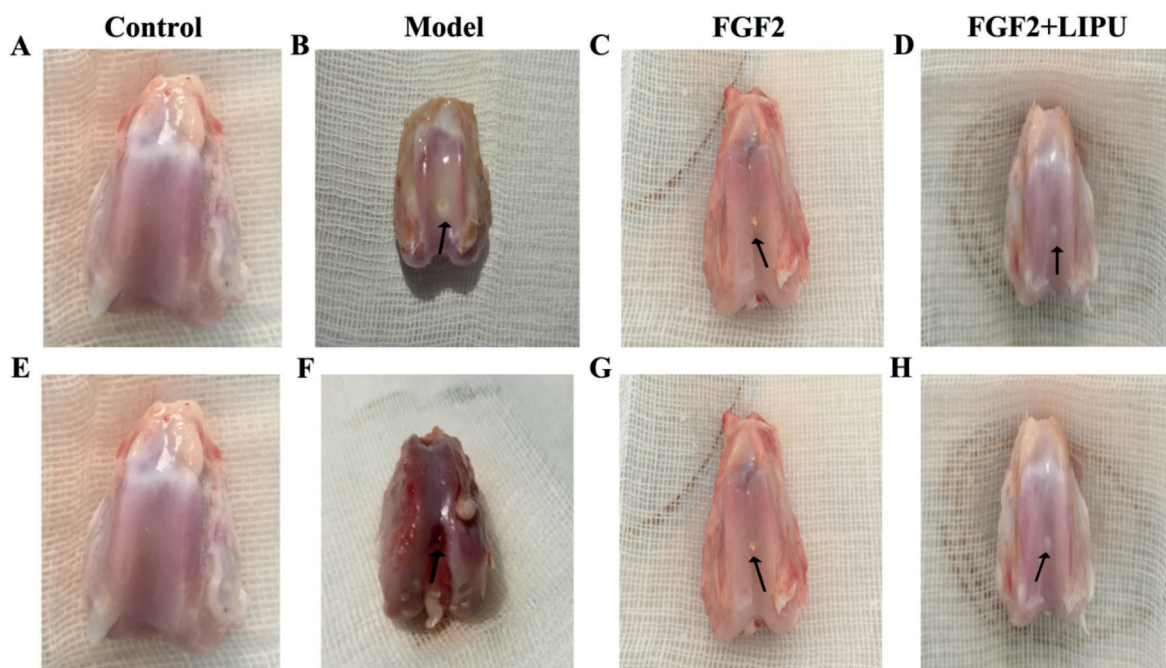


Figure 1. General appearance of knee articular cartilage at 4, 8 weeks. The first line represented the change of knee articular cartilage at 4 weeks (n=5). *A*, control group, *B*, model group, *C*, FGF2 group, *D*, FGF2 + LIPU group. The second line represented the observation of knee articular cartilage at 8 weeks (n=5). *E*, control group, *F*, model group, *G*, FGF2 group, *H*, FGF2 + LIPU group.

the defects were fibrous granulation tissue filled, but still left a large defect (Figure 2B). In the FGF2 group (FGF2), the surface of the repair tissue was flatter and part tissues were higher than the surrounding cartilage. The repair tissue was filled with a large number of cartilage-like cell clusters (Figure 2C). After 4 weeks, the cartilaginous bones were filled with cartilage-like cells in FGF2 + LIPU group (Figure 2D). At 8 weeks, the defect area was filled by the fibrous tissue. However, the surrounding cartilage surface was rough and cracked (Figure 2F). In the FGF2 group, the defect repair tissue surface was flat and filled with cartilage-like cells (Figure 2G). In the FGF2+ LIPU group, repair area tissue was hyaline cartilage, the boundaries were not obvious (Figure 2H). The results suggested that FGF2 + LIPU repair were better than FGF2 on histopathological changes. The outcomes of Wakitani were consistent with the HE results. Both the FGF2 and FGF2 + LIPU group had lower scores than the model group ($p < 0.05$). Compared with the FGF2 group, the FGF2 + LIPU group had lower scores.

The Expression of FGF2 in Each Group

After 4 weeks and 8 weeks, the expressions of FGF2 mRNA in FGF2 and FGF2 + LIPU group were significantly higher than that in model group

($p < 0.01$). Compared with FGF2 group, the levels of FGF2 mRNA had higher expression in FGF2 + LIPU group (Figure 3). Remarkably, the levels of FGF2 mRNA had no significant different in FGF2 + LIPU group compared with that of control group ($p > 0.05$).

Collagen I, Collagen II Protein Expression Levels

Collagen I/ β -actin and Collagen II/ β -actin were used to show the protein expression. After rabbit knee joint injury, the Collagen I and Collagen II protein expressions in FGF2 and FGF2 + LIPU group were significantly higher than those in model group ($p < 0.01$). Further, the levels of Collagen I and Collagen II proteins in FGF2 + LIPU group were higher than that of FGF2 group. That showed the elevated expression of FGF2 could promote the expression levels of Collagen I and Collagen II protein (Figure 4).

Discussion

As a member of FGFs, FGF2 could mediate mitogenic activities and stimulate tissue growth^{12,13}. Therefore, FGF2 has a potential for repair cartilage

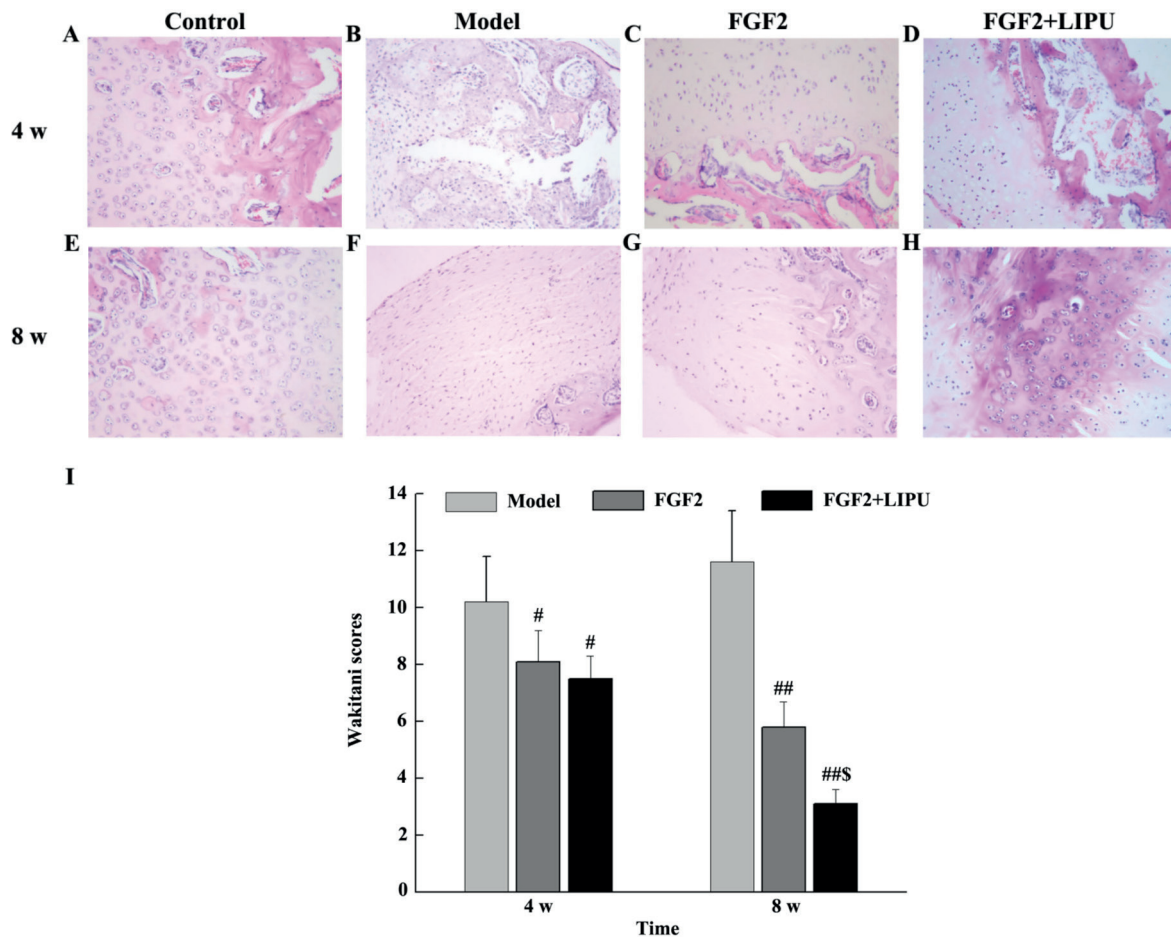


Figure 2. Histopathological changes of knee cartilage ($\times 200$) in all groups. The first line represented the change of knee articular cartilage at 4 weeks ($n=5$). **A**, control group, **B**, model group, **C**, FGF2 group, **D**: FGF2 + LIPU group. The second line represented the observation of knee articular cartilage at 8 weeks ($n=5$). **E**: control group, **F**, model group, **G**, FGF2 group, **H**, FGF2 + LIPU group. **I**: Waktani score. Compared with the model group, [#] $p<0.05$, ^{##} $p<0.01$. Compared with the FGF2 group, ^{\$} $p<0.05$.

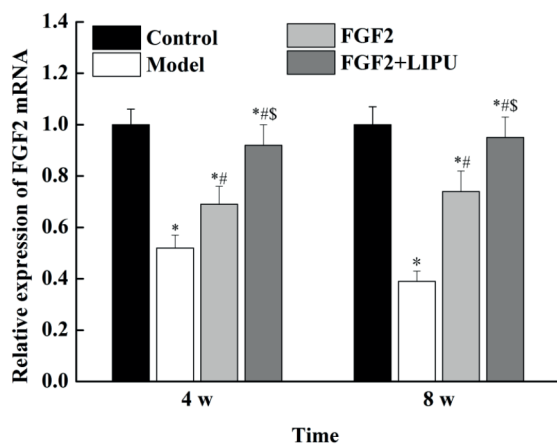


Figure 3. The levels of FGF2 mRNA in different groups at 4, 8 weeks detected by RT-PCR. Compared with control, ^{*} $p<0.05$. Compared with model, [#] $p<0.05$. Compared with the FGF2 group, ^{\$} $p<0.05$.

injury. We found that FGF2 could promote the repair of knee articular cartilage in rabbits. However, its effect was not very significant due to the lack of an efficient delivery system. LIPU is a new form of ultrasound therapy, that has been demonstrated beneficial effects on bone fracture healing and repair of bone fracture¹⁴. LIPU has also been shown to have a positive effect on the healing of other tissues, including ligament, muscle, and cartilage¹⁵⁻¹⁷. Research found that low-intensity pulsed US protects cartilage from damage in early-stage osteoarthritis via the integrin/FAK/MAPK pathway¹⁸. Extracellular signal-regulated kinase1/2 and p38 signaling pathway were also involved in abating this damage¹⁹. However, there is little study about FGF2 and LIPU combination application. We observed the effect of FGF2 combined with LIPU on repair of knee articular cartilage in rabbits. The results suggested the

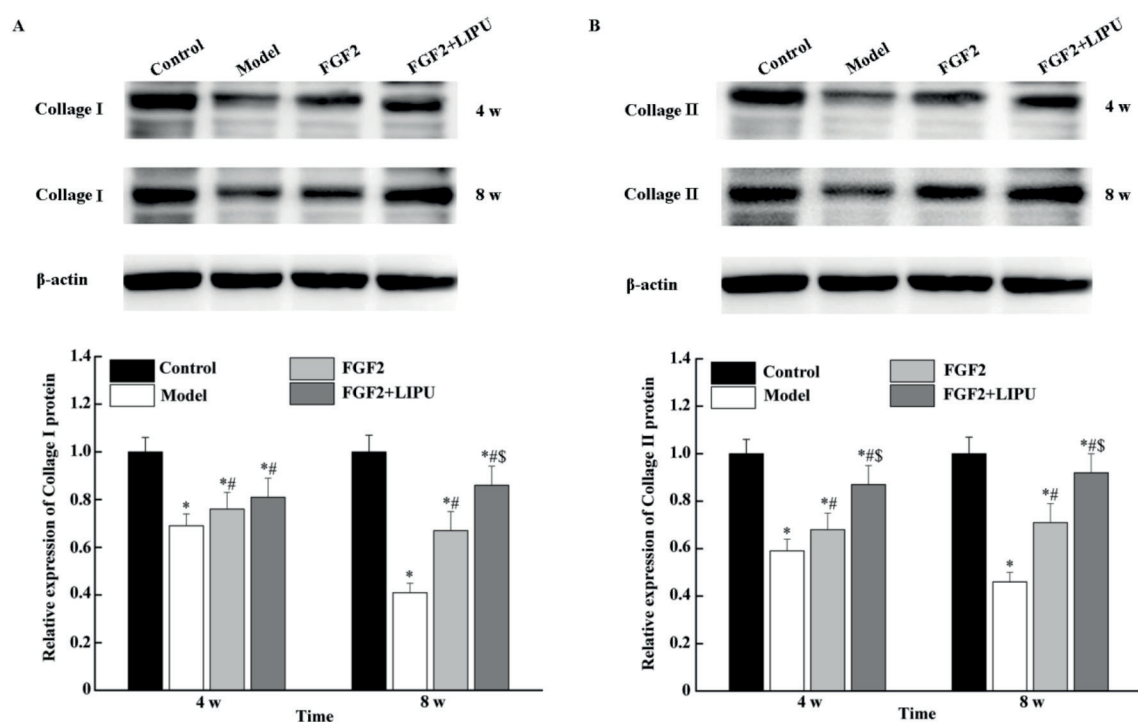


Figure 4. Western blot assay were used to detect Collagen protein level at 4 and 8 weeks. (A) The levels of Collagen I in control, model, FGF2 and FGF2+LIPU groups; (B) The levels of Collagen II proteins in control, model, FGF2 and FGF2+LIPU groups. Compared with control, * $p < 0.05$. Compared with model, # $p < 0.05$. Compared with the FGF2 group, \$ $p < 0.05$

effect of combination was more efficiently than that of FGF2 alone. Further investigation demonstrated that the expression of FGF2 mRNA was higher in FGF2 + LIPU group than that in FGF2 group, which meant the LIPU could stimulate FGF2 expression in articular cartilage. The results were consistent with the previous findings^{13,17}. Collagens are a major component of connective tissue in animals, including I, II, III, V and XI²⁰. Collagen I is mainly distributed in the skin, tendon and other tissues, and Collagen II is produced by chondrocytes²¹. A research²² showed that TGF- β and fibroblast growth factor could active chondrocyte differentiation and expression type of collagen. We demonstrated that the expression of Collagen I and Collagen II increased both in FGF2 + LIPU and FGF2 group, showing that Type I and type II collagens played a crucial role in the regeneration of articular cartilage in rabbits. The result was consistent with the findings of Wang et al²³.

Conclusions

We found that FGF2 + LIPU combination was superior to FGF2 alone in repairing the rabbit knee

joint. We provide a basis for further research and novel treatment option in clinical knee joint repair.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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