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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 area were filled with cartilage-like cells and the surface was fused with surrounding cartilage in FGF2 and FGF2 + LIPU groups. Wakitani scores were consistent with the 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 the model group.

CONCLUSIONS: FGF2 and LIPU combined application on rabbit knees joint repair is better than FGF2 alone. FGF2 and LIPU combination promote the synthesis and secretion of collagen in chondrocytes, promote the differentiation and maturation of chondrocytes during 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 another without rubbing. The most common type of osteochondral lesions is trauma in the United States[1]. Articular cartilage wear away when osteoarthritis affects a joint, allowing 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 absorption[2].

It 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 tissues[6]. 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 research[9] 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 tissues[10]. The LIPU application on treatment of articular cartilage injury has been drawn the attention. Previous investigation[11] indicated that LIPU had significant therapeutic potential in treating a severe articular cartilage injuries in our animal study. We inve-

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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 [illegible] was cut about 2 cm long. Holes of 3.0-mm diameter, evenly spaced, were drilled using [illegible] and drill (Synthes, Mathys AG, Bettlach, Switzerland), until the articular surface of the intercondylar fossa of femur was exposed. After hemostasis, [illegible] was washed with isotonic [illegible] penicillin sodium (Lukang Pharmaceutical Limited by Share Ltd., Shandong, China) injected at 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 only. The collagen membrane contained 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/cm^2 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% formaldehyde (SolaiBao Biotechnology, [illegible]). The color, cartilage surface and defect area of joint were observed in different [illegible].

Histopathological Changes

A tissue slide was randomly selected from each group and stained with hematoxylin and eosin (HE, Bolait Chemical [illegible], Ltd. [illegible] China). The repair of articular cartilage was observed under light microscope (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 cartilage and the surrounding cartilage. Aspects of the ability to repair the situation, the highest 14 points were the poor repair, the lowest 0 points 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 $2^{-\cdots Ct}$ 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, trans[parent] and viscous, while the appearance of arti[cular] cartilage was milky and shiny i[n the] control g[roup] (Figure 1A, E). After 4 weeks [there] was a l[ight] adhesion in the knee joint, [the] a[rticu]lar flu[id] 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 surf[ace] (Figure 1C). In the FGF2 + LIPU group, th[e] repair t[issue] was filled with milky white trans[parent] cartila[ge]-like tissue, and neonatal cartil[age] su[rface] was [s]mooth. The outline of the re[pair] tion ar[ea] blurred (Figure 1D). After [4] weeks, compa[red] with the control group, the [color] was light yellow, and the depression wa[s] obv[iously] mo[re] group (Figure 1F). In the FGF2 grou[p], [the] joint was no adhesions and [the] structure, ar[ticular] cartilage defect repair tissue [was] fi[lled] completely (Figure 1G). In the FGF2 + LIPU gr[oup], defect zone repair tissue was c[lose] to normal hyaline cartilage with surface smoothness. 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 Waki[...]

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

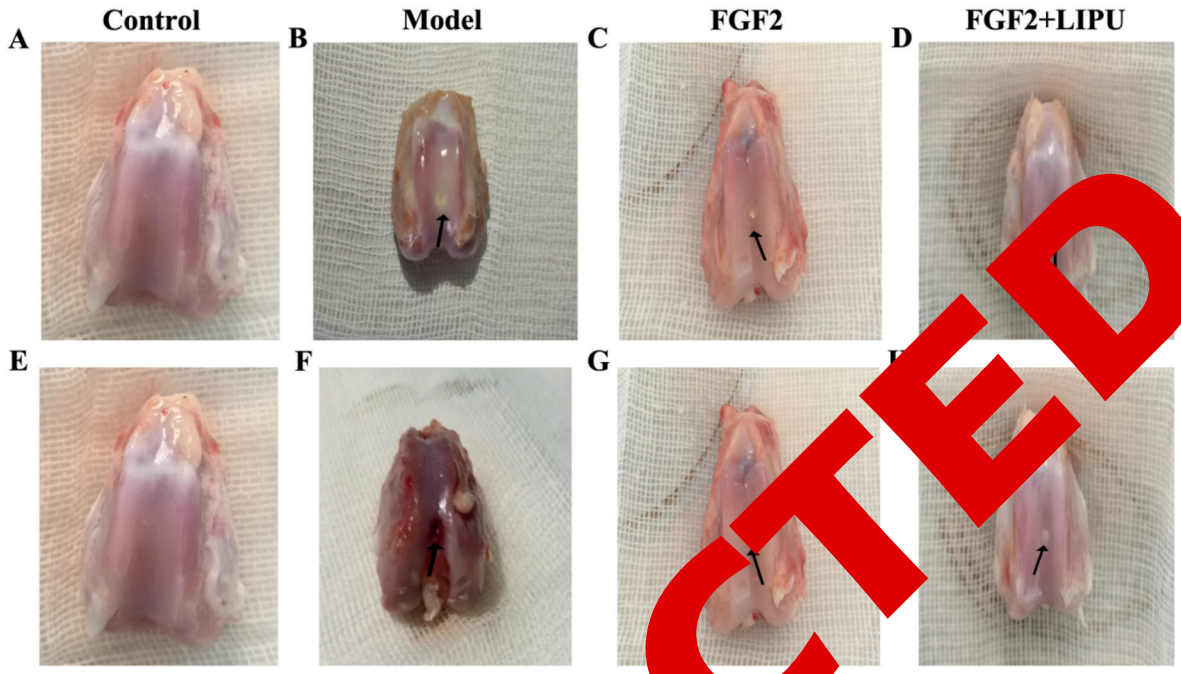

Figure 1. General appearance of knee articular cartilage at 4, 8 weeks. The first line represented the change of knee articular cartilage at 4 weeks (n=5). ***A,*** control group, ***B,*** model group, ***C,*** FGF2 group, ***D,*** FGF2 + LIPU group. The second line represented the observation of knee articular cartilage at 8 weeks. ***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 FGF2 group (FGF2), the surface of the tissue was flatter and part tissues were higher than surrounding cartilage. The repair tissue was filled with a large number of cartilage-like cell clusters (Figure 2C). After 4 weeks, the cartilage 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 similar to 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

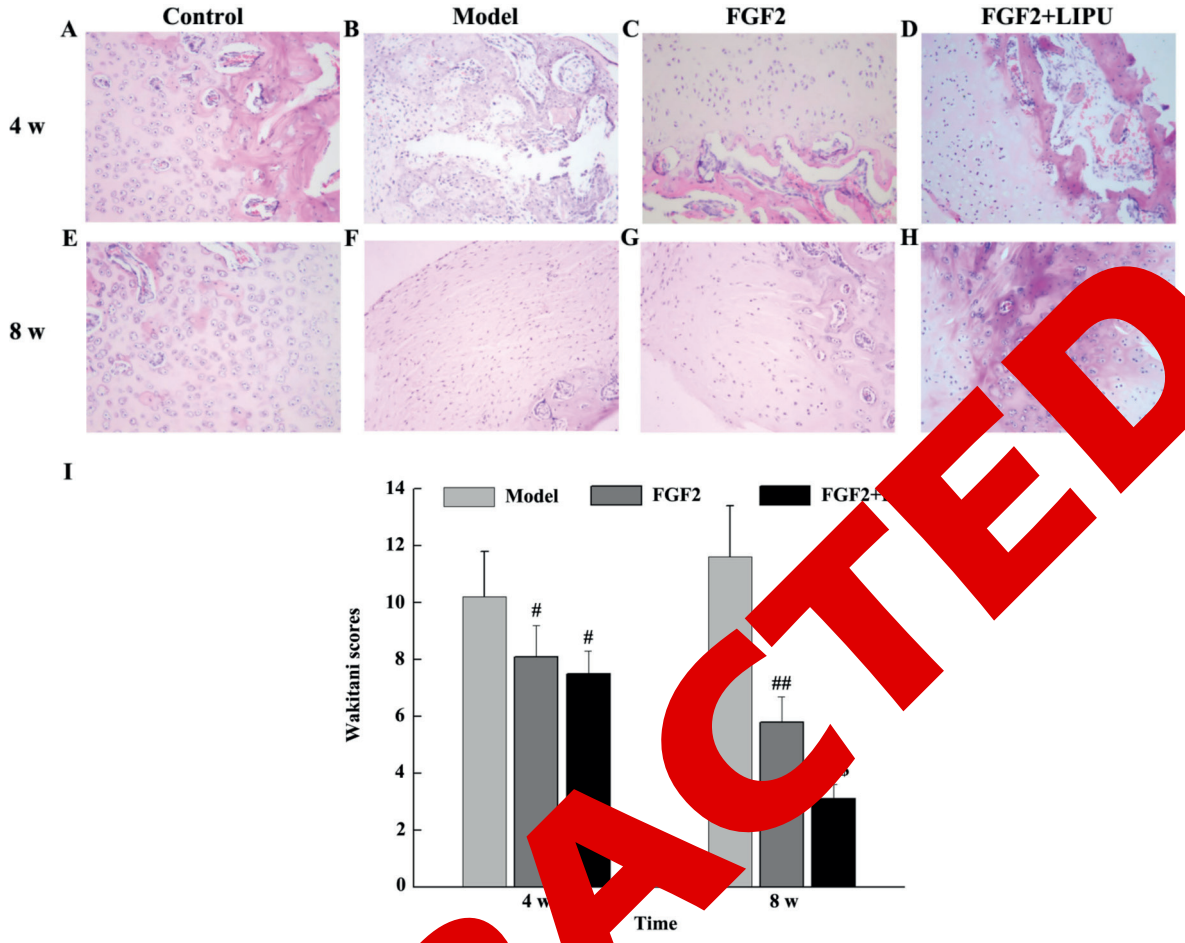

Figure 2. Histopathological changes of k... arti... (× 200... ll groups. The first line represented the change of knee articular cartilage at 4 weeks (n=5). ***A,*** contr... up, ***B*** ...del gro..., ***C,*** FGF2 group, D: FGF2 + LIPU group. The second line represented the observation of knee arti...dla... ...ge... ...n=5). E: control group, ***F,*** model group, ***G,*** FGF2 group, ***H,*** FGF2 + LIPU group. I: Wakitani score. C...pared ...he mo... group, $^{\#}p<0.05$, $^{\#\#}p<0.01$. Compared with the FGF2 group, $^{\$}p<0.05$.

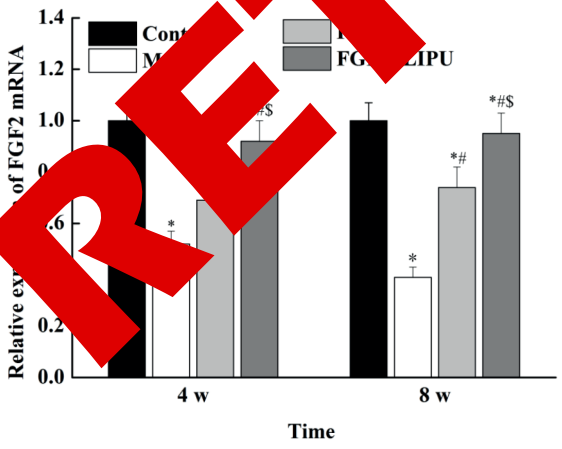

Figure 3. The levels of FGF2 mRNA in different groups at 4, 8 weeks detected by RT-PCR. Compared with control, $^{*}p<0.05$. Compared with model, $^{\#}p<0.05$. Compared with the FGF2 group, $^{\$}p<0.05$.

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 fracture[14]. LIPU has also been shown to have a positive effect on the healing of other tissues, including ligament, muscle, and cartilage[15-17]. Research found that low-intensity pulsed US protects cartilage from damage in early-stage osteoarthritis via the integrin/FAK/MAPK pathway[18]. Extracellular signal-regulated kinase1/2 and p38 signaling pathway were also involved in abating this damage[19]. 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

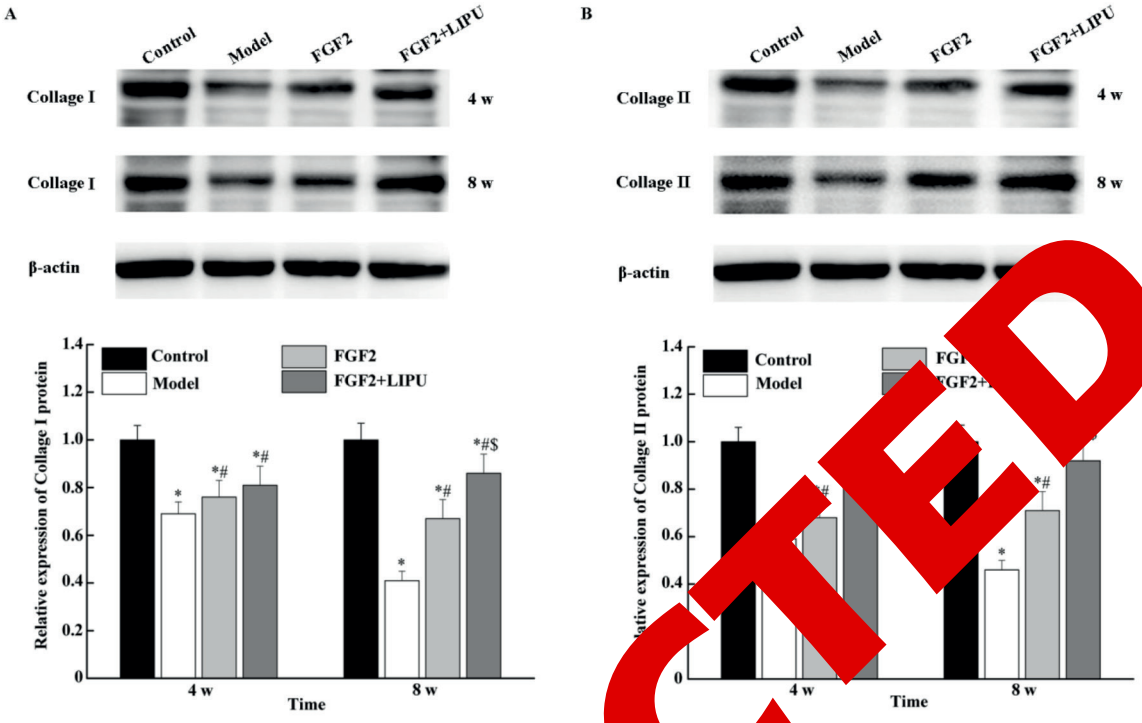

Figure 4. Western blot assay were used to detect Collagen protein[illegible] weeks. ***(A)***The levels of Collagen I in control, model, FGF2 and FGF2+LIPU groups; ***(B)*** The le[illegible] Collage[illegible]ns in control, model, FGF2 and FGF2+LIPU groups. Compared with control, $^{*}p<0.05$. Compared[illegible]<0.05. Compared with the FGF2 group, $^{\$}p<0.05$

effect of combination was more effi[illegible] than of FGF2 alone. Further investiga[illegible]nstra[illegible] that the expression of FGF2 p[illegible]A wa[illegible]igher FGF2 + LIPU group than th[illegible] in[illegible]2 g[illegible]ch meant the LIPU could s[illegible]late [illegible] expression in articular cartilage[illegible] results w[illegible]onsistent with the previous fi[illegible]g[illegible] Collagen[illegible]are a major component of c[illegible]nective [illegible] in animals, including I, II, III[illegible]nd XI[20]. C[illegible]n I is mainly distributed i[illegible] ski[illegible] tendon and other tissues, and Collagen[illegible]r[illegible]ed by chondrocytes[21]. A research[22] showe[illegible]GF[illegible]d fibroblast growth factor[illegible] acti[illegible]rocyte differentiation and [illegible]pres[illegible]n type[illegible] collagen. We demonstrated [illegible]at th[illegible]ression of Collagen I and Collagen II [illegible]ase[illegible] in FGF2 + LIPU and FGF2 group, si[illegible]g that Type I and type II collagens played a cr[illegible]ole in the regeneration of articular cartilage in rabbits. The result was consistent with the findings of Wang et al[23].

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.

References

1) Felson DT, Lawrence RC, Dieppe PA, Hirsch R, Helmick CG, Jordan JM, Kington RS, Lane NE, Nevitt MC, Zhang Y, Sowers M, McAlindon T, Spector TD, Poole AR, Yanovski SZ, Ateshian G, Sharma L, Buckwalter JA, Brandt KD, Fries JF. Osteoarthritis: new insights. Part 1: the disease and its risk factors. Ann Intern Med 2000; 133: 635-646.

2) Fernández-Torres J, Zamudio-Cuevas Y, Martinez-Nava GA, López-Reyes AG. Hypoxia-inducible factors (HIFs) in the aricular cartilage: a systematic review. Eur Rev Med Pharmacol Sci 2017; 21: 2800-2810.

3) Sak ME, Gul T, Evsen MS, Soydinc HE, Sak S, Ozler A, Alabalik U. Fibroblast growth factor-1 expression in the endometrium of patients with repeated implantation failure after in vitro fertilization. Eur Rev Med Pharmacol Sci 2013; 17: 398-402.

4) Finklestein SP, Plomaritoglou A, Miller LP, Hayes RL, Newcomb JK. Head trauma: basic, preclini-

cal, and clinical directions. New York, Wiley 2001; 165-187.

5) Pan ZC, Wang SP, Ou TT, Liu H, Ma JW, Wang WX, Fang WY, Qu XK, Zhang M. A study on the expression of FGF-21 and NF-κB pathway in the tissues of atherosclerotic mice. Eur Rev Med Pharmacol Sci 2017; 21: 102-107.

6) Zhou Y, Wang Z, Li J, Xiao J. Fibroblast growth factors in the management of spinal cord injury. J Cell Mol Med 2018; 22: 25-37.

7) Cao R, Brakenhielm E, Pawliuk R, Wariaro D, Post MJ, Wahlberg E, Leboulch P, Cao Y. Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. Nat Med 2003; 9: 604-613.

8) Stegmann, TJ. New approaches to coronary heart disease: induction of neovascularisation by growth factors. Bio Drugs 1999; 11: 301-308.

9) Chuma H, Mizuta H, Kudo S, Takagi K, Hiraki Y. One day exposure to FGF-2 was sufficient for the regenerative repair of full-thickness defects of articular cartilage in rabbits. Osteoarthritis Cartilage 2004; 12: 834-842.

10) Hadjiargyrou M, McLeod K, Ryaby JP, Rubin [illegible] Enhancement of fracture healing by low int[illegible] ty ultrasound. Clin Orthop Relat Res 1998; [illegible] S216-S229.

11) Lu H, Liu F, Wang Z, Chen H, Qu J, [illegible], Xu [illegible] Hu J. Low-intensity pulsed ultras[illegible]ulatio[illegible] for tendon-bone healing: a do[illegible]epe[illegible]nt stu[illegible] dy. Am J Phys Med Rehabil [illegible]7: [illegible]

12) Henson FM, Bowe EA, Da[illegible] ME. [illegible]otion[illegible]e intrinsic damage-repai[illegible]sponse [illegible]cular cartilage by fibroblastic[illegible]h factor-2.[illegible]arthritis Cartilage 200[illegible]14.

13) Zhao YF, Luo [illegible], Xiong [illegible]g W, Li YR, Zhao W, Zeng HZ[illegible] HC, Wu X[illegible]enchymal stem cell-base[illegible]GF2 gene thera[illegible]y for acute lung injury i[illegible]ed b[illegible]opolysaccharide in mice. Eur Rev Me[illegible]col S[illegible]2015; 19: 857-865.

14) [illegible] Qin [illegible]P, [illegible]g WH, Lee K, Guo X, Wong [illegible]ng K[illegible]ensity pulsed ultrasound ac[illegible]cele[illegible] bone-[illegible]ion junction healing: a partial pate[illegible]my model in rabbits. Am J Sports Med [illegible]006; [illegible]7-1296.

15) Watanabe M, Kojima, S, Hoso M. Effect of low-intensity pulsed therapy on a rat knee joint contracture model. J Phys Ther Sci 2017; 29: 1567-1572.

16) Schofer MD, Block JE, Aigner J, Schmelz A. Improved healing response in delayed u[illegible]f the tibia with low-intensity pulsed ultra[illegible]ults of a randomized sham-controlle[illegible]al. Bi[illegible]sculoskelet Disord 2010; 11: 2[illegible]

17) Liao JC, Chen WJ, Niu CC, [illegible] Li[illegible]ct of lo[illegible]tensity pulsed ultrasou[illegible] spina[illegible]ro-sis created by nicotin[illegible]ministratio[illegible]del of lumber posterolate[illegible]seud[illegible]rosis i[illegible]abbits. J Ultrasound Med 20[illegible]43-10[illegible].

18) Xia P, Shen S[illegible]Q, [illegible], P[illegible], Gao M, Li X. Low-inten[illegible]ulsed u[illegible]treatment at an early os[illegible]itis stage [illegible]ects rabbit cartilage fr[illegible]dar[illegible]via the integrin/focal adhesion kin[illegible]a/mitoge[illegible]ated protein kinase signaling [illegible]way. J Ultras[illegible]Med 2015; 34: 1991-1999.

19) [illegible]X, Lin Q, Wang D, Dai Y, Cheng K, Yu J, Lin A, [illegible]ao M, Chen [illegible]A. The effects of low-intensity pulsed [illegible]rasound[illegible]d nanomagnet applications on the [illegible] MMP-13 and MAPKs in rabbit knee o[illegible]tis. J Nanosci Nanotechnol 2013; 13: 722-727.

[illegible]g CC, Chiu LH, Lai WF, Tsai TT, Fang CL, Chen [illegible], Tsai YH. Phenotypic re-expression of near quiescent chondrocytes: the effects of type II collagen and growth factors. J Biomater Appl 2010; 25: 75-95.

21) Aitken KJ, Bagli DJ. The bladder extracellular matrix. Part I: architecture, development and disease. Nat Rev Urol 2009; 6: 596-611.

22) An G, Xue Z, Zhang B, Deng QK, Wang YS, Lv SC. Expressing osteogenic growth peptide in the rabbit bone mesenchymal stem cells increased alkaline phosphatase activity and enhanced the collagen accumulation. Eur Rev Med Pharmacol Sci 2014; 18: 1618-1624.

23) Wang KH, Chan WP, Chiu LH, Tsai YH, Fang CL, Yang CB, Chen KC, Tsai HL, Lai WF. Histological and immunohistochemical analyses of repair of the disc in the rabbit temporomandibular joint using a collagen template. Materials (Basel) 2017; 10: pii: E924.