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*Original Article*

# Experimental study of biologically active icariin combined with a porous PLGA/TCP scaffold to repair cartilage and subchondral bone in articular cartilage defect in rabbits

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**Abstract:** In this study, we designed a porous PLGA/TCP scaffold to carry and slowly release biologically active icariin, then observed the effect of the scaffold on the integral repair of articular cartilage defect in rabbits. We used New Zealand white rabbits to establish an articular cartilage defect animal model with a 5 mm diameter by 4 mm depth defect in the femoral intercondylar fossa. Gross specimen scores, Mankin scores, HE staining, AB-PAS staining, Safranin O staining, immunohistochemical staining of type II collagen, and CT scans were used to evaluate the repair conditions 6 months after the model was established. The result showed that there were more chondrocytes, matrix, and type II collagen around and covering the defect and the subchondral bone was in better shape in the bioactive icariin porous PLGA/TCP scaffolds repairing group. CT scans showed that the trabecular bone structure was intact. Gross specimens and Mankin scores were significantly lower. These findings suggested that the use of bioactive icariin porous PLGA/TCP scaffolds promoted the integral repair of articular cartilage defect from the cartilage to the subchondral bone.

**Keywords:** Icariin, PLGA/TCP scaffold, chondrocytes, cartilage defect

## Introduction

Articular cartilage defect is a common clinical disease and is mainly caused by trauma, inflammation, or a tumor, with pathological cartilage loss or degeneration. It can be divided into partial defect or full-thickness defect [1]. Nevertheless, the capacity of cartilage to be repaired is still limited. When the diameter of the defect is greater than 4 mm, the cartilage cannot repair itself. For this reason, articular cartilage defect has become one of the most concerning problems in the medical field [2]. Most doctors use bone tissue engineering [3], cartilage transplantation [4] or chondrocyte transplantation [5] to repair articular cartilage defect. Although some beneficial effects have been achieved through the use of these treatments, the entire therapeutic process is complex, time-intensive, and expensive. At the same time, these therapeutic processes are just like “pad pasting”, with a direct repair of the cartilage defect. The regenerative cartilage still has no connection with the subchondral bone tissue. Thus, after the repair, the cartilage and the subchondral bone are not integrated, which causes a poor postoperative outcome in some patients [6]. In modern medical research, more attentions have focused on the concept of entirety. Sherwood and his colleagues [7] used their 3D printing method to develop a new three-dimensional scaffold for osteochondral injuries, which could form a gradient of composition and porosity changes at the junction area of the bone and cartilage. This enabled the scaffold to avoid interfacial delamination, as seen in *in vitro* cultures and *in vivo* implantation, and could form a good interface between regenerative bone and regenerative cartilage.

Epimedium is a perennial herbaceous plant belonging to dry berberidaceae. It is also a traditional Chinese medicine, which has been used to tonify kidney yang, strengthen bones

and muscles, and dispel wind-damp [8]. Icariin is the main active component of epimedium. Many studies have shown that icariin has shown promise in anti-osteoporosis treatments [9], improving liver function [10], and regulating the immune system [11]. Some scholars have confirmed that icariin has a certain role in cartilage repair [12, 13], but the specific mechanism requires further elucidation.

Poly lactic acid-glycolic acid (PLGA) is made from different proportions of polylactic acid (PLA) and polyglycolic acid (PGA), and is widely used in the study of tissue engineering [14, 15]. It has good biodegradability and bioabsorbability *in vivo*, and is neither toxic nor antigenic at a certain mechanical strength. It can also control the slow release of the drugs it carries. PLGA, therefore, is favored in orthopedics. Tricalcium phosphate (TCP) is a new type of substitute bone graft material with good biocompatibility and bone conductivity, which has garnered it increasing attention [16]. The degradation rates of PLGA and TCP are similar and their combination can improve strength and toughness and promote their individual biological effects compared with each material alone [17].

In order to study knee joint articular cartilage defect, we designed a porous PLGA/TCP scaffold that could carry and slowly release biologically active icariin and we established an articular cartilage defect animal model with a 5 mm diameter by 4 mm depth-defect in the femoral intercondylar fossa using microfracture surgery. The porous PLGA/TCP scaffold carrying biologically active icariin was applied to the defect and its repair abilities were observed. We investigated the effects of the scaffold on the transformation, proliferation, and regeneration of chondrocytes in knee joint articular cartilage defect, thereby providing a basis for future therapies for articular cartilage defect repair.

## Materials and methods

### *Reagents*

The main reagents used were type II collagen antibody (Abcam), biotin-labeled goat anti-rabbit IgG, SABC-POD, the DAB reagent kit (Boster, China), iodophor (adf, China), sodium pentobarbital, gentamicin, other antibiotics, saline (Sichuan Kelun Pharmaceutical Co., Ltd.), Alcian blue reagent, Schiff reagent, and Safranin O reagent (Scytek, USA).

### *Preparation of the porous PLGA/TCP scaffold carrying biologically active icariin*

The porous PLGA/TCP scaffolds with and without biologically active icariin were made with a biological materials rapid formation machine, which was designed and manufactured by Shenzhen-Hongkong Joint Musculoskeletal Health Science Center of Innovation and Cooperation Platform with proprietary intellectual property rights (such as Tiss Form, Med Form, and biological materials rapid prototyping system). In -10-20°C, PLGA/TCP was mixed with icariin at $1 \times 10^{-5}$ mol/L [18]. The frozen dry pumping machine was then used to give the porous material a certain mechanical strength, biological activity, and good biodegradability, which were beneficial to the growth of osteoblasts, chondrocytes, and blood vessels [19]. The material was treated with UV disinfection before use.

### *Experimental animals*

27 6-month-old male New Zealand rabbits were purchased from Dilepu Biological Resources Development Co., Ltd. (Xian Province [production license: SCXK (shan) 2010-0106]). Each weighed 2-2.5 kg and had a clear genetic background. The rabbits used in the experiment were housed in clean rabbit cages with continuous individual air filtration and free access to food and sterile water at the Experimental Animal Center in Shenzhen PKU-HKUST Medical Center at a room temperature of 23 ± 2°C and relative humidity of 60%~65%. All experimental procedures and treatments were performed according to the experimental animal management regulations.

### *Treatment and grouping of rabbits*

An injection of sodium pentobarbital in the ear vein served as anesthesia with iodophor as a disinfectant. Under sterile conditions, an incision was made into the left knee joint to fully expose the femoral condylar articular surface. A hole was drilled in the middle of the knee joint femoral condyle non-weight-bearing area at a

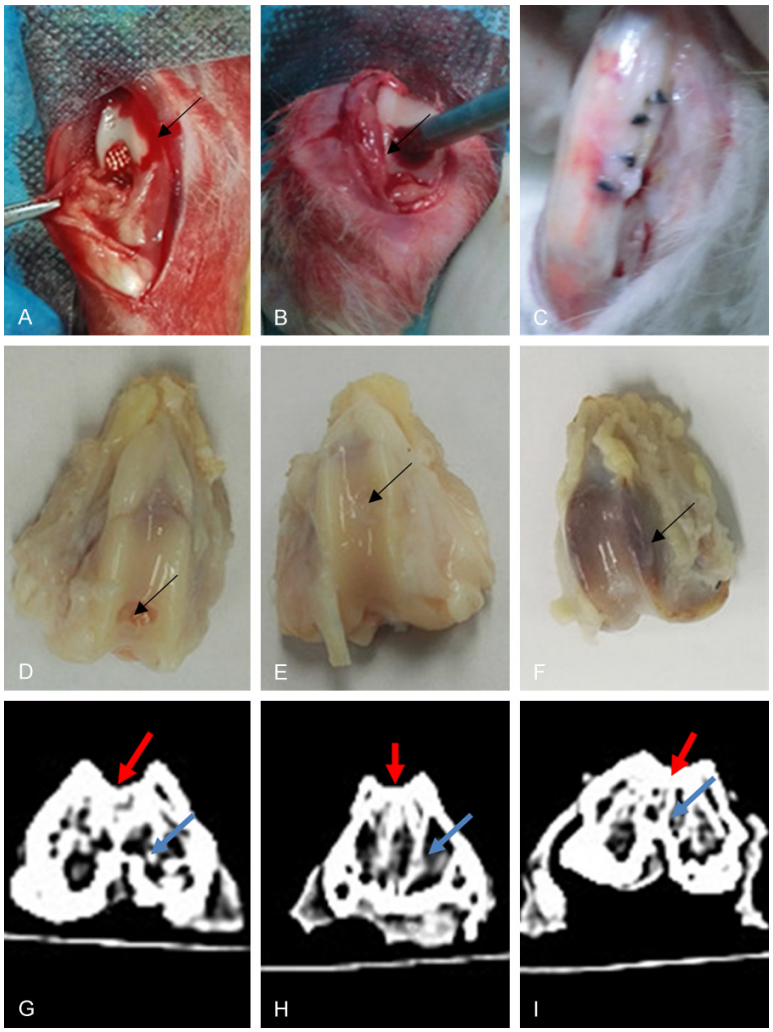


**Figure 1.** Macroscopic images of knee joint specimens. A-C. Are from the microfracture operation option d and scaffold implantation. Since the scaffold is porous, BMSCs in the blood can cover the surface of the scaffold and provide optimal conditions for the BMSCs to transformation into chondrocytes. D-F. Are the gross specimens of the no scaffold group, the porous PLGA/TCP scaffold group, and the porous PLGA/TCP scaffold combined with biologically active icariin group, respectively. G-I. Are the CT images of the no scaffold group, the porous PLGA/TCP scaffold group, and the porous PLGA/TCP scaffold combined with the biologically active icariin group, respectively. The arrows are the repair area and trabecula.

diameter of 5 mm and a depth of 1 mm. We then used microfracture surgery to drill a hole at a depth of 3 mm with 4.5 mm Kirschner wire in the defect, which reached the subchondral bone. The rabbits were randomly divided into 3 groups: in group A, no scaffold was inserted in the defect; in group B, a porous PLGA/TCP scaffold was inserted in the defect; and in group C, a porous PLGA/TCP scaffold carrying biologically active icariin was inserted in the defect. Conventional sutures were used and the joint was properly fixed and pressed. The wound was closed after washing the articular cavity with $2 \times 10^4$ U gentamicin. Antibiotics were used for 3 days after the operation (**Figure 1**).

*Observation of the gross specimens*

Rabbits from each group were sacrificed with an overdose of anesthesia 6 months after the model was established. After opening the bilateral knee joint capsule, the cartilage of the knee joint femoral condyle and trochlea areas were observed. Knee joint effusion, synovial swelling, and other manifestations of synovitis were also noted. Pathological changes on the articular surface in the medial condyle of the femur were observed under an anatomical microscope. Gross specimen scores were determined in this way: 0 points: joint surface is continuous with color as usual; 1 point: the articular surface is rough, with small cracks, and is dark gray in color; 2 points: articular cartilage was eroded and the defect depth reached to the middle layer; 3 points: presence of the articular surface ulcer and the defect depth reached to the deep layer; 4 points: cartilage was stripped and the subchondral bone was exposed [20].

*HE staining*

Tissue specimens were fixed in 10% paraformaldehyde for 24 h and then decalcified and embedded in paraffin. Serial 5-μm specimen sections were obtained and stained with HE. The tissue sections were then treated with campeachy for 15 min, rinsed with water for 1 min, and immersed in static water for 5 min. Next, the sections were treated with 0.5% eosin for 3 min and were then rinsed with water.

*AB-PAS staining*

Tissue specimens were fixed in 10% paraformaldehyde for 24 h and subsequently decalcified and embedded in paraffin. Serial 5-μm specimen sections were obtained and stained with AB-PAS. The tissue sections were treated with 1% Alcian blue-3% acetic acid for 40 min, 1% periodic acid for 10 min, Schiff liquid dye for 30 min, and finally were treated again with campeachy.

*Safranin O staining*

Tissue specimens were fixed in 10% paraformaldehyde for 24 h and subsequently decalcified and embedded in paraffin. Serial 5-μm specimen sections were obtained and stained with Safranin O. The tissue sections were treated with Safranin O reagent for 20 min.

*Mankin scores*

We calculated the Mankin scores according to the cartilage structure, chondrocytes, safranin O staining, and the integrity of the tidal line. The specific scoring rules have been previously described [21].

*Immunohistochemical analysis*

Tissue specimens were fixed in 10% paraformaldehyde for 24 h, decalcified, and embedded in paraffin. Serial 5-μm sections were obtained. The tissue sections were baked for 3 min, rinsed twice with water, and subjected to antigen retrieval. They were then washed three times with PBS, treated with peroxidase blocker, and washed again three more times with PBS. The sections were treated with normal goat serum and were then incubated with type II collagen antibody. The sections were washed three times with PBS and then incubated with secondary antibody, before being treated with SP solution, followed by two drops of fresh DAB solution. The brown yellow area was positive. We calculated the positive area under the microscope.

*CT examination*

The cartilage specimens of sacrificed New Zealand rabbits were observed under CT scan. CT values were selected as SPOV 32 cm, 120 KV, 100 mA, and TI0.5, in order to observe the internal structure.

*Statistical analysis*

All data were expressed as $\bar{x} \pm s$. SPSS 20.0 was used for statistical analysis. Comparisons between groups were made with nonparametric tests. The image processing software package Image Pro-Plus 6.0 was used for image analysis. A $P$ value < 0.05 was considered to be statistically significant.

## Results

*Observation of rabbit knee joint gross specimens, articular cartilage slices, and Mankin scores*

In the observation 6 months after the repair, we found that the gross specimen scores of groups A, B, and C were (3.7778 ± 0.4410), (1.1111 ± 0.7817), and (0.2222 ± 0.4410) and the Mankin scores were (3.1667 ± 1.1691), (6.1667 ± 1.1691), and (11.1667 ± 2.3166), respectively. In group C, a smooth cartilage surface was observed with good color and luster. Under microscopy, the scaffold was shown to be predominantly degraded. The defect was mostly repaired by cartilage tissue and had been completely repaired with the spread of chondrocytes to the defect matrix layer. There were more polysaccharides in the matrix layer than in the other groups and there was almost no difference compared with normal cartilage tissue. Regenerated cartilage was obvious in the area surrounding the degraded scaffold. The surface, transitional, radiation, and calcified layers of the cartilage were clearly distinguishable. The size and shape of the chondrocytes were uniform with a scattered distribution. The tidal line was neat, the subchondral bone was regular, and the regenerated cartilage was tightly combined with both the subchondral bone and with the cartilage surrounding the defect. The gross specimen and Mankin scores had obviously decreased. Compared with groups A and B, the gross specimen and Mankin scores decreased significantly ($P < 0.05$). In group B, a slightly rough surface was observed with a darker color. The defect had been partly repaired by regenerated cartilage, although there was less cartilage surrounding the scaffold than in group C. The cartilage tissue surrounding the defect extended to the defect with fewer regenerated chondrocytes and polysaccharides. A certain level of repair was evident, but a shallow defect with some erosion still

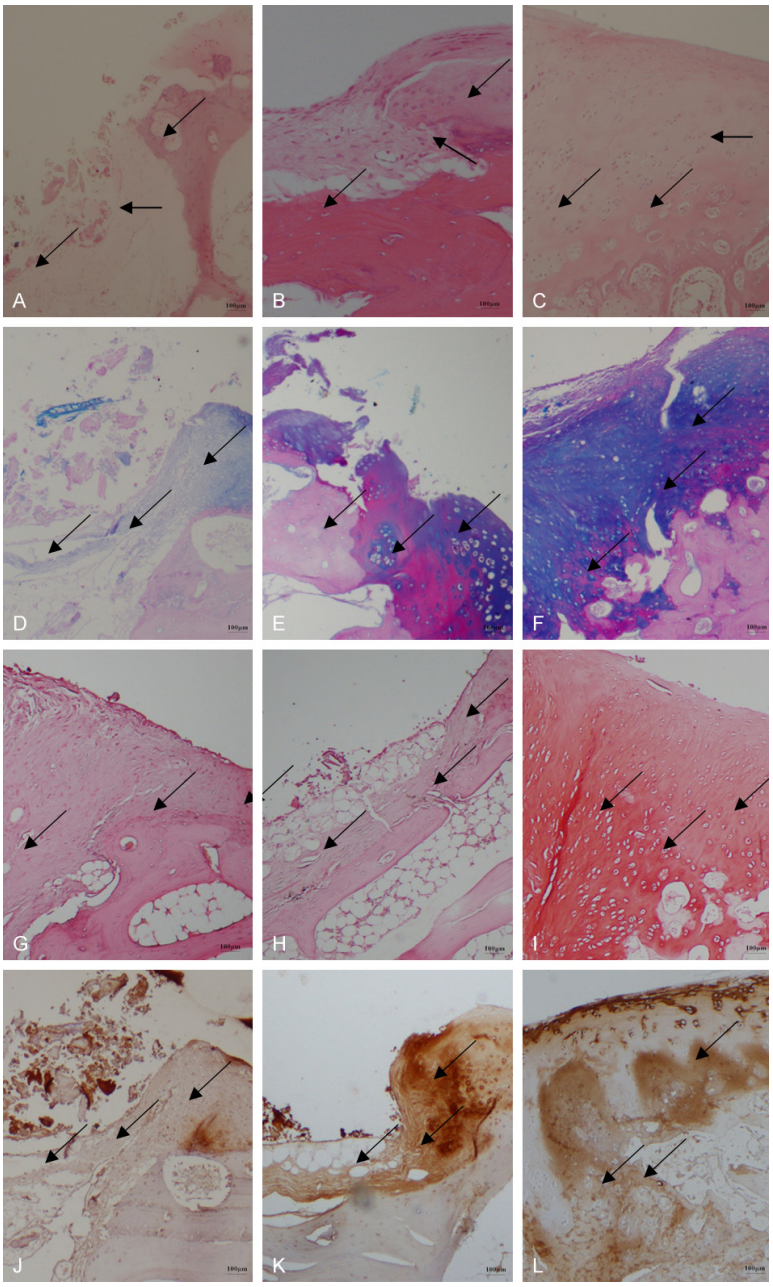


**Figure 2.** Microcosmic images of knee joint specimens (Scale bar = 100 μm). A-C. Are the HE staining images of the no scaffold group, the porous PLGA/TCP scaffold group, and the porous PLGA/TCP scaffold combined with biologically active icariin group, respectively. D-F. Are the AB-PAS staining images of the no scaffold group, porous PLGA/TCP scaffold group, and porous PLGA/TCP scaffold combined with biological active icariin group, respectively. G-I. Are the Safranin O staining images of the no scaffold group, the porous PLGA/TCP scaffold group, and the porous PLGA/TCP scaffold combined with biologically active icariin group, respectively. J-L. Are the type II collagen immunohistochemical staining of the no scaffold group, porous PLGA/TCP scaffold gro up, and porous PLGA/TCP scaffold combined with biological active icariin group, respectively. The arrows show the repair area, the surrounding repair area, and the combined area of regenerated cartilage and subchondral bone.

remained, the surface was uneven, the four layers of cartilage were poorly distinguishable, and many of the chondrocytes had a less uniform distribution. The tidal line was less regular, the subchondral bone was irregular, and there was a gap between the regenerated cartilage and subchondral bone. The gross specimen and Mankin scores decreased. Compared with group A, the gross specimens and Mankin scores decreased significantly ($P < 0.05$). In group A, the surface was rough with a darker color and an obviously discernable defect under the microscope. A greater amount of the vagiform substance remained. The surrounding cartilage tissue hardly reached the defect. No obvious polysaccharides were secreted and the repair was poor. The four layers of cartilage were indistinguishable, the chondrocytes size was irregular, with a disordered or clustered distribution, the tidal line was irregular, blood vessels evenly crossed the tidal line, and the subchondral bone was more irregular. There was a large gap between the regenerated cartilage and the subchondral bone and the gross specimen and Mankin scores were still high (**Figures 2**, **3**).

*Immunohistochemical staining*

In the type II collagen immunohistochemical staining 6 months after the repair, we found that the positive areas from groups A, B and C were different. In group C, the positive type II collagen area was the largest and had a deep color. Type II collagen experienced the greatest level of

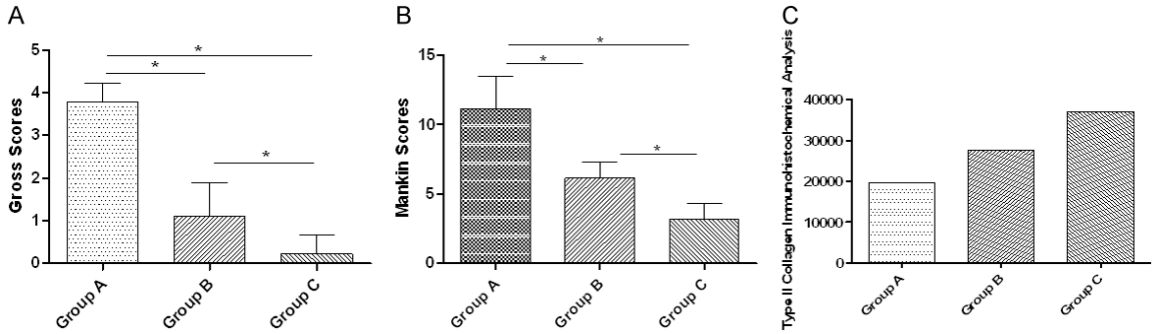


**Figure 3.** Scores and type II collagen of the knee joint. A. Is the gross score. B. Is the Mankin score. C. Is a type II collagen immunohistochemical analysis.

secretion in this group, especially in the area surrounding the defect and on the surface of the matrix layer. It showed no differences with normal cartilage. We confirmed that there was a large number of regenerated chondrocytes. In group B, the positive type II collagen area was the second largest and had a moderate color. Type II collagen experienced moderate secretion in this group; it was present surrounding the defect and in the matrix layer, but was still less than in group C. In group A, the positive type II collagen area was the smallest and had a light color. Type II collagen was secreted at the lowest level of all the groups. A larger amount of the vagiform substance remained. Less type II collagen had been secreted in the area surrounding the defect and in the matrix layer (**Figures 2**, **3**).

*Iconography observation*

The CT observations revealed that, 6 months after the repair, the scaffold in group C had integrated well with the knee joint. The regenerated cartilage in the defect was flush and integrated with the surrounding cartilage. There was a large number of trabecular under bone and the trabecular structural was strong with good porosity. In group B, the regenerated cartilage was slightly concave and was basically integrated with the surround cartilage. Some of the trabecular under bone had a loose structure. There was a slight collapse in the regenerated cartilage of group A, but there was probably still some integration with the surrounding cartilage (**Figure 1**).

## Discussion

Icariin is the main active component of epimedium, which has significant effects on the treatment of osteoarthritis [22], peripheral nerve injury [23], spinal cord injury [24], and other orthopedic diseases. These studies have confirmed that icariin is an osteoinductive factor that plays an important role in promoting the osteogenic differentiation [25] of bone marrow mesenchymal stem cells (BMSCs) [26] and adipose derived mesenchymal stem cells [27]. Icariin can also promote the differentiation of BMSCs into chondrocytes, which may be the possible mechanism by which it repairs cartilage. The Wnt/β-catenin signaling pathway plays an important role in this process [28]. Icariin can also slow the development of osteoarthritis through the inhibition of the NF-κB signaling pathway [29]. The PI3K/AKT signaling pathway can also promote BMSC differentiation into osteoblasts [30]. In addition, low concentrations of icariin have toxic effects on rabbit chondrocytes, while high concentrations can inhibit their proliferation. Only a suitable concentration can be used in the study of chondrogenesis in tissue engineering, such as $1 \times 10^{-5}$ M [18]. When injected into the articular cavity of rats, icariin significantly reduces the cartilage degeneration of osteoarthritis, which may be related to changes in the MAPK signaling pathway and the low expression of matrix metalloproteinase [31]. In cartilage repair and inflammation, icariin can activate HIF-1α in chondrocytes to repair cartilage [12], reduce the LPS-induced inflammatory response, and reduce the degradation of the extracellular matrix [32], while promoting the synthesis of the cartilage matrix [18]. Icariin can also reduce the incidence of rheumatoid arthritis by regulating Th17 [33], which may be achieved by inhibiting the expression of cathepsin K [34]. These studies show the importance of icariin in the repair of cartilage defect.

The PLGA/TCP scaffold is a new kind of composite material that is widely used in tissue engineering, for its ability to control the release of drugs. This scaffold has moderate hardness, and its porosity promotes cell migration and the permeation of growth factors. It can provide support for the attachment of cartilage, while at the same time being able to degrade *in vivo* with extended repair time. It will not cause acid-base balance disorders and has no effect on the normal physiological function of the body. It is, ultimately, a good scaffold for cartilage repair. Therefore, we combined icariin with the porous PLGA/TCP scaffold in order to take advantage of the slow release as the scaffold degraded and achieve long-term stimulation. In the process of making this scaffold, we dissolved icariin in organic solvent and ensured its uniform distribution. In our study, cartilage rapidly formed due to the stimulation of icariin. Underneath the cartilage, the scaffold closely combined with the surrounding bone tissue, so that the cartilage tissue could become integrally fixed and provide support without collapse. At the same time, the cartilage also closely combined with the autogenous bone, which promoted further repair of the cartilage and subchondral bone. With the absorption and degradation of the scaffold, the bone and cartilage became integrally connected to repair articular cartilage defect.

In our study, we found that biologically active icariin combined with the porous PLGA/TCP scaffold had a significant effect on the repair of cartilage defect 6 months after the repair. A smooth cartilage surface was observed with good color and luster, which was better than was seen in the group with the PLGA/TCP scaffold alone and the group with no scaffold. The scaffold in group C had almost degraded under the microscope, the defect was mostly repaired by cartilage tissue, and was completely repaired with chondrocyte spread to the defect's matrix layer. There were more polysaccharides and type II collagen in the matrix layer and it showed almost no difference compared with normal cartilage tissue. Regenerated cartilage was more obvious surrounding the degraded scaffold. Cartilage tissue surrounding the defect extended clearly beyond the defect with more regenerated chondrocytes and higher levels of polysaccharides and type II collagen. The surface, transitional, radiation, and calcified layers of the cartilage could be easily distinguished. The size and shape of the chondrocytes were uniform with a scattered distribution. The tidal line was neat, the subchondral bone was regular, and the regenerated cartilage had tightly combined with the subchondral bone and the cartilage surrounding the defect. The gross specimen and Mankin scores decreased obviously. CT scans showed that the scaffold had integrated well with the knee joint. Regenerated cartilage in the defect was flush and integrated with the surrounding cartilage. The trabecular structural had a high integrity and good porosity. The group with only the PLGA/TCP scaffold showed a slightly rough surface with a dark color. The defect was partly repaired by regenerated cartilage, but less cartilage surrounded the scaffold and the cartilage tissue extended to the defect, but with fewer regenerated chondrocytes, polysaccharides and type II collagen than in the icariin group. Repair was evident, but a shallow defect with some erosion remained. The surface was uneven and the four layers of cartilage were poorly distinguishable. The tidal line was less regular, as was the subchondral bone. There was a gap between the regenerated cartilage and the subchondral bone and CT scans showed that the regenerated cartilage was slightly concave and was basically integrated with the surround cartilage. There were some trabeculae under the bone with a structure too loose to provide support for the cartilage. The gross specimen and Mankin scores were decreased moderate. In the no scaffold group, 6 months after the repair, a rough surface with a darker color was evident and an obvious defect was observable under the microscope. The cartilage tissue surrounding the defect barely reached the defect. No obvious polysaccharides and type II collagen were secreted, and the repair was poor. The four layers of cartilage were indistinguishable and chondrocyte size was irregular with a disordered or cluster distribution. The tidal line was irregular, with even blood vessels crossing through. The subchondral bone was more irregular and there was a big gap between the regenerated cartilage and the subchondral bone. CT scans showed that there was a slight collapse in the regenerated cartilage. There was probably some sort of integration with the surrounding cartilage. The trabecular tissue was disordered and had lost its ability to support the cartilage and communicate with the cartilage surface. The gross specimen and Mankin scores were still high. All this might cause by biologically active icariin combined

with porous PLGA/TCP scaffold. BMSCs were shown to migrate through the holes of the scaffold. There were a number of BMSCs in the blood of the defect after the model was established. The BMSCs were uniformly distributed in the scaffold through the blood flow, due to the porosity. Coupled with the effects of icariin, the scaffold promoted the transformation of BMSCs into chondrocytes. The cartilage and subchondral bone were repaired into an organic whole and, at the same time, the scaffold promoted the chondrocytes surrounding the scaffold to secrete more cartilage matrix. Therefore, the porous PLGA/TCP scaffold combined with biologically active icariin was conductive to bone formation and provided support for the regeneration of the cartilage matrix. The porous PLGA/TCP scaffold combined with biologically active icariin repaired the cartilage as a whole.

In summary, we confirmed that biologically active icariin combined with the porous PLGA/TCP scaffold could promote the transformation, proliferation, and regeneration of chondrocytes and increased the cartilage defect repair in the New Zealand rabbit model. At the same time, the scaffold could also promote the construction of cartilage tissue. The cartilage and subchondral bone were repaired into an organic whole. These results provided a theoretical basis for combining biologically active icariin with a porous PLGA/TCP scaffold in repairing articular cartilage defect in a clinical setting and laid the foundation for research on the related mechanisms.

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## Disclosure of conflict of interest

None.

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