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Effect of diclofenac sodium on the cartilage-regeneration potential of the chitosan-gelatin-chondroitin sulfate scaffold

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Abstract

Osteoarthritis (OA) is a joint disorder that results in decreased chondrocyte function, leading to degradation of the extracellular matrix. Tissue engineering, mainly using scaffolds as implants at the affected joints, has emerged as an effective way to treat OA. The chitosan gelatin chondroitin sulfate scaffold possesses a structure that mimics the cartilage extracellular matrix, enabling it to trigger chondrogenesis. This study aimed to determine the effect of adding diclofenac sodium to a gelatin-chitosan-chondroitin sulfate scaffold on cartilage regeneration. The study was performed by implanting chitosan-gelatin-chondroitin sulfate-diclofenac sodium scaffolds or chitosan-gelatin-chondroitin sulfate scaffolds in New Zealand White rabbits' strain in which cartilage defects had been surgically created. Based on immunohistochemical analysis, cartilage regeneration was determined by observing chondrocytes by histological examination using hematoxylin and eosin staining and TNF-α detection. Visual examination showed that both the scaffold-treated groups had better healing than the control group, with the diclofenac-containing scaffold group showing the best healing. These results showed that there was a significantly higher number of chondrocytes ($P<0.05$) in the chitosan-gelatin-chondroitin sulfate-diclofenac sodium scaffold group, which indicated cartilage regeneration due to its anti-inflammatory activity by reducing TNF-α levels shown by the immunoreactive score. From this study, it can be concluded that adding diclofenac sodium to the gelatin-chitosan-chondroitin sulfate composite scaffold suppresses the inflammatory response, inhibiting cartilage degradation and aiding in the rapid healing of cartilage defects. This can be viewed as a therapeutic approach for OA in the future.

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Introduction

Osteoarthritis is a degenerative disease of the joints characterized by damage to the cartilage (1,2). Cartilage is a slippery tissue surrounding the end of a joint's hard bone. This tissue is a shock absorber for movement between bones and a damper when the joints perform activities or movements. An estimated 240 million people worldwide suffer from OA, where the prevalence of OA is 10% and 18% among men and women aged 60 years and above, respectively (3). The prevalence of osteoarthritis in Indonesia reaches 5% at the age of 61 years (4). OA most often occurs in the knees, hips, and hand joints. Abnormalities in joints in patients with OA are caused by decreased chondrocyte function, which results in degradation of the extracellular matrix. Under normal conditions, chondrocytes maintain joint homeostasis. However, in joints affected by osteoarthritis, there is an imbalance between anabolic and catabolic processes in the articular joint tissue, where catabolic activity exceeds anabolic activity, resulting in tissue degeneration (5,6). Articular cartilage comprises chondrocytes—the cellular component of cartilage—and extracellular matrix (ECM) components, such as type II collagen and proteoglycans. In

OA conditions, there is an increase in the levels of proinflammatory mediators (including tumor necrosis factor [TNF]-α, interleukin [IL]-1β, IL-6, IL-15, IL-17, and IL-18). IL-1β and TNF-α are the most critical proinflammatory mediators in the inflammatory process in OA (7). IL-1β and TNF-α have complex mechanisms through the NF-κB and MAPK signaling pathways. Activation of NF-κB causes systemic inflammation in cartilage, synovial membrane, subchondral bone, and ligaments (8,9). In the MAPK pathway, there is an increase in matrix metalloproteinase (MMPs) and aggrecanases (ADAMTS), which are responsible for ECM degradation. The mechanisms of IL-1β and TNF-α differ in that IL-1β, in addition to activating the NF-κB and MAPK signaling pathways, can also upregulate the expression of TNF-α (10). Therefore, in OA, TNF-α levels increase in the synovial fluid, synovial membrane, cartilage, and subchondral bone and thereby induce further inflammation. In cartilage regeneration, there are 3 phases, namely the initial phase (cell death and inflammation), the intermediate (potential balance between decreased catabolic response and increased anabolic response), and remodeling (11,12). OA causes pain in the affected joints; therefore, treatment for OA generally focuses on relieving pain and reducing swelling. The primary therapeutic options for OA include oral nonsteroidal anti-inflammatory drugs (NSAIDs) and intra-articular injections. Another potential treatment is autograft transfer therapy, where joint replacement surgery is performed in severe cases of OA. However, this treatment cannot restore the tissue to its normal condition and is associated with post-therapy side effects (13,14). Cartilage cannot regenerate because it is avascular and lacks innervation (15,16). Not only this problem but for osteoarthritis treatment, corticosteroid injections or current surgical procedures cannot restore the tissue to normal conditions (17). This has triggered the development of methods to overcome problems related to osteoarthritis. Tissue engineering is a method for regenerative medicine that offers scaffold elements as materials that enable the development and acquisition of biological substitutes. The role of scaffolds in cartilage tissue engineering is to provide a suitable environment for cells and ensure success in the tissue regeneration process, and this is possible by providing an environment similar to native articular cartilage (18). The significant advantages of tissue engineering scaffolds are their biocompatibility, biodegradability, and three-dimensional (3D) structure similar to native cartilage (18). Porous 3D scaffolds are used for substitution and to facilitate the transfer of oxygen and nutrients in cartilage repair. This study presents a chitosan, gelatin, and chondroitin sulfate scaffold. The role of gelatin in this scaffold is to increase cell adhesion and chondrocyte proliferation. Chitosan has a structure similar to glucosamine, which is widely distributed in connective tissue, including cartilage (19-21). The similarity of this structure in the same environment stimulates chondrogenesis. Chondroitin sulfate is a bone lesion filler and is biodegradable as it has a structure similar to the cartilage matrix (22). Mixing gelatin with natural or synthetic polymers produces a very effective matrix with improved biomechanics and affinity (23,24). Chitosan-gelatin-chondroitin sulfate scaffolds have advantages, including comparative properties of high porosity, optimum pore size distribution, and low density with good tensile mechanical properties. Diclofenac is the most widely used NSAID in the treatment of musculoskeletal disorders (25) by inhibiting PGE2 production through cyclooxygenase-2 (COX-2), which is induced and produced in response to proinflammatory cytokine signaling such as IL-1 and TNF-α (26). This inhibition causes neutrophils and macrophages to decrease in the injured area (27).

So, in this study, a scaffold was made as a drug delivery system, and crosslinking was carried out with the crosslink agent glutaraldehyde to control the release of diclofenac (28,29). Therefore, the diclofenac scaffold can be gradually released locally over a long period and does not interfere with cartilage regeneration. We performed *in vivo* cartilage repair studies in rabbits using the chitosan-gelatin-chondroitin sulfate scaffolds with diclofenac. Cartilage repair was monitored using TNF-α activity and histopathology.

Materials and methods

Ethical approve

The Veterinary Ethics Committee of the Faculty of Veterinary Medicine, Universitas Airlangga (No. 2.KEH.181.12.2023) approved the protocol for all animal experiments. They were conducted according to the Institutional Regulations for Animal Trials guidelines and the Basic Guidelines for Good Animal Experimental Behaviour.

Animal experiments, materials, and instruments

The animals were acclimated for a week at 22.5 ± 2.5 ^{o}C with a 12-h light cycle before experiments, and food and water were available *ad libitum*. A total of 27 New Zealand female white rabbits from the animal laboratory, Faculty of Pharmacy, Universitas Airlangga, aged 4-8 months and weighing between 2.0 and 3.0 kg, were used in this study. Materials used in this study were: BHA (PT. Inobi, Surabaya, Indonesia), diclofenac sodium, chondroitin sulfate (Elam Pharma Pvt. Ltd., Ankleshwar, Gujarat, India), chitosan, gelatin, glutaraldehyde (Sigma-Aldrich, Darmstadt, Germany), anti-TNF-α antibody (Antibodies-online, Limerick PA, USA), xylazine (Xyla; Interchange, Venray, Netherland), ketamine (PT. Dexa Medica, Cikarang, Indonesia), ampicillin (PT. Meiji, Jakarta Selatan, Indonesia), and povidone (PT. Afi Farma, Kediri, Indonesia). Instruments used were: freeze-dryer (PT. Buchi, Tangerang, Indonesia), syringe (PT. Bukit Bersemi Abadi, Medan, Indonesia), analytical stain Ohaus CP 214 (Sigma-

Aldrich, Darmstadt, Germany), Drill Bosh GBM350 (PT. Bosch Rexroth, Surabaya, Indonesia), scanning electron microscope (SEM; Inspect S-50, FEI, Japan), computed tomography (CT) scanner (GE Revolution EVO 64/129 Slice, USA), microscope (Nikon, Japan), and multiple reader (Biochrome EZ-2000, Cambridge, UK).

Preparation of the scaffold

The scaffold was prepared by gently mixing the gelatin solution in water with the chitosan solution in acetic acid. The solution was then neutralized by adding NaOH (5%) till the pH reached approximately 7. A diclofenac sodium solution in PEG-400 was added to the previous solution to make the chitosan-gelatin-chondroitin sulfate scaffold. The 0.5% glutaraldehyde crosslinker was added to the solution and mixed for 1-2 h. The solution was then frozen and dried using a freeze-dryer, and the dried scaffolds are shown in figure 1. The scaffolds were 5 mm in diameter and 4 mm in thickness.

Evaluation of the scaffolds in *in vivo* defect models

The 27 New Zealand white rabbits obtained for this study were divided into negative control, scaffold without diclofenac, and scaffold with diclofenac groups. Each group was then divided into three subgroups for observation on days 2, 7, and 14 of the study (n=3, per interval). The defect model was created by drilling the femur with a diameter of 4.2 mm and a depth of 2.0 mm. The scaffold was then inserted into the defect. Post-operative care was provided to eliminate the possibility of infection till wound healing was complete. The rabbits were euthanized on days 2, 7, and 14, and the femur bone and cartilage were removed and decalcified using 10% EDTA solution (pH 7.4). Macroscopic observation, hematoxylin and eosin staining for chondrocytes, and immunohistochemical staining for TNF-α were performed. Semi-quantitative immunohistochemical analysis was performed using the Immunoreactive Score (IRS) (30), as shown in table 1.

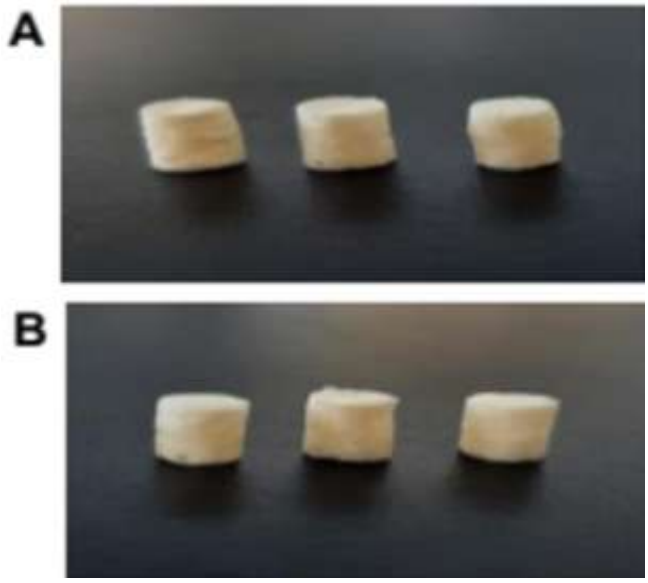

Figure 1: (A) Chitosan-gelatin-chondroitin sulfate-diclofenac sodium scaffolds and (B) Chitosan-gelatin-chondroitin sulfate scaffolds.

Table 1: Semi-quantitative IRS (30)

A	B	A x B
0: no positive cell	0: no color after staining	0 - 1: Negative
1: ≤ 10% positive cells	1: weak intensity	2 - 3: low
2 : < 10% - ≤ 50% positive cells	2: medium intensity	4 - 8: medium
3 : < 50% - > 80% positive cells	3: strong intensity	9 - 12: High
4 : ≥ 80% positive cells		

Statistical analysis

GraphPad Prism 8 (GraphPad Software, Boston, USA) statistics software was used for analysis. Data are expressed as a mean ± SD. The significance of the differences between groups was tested using a non-parametric Kruskal-Wallis test. $P<0.05$ was considered to indicate statistical significance.

Results

Efficacy of the scaffolds in promoting the *in vivo* healing of cartilage

After the rabbits were euthanized at the mentioned intervals, the femur was removed, and the defects were visually observed to note macroscopic changes caused by the treatment (Figure 2). The control group showed no signs of healing on day 2, minor healing effects on day 7, and partial healing on day 14. Both groups treated with scaffolds showed better healing at all time points. Of note, the diclofenac-containing scaffold had the best healing performance, with excellent defect closure observed on day 14, which was substantially better than the healing effect of the scaffold without diclofenac.

After macroscopic observation, the tissues were sectioned and subjected to H&E staining to note chondrocyte migration. Chondrocytes have a round shape with the old purple nucleus. Immature chondrocytes are elliptical and grow more significantly with intensive production of ECM

(31). On days 2, 7, and 14, there was an increasing number of chondrocytes in all groups, as shown in table 2 and figure 3. However, the number of chondrocytes was significantly higher in the group that received the diclofenac-containing scaffold ($P<0.05$).

TNF-α plays a vital role in inducing PGE2 and MMP, which result in cartilage degradation (32,33). Decreasing levels of TNF-α indicate a substance's anti-inflammatory activity. Figure 4 shows the images of immunohistochemical analysis, and table 3 shows the corresponding IRS for each group. As shown in table 3, the IRS was the lowest for the diclofenac-containing scaffold group compared to the control group and the group that received the scaffold without diclofenac.

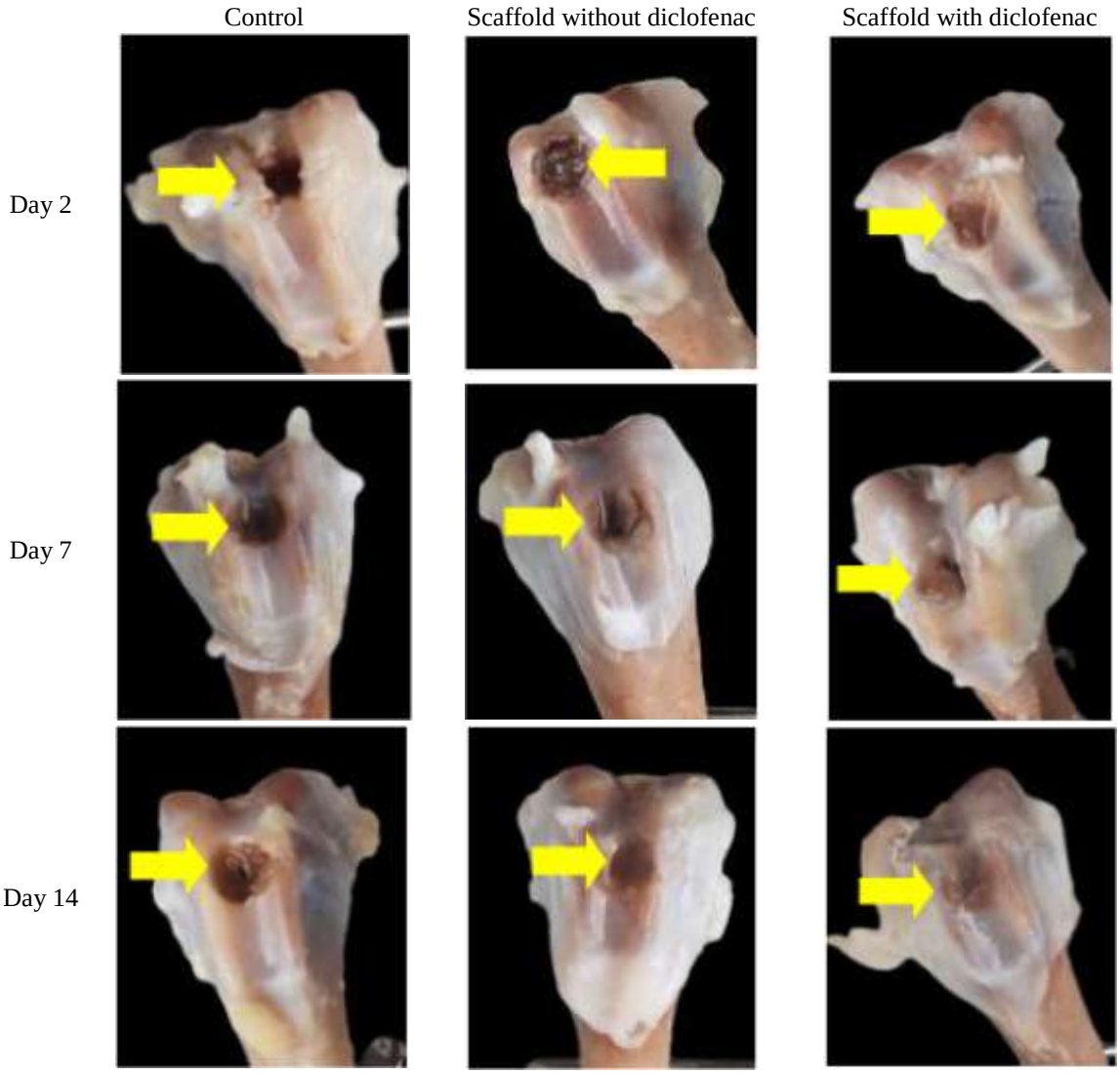

Figure 2: Photographs of the femur with cartilage showing healing of the created defects on days 2, 7, and 14.

Table 2: Each group's number of chondrocytes on days 2, 7, and 14. The data are shown as mean ± SD. The different letters show different significant groups

Group	Day 2	Day 7	Day 14
Control	62.67 ± 4.51^{c}	53.00 ± 3.61^{c}	47.33 ± 2.08^{c}
Scaffold without diclofenac	69.00 ± 4.00^{b}	57.00 ± 4.58^{b}	83.33 ± 4.04^{b}
Scaffold with diclofenac	100.67 ± 3.51^{a}	112.33 ± 4.04^{a}	96.00 ± 5.00^{a}

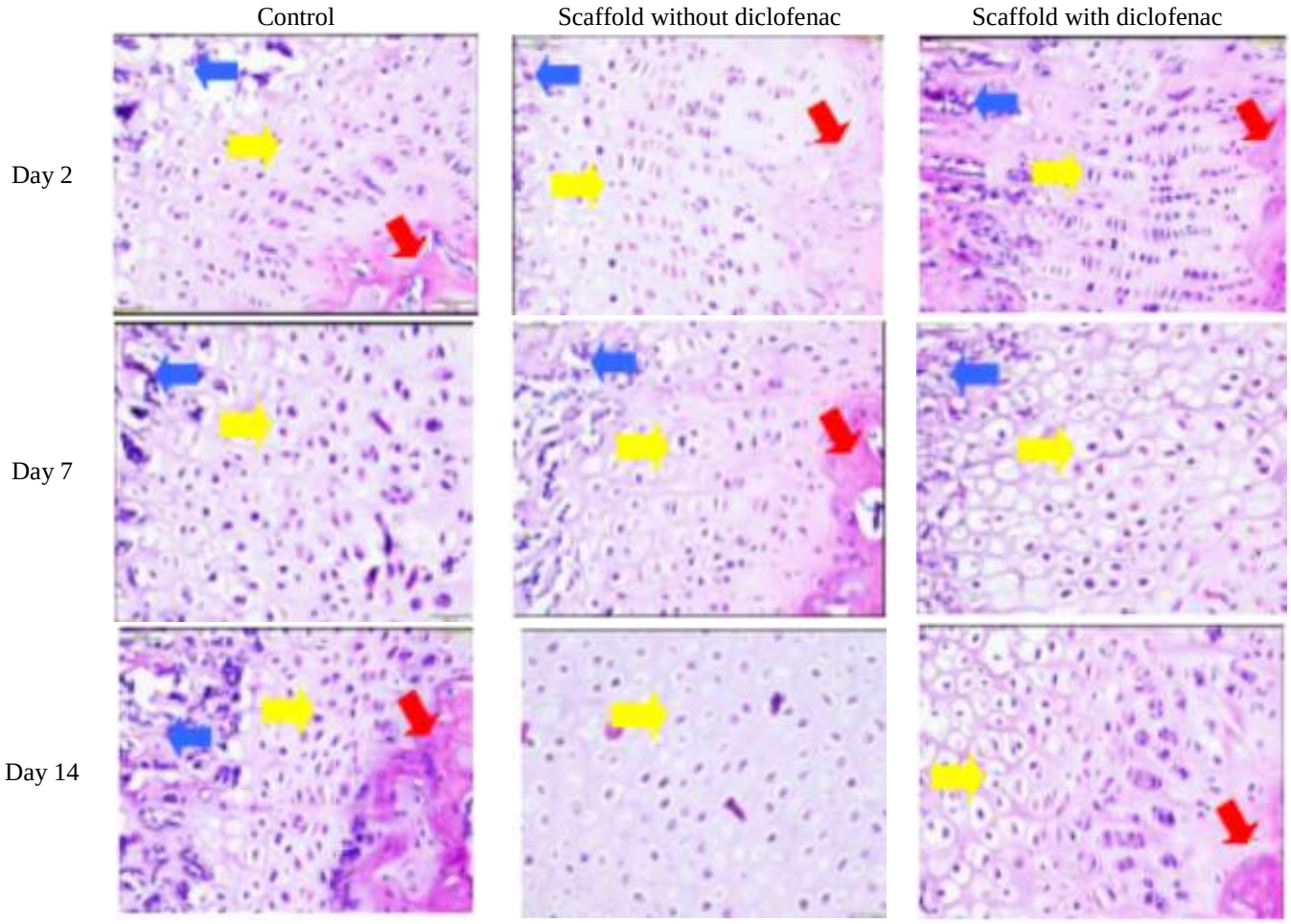

Figure 3: H&E staining images of tangential cartilage slices of each group on days 2, 7, and 14. Blue arrows indicate fibrous tissue; yellow arrows indicate chondrocytes; red arrows indicate bone.

Table 3: Each group's IRS on days 2, 7, and 14. The data are shown as mean ± SD. The different letters show different significant groups

Group	Day 2	Day 7	Day 14
Control	11.00 ± 1.73^{c}	5.33 ± 3.06^{b}	5.67 ± 2.52^{b}
Scaffold without diclofenac	8.67 ± 0.58^{b}	4.67 ± 1.15^{b}	3.33 ± 1.15^{ab}
Scaffold with diclofenac	2.00 ± 0.00^{a}	2.33 ± 0.58^{a}	2.67 ± 1.15^{a}

Discussion

Zhao *et al.* (11) used a gelatin-diclofenac scaffold and observed healing over four weeks. They noted that the control group had a pink fibrous appearance. In contrast, the treatment group had a porcelain-white cartilage appearance, although the closure of the defect was not optimal. In another study, autologous rabbit synovial fluid-derived mesenchymal stem cells were used to treat a rabbit cartilage defect, resulting in 80% closure by cartilage growth within eight weeks and complete repair by 12 weeks (31-34). In this study, it was observed that on day 14, the defect was still clearly visible in the control group, whereas the group implanted with the scaffold showed partial healing.

Widhyanto *et al.* (35) showed that the cartilage defect healing in the rabbits occurred in week 12. Two weeks of observation are needed to deeply observe the proinflammatory cytokine production in cartilage regeneration, such as TNF-α. TNF-α plays a crucial role in the inflammation phase from Day 1 until Day 3. Correlated to the study by Yan *et al.* (36), day 14 will hold the matrix healing. Upon H&E staining, chondrocytes exhibited a round

shape with a dark purple nucleus. The bright pink indicates the matrix, while the deep purplish pink indicates bone. Juvenile chondrocytes have an elliptical shape and can be seen on the edge of the cartilage. During their development, chondrocytes increase in size to 20-30 μm to support more intensive ECM production (31). After growth, chondrocytes tend to shrink to irregular shapes seen in the cartilage. Differentiated/mature chondrocytes are smaller than juvenile chondrocytes, which are around 10-20 μm and are found in the deeper part (37).

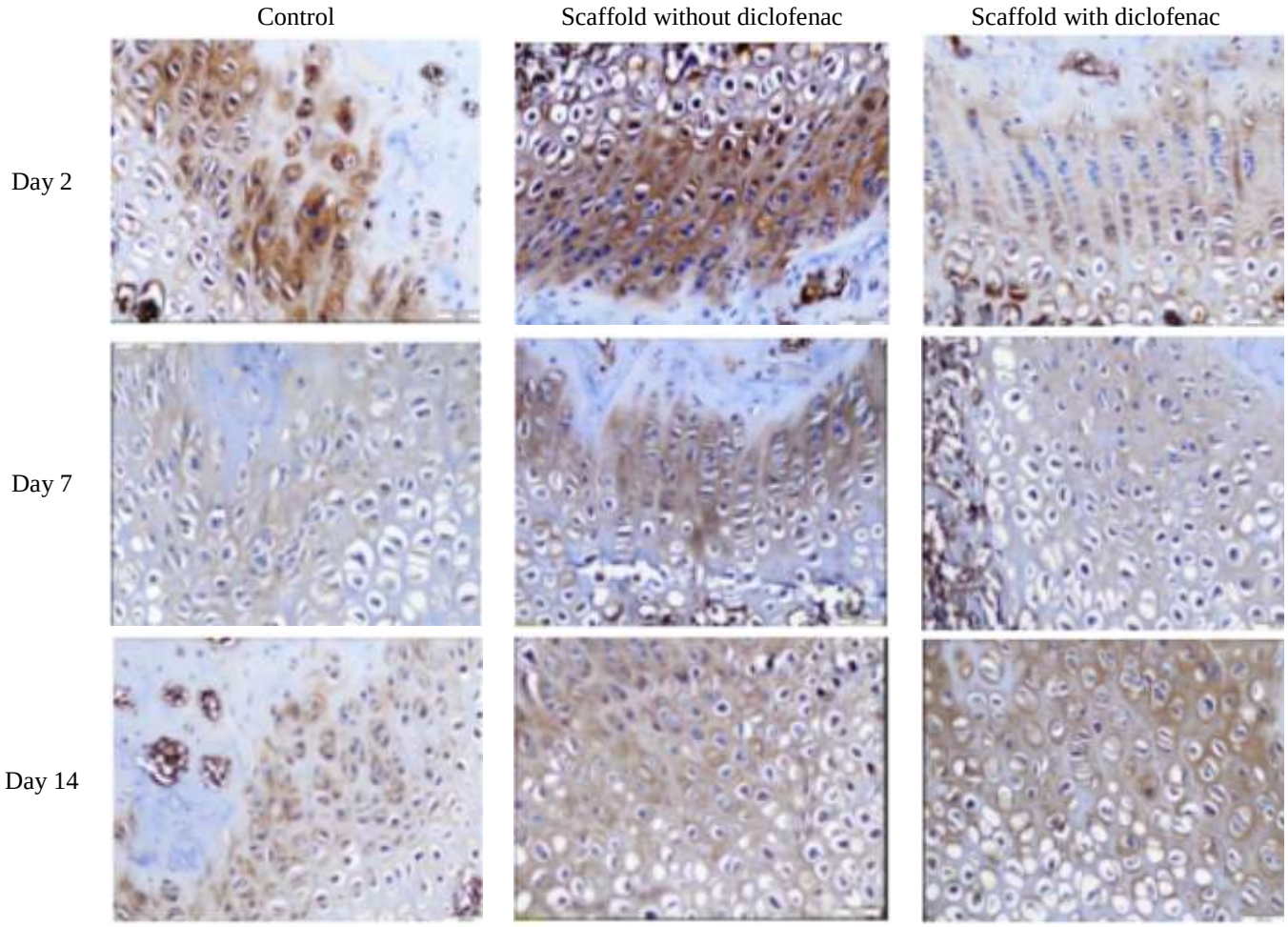

Figure 4: Immunohistochemical analysis images of the tangential cartilage slices from each group on days 2, 7, and 14 (400x magnification). Brown parts indicate chondrocytes.

In the natural course of healing of a cartilage defect, the inflammatory response occurs in the first week after drilling, where the highest levels of inflammation usually happen around the first three days. During the initial stages of repair, an inflammatory process can trigger ECM degradation and inhibit chondrogenesis (38,39). The inflammatory process triggers the release of proinflammatory cytokines, such as IL-1 and TNF-α. Both cytokines are inducers of cartilage matrix degradation because of their ability to induce the expression of genes encoding MMP and aggrecanase. TNF-α induces the production of PGE2, which acts as a solid catabolic signal in cartilage by increasing chondrocyte injury and the potential for chondrocyte apoptosis (40,41). In this study, diclofenac sodium in the scaffold inhibits the release of PGE2 via the COX pathway, which prevents chondrocyte apoptosis. Therefore, the group treated with the chitosan-gelatin-chondroitin sulfate-diclofenac sodium scaffold implants can accelerate the growth of chondrocyte cells (27). In the control group and the chitosan-gelatin-chondroitin sulfate scaffold group, there was a decrease in the number of chondrocytes on days 2 and 7, which could result from apoptosis.

Based on the results of immunohistochemical staining, it can be seen that the presence of diclofenac sodium in the scaffold can reduce TNF-α activity, which is significantly high during the inflammatory process. Adding diclofenac is

intended to relieve pain and inhibit the production of proinflammatory cytokines that can worsen cartilage damage. It has been reported that scaffold implantation causes pain and inflammation during and after surgery, owing to local tissue damage triggered by macrophage activation (42,43). Macrophages have different phenotypes depending on the local tissue environment. The classically activated M1 phenotype appears during acute inflammation and is associated with increased regulation of proinflammatory cytokines and enzymes inhibiting chondrogenesis, whereas activated M2 triggers chondrogenesis. Macrophages accumulate and become polarized (M1 or M2) in the synovium and articular cavity during the development of OA (44).

Zhang *et al.* (45) reported an imbalance of M1 and M2 macrophages where polarized M1 but unpolarized M2 accumulated in human and mouse OA synovial tissue. In 2018, Guo *et al.* (46) found that activated synovial macrophages in patients with OA were mainly M1 macrophages, not M2 macrophages. These findings indicate increased M1 synovial macrophages in cartilage, which can secrete proinflammatory cytokines such as TNF-α and IL-1. Increased levels of TNF-α upregulate the expression of MMP, A Disintegrin, and ADAMTS-4 while downregulating the expression of Aggrecan and type II collagen (11). In addition, increased TNF-α levels also result in COX stimulation and increased PGE2 expression, where PGE2 can also degrade cartilage. During tissue repair, macrophages transition to the M2 phenotype, which plays a role in suppressing inflammation through the production of anti-inflammatory cytokines.

Suyatno *et al.* (47) showed that administering diclofenac sodium as a bilayer scaffold can decrease the bone closure time while increasing the bone growth factor. The linear result was demonstrated by Khodir *et al.* (48). The cartilage regeneration mechanism must promote the inflammation process to induce the growth factor. The use of a non-selective COX-2 inhibitor will decrease the amount of prostaglandine (PGE-2) production and neutrol and macrophage uptake. The COX-2 pathway has been associated with the transition of M1 macrophages to the M2 phenotype, and the presence of diclofenac may delay the transition of M1 macrophages to M2 macrophages. Therefore, glutaraldehyde was added to the scaffold as a crosslinking agent to control the release of diclofenac sodium at a rate that does not interfere with the cartilage repair process (49). Other studies have shown that diclofenac sodium can form an anti-inflammatory microenvironment by increasing the polarization of M2 macrophages, thereby reducing inflammation and delaying cartilage degradation (11,27,50). Therefore, diclofenac in small doses in the scaffold will not interfere with the cartilage repair process.

Conclusion

The inflammatory process is still needed to help migrate immune response and growth hormone to induce cell differentiation and proliferation. However, the inhibition of the inflammatory process by the addition of diclofenac sodium to the scaffold has shown promising outcomes in terms of cartilage healing. The different mechanisms of the inflammatory pathway were the key to keeping the inflammatory acute phase to conduct cartilage healing through the administration of the scaffold.

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Conflict of interest

There is no conflict of interest.

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تأثير ديكلوفيناك الصوديوم على إمكانية تجديد الغضروف في سقالة الكيتوزان والجيلاتين وكبريتات الكوندرويتين

أنيك سيتيا بودياتين، موتيارا بوتري براميستي، وا أودي نورفينتي، يوسف أليف براتاما، ديندا مونيكا نوسانتارا راتري و كريسماوان أرديانتو

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الخلاصة

هشاشة العظام هو اضطراب في المفاصل يؤدي إلى انخفاض وظيفة الخلايا الغضروفية ، مما يؤدي إلى تدهور المصفوفة خارج الخلية. ظهرت هندسة الأنسجة، التي تستخدم السقالات بشكل أساسي كغرسات في المفاصل المصابة، كطريقة فعالة لعلاج الزراعة العضوية. تمتلك سقالة كبريتات الجيلاتين الشيتوزان كوندرويتين بنية تحاكي مصفوفة الغضروف خارج الخلية، مما يمكنها من تحفيز تكوين الغضروف. هدفت هذه الدراسة إلى تحديد تأثير إضافة ديكلوفيناك الصوديوم إلى سقالة كبريتات الجيلاتين الشيتوزان شوندروتن على تجديد الغضروف. أجريت الدراسة عن طريق زرع سقالات الصوديوم الشيتوزان-الجيلاتين-شوندروتن أو كبريتات الديكلوفيناك أو سقالات كبريتات الشيتوزان-الجيلاتين-شوندروتن في سلالة الأرانب البيضاء النيوزيلندية التي تم فيها إنشاء عيوب في الغضروف جراحيا. بناء على التحليل الكيميائي المناعي، تم تحديد تجديد الغضروف من خلال مراقبة الخلايا الغضروفية عن طريق الفحص النسيجي باستخدام صبغة الهيماتوكسيلين والأيوزين والكشف عن TNF-α. أظهر الفحص العياني أن كلا المجموعتين اللتين عولجان بالسقالات كان لديهما شفاء أفضل من مجموعة السيطرة، حيث أظهرت مجموعة السقالة المحتوية على الديكلوفيناك أفضل شفاء. أظهرت هذه النتائج أن هناك عددا أعلى بكثير من الخلايا الغضروفية ($P<0.05$) في مجموعة سقالة الصوديوم الشيتوزان-الجيلاتين-شوندروتن كبريتات-ديكلوفيناك، والتي أشارت إلى تجديد الغضروف بسبب نشاطها المضاد للالتهابات عن طريق تقليل مستويات α عامل نخر الورم التي أظهرتها درجة التفاعل المناعي. من هذه الدراسة، يمكن الاستنتاج أن إضافة ديكلوفيناك الصوديوم إلى السقالة المركبة من الجيلاتين والشيتوزان والكروندروتن تثبط الاستجابة الالتهابية ، وتثبيط تدهور الغضروف وتساعد في الشفاء السريع لعيوب الغضروف. يمكن اعتبار هذا نهجا علاجيا لالتهاب المفاصل في المستقبل.

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