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Research Article

Collagen type I and type II expression evaluation on cartilage defect regeneration treated with Dwikora–Ferdiansyah–Lesmono–Purwati (DFLP) scaffold supplemented with adipose–derived stem cells (ASCs) or secretome: an in-vivo study

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Abstract

Cell-based therapies such as Scaffold, stem cells, and secretome, are one of the alternatives to enhance the regeneration of hyaline-like cartilage in cases of cartilage defects. This study is an in-vivo experiment using animal models, in which we apply a composite of DFLP (Dwikora-Ferdiansyah-Lesmono-Purwati) Scaffold and Adipose-Derived Stem Cells (ASCs) or Secretome to an injury model on the distal femoral trochlea of New Zealand White Rabbits. The animals were divided into four groups: (1) control (K); (2) Scaffold only (S); (3) Scaffold + ASCs (SA); (4) Scaffold + Secretome (SS). Animals were terminated in the 12th week, and an immunohistochemistry (IHC) evaluation for Collagen type I and II were done. Statistical analysis shows that collagen type I IHC between groups shows no significant difference ($p = 0.546$). Collagen type II IHC shows significant difference between groups ($p = 0,016$). The findings in this study showed that Scaffold + ASCs group and Scaffold + Secretome have better collagen type II expression compared to the control group. DFLP Scaffold composite with ASCs or Secretome shows potential for cartilage regeneration therapy by increasing type II collagen expression as in hyaline-like cartilage which may be used for regenerative therapy for cartilage defects.

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INTRODUCTION

Articular cartilage is an avascular, aneural, and alymphatic tissue. This causes problems in the treatment of cartilage defects. Initially, inflammatory cytokines IL-1 and TNF-α will appear. IL-1 will block the stimulation of proteoglycan synthesis and stimulates MMP production, TNF-α will stimulate the production of cartilage degrading enzyme and block collagen synthesis (Fritz, Janssen, Gaissmaier, Schewe, & Weise, 2008; Schmitz, DeHart, Qazi, & Shuler, 2016).

If the defect is only at the chondral level, regeneration cannot occur, if it is at the subchondral/osteochondral level, fibrocartilage regeneration will occur, this is caused by collagen type III production by the chondrocytes. The most commonly used and developed therapy is by microfracture and cellular-based therapy, as in ACI and Scaffold. Compared to the need for 2 surgeries in ACI therapy, Scaffold based therapy only needs a single event surgery (Bedi, Feeley, & Williams, 2010; Beer, Mildner, & Ankersmit, 2017; Jang, Jung, & Ju, 2017; Mancuso, Raman, Glynn, Barry, & Murphy, 2019).

An earlier study using Freeze Dried Bovine Cartilage (FDCB) composite using Mesenchymal Stem Cells and Platelet Rich Plasma in 2017 shows good results in an animal model. The FDBC scaffold is a precursor for this study's DFLP (Dwikora–Ferdiansyah–Lesmono–Purwati) Scaffold, which in an undecellularized cartilage bovine scaffold. (Dwikora Novembri Utomo, Abdul Rantam, Ferdiansyah, & Purwati, 2017) Scaffold functions to isolate the stem cells and to capture the extracellular matrix produced by the chondrocyte to increase the mechanical properties of the regenerated material (Tuan & Mauck, 2013).

The addition of ASCs or Secretome is expected to produce more hyaline-like cartilage which has more collagen type II than collagen type I expression, which is one of the characteristics of hyaline-like cartilage rather than fibrocartilage (Henderson, Lavigne, Valenzuela, & Oakes, 2007; Ulrich-Vinther, Maloney, Schwarz, Rosier, & O'Keefe, 2003). After this therapy, the result is expected to be hyaline-like cartilage.

METHODS

This study is in an in-vivo post-test only controlled animal laboratory study done at the Stem Cell Research and Development Center Laboratory. In this study a model cartilage defect of 4.5 mm^2 was made on 24 New Zealand White Rabbits (*Oryctolagus cuniculus)* which were randomly divided into four groups: (1) Control group (K); (2) Scaffold only group (S); (3) Scaffold + ASCs group (SA); (4) Scaffold + secretome group (SS). The randomization was done using physical methods by shuffling designated groups in each subject. The sample size was quantified using the degree of freedom of ANOVA (Charan & Kantharia, 2013; Ilyas, Adzim, Simbak, & Atif, 2017). The rabbits were aged 8-12 months, weighs 2400-3200 grams, and are healthy until the end of the study. Each group consists of 6 rabbits. This experiment was ethically approved by the Animal Care and Use Committee of Airlangga University Veterinary Medicine Faculty (Certificate number: 2.KE.060.04.2019). Each rabbit was placed in a cage 100 cm x 50 cm x 70 cm, free access to food (300 grams daily), and water. Before the study, the rabbits were acclimated to the conditions for 1 week. Rabbits that were deemed not healthy or failed to acclimate with the conditions within the acclimatization period were excluded from this study and replaced.

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Anesthesia

Anesthesia was done initially by premedication using 0.2 mg/kg atropine sulfate and 1.0 mg/kg diazepam, each was given intramuscular, continued by ketamine 20 mg/kg, and then maintained using additional 10 mg/kg dose if there was a reaction from the rabbits.

ASCs Preparation

The stem cells used were allogenic and were procured in advance of this study, Cryopreserved in the Airlangga University Stem Cell Research and Development Center, and thawed to be used for this study. It was retrieved from the scapular adipose tissue of New Zealand White Rabbits not included in this study, therefore the stem cells were allogenic. Lipoaspirates are collected in an aspiration container and may be comprised of three distinct layers: 1) an upper layer of oil due to the lysis of mature adipocytes, 2) a middle layer of adipose tissue, and 3) a bottom, liquid infranatant containing saline and contaminating cell types such as red blood cells (RBCs). Washing the lipoaspirate will remove the greater majority of contaminating red blood cells and saline. Following this initial culture period, the culture media may be aspirated, and any contaminating red blood cells gently removed by washing with sterile 1X PBS (Phosphate Buffer Saline). Replace with culture medium and continue to culture the ASCs as needed (Bhang et al., 2014; Jang et al., 2017).

The tissue cells were then processed and cultured until the 5^{th} passage. Phenotype confirmation was done using CD 45, CD 73, CD 90, CD 105 markers. We used 2 x 10^6 cells for each application in the stem cell group, and the cells were marked using PKH26 and traced using a fluorescence microscope.

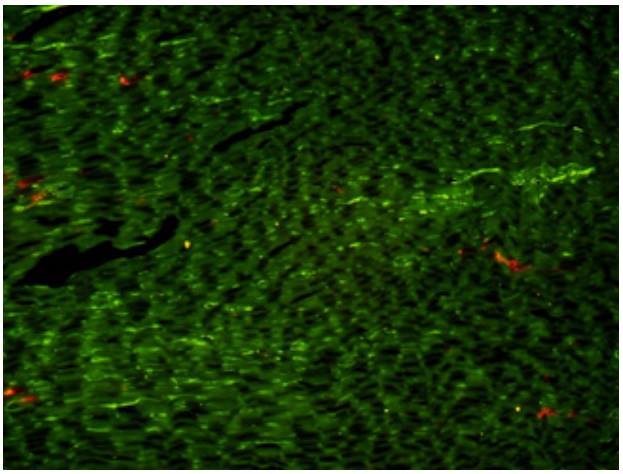

Figure 1. White arrow shows PKH26 stained cells (orange colored) within regenerated cartilage tissue

Secretome Preparation

Secretome used in this study were retrieved from the stem cells processing. Secretome was collected after 24 hours and centrifuged at 700 ×g for 8 min to remove cell debris. Retrieval was done in 70-80% stem cell confluent cultures. It was then centrifuged and used in a ±1 mm solution for the application in each treatment.

DFLP Scaffold Preparation

The base materials for this scaffold was taken from the femoral head and condyles of Ongole cattle aged 24 months. These cattle were certified healthy and retrieved from the Pegirian slaughterhouse in Surabaya. The cartilages were then separated from the subchondral bone by using bone rongeurs. The separated cartilage was flushed with NaCl 0.9% solution or distilled water until it was clean, and ground into powder. These powders were then mixed with NaCl 0.9% solution or distilled water under 1:1 ratio and then put into a 5 cm diameter mold.

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These molds were then deep-frozen in –80^{0}C for at least 24 hours. Afterward, it was dried by a sublimation technique using a freeze-dry machine. These dried molds were then re-molded into 5 mm diameters. The scaffold used in this study is as in Fig. 1. An earlier preliminary study has shown that this material was biocompatible and may be used as a biomaterial (Dwikora Novembri Utomo & Fachrizal, 2017; D. N. Utomo et al., 2019; Wirashada, Utomo, Purwati, Widhiyanto, & Hernugrahanto, 2019).

Figure 2. *DFLP Scaffold*

Articular Injury Model

The site used for the injury model is at the femoral trochlea. Surgery was done under standard sterile protocols. A midline incision and a medial parapatellar approach were done, and a full-thickness 4.5 mm^2 lesion was made. Each lesion was treated as per groups divided accordingly. In the control group, the defect was left as is. The surgeries were done by the author, experienced in the procedure. The timing of surgery was divided daily between groups, in which every group was done in a single day, followed by the other groups in respective days. We consider this to mimic microfracture by stimulating the subchondral bone marrow. When DFLP Scaffold was used, to fix the scaffold, a thin film of fibrin glue (Beriplast® P Combi-Set) was applied. These are shown in Fig. 2 The rabbits were then put inside cages and environment mentioned above until the 12th week. No specific immobilization system to the affected limb was applied to the animals. As the pain subsided, they start to mobilize with both legs inside their respective enclosures. They were then terminated on the 12th week each group according to the dates the surgery was done, and immunohistochemistry staining for collagen type I and type II was performed.

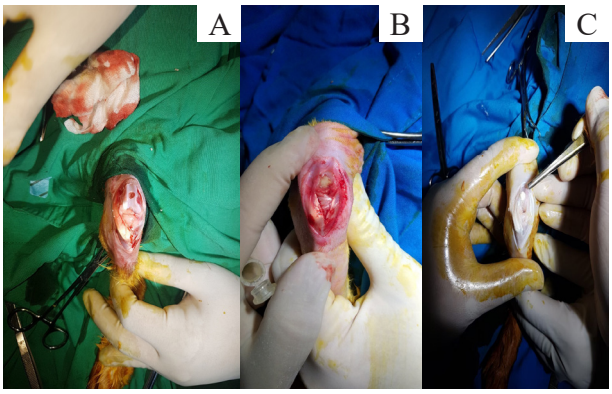

Figure 3. a. The defect on the femoral trochlea (Control group); b. Implanted scaffold (Scaffold group); c. Implanted scaffold saturated with ASCs/Secretome (Scaffold + ASCs/Secretome group with similar clinical picture between both groups)

Statistical Analysis

We did a statistical analysis using SPSS 26. Data normality was then tested using Shapiro-Wilk. All collagen type I expression score has $p > 0.05$, thus considered having normal distribution, and further statistical analysis was done using Oneway ANOVA. Collagen type II expression for the K group has $p = 0.023$. The statistical analysis for the Collagen type II group was then done using Kruskall-Wallis.

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RESULTS

Table 1. IRS Scoring System, final score = A x B

A	B
0 pts – no cells with positive reaction 1 pt – to 10% cells with positive reaction 2 pts – 11-50% cells with positive reaction 3 pts – 51-80% cells with positive reaction 4 pts – > 80% cells with positive reaction	0 pts – no color reaction 1 pt – low intensity of color reaction 2 pts – moderate intensity of color reaction 3 pts – intense color reaction

Table 2. Collagen type I and type II mean comparison between groups

(K=Control, S=Scaffold, SA= Scaffold + ASCs, SS=Scaffold + Secretome)

	Group	Mean (points)	Std. Deviation	P-Value (α =0.05)
Col-1	K	3.33	2.04	0.546*
	S	4.30	2.00	
	SA	5.35	2.64	
	SS	3.85	2.05	
Col-2	K	2.20	1.17	**0,016#**
	S	4.17	2.73	
	SA	7.30	1.37	
	SS	6.35	2.35	

Note : *: *Oneway* ANOVA Test

#: Kruskal Wallis Test

Table 3. Kruskal-Wallis Pairwise Comparison Between Group for Collagen Type II

(K=Control, S=Scaffold, SA= Scaffold + ASCs, SS=Scaffold + Secretome)

Group Col-2	Statistic Test	Std. Error	Std. Statistic Test	Sig. (α =0.05)
K-S	-4.92	3.41	-1.44	0.149
K-SA	-11.25	3.81	-2.95	**0.003**
K-SS	-8.88	3.81	-2.33	**0.020**
S-SA	-6.33	3.81	-1.66	0.097
S-SS	-3.96	3.81	-1.04	0.299
SS-SA	2.38	4.18	0.57	0.569

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To evaluate the immunohistochemistry expression of collagen type I and type II, we use the (Immuno Reactive Score) IRS scoring system (Table 1). This is a semiquantitative scoring system in which the final score is a multiplication between the percentage of immunoreactive cells and the color intensity of immunoreactive cells. Each sample scores were the mean results of 5 different field of view in 1000x magnification.(Nowak, Madej, & Dziegiel, 2007; Remmele & Stegner, 1987). To avoid bias in evaluation, we do a blinded evaluation between two independent observers competent and experienced in the field of histology and cartilage.

Table 2 shows the mean values and standard deviation for each group's collagen type I and type II expression. The mean value for collagen type I for groups K, S, SA, and SS are as follows: 3.33±2.04, 4.50±2.00, 5.35±2.64, and 3.85±2.05. Collagen type I expression comparison between treatment groups shows no significant difference ($p = 0.546$). Collagen type II expression Kruskal-Wallis across groups comparisons shows the statistical difference between groups ($p = 0.016$), the analysis was then continued using Kruskal-Wallis pairwise comparison (Table 3) shows significant difference between K–SA ($p = 0.003$) and K – SS ($p = 0.020$).

The IHC scoring analysis correlates to the mean values of collagen type II groups. Scaffold + Stem Cell group and Scaffold + Secretome group shows better results than the control group. Fig. 3 shows normal cartilage, and Fig. 4 and 5 shows IHC evaluation for Collagen type I and Collagen type II for each group respectively.

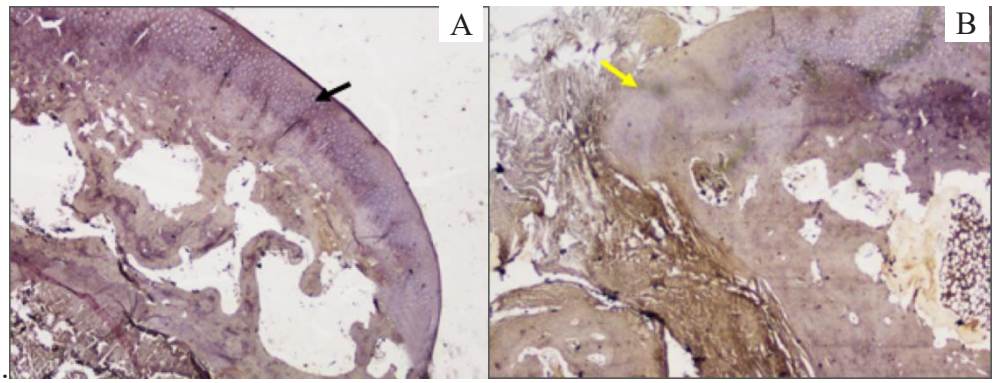

Figure 4. A. Normal Cartilage, B. Defect area. Black and yellow arrows were the areas of evaluation (Immunohistochemistry staining, 40x magnification; Nikon H600L microscope; DS Fi2 300 megapixel camera)

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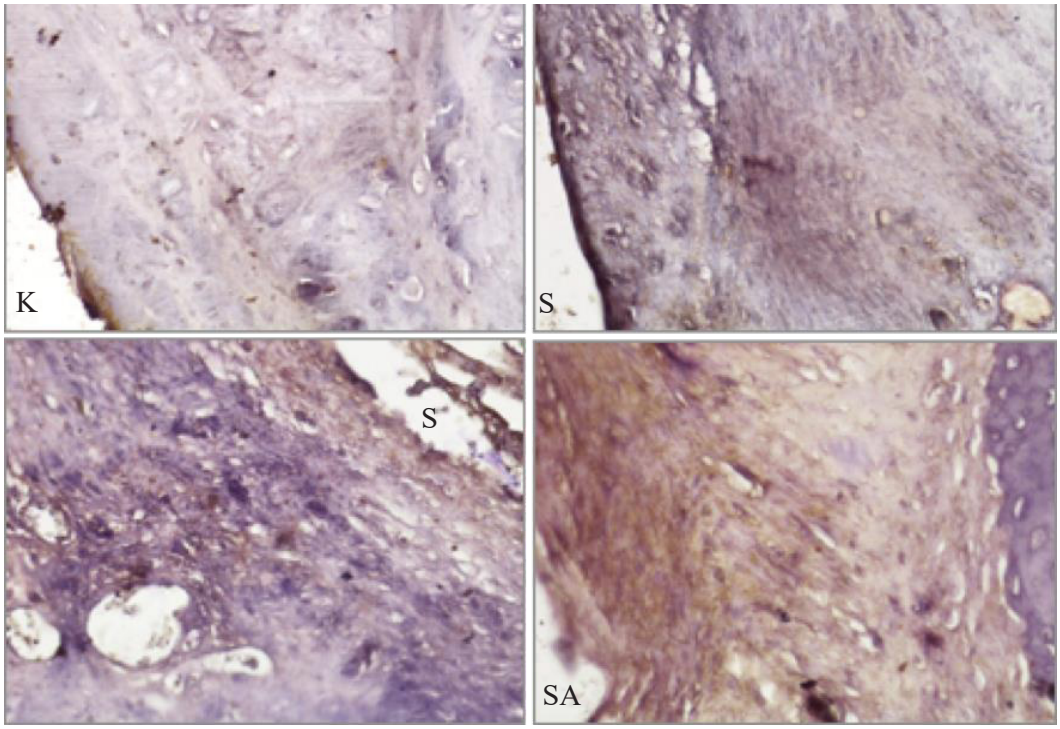

Figure 5. Comparison for Col-1 IHC staining for each group on chondrocytes and epiphyseal matrix for evaluation. (Immunohistochemistry staining, 40x magnification; Nikon H600L microscope; DS Fi2 300 megapixel camera)

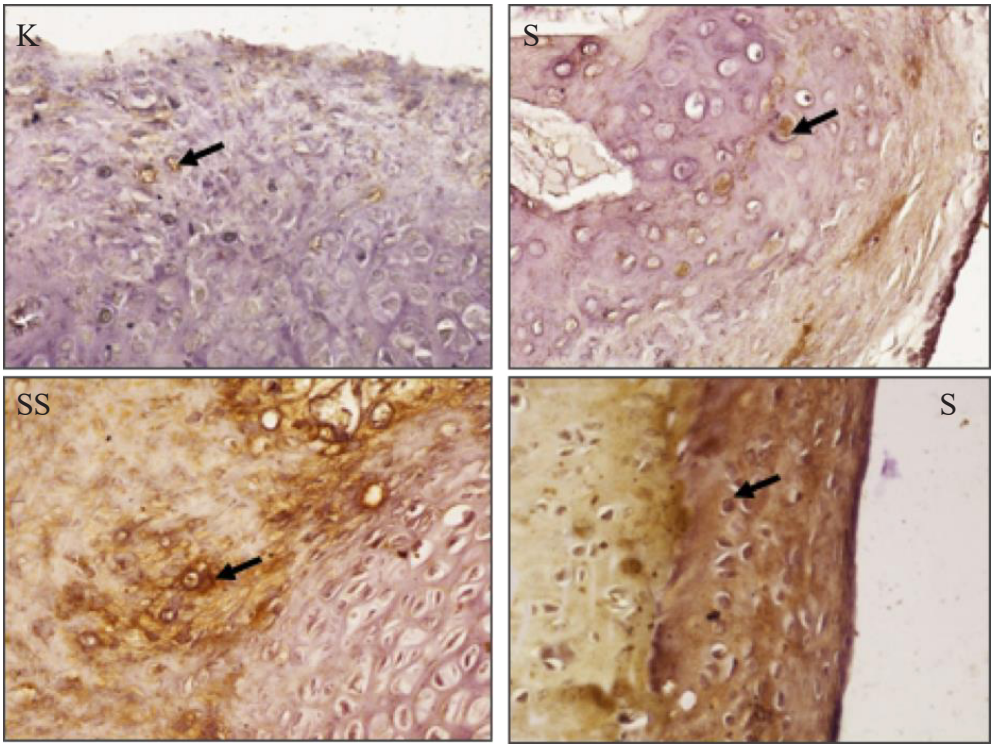

Figure 6. Comparison for Col-2 IHC staining for each group on chondrocytes and epiphyseal matrix for evaluation. (Immunohistochemistry staining, 40x magnification; Nikon H600L microscope; DS Fi2 300 megapixel camera)

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DISCUSSION

A Scaffold should be biodegradable, biocompatible, non-toxic, and has the ability to integrate with the host tissue and to sustain the life and phenotype of the cells during in-vitro and in-vivo implantation. Based on these results, the SA group has the highest mean value. For the collagen type II mean values for each group, K, S, SA, and SS are as follows: 2.20±1.17, 4.17±2.73, 7.30±1.37, and 6.35±2.35. These results showed that the SA group has the best mean value. DFLP Scaffold composite with ASCs or Secretome shows potential for cartilage regeneration therapy by increasing type II collagen expression as in hyaline-like cartilage.

Scaffold functions for cell isolation and to capture the extracellular matrix produced by the chondrocyte. Tissue engineering is considered to increase the mechanical characters of the end product. A chondro-inductive trigger is also needed to start chondrogenesis and chondrogenic characteristics of the implanted cells. DFLP Scaffold has been tested for biocompatibility, physicobiochemical properties, and its immunogenicity. Its results showed that DFLP Scaffold has the characteristics a scaffold should have. Compared with readily available synthetic scaffolds in the market, DFLP Scaffold is easier to produce and maybe a more economical alternative. In this study, we noted that there were no adverse effects related to the treatment (Dwikora Novembri Utomo & Fachrizal, 2017; D. N. Utomo et al., 2019; Wirashada et al., 2019).

Studies using secretome as biotherapy to overcome bone defects showed an increase of cartilage volume and quality, showed by mineralization and connectivity increase, and also angiogenesis. These results imply that the potential for secretome biotherapy for musculoskeletal problems, specifically, cartilage problems for this study (Khatab et al., 2018; Satue, Schuler, Ginner, & Erben, 2019).

The increase of cartilage formation might be caused by the decrease of chronic inflammation by secretome biotherapy. Secretome from various sources contains many growth factors that can promote migration, proliferation, and induction. After the discovery of mesenchymal stem cells (MSCs) in the bone marrow by Becker et al. in the 1960s, regenerative medicine discipline has developed. Many stem cells have been discovered since then. Adipose tissue has been proven to be a readily and abundant source for multipotent stem cells. These cell population of Adipose-Derived Stem Cells (ASCs) has also been proven to be safe (Satue et al., 2019; Vizoso, Eiro, Cid, Schneider, & Perez-Fernandez, 2017).

Adipose-derived stem cells (ASCs) were retrieved from adipose tissue that has the potency to differentiate into adipocyte and some other cells. ASCs are also similar to MSCs that can be cultured in-vivo or in-vitro for various therapy options, cartilage injuries included. ASCs were often taken from the white subcutaneous adipose tissue of the gluteus, abdomen, or infrapatellar fat pad by lipoaspiration or open excision. Subcutaneous adipose tissue contains adipocyte and a various stromal vascular fraction (SVF), including fibroblast, endothelial cells, pre-adipocyte, vascular muscle cells, monocyte, and ASCs themselves (Fukuda, Chikama, Nakamura, & Nishida, 1999; Khatab et al., 2018).

Cell-Based Therapies such as Autologous Chondrocyte Implantation (ACI) and its further developments and Bone Marrow Stimulation (BMS) such as Microfracture are types of surgical options for articular cartilage to achieve regenerated tissue with biomechanics and structure as close as normal hyaline cartilage. ACI and its developments

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give good results for cartilage lesions > 4 cm^2, whereas Microfractures are used for smaller lesions. Studies comparing these 2 techniques showed that long-term outcomes of these tissue engineering techniques do not show significant differences (Harrell et al., 2019; Jang et al., 2017).

The expected regenerated tissue after cartilage defect therapy is more hyaline-like cartilage and achieved with a single event surgery. This expectation gives rise to the usage of Scaffold + Stem cell/Secretome therapy for cartilage defects in this study. Other studies using scaffold primarily uses synthetic material; one of them uses atelocollagen and poly-L-lactic acid (PLLA), evaluated at the 12^{th} week, hyaline-like cartilage was seen (Harrell et al., 2019; Xu et al., 2019).

In this study, we found that the secretome group also increases the proliferation of collagen type II compared to the control group. This may correlate with several growth factors identified in earlier studies within secretomes, such as PDGF-BB, TGFβ2, VEGF, TIMP-1, TIMP-2, angiogenin, and other unidentified factors such as exosomes. VEGF and angiogenin are potent inducers of angiogenesis, VEGF has also been proven to increase proliferation and osteoblast migration. PDGF-BB and TGFβ2 increase bone regeneration by acting as a chemotactic and proliferating agent for MSC recruited towards the defect to differentiate into osteoblast. Finally, TIMP-2 has also been proved to induce the proliferation of primary osteoblast culture in murine and increase bone defect healing. The in-vitro analysis showed that secretome therapy might induce cartilage regeneration (Kim, Kim, Lim, Lee, & Yun, 2010; Mancuso et al., 2019).

In this study, collagen type II expression is higher after scaffold + ASCs/secretome, and this has similar outcome as another study by Satue et al. (Satue et al., 2019) in which intraarticular MSCs injection was done to a murine knee full-thickness cartilage defect model, the study also found an increase of collagen type II expression compared to the control group (Buttgereit, Burmester, & Bijlsma, 2015; Janssens, ten Dijke, Janssens, & Van Hul, 2005). These results showed that stem cell also has the potential for regenerative therapy for chondrogenesis for cartilage defects. Neo-chondrogenesis is the expected result of multipotent MSC and Secretome therapy. In this study, we found that the addition of ASCs or Secretome in Scaffold therapy increases collagen type II expression significantly compared to the control group, although for collagen type I, there is no significant difference for its expression.

The limitation of this study is that we only evaluate two variables of determining the characteristics of regenerated cartilage tissue. Further studies regarding the orientation of the collagen in each layer of cartilage or other IHC markers should be done to further determine the characteristics of the regenerated cartilage tissue.

CONCLUSION

Implantation of DFLP Scaffold and supplemented with ASCs or Secretome in animal model shows a positive effect for cartilage defect treatment towards a hyaline-like cartilage result by increasing collagen type II expression.

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