

Research Article

Evaluation of Polymorphonuclear Immune Cells in Biocompatibility Test DFLP Sponge Cartilage Scaffold, Adipose-Derived MSC and Secretome with Cartilage Injection Model

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ABSTRACT

Background: In recent years, Freeze-Dried Scaffold Bovine Cartilage has been widely used as an alternative therapy for joint cartilage defects. This study aims to determine the biocompatibility of scaffold without involving implantation which provides clinical reports as expected through the evaluation of post-implantation chondrocytes regeneration, biocompatibility markers of the scaffold, and biocompatibility of sponge cartilage scaffold involving cartilage defects New Zealand White Rabbit.

Methods: This experimental in-vivo study was conducted for four weeks. Rabbits were divided into 4 treatment groups: microfracture defect group with DFLP sponge cartilage scaffold (P1) implantation; Microfracture defect group with DFLP sponge cartilage scaffold-secretome implantation (P2); Microfracture defect group with DFLP sponge cartilage scaffold-adipose derived Mesenchymal Stem Cells (ADMSCs) (P3); Microfracture defect group without implantation (control). The evaluations of basophil, eosinophil, neutrophil, and polymorphonuclear (PMN) cells were done in the first 24 hours, 3 days, and 1 week after the treatment. The collected data will be analyzed statistically.

Results: Research observations performed three times in the first, third, and seventh days. The results showed a small number of average Neutrophil (Neutrophil granulated) and PMN (segmented Neutrophils) cells both in the P2 and P3 groups compared with the control and the P1 group.

Conclusion: In general, biocompatibility is not included on the cytotoxic effects including inflammatory reactions and post-cartilage scaffold sponge implantation (DFLP) with or without the addition of ADMSC and secretome in the white rabbit New Zealand cartilage defect associated with differences seen in eosinophils, basophils, neutrophils, also total PMN cells in four groups.

Keywords: Scaffold; Cartilage joint; ADMSCs; Secretome; Human and Medicine

INTRODUCTION

Hyaline cartilage is a joint cartilage and is a structure that lines the diarthrodial joint in the joint. The main functions of cartilage are to promote the transmission of loads with the minimum coefficient of friction and to provide a smooth and lubricated surface for the joints.^{1,2} A small prevalent chondral lesion even <1cm

lesion can increase the incidence of cartilage damage in other subregions of the knee joint and new cartilage damage in another subregion of the same knee joint can also be found. The main goal of therapy in joint hyaline cartilage injury is to get strong biomechanics as they originate. From the various therapeutic options available, which is often used and give quite good results

are Autologous Chondrocyte Implantation (ACI) and Microfracture.^{3,4}

This rapidly developing technique of using a scaffold has become an alternative therapy for joint cartilage defects. In tissue engineering, scaffold acts as a cover media for defects, proliferation, distribution, cellular differentiation and can integrate with host tissue. Prolonged contact between the receiving tissue and the implanted scaffold indicates the need for an excellent examination before the scaffold is clinically applied to humans. For more than 40 years, biocompatibility has been used as a term used in the biomaterial field to differentiate biomaterials that can be used clinically from other materials and to depict the performance of material after implantation.

However, *in vivo* research is still needed further research to prove whether this scaffold has desired clinical picture and shall not have the effect of implantation rejection, by evaluating the regeneration of chondrocytes after implantation and infection markers to determine the biocompatibility of the scaffold.⁵ A scaffold should have biocompatible properties without causing radical changes in the intensity and period of the optimal wound healing process when implanted in the human body. The inflammatory response is significantly influenced by the composition of biomaterials, surface charge and roughness, porosity and biodegradability.^{1,2}

Over the past 10 years, many studies have proven that polymorphonuclear neutrophils (PMNs) can release a variety of cytokines that show the role of PMN in the pathophysiological process of inflammation.

PMN is the most circulating blood leukocytes. Biocompatibility, where the body's immune reaction to foreign biomaterials given to the body is considered to be very important because scaffold that is not suitable and toxic to the body will cause a rejection response from the body characterized by inflammation and produce secondary fibrocartilage tissue which biomechanically will affect the quality of life patient.^{1,2,5}

METHODS

The design of this study was *in vivo* experiments. This study was conducted in our institution. In this research, DFLP sponge cartilage scaffold with adipose-derived Mesenchymal Stem Cells (ADMSCs) as well as secretome from New Zealand White Rabbits will be implanted on cartilages in the knee that were previously injured on the joint surface in the form of a defect of 4.5 mm² which is considered equivalent to defects of 6.75 cm² in humans to the subchondral.⁶

Rabbits were then divided into 4 treatment groups: (P1) Microfracture defect group with DFLP sponge cartilage scaffold implantation; (P2) Group of microfracture defects with implantation of DFLP sponge cartilage scaffold-secretome; (P3) Group of microfracture defects with implantation of DFLP sponge cartilage scaffold-adipose derived Mesenchymal Stem Cells (ADMSCs), and (Control) microfracture defect groups without implantation. The evaluations of basophil, eosinophil, neutrophil, and polymorphonuclear (PMN) cells were done in



the first 24 hours, 3 days, and 1 week after the treatment.

Preparation

Acclimatization for experimental animals was performed for 1 week in our institution. If the animals are sick, they will be exempt from this study. The study was undertaken experimentally at Institute Tropical Disease Center in our region. This study used 4 groups of rabbits. Each group would have defects in the right knee cartilage. Premedication using atropine sulfate 0.2 mg/kg and diazepam 1.0 mg/kg intramuscularly. Then proceed to use ketamine (Ketalar) at a dose of 20 mg/kg intramuscularly in the left quadriceps muscle (effect of 3-6 minutes), followed by maintenance of 10 mg/kg intramuscularly if there is any reaction from rabbits.

Isolation of Adipose-Derived Mesenchymal Stem Cells (ADMSCs)

Adipose tissue is acquired by biopsy needles or liposuction aspiration. Adipose samples are stored at room temperature for less than 24 hours before use. Adipose stem cells (ASCs) are isolated from adipose tissue by extensive tissue washing using phosphate-buffered saline (PBS) containing 5% penicillin/streptomycin (P/S). The sample with debris removal was enclosed in a culture plate with sterile tissue with 0.075% type 1 collagenase prepared in PBS containing 2% P/S for tissue digestion. Furthermore, adipose tissue is chopped utilizing 2 scalpels, then pipette sampling is carried up and down using a 25 ml or 50 ml pipette to promote digestion. The sample was incubated for 30

minutes at 37°C, 5% CO₂, then neutralized type 1 collagenase activity by adding 5 ml α -MEM containing 20% heat-inactivated fetal bovine serum (FBS) to the tissue sample. Subsequently, the pipette is sampled up and down to further disintegrate the adipose tissue. After disintegration, transmit the sample into a 50 ml tubes. Stromal Vascular Fractions (SVF) containing ASCs were obtained by centrifugation of the sample at 2000 rpm for 5 minutes.

Next, take a sample from the centrifuge and do a strong shake to fuse the cells and disrupt the pellet. This step achieves the process of separating stromal cells from primary adipocytes. After spinning, repeat the centrifugation step, aspirate the collagenase solution on the pellet without distracting the cell. This pellet was then re-suspended in 1 ml of lysis buffer then incubated in ice and washed with 20 ml PBS / 2% P/S followed by centrifugation at 2000 rpm in 5 minutes. The supernatant formed is aspirated and the cell pellet is re-suspended in 3 ml stromal medium (α -MEM, Mediatech, Hemdon, VA) plus 20% FBS, 1% L-glutamine (Mediatech), 1% penicillin/streptomycin (Mediatech), then cell suspension was filtered through a 70 μ m cell filter.

It is necessary to understand that FBS must be filtered first to support adipocyte proliferation and differentiation. Therefore, arrange the sample containing cells in a culture plate coated with lysine and incubate at 37°C, 5% CO₂. Inoculate cells in 12 plates for an amount of about 500mg of adipose tissue.



Cell Culture from Adipose-Derived Mesenchymal Stem Cells (ADMSCs)

Aspirate all media from the well within 72 hours after plating. Afterward, wash cells with warm PBS (1% antibiotic can be added to the solution). Pipette the solution over the cell layers several times to clear cells from tissue fragments and blood cells. Next, add the volume of the fresh stromal medium according to the capacity of the culture plate. Thus, these cells are subsequently maintained in a tissue culture incubator at 37°C with 5% CO₂. The medium is replaced every second day until it reaches 80-90% confluences. Consequently, it generates two choices, either harvesting cells or directly inducing adipocyte differentiation. To harvest ASCs, add (250-500 µl) sterile warm PBS to the well for 2 minutes, then replace the PBS with 500 µl of Trypsin/EDTA solution (0.5%). Incubate in the incubator for 5-10 minutes, then verify with a microscope that 90% of the cells have separated and added 500 µl stromal medium to allow the serum contained in the solution to neutralize the trypsin reaction.

Next, transmit the media containing the cell suspension from the well to a 2 ml sterile tube, centrifugation at 1200 rpm for 5 minutes. Supernatant aspiration formed and the cell suspension in 250 µl stromal medium. Cell counting was done by taking cell aliquots diluted in trypan blue (for 1:8 dilution, add 12.5 µl cell suspension to 87.5 µl trypan blue). Cells are counted using a hemocytometer, then replace according to the culture plate cell capacity. When the cells are taken from frozen vials, cells should be immediately thawed at 37°C and arranged in 28 cm² or 175 cm² plates

complete with the stromal medium. The medium is replaced one day after and every second day.

Sponge Synthesis

The bovine scaffold sponge material is taken from the head femur and condyle femur of Ongole cattle at least 24 months old provided by Pegirian Animal Abattoir which has been certified as healthy. Cartilage is separated from the bone using knabel (bone rongeur). The cartilage that has been separated from the bones is washed with 0.9% NaCl solution or aquadest until clean and then processed mashed into flour. The cartilage that has been processed in the form of flour is then mixed with distilled water or 0.9% NaCl with cartilage: aquadest or 0.9% NaCl flour ratio 1: 1 then put into a sponge mold with a diameter of 4.5mm first. The mixture of cartilage flour and distilled water for producing mold is then be frozen in a freezer machine with a temperature of -800°C for at least 24 hours with deep-frozen technique. After being frozen, then dried using the sublimation technique by freeze-dried using the freeze-dried machine. After drying, the cartilage sponge is reprinted with a three-dimensional sponge measuring 5mm in diameter. The decellularized sponge bovine cartilage scaffold (DSBCS) group performed physical freeze-thaw (chemical freeze-thaw) and sodium dodecyl sulfate (SDS) for 72 hours.⁶

Preparation of the Articular Injury Model

Rabbits were premedicated using atropine sulfate 0.2 mg/kg and diazepam 1.0 mg/kg intramuscularly. Then proceed to use ketamine



(Ketalar) at a dose of 20 mg/kg intramuscularly in the left area of the quadriceps muscle (effect about 3-6 minutes), followed by maintenance of 10 mg/kg intramuscularly every time there is a reaction from rabbits. Rabbits have fasted for 6 hours postoperatively. Rabbit's hind limbs are prepared by shaving rabbit hair using a razor to a distance of 5cm from the surgical area.

The area to be treated as an injury model is on the trochlea femur. A parapatellar approach was done in the medial incision. A full-thickness lesion was performed on the articular cartilage of 4.5cm². Each rabbit was given treatment according to the group. In the group that was implanted with the scaffold, fibrin glue was given to fix the scaffold.⁶

RESULTS

Rabbits were divided into 4 treatment groups namely microfracture defect group with DFLP sponge cartilage scaffold (P1) implantation; Microfracture defect group with DFLP sponge cartilage scaffold-secretome implantation (P2); Microfracture defect group with DFLP sponge cartilage scaffold-adipose derived Mesenchymal Stem Cells (ADMSCs) (P3); Microfracture defect group without implantation (Control). Evaluation is done in the first 24 hours, 3 days, and 1 week after the treatment.

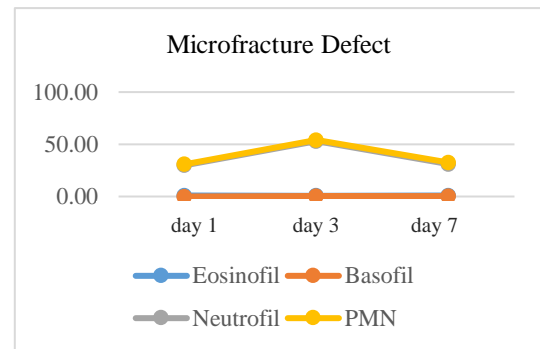


Figure 1. Development of the average number of cells observed in the microfracture defect treatment group in three-time observations.

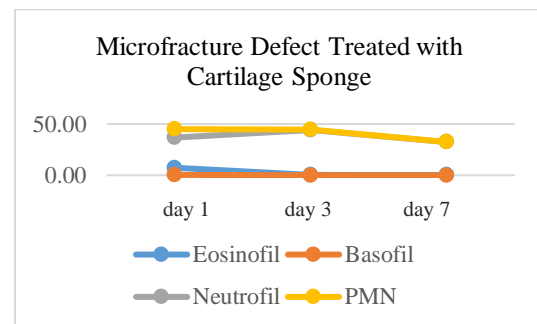


Figure 2. Development of the average number of cells observed in the microfracture defect treatment group with cartilage sponge in three-time observations

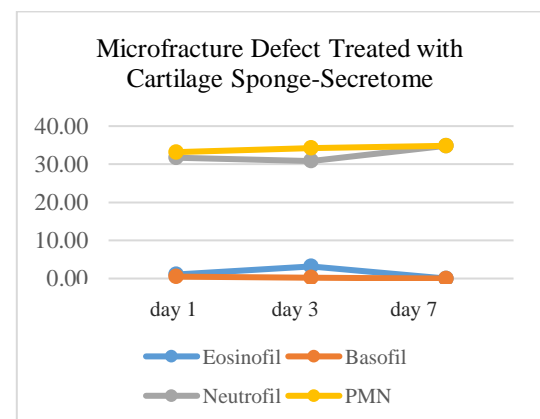


Figure 3. Development of the average number of cells observed in the microfracture defect treatment group with cartilage sponge-secretome in three-time observations

Table 1. Mean \pm Standard Deviation of Observation Results of Total Eosinophil, Basophil, Neutrophil and PMN on the First Day

Type of test	Microfracture Defect (cell/ field of view)	Microfracture Defect Treated with Cartilage Sponge (cell/ field of view)	Microfracture Defect Treated with Cartilage Sponge-Secretome (cell/ field of view)	Microfracture Defect Treated with Cartilage Sponge-ADMSC (cell/ field of view)
Eosinophil	1.00 \pm 0.63	7.33 \pm 7.68	1.00 \pm 2.00	6.67 \pm 5.35
Basophil	0 \pm 0	0.33 \pm 0.51	0.50 \pm 0.83	1.33 \pm 2.16
Neutrophil	29.83 \pm 17.62	37 \pm 21.62	31.67 \pm 11.07	36.33 \pm 16.28
PMN	30.83 \pm 17.56	45 \pm 21.53	33.17 \pm 12.15	44.33 \pm 13.63

Table 2. Mean \pm Standard Deviation of Observation Results of Total Eosinophil, Basophil, Neutrophil and PMN on the Third Day

Type of test	Microfracture Defect (cell/ field of view)	Microfracture Defect Treated with Cartilage Sponge (cell/ field of view)	Microfracture Defect Treated with Cartilage Sponge-Secretome (cell/ field of view)	Microfracture Defect Treated with Cartilage Sponge-ADMSC (cell/ field of view)
Eosinophil	0.67 \pm 0.81	0.5 \pm 0.54	3.17 \pm 6.79	1.67 \pm 3.14
Basophil	0.50 \pm 0.54	0 \pm 0	0.17 \pm 0.40	0.17 \pm 0.40
Neutrophil	53 \pm 17.14	44.17 \pm 10.59	30.83 \pm 7.85	47.33 \pm 11.39
PMN	54.17 \pm 17.41	44.67 \pm 10.46	34.17 \pm 8.35	49.17 \pm 11.08

Table 3. Mean \pm Standard Deviation of Observation Results of Total Eosinophil, Basophil, Neutrophil and PMN on the Seventh Day

Type of test	Microfracture Defect (cell/ field of view)	Microfracture Defect Treated with Cartilage Sponge (cell/ field of view)	Microfracture Defect Treated with Cartilage Sponge-Secretome (cell/ field of view)	Microfracture Defect Treated with Cartilage Sponge-ADMSC (cell/ field of view)
Eosinophil	1 \pm 2.44	0.17 \pm 0.40	0 \pm 0	0.5 \pm 1.22
Basophil	0.17 \pm 0.40	0 \pm 0	0 \pm 0	0.17 \pm 0.40
Neutrophil	31.17 \pm 18.38	32.33 \pm 11.99	34.83 \pm 9.96	40.5 \pm 3.01
PMN	32.33 \pm 18.86	32.5 \pm 12.14	34.83 \pm 9.96	41.17 \pm 3.86

DISCUSSION

Our study found that there were no significant differences in the average total number of PMNs between the Microfracture Defect groups only, Microfracture Defects with Cartilage

Sponge, Microfracture Defects with Cartilage Sponge-Secretome, and Microfracture Defects with Cartilage Sponge-ADMSC. On the observations of the first day, the third day, and the seventh day did not demonstrate any



significant difference in the average number of PMNs in each group. This shows that compared to the control group, the use of cartilage sponge, secretome, and ADMSC did not provide a body rejection reaction. The body's rejection reaction marked by an increasing number of PMNs because the mechanism of PMN calling by chemical mediators increases during rejection.

Statistically, the microfracture defect treatment group with cartilage scaffold (DFLP)-secretome and the microfracture defect treatment group with cartilage scaffold (DFLP)-ADMSC show that the average number is lower compared to the difference in the average number in the microfracture defect treatment group and microfracture defect with the cartilage scaffold (DFLP) is not significant. However, based on the observation, the microfracture defect treatment group with cartilage scaffold (DFLP)-secretome and the microfracture defect treatment group with cartilage scaffold (DFLP) and ADMSC can suppress the inflammatory reaction.

The eosinophil amount in the microfracture defect treatment group with cartilage scaffold (DFLP)-secretome was lower than the other two treatment groups: microfracture defect group with cartilage sponge and microfracture defect group with cartilage sponge-ADMSC. In the eosinophil activation process, these components deliver and synthesize a large number of active mediators biologically that each individual has a positive or negative potential effect on various target cells. Eosinophils act as guardians of the surrounding environment, quickly understanding tissue damage and starting to

activate biochemical reactions to trigger the inflammation or repair process.⁷

During the acute phase of infection, IL-1 stimulates the release of cytokines and stimulates bone marrow resulting in neutrophilia, eosinopenia, and lymphopenia. Neutrophil segments are released earlier than stem cells, but if the release increases then the stem cells are released and increased followed by other young cells such as metamyelocytes, myelocytes, promyelocytes, and myeloblasts. The increasing of young cells also occurs due to the temporary cessation of neutrophil cells maturation by TNF- α mediators so that young neutrophil cells become numerous and can even be an absolute increase in neutrophil young cells.⁸

Although many studies mention that scaffold is a biomaterial that can be used in tissue engineering applications, some studies also mention the ineffectiveness of scaffold in tissue regeneration and the potential for healing in the scaffold is doubtful because it has low biodegradability and limited osteoconductivity (in the bone healing process) and the presence of fiber-binding tissue, inflammatory cells, and not new tissue formation but scaffold remains. However, several studies also mention that scaffold augmentation by adding biodegradable and biocompatible biomaterials such as gels and by increasing growth factors on platelets can increase the regeneration in bone defects.⁹⁻¹¹

CONCLUSION

There is no cytotoxicity effect in the form of inflammatory or allergic reactions after implantation of DFLP sponge Cartilage



Scaffold (DFLP) with or without the addition of ADMSC and Secretome on New Zealand white rabbit cartilage defects marked by no significant increase in observations of eosinophil, basophil, neutrophils, or total PMNs in all four treatment groups. Future studies are expected to be able to find out the results of regenerating sponge cartilage scaffold (DFLP) with or without the addition of ADMSC and secretome implanted on experimental animals so that the results of these studies can be used as a research foundation on human subjects with cartilage defects which are often found in the field of orthopedic surgery.

REFERENCES

1. Sophia Fox AJ, Bedi A, Rodeo SA. The basic science of articular cartilage: Structure, composition, and function. *Sports Health*. 2009;1(6):461–8.
2. Fritz J, Janssen P, Gaissmaier C, Schewe B, Weise K. Articular cartilage defects in the knee-Basics, therapies, and results. *Injury*. 2008;39(1 SUPPL.):50–7.
3. Mistry H, Connock M, Pink J, Shyangdan D, Clar C, Royle P, et al. Autologous chondrocyte implantation in the knee: Systematic review and economic evaluation. *Health Technol Assess*; 2017.
4. Van Arkel R, Amis A. (i) Basics of orthopaedic biomechanics. *Orthopedics Trauma*. 2013;27(2):67–75.
5. Taipaleenmäki H. Factors Regulating Chondrogenic Differentiation. *Annales Universitatis Turkuensis*; 2010.
6. Utomo DN, Mahyudin F, Wardhana TH, Purwati P, Brahmana F, Gusti AWR. Physicobiochemical characteristics and chondrogenic differentiation of bone marrow mesenchymal stem cells (hbm-mscs) in biodegradable porous sponge bovine cartilage scaffold. *Int J Biomater*. 2019;2019.
7. Varricchi G, Senna G, Loffredo S, Bagnasco D, Ferrando M, Canonica GW. Reslizumab and eosinophilic asthma: One step closer to precision medicine? *Front Immunol*. 2017;8:242.
8. Krueger JM, Majde JA, Rector DM. Cytokines in immune function and sleep regulation. *Handb Clin Neurol*. 2011;98:229-40.
9. Oryan A, Sahviah S. Effectiveness of chitosan scaffold in skin, bone and cartilage healing. *Int J Biol Macromol*. 2017;104(Pt A):1003-11.
10. Nakamuta Y, Todo M, Arahira T. Improvement of Collagen Gel/Sponge Composite Scaffold by Gel Wrapping for Cartilage Tissue Engineering. *Int J Biosci Biochem Bioinforma Title*. 2017;7(2):102–9.
11. Steward AJ, Liu Y, Wagner DR. Engineering cell attachments to scaffolds in cartilage tissue engineering. *J Miner Met Mater Soc*. 2011;63(4):74–82.

