Biocompatibility Test of Decellularized Cartilage Bovine Scaffold in Vitro and In Vivo

Achmad Fachrizal[1], Dwikora Novembri Utomo[1]

[1]Department of Orthopedics and Traumatology, Faculty of Medicine, Universitas Airlangga, Surabaya (60131), Indonesia

Abstract

Background: Incidence of cartilage defects was reported at 65.00% of the routine arthroscopy procedures. Dr.Soetomo Teaching Hospital Network Bank developed a cartilage scaffold from bovine that has been deselularized decellularized cartilage bovine scaffold (DCBS). It takes a study of biocompatibility test to prove that DCBS is not cytotoxic to cells and not generate a host response.

Objective: To analyze the biocompatibility of decellularized cartilage bovine scaffold (DCBS) in vivo and in vitro.

Methods and Materials: The subjects were experimental rats and experimental with in vitro and in vivo trials. The subjects were treated by toxicity test with MTT assay, irritation test using Draize Scale, acute/ pyogenic systemic toxicity test by observing changes in body weight and temperature, and implantation test by observing fibrous capsule formation and Immunoglobulin G. Data was analyzed by using Kolmogorov-smirnov, Independent T-test, Mutivariate Analysis of Variance (MANOVA), and Mann Whitney.

Result: The percentage of viable DCBS (81.78%) and CBS (92.45%) was ≥70% of the control; thus, it is non-toxic to the cell. Draize scale in each group was in grade 0. Changes in body weight ($p = 0.981$) and temperature ($p > 0.05$) had no significant association between groups. There was a significant difference mean of the fibrous capsule ($p = 0.000$) thickness and no significant difference in the mean number of Immunoglobulin G which was formed at week 1 ($p = 0.87$) and week 4 ($p = 0.63$) after implantation between CCBS and DCBS.

Conclusion: The biocompatibility test shows that DCBS is compatible as a biomaterial.

Keywords: *DCBS, biocompatibility, In vivo, In vitro.*

Introduction

Articular cartilage is a structure that coats the diarthrodial joint and serves to protect the subchondral bone from the axial force loading [1]. Damage to the articular cartilage, either due to trauma, degenerative, or due to congenital abnormalities causes joint pain and decreased quality of life [2]. Incidence of cartilage defects was reported at 65.00% of routine arthroscopy procedures. If lesions in the cartilage are not treated, it will cause joint pain, joint dysfunction and osteoarthritis (OA). Currently, there is no single effective therapy for osteoarthritis treatment [3].

Current treatment options are bone marrow stimulation (microfracture), osteochondral autograft transfer system (OATS) or mosaicplasty, and autologous chondrocyte transplantation (ACT) [1]. All of the above therapies do not produce enough hyaline cartilage tissue; thus, it continues on tissue engineering to develop strategies for cartilage repair and regeneration with biomechanical characteristics, biological compositions,

Corresponding Author:
Dwikora Novembri Utomo
Department of Orthopedics and Traumatology, Faculty of Medicine, Universitas Airlangga, Surabaya (60131), Indonesia E-mail: novembridwikora@gmail.com,
Phone: (+6231) 5020251

and the same organization as the original articular cartilage. Trias engineering network consists of 3 components, namely cell, signal, and scaffold [4].

Scaffold serves as a medium for closing defects and cell regeneration media [5]. The cells used are mesenchymal stem cells. In vitro research proved that mesenchymal stem cell on the appropriate media (scaffold) can regenerate and decellularized biomaterial scaffold limited the use of growth factor resulting in better cost and resource efficiency [1]. Dr.Soetomo Teaching Hospital Network Bank developed a cartilage scaffold from bovine that has been deselularized (decellularized cartilage bovine scaffold (DCBS). Based on the deselulerization process, it is expected that cellular components that can induce an immune response can be eliminated and provided scaffold construction that may allow penetration of cultured cells [6]. Therefore, we need to conduct a study of biocompatibility test to prove that the DCBS is not cytotoxic to the cell and not cause response from the host.

Methods

This study used an experimental study that was conducted in 3 months (November 2016-January 2017). Before being tested, replication was conducted by generating a large sample of 9 experimental rats in 1 group. In the in vitro test, samples of mesenchimal stem cells were divided into three groups, namely, a group of DCBS Sodium Dodecyl Sulfate (RSDS) products, a group cultured with Cartilage Bovine Scaffold (CBS), and a group cultured with media (control group). All three groups were tested by its viability using MTT Assay. In vivo trials, the experimental rats were divided into three groups: the implanted group of DCBS RSDS products, CBS implanted group, and placebo implanted group. Biocompatibility test results included irritation test, acute systemic & pyrogenicity toxicity test, and implantation test.

The treatment stage was toxicity test with MTT assay (in vitro), irritation test (in vivo), acute/pyogenic toxicity test (in vivo), and implantation test (in vivo). In the cultured bone marrow mesenchimal stem cells (BM-MSCs), cytotoxicity test was added DCBS+MTT reagent on culture plate. Then, it was incubated within 48 hours at 37° C in an incubator containing 5% of CO2. After that, cell viability was quantitatively calculated based on the color change of the formazan. The tested material was not assessed by cytotoxic if the percentage of living cells was ≥70% compared to the control group.

The irritation test (in vivo) was performed by intracutaneous injection of a 0.2 ml scaffold extract on the back skin of experimental animals that had been shaved at 5 sites. Evaluation was conducted within 24 hours, 48 hours, 72 hours using Draize Scale on a scale of 0 (no erythema/edema) to 4 (severe erythema/edema).

Acute/pyrogenic toxicity test (in vivo) was performed by a 1x1x10 mm scaffold sample. This process subjects (experimental animals) were performed by intramuscular implantation. Thirty minutes before implantation was conducted rectal animal temperature measurement try. If there was a difference of 1° C from basal temperature or temperature over 39.8° C, the experimental animal was excluded from 61 toxicity tests. The evaluation was performed after implantation of 72 hours through weight and rectal temperature.

At the time of sample implantation (1x1x10 mm scaffold) inserted by injection with needle no. 16, the subjects were observed periodically at week 1 and week 4. The subjects were observed at the macroscopic implantation site (observation on fibrous capsule formation around the implant) and microscopic [7]. Reactive materials can form capsules of 2-4 mm while non-reactive materials can form invisible capsules. For tissue sampling, the rats were tried to be terminated in every interval of examination. Then, tissue samples along with the implants were taken and inserted in a 10% formalin solution [8].

The data recorded were grouped, analyzed, and conducted normality test by using Kolmogorov-smirnov test, homogeneous data with Mutivariate Analysis of Variance (MANOVA) test, Independent T-test and Mann Whitney with SPSS program (SPSS, Inc., Chicago, IL).

Result

In acute systemic and pirogenisity toxicity test, subjects were performed weight measurements before and 72 hours after implantation. Average weight change was 9.67 gr, 9.33 gr, and 8.78 gr for DCBS, CBS, and control group respectively. There was no significant weight difference in all groups ($p = 0.981$) (Table 1).

Table 1. One Way Anova Acute Toxicity Test (weight change)

Group	Mean±SD body weight change	One Way ANOVA (p-value)
DCBS	9.67±14.07	0.981
CBS	9.33±7.39	
Control	8.67±10.97	

The subjects were also performed rat temperature measurements before implantation, 30 minutes, 60 minutes, 90 minutes, 120 minutes, 150 minutes, and 180 minutes' post implantation. The statistical test results showed no significant difference from the mean of temperature change in all groups (p> 0.05) (Table 2).

Table 3. One Way Anova Temperature Change Test (pyrogensity test)

Group	One Way ANOVA (p-value)					
	30 minutes	60 minutes	90 minutes	120 minutes	150 minutes	180 minutes
DCBS	0.502	0.511	0.723	0.418	0.410	0.650
CBS						
Control						

In the implantation test, the average fiber fibrous caps were obtained at 1 week post implantation of 16.52 µ and 8.92 µ in Cellularized Cartilage Bovine Scaffold (CCBS) and DCBS. At week 4 post implantation, the average thickness of the fibrous capsule was 10.47 µ (CCBS) and 2.93 µ (DCBS). There was a significant difference mean of fibrous capsule thickness at 1 and 4 weeks post implantation between CCBS and DCBS (p = 0.000) (Table 3).

Table 3. Independent T-test of fibrous capsule thickness at week 1 and 4 post implantation

Group	Mean±SD body weight change		Independent T-Test (p-value)	
	Week 1	Week 4	Week 1	Week 4
CCBS	16.52±2.88	10.47±2.81	0.000	0.000
DCBS	8.92±2.38	2.93±0.84		

Immunoglobulin G was performed on Immunoglobulin G test. The mean of Immunoglobulin G was formed at 1 week post implantation of 0.05 (CCBS) and 0.03 (DCBS). At week 4 after implantation, the average number of Immunoglobulin G in CCBS group was equal to DCBS of 0.04. There was no significant difference in mean Immunoglobulin G number at week 1 (p = 0.87) and week-4 (p = 0.63) after implantation between CCBS and DCBS (Table 4).

Table 4. Mann-Whitney U Test of the number of Immunoglobulin G at week 1 and week 4 post implantation

Group	Mean±SD Body weight change		Independent T Test (p-value)	
	Week 1	Week 4	Week 1	Week 4
CCBS	0.05±0.10	0.04±0.05	0.87	0.63
DCBS	0.03±0.05	0.04±0.05		

Discussion

The cytotoxicity test shows a viable percentage of 70% of the control. The irritation test with Draize scale in each group is in grade 0. The acute toxicity/pyrocillicity test states that changes in body weight and temperature have no significant relationship between groups. The biocompatibility test that has been studied in previous studies on CBS showed that scaffolds have low toxicity levels in vitro and in vivo [9]. It shows in vitro DCBS and CBS non-toxic to cells, according to the standard MTT assay that non-toxic in cytotoxicity test in vitro when the percentage of cells viable ≥70% of untreated controls [7].

The percentage of cell viability obtained in the DCBS group was fewer than the CBS group. This is probably because the SDS concentration used in this study is 5%, in contrast to previous studies where SDS used was 2% [10]. In another study, it was using 5% SDS, it also obtained a good microscopic deselularization result but because SDS is cytotoxic, it is necessary to conduct additional leaching which takes longer time with Phospat Buffered Salinese (PBS) for 4 days. However, this washing extension has an adverse effect on Extra Cellular Matrix (ECM) cartilage, which causes ECM to appear broken between the second and third days [11].

Irritation test in DCBS, CBS, and control group showed no irritation reaction in the form of edema and erythema in all three groups (draize score in all three groups were in grade 0). This shows that DCBS is a non-irritant material according to grading at ISO [3]. Acute systemic toxicity and pyrogenicity test showed that DCBS, CBD. The control did not result in acute systemic toxicity and pyrogenicity reactions. This can be seen from the results of clinical observations of changes in body weight and rectal temperature of rats tested for 72 hours. There was no significant change in body weight and temperature between the 2 groups and the controls [11].

In this study, the evaluated implantation test was the fibrous and immunoglobulin tissue that was formed. In the evaluation of fibrous tissue of week 1 and week 4 showed that in the DCBS group, there was a better implantation test compared to CCBS which was characterized by smaller fibrous tissue formed in DCBS group although macroscopically both groups did not show any tissue reaction the formation of fibrous tissue in both groups both first week and second week does not exceed 2-4 mm. Previous studies comparing autologous ECM, allogenic ECM, xenogenic bovine collagen sponge (BCS), and synthetic scaffold (PLGA) implanted in rats showed that in the first week there was no fibrous tissue surrounding the autologous and allogenic ECM whereas in the BCS and PLGA groups formed the fibrous tissue that surrounds it [12].

The evaluation of the first week and the fourth week showed that the formation of Immunoglobulin G in CBS and DCBS groups. Despite the formation of Immunoglobulin G,statistically there is no significant difference. This suggests that CBS and DCBS do not generate a significant immune response but in the first week the number of Immunoglobulin G formed in the DCBS group was less than that of the CBS group.

The decellularization process is crucial in the elimination of cellular and antigenic components of tissue with the aim of preventing the transmission of the disease, reducing the immune response and inflammation, particularly in the xenogenic or allogenic donor tissue [13]. Unlike cellular materials, ECM components are commonly found in all species. Therefore, this is tolerable enough when it is used as an allograft or xenograft [1]. It can be seen that the DCBS used as xenograft did not result in a difference in the number of Immunoglobulin G depicting the absence of a meaningful immune response.

The previous studies of the biocompatibility of freeze-dried bovine cartilage in vivo in rabbits, it showed

that the biomaterials were compatible and no significant immune response was obtained [9]. Previous studies that examined the immunogenicity of chondrocytes and meniscus bovine and leporine also showed no immune response formed when chondrocytes and meniscus bovine and leporine were implanted in leporine [14]. It is also seen that CBS and DCBS used as xenograft are also compatible and not stimulate the immune response. This study was comparing autologous ECM, allogenic ECM, xenogenic bovine collagen sponge (BCS), and synthetic scaffold (PLGA) implanted in mice, it showed that in the first week no macrophages were formed in all four groups [15].

However, the lowest was found in the autologous ECM group with the number of PLGA macrophages was equal to the number in the BCS group, but significantly larger than the allogenic ECM group. Besides that, this study also investigated the cytokines formed represented by interleukin-10 (Il-10), interleukin-2 (Il-2), interleukin-4 (Il-4), and TNF-α in the first week after implantation. From this study, it was found that Il-10 expression was lower in autologous ECM group than allogenic ECM, BCS, and PLGA. There was no significant difference in TNF-α transcription between the four groups although the number of TNF-α formed was lower in autologous ECM than in the other three groups. No Il-2 and Il-4 expressions were formed. It was concluded that autologous ECM stimulated the minimal cytokine against host response modulation [3]. It is also seen in this study that the host response formed evaluated through Immunoglobulin G in the DCBS group which was the ECM xenograft was very minimal.

Conclusion

The cytotoxicity test shows a viable percentage of 70% of the control. The irritation test with Draize scale in each group is in grade 0. The acute toxicity/ pyrocillicity test states that changes in body weight and temperature have no significant relationship between groups. On the other hand, implantation test states that there is significant difference mean of fibrous capsule thickness and no significant difference mean of the amount of Immunoglobulin G which is formed at week 1 and week 4 post implantation between groups.

Ethical Clearance

This research involves animals in the process using a questionnaire that was accordant with the ethical research principle based on the regulation of research ethic regulation. The present study was carried out in accordance with the research principles. This study implemented the basic principle ethics of respect, beneficence, non-maleficence, and justice.

Conflict of Interest : Authors guarantee that there is no conflict of interest of this paper in the future

Source of Funding : All of expenses for this research process are paid by authors without any sponsorship

References

1. Mahyudin F, Utomo DN, Martanto TW, Hidayat AR, Putri LM. Effect of Decellularized Cartilage Bovine Scaffold and Hypoxic Condition on Stem Cell Differentiation to Chondrocyte: An In Vitro Study. In: Journal of Biomimetics, Biomaterials and Biomedical Engineering. Trans Tech Publ; 2018. p. 67–76.
2. Sari GM. THE EFFECT OF LONG TERM ADMINISTRATION OF GLUCOCORTICOID TO BONE LINING CELLS APOPTOSIS. Folia Medica Indones. 2017;52(4):251–7.
3. Castiglione F, Hedlund P, Weyne E, Hakim L, Montorsi F, Bivalacqua TJ, et al. Intratunical injection of human adipose tissue–derived stem cells restores collagen III/I ratio in a rat model of chronic Peyronie's disease. Sex Med. 2019;7(1):94–103.
4. Mentari IA, Naufalina R, Rahmadi M, Khotib J. Development Ischemic Stroke Model by Right Unilateral Common Carotid Artery Occlusion (RUCCAO) Method. Folia Medica Indones. 2018;54(3):200–6.
5. Gikas PD, Aston WJS, Briggs TWR. Autologous chondrocyte implantation: where do we stand now? J Orthop Sci. 2008;13(3):283–92.
6. Zilberman M. Active implants and scaffolds for tissue regeneration. Springer; 2011.
7. Nasution DAA, Artaria MD. Soft tissue markers thicknesses of batak toba for forensic facial approximation from skull. Int J Med Toxicol Leg Med. 2018;21(3and4):225–7.
8. Kusumaningsih T, Luthfi M, Moffan MDB. Macrophages Analysis on Gingival Tissue of Diabetic Rats after Insulin Leaf Extract Administration. J Int Dent Med Res. 2018;11(1):308–11.
9. Baskara MS, Utomo DN. Biokompatibilitas dari

bubuk freeze dried bovine cartilage dengan platelet rich plasma (PRP) sebagai scaffold untuk regenerasi kerusakan tulang rawan secara in vivo pada hewan kelinci.

10. Bertanha M, Moroz A, Jaldin RG, Silva RAM, Rinaldi JC, Golim MA, et al. Morphofunctional characterization of decellularized vena cava as tissue engineering scaffolds. Exp Cell Res. 2014;326(1):103–11.
11. Sutjiati E, Wirjatmadi B, Kalim H. Effect of Low Methionine Formula on Levels of IL-1β Serum and IL-1β Gene Expression in Knee Joint Cartilage Tissues of Normal Rabbits and ACL Induction OA Models. Indian J Public Heal Res Dev. 2018;9(10).
12. Lu H, Hoshiba T, Kawazoe N, Chen G. Autologous extracellular matrix scaffolds for tissue engineering. Biomaterials. 2011;32(10):2489–99.
13. Badylak SF, Taylor D, Uygun K. Whole-organ tissue engineering: decellularization and recellularization of three-dimensional matrix scaffolds. Annu Rev Biomed Eng. 2011;13:27–53.
14. Huey DJ, Sanchez-Adams J, Willard VP, Athanasiou KA. Immunogenicity of bovine and leporine articular chondrocytes and meniscus cells. Tissue Eng Part A. 2011;18(5–6):568–75.
15. Kartikasari N, Yuliati A, Listiana I, Setijanto D, Suardita K, Ariani D, et al. Characteristic of bovine hydroxyapatite-gelatin-chitosan scaffolds as biomaterial candidate for bone tissue engineering. In: 2016 IEEE EMBS Conference on Biomedical Engineering and Sciences (IECBES). IEEE; 2016. p. 623–6.