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THE EFFECT OF USING A BOVINE PERICARDIUM SCAFFOLD ON THE GROWTH AND DIFFERENTIATION OF ADIPOSE-DERIVED MESENCHYMAL STEM CELLS (ADMSC)

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Abstract

Introduction: Decellularized pericardium seeded with adipose-derived mesenchymal stem cells is being developed in cardiac surgery to overcome closure difficulties and reduce adhesion risks. This study aims to explain the role of bovine pericardium scaffold as a medium for growth, proliferation and differentiation of ADMSC. **Methods:** The research is a true experimental type of research using in vitro tests through the posttest-only control group design. Variables studied included viability, TGF-B, NO, calretinin, Wilms Tumor I (WT I), vimentin, and FSP1. **Results:** The average live cell has a diameter of 23.56 µm, while the average dead cell has a cell diameter of 10.96 µm. The scaffold decellularized with ASB-16 had anti-inflammatory activity as indicated by the presence of TGF-β (p= 0.000164) and Nitric Oxide (NO) (p= 0.033031). It was detected the presence of mesothelial progenitors as indicated by the presence of the WT1 marker (p= 0.000374). The presence of mesothelium is indicated by the presence of the Calretinin (p= 0.003). The presence of fibroblast progenitors as indicated by the presence of the FSP1 (p= 0.021910). Fibroblasts as indicated by the detectable Vimentin marker (p= 0.122889). **Conclusion:** The bovine pericardium scaffold can be used as a growth medium for ADMSC to be able to proliferate and differentiate into mesothelial as evidenced by the presence of WT1 and Calretinin markers, and into fibroblasts as evidenced by the presence of FSP1 and Vimentin markers.

Keywords: Bovine Pericardium, Scaffold, Growth, Differentiation, ADMSC.

INTRODUCTION

Heart surgery is one of the most frequently performed operations. In the field of congenital heart surgery, 67% of operations performed are recorded in the Society of Thoracic Surgery (STS) database[1]. In cardiac surgery, opening the pericardium is often necessary to access the heart and create an autologous patch.

This can result in difficult primary closure, increase the risk of intrapericardial adhesions, and increase treatment costs[2]. To face the problem of these limitations, tissue engineering is currently being developed using decellularized pericardium and seeded with stem cells. This is for the reason that stem cells have the potential to be developed because the cell number and proliferation properties are good.

The source of stem cells with the largest number of cells, good cell proliferation and easier tissue harvesting techniques is adipose-derived mesenchymal stem cells (ADMSC)[3].

This research develops tissue engineering technology in the field of cardiac surgery. Bovine pericardium scaffolds were made using ASB-16 decellularization, then seeded with ADMSC. This method has never been used before in pericardial research in the field of cardiac surgery. This study aims to explain the role of bovine pericardium scaffold as a medium for growth, proliferation and differentiation of ADMSC.

MATERIALS AND METHODS

Study Design, Sampling and Population

This research was a true experimental type of research that utilized in vitro tests through the posttest-only control group design. There were two groups in this study.

The first group consisted of ADMSCs in αMEM media, and the second group consisted of a scaffold that was decellularized with ASB-16 + ADMSCs in MEM α media. The sample size in this study was calculated using the "Resource Equation Approach" formula, and a total of 8 samples were obtained for each group.

Inclusion and Exclusion Criteria

Inclusion criteria were pericardium taken from beef cattle that have been certified as healthy by veterinarians at slaughterhouses, scaffolds that have been tested as non-toxic, and ADMSC cells that have gone through a preparation process that has been characterized.

Samples were excluded from this study if the scaffold was defective due to the decellularization process, toxicity tests, and ADMSC cells were not viable and could not be seeded on the scaffold.

Research Material and Method

Fat Tissue Sample Preparation

This is done by preparing a MEM α transport medium to carry samples in the form of fat tissue with a size of 1 cm3 and no connective tissue or blood clots.

Isolation of Mesenchymal Stem Cells From Fat Tissue

Fat tissue was excised then cleaned with PBS solution, chopped, and mixed with colagenase. After incubation on a hot plate, the homogeneous solution was poured into a 50 ml container with a filter, then centrifuged to form pellets.

Pellets were resuspended in MEM α medium and planted in petri dishes, incubated for 24 hours in CO2. The medium was changed every 2 days until the cells formed colonies with 80% confluent. This process ensures optimal cell growth for further research in a controlled culture environment.

Mesenchymal Stem Cell Multiplication

Cells that reached 80% confluency in the petri dish were rejuvenated by passage. The medium was removed, the monolayer was rinsed with PBS solution, then triple express enzyme was added and incubated until it separated from the petri base.

After that, a medium stopper is added, resuspension is carried out until it becomes a single cell. The single cell was centrifuged, forming a pellet which was then resuspended in MEM α medium.

The homogeneous solution is grown in a new petri dish to increase the number of cells, ensuring the dose required for treatment is achieved.

Viability Test

After reaching 80% confluence, cells were treated by washing with PBS solution to remove residual serum, debris, and dirt. Next, the cells are converted into single cells using the triple express enzyme.

After this process, the cells were resuspended to form a homogeneous solution and poured into a 15 mL conical tube. Through centrifugation for 5 minutes, a pellet was formed at the bottom of the tube, and the supernatant was discarded, leaving the pellet which was resuspended by adding culture medium.

A total of 10 µL of solution was taken, mixed with trypan blue dye, and the results were calculated using a cell counter to determine cell viability. Next, the calculation results and cell images appear on the screen after a while.

Scaffold Preparation

Washed with PBS 3 times to remove residue, then immersed in culture medium for 15 minutes.

Seeding stem cells on a scaffold

Stem cells that have reached 80% confluency and are in a healthy condition are converted into single cells with triple expression enzymes. After centrifugation, the cell pellet is mixed with the culture medium and poured into a plate containing the scaffold.

Incubation was carried out for 3 x 24 hours in a CO2 incubator, with medium changes every 3 x 24 hours. This process lasts for 7 days, causing morphological changes in the cells.

Harvesting Secretom

After planting the cells on the scaffold and incubating for 7 x 24 hours, evaluation was carried out for 3 x 24 hours. At this stage, the secretome from the cells is collected after changing the color of the medium.

The secretome was taken slowly with a micropipette, placed in a 2 ml tube, and stored in the refrigerator for further analysis. Next, MSCs were isolated from adipose cells and characterized with markers CD 105, CD 73, and CD 190.

The appropriate ADMSCs were cultured in DMEM medium with the addition of 10% FCS. Harvesting of ADMSC was carried out, followed by seeding of ADMSC that had been replicated in an incubator at optimal temperature, and administration of PBS and antibiotics to prevent contaminant infection.

Data Analysis

The data obtained was tabulated, then normality and homogeneity of variance tests were carried out. If the test results show a normal distribution, then continue with the parametric statistical test using the unpaired T test with a significance level of 95%. A P value of less than 0.05 was considered a statistically significant difference.

RESULTS

Cell Viability Test via Trypan Blue Staining and MTT Assay on Bovine Pericardium Scaffold as ADMSC Growth Media

Viability Test is used to see the growth of cells grown in the scaffold. From this test, 91% of the cells were alive. The average live cell has a diameter of 23.56 µm, while the average dead cell has a cell diameter of 10.96 µm.

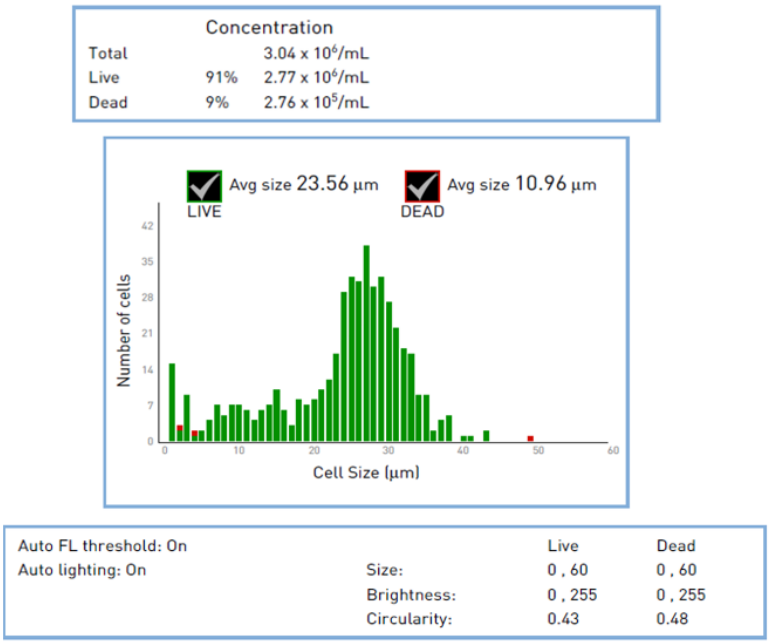

Figure 1: Viability Test

Anti-Inflammatory Activity Test on Bovine Pericardium Scaffold as ADMSC Growth Media

Marker TGF β

From the ELISA test, it was found that the scaffold decellularized with ASB-16 had anti-inflammatory activity as indicated by the detectable presence of TGF-β. The data was then tested for normality.

From this test, the data obtained were normally distributed (Sig. < 0.05), so the data was then processed using the Independent T-Test.

There was a significant difference (p-value: 0.000164) in the scaffold decellularized with ASB-16 compared to the control.

Table 1: Results of TGF β Detection on Scaffolds Decellularized with ASB-16 Compared to Controls

No	Control (ng/mL)	Mean ± SD	Treatment Group (ng/mL)	Mean ± SD	
1	17.483	19.66 ± 2.59	27.000	26.71 ± 2.93	t-value: -5.09132. p-value: 0.000164.
2	19.138		27.586		
3	19.724		29.772		
4	18.207		30.417		
5	24.241		27.117		
6	23.034		26.738		
7	18.103		21.807		
8	17.379		23.248		

Marker NO

Table 2: Results of NO Detection in Scaffolds Decellularized with ASB-16 Compared to Controls

No	Control (ng/mL)	Mean ± SD	Treatment Group (ng/mL)	Mean ± SD	
1	18.055		50.685		
2	25.313		63.666		
3	19.708		101.724		t-value : -2.36459. p-value : 0.033031.
4	22.658		46.555	49.03 ± 25.50	
5	13.512	26.07 ± 10.19	50.095		
6	37.999		30.624		
7	43.605		23.248		
8	27.673		25.608		

From the ELISA test, it was found that the scaffold decellularized with ASB-16 had anti-inflammatory activity as indicated by the presence of Nitric Oxide (NO) which was detected.

The data was then tested for normality. From this test, the data obtained were normally distributed (Sig. < 0.05), so the data was then processed using the Independent T-Test. There was a significant difference (p-value: 0.033031) in the scaffold decellularized with ASB-16 compared to the control.

WT1 Mesothelial Cell Progenitor Marker Test on Bovine Pericardium Scaffold as ADMSC Growth Media

Table 3: Results of WT1 Detection on Scaffolds Decellularized with ASB-16 Compared to Controls

No	Control (ng/mL)	Mean ± SD	Treatment Group (ng/mL)	Mean ± SD	
1	97.935		192.069		t-value : -4.65199. p-value : 0.000374.
2	156.766		199.484		
3	115.372		200.980		
4	105.669	127.92 ± 21.67	194.137	178.67 ± 21.96	
5	116.473		141.138		
6	147.701		156.317		
7	142.660		177.397		
8	140.762		167.834		

From the ELISA test, it was found that the scaffold decellularized with ASB-16 detected the presence of mesothelial progenitors as indicated by the presence of the WT1 marker which was detected. In this test, the WT1 marker on scaffolds that have been implanted with ADMSC is compared with the control, namely ADMSC cells only. The data was then tested for normality.

From this test, the data obtained were normally distributed (Sig. < 0.05), so the data was then processed using the Independent T-Test. There was a significant difference (p-value: 0.000374) in the scaffolds decellularized with ASB-16 compared to the control, where the scaffold group decellularized with ASB-16 expressed the WT1 marker to a greater extent than the control group.

Calretinin Mesothelial Cell Marker Test on Bovine Pericardium Scaffolds as ADMSC Growth Media

Table 4: Calretinin Detection Results on Scaffolds Decellularized with ASB-16 Compared to Controls

No	Control (ng/mL)	Mean ± SD	Treatment Group (ng/mL)	Mean ± SD	
1	118.430		398.860		
2	131.151		413.194		
3	116.711		326.360		
4	118.486	125.72 ± 13.19	356.842	125.72 ± 13.19	p-value : 0.003.
5	154.668		322.241		
6	118.430		326.494		
7	131.151		128.134		
8	116.711		140.168		

From the ELISA test, it was found that the scaffold decellularized with ASB-16 detected the presence of mesothelium as indicated by the presence of the Calretinin marker which was detected. In this test, the Calretinin marker on scaffolds that have been implanted with ADMSC is compared with the control, namely ADMSC cells only. The data was then tested for normality. From this test, it was found that the data was not normally distributed (Sig. < 0.05), so the data was then processed using Mann-Whitney. There was a significant difference (p-value: 0.003) in the scaffolds decellularized with ASB-16 compared to the control, where the scaffold group decellularized with ASB-16 expressed the marker Calretinin to a greater extent than the control group.

FSP1 Fibroblast Cell Progenitor Marker Test on Bovine Pericardium Scaffold as ADMSC Growth Media

Table 5: Results of FSP1 Detection on Scaffolds Decellularized with ASB-16 Compared to Controls

No	Control (ng/mL)	Mean ± SD	Treatment Group (ng/mL)	Mean ± SD	
1	3.227		7.523		t-value : 2.57763. p-value : 0.021910.
2	11.302		5.859		
3	8.346		6.698		
4	7.814	8.80 ± 3.33	4.437	5.57 ± 1.20	
5	9.912		5.416		
6	8.910		5.993		
7	14.464		4.660		
8	6.385		3.966		

From the ELISA test, it was found that the scaffold decellularized with ASB-16 detected the presence of fibroblast progenitors as indicated by the presence of the FSP1 marker which was detected. In this test, the FSP1 marker on scaffolds that have been implanted with ADMSC is compared with the control, namely ADMSC cells only. The data was then tested for normality.

From this test, the data obtained were normally distributed (Sig. < 0.05), so the data was then processed using the Independent T-Test. There was a significant difference (p-value: 0.021910) in the scaffolds decellularized with ASB-16 compared to the control where the control group expressed the FSP1 marker to a greater extent than the scaffold group decellularized with ASB-16.

Vimentin Fibroblast Cell Marker Test on Bovine Pericardium Scaffolds as ADMSC Growth Media

Table 6: Vimentin Detection Results on Scaffolds Decellularized with ASB-16 Compared to Controls

No	Control (ng/mL)	Mean ± SD	Treatment Group (ng/mL)	Mean ± SD	
1	5.447		8.398		t-value : -1.64183. p-value : 0.122889.
2	1.687		9.778		
3	2.830		8.921		
4	9.968	7.80 ± 4.81	9.445	10.87 ± 2.19	
5	13.205		13.157		
6	15.061		10.587		
7	5.209		12.110		
8	8.969		14.537		

From the ELISA test, it was found that the scaffold decellularized with ASB-16 detected fibroblasts as indicated by the detectable Vimentin marker. In this test, the Vimentin marker on scaffolds that have been implanted with ADMSC is compared with the control, namely ADMSC cells only. The data was then tested for normality. From this test, the data obtained were normally distributed (Sig. < 0.05), so the data was then processed using the Independent T-Test. There was a non-significant difference (p-value: 0.122889) in the scaffold decellularized with ASB-16 compared to the control.

DISCUSSION

This research aims to analyze the use of pericardial scaffolds as ADMSC growth media which can support cell growth, anti-inflammatory activity and cell differentiation.

Cell Viability Test via Trypan Blue Staining and MTT Assay on Bovine Pericardium Scaffold as ADMSC Growth Media

There were no statistical differences in cell viability or cytotoxicity between antigen-removed scaffolds (with ASB-14 or ASB-16) and native tissue.[4]

Anti-Inflammatory Activity Test on Bovine Pericardium Scaffold as ADMSC Growth Media

Marker TGF β

TGF-β plays an important role in the transformation of epithelial cells into mesenchymal cells (EMT). This transformation process is key in the regulation of embryonic cells, including the formation of heart cells, including coronary blood vessels, valves and pericardium. Apart from that, disease progression can also be influenced by the occurrence of EMT. Lack of TGF-β cells as an anti-inflammatory can cause problems in heart and pericardium cells, as well as during the healing process of post-operative cells. In this case, adequate TGF-β, which is indicated by the presence of BMP2 signals in epithelial cells, is very necessary in maintaining EMT integrity. Apart from the anti-inflammatory effect, TGF-β was also proven to be a pathway in modulating cardiogenesis in AMSc Brown seeded scaffolds. This cardiogenesis can be seen from increasing cardiomyocyte markers (cardiac troponin c and α-actinin).[5] In this study, TGF-β was significantly increased in bovine pericardium scaffolds decellularized with ASB-16 compared with controls. This supports ASB-16 being an optimal decellularization method because it increases the

expression of TGF-β as an anti-inflammatory component to reduce the risk of post-operative adhesions.

Furthermore, other evidence that a significant increase in TGF-β can help cell proliferation and tissue regeneration was also revealed in in vitro and in vivo studies. Huang et al. (2019)[6] on a hybrid scaffold that was seeded with synovium-derived mesenchymal stem cells.

Marker NO

Another anti-inflammatory that plays an important role in post-operative cell regeneration is NO. NO was expressed significantly more in bovine pericardium scaffolds decellularized with ASB-16 than in controls in this study. Even better, NO not only provides anti-inflammatory benefits and accelerates healing of damaged tissue, but also angiogenesis in organ transplants. This is proven by in vitro and in vivo studies on mesenchymal stem cells (MSCs) by Bandara et al. (2019).[7] In this study, NO was found to produce significant anti-inflammatory effects and generate angiogenesis in endothelial cells through the Wnt signaling pathway and β-catenin protein. Scaffold transplantation that had seeded MSCs which can produce NO was successful and significant and adequate angiogenesis occurred.[7] In another similar study, AMSc seeding was carried out on blood vessel scaffolds. This decellularization is given to human great saphenous vein allografts to remove foreign antigens and reduce tissue immunogenicity. Furthermore, this study not only provides a significant angiogenesis effect, but also shows a non-thrombogenic effect on the surface of the transplanted scaffold.[8] From the results of these studies, it can be concluded that ASB-16 decellularization is important to carry out on bovine pericardium scaffolds seeded with stem cells because it has been proven to produce significant amounts of NO so that it can help the pericardium regeneration process after surgery.

WT1 Mesothelial Cell Progenitor Marker Test on Bovine Pericardium Scaffold as ADMSC Growth Media

WT1 regulates the maintenance of mesothelial cell phenotypes during the development of certain mesodermal tissues. Analysis of WT1 function during normal development and homeostasis of adult tissue is a balance between epithelial and mesenchymal cell states. In studies with pleural mesothelial cells (PMC), loss of WT1 abrogated the PMC phenotype and showed evidence of mesothelial-to-mesenchymal transition.[9] This study showed that the expression of the mesothelial cell progenitor marker WT1 was significantly higher in bovine pericardial scaffolds decellularized with ASB-16 than in controls. Similar results were proven in studies of decellularized renal scaffolds.[10]

Calretinin Mesothelial Cell Marker Test on Bovine Pericardium Scaffolds as ADMSC Growth Media

The presence of calretinin protein in mesothelial cells is an important component in internal organ transplantation so that the risk of adhesions can be minimized and trigger cell proliferation. Moreover, as specific epithelial cells in internal organs and serosal cavities, mesothelial cells also have a protective function.[11,12] In in vitro research Konar et al. (2017),[12] decellularized scaffolds can express calretinin and trigger cell growth in vitro. In vivo, decellularized scaffolds were also shown to persistently express mesothelial cells via the calretinin gene.[11] Both studies support

the results of the present study showing that mesothelial cell expression of calretinin was higher in bovine pericardial scaffolds decellularized with ASB than controls.

FSP1 Fibroblast Cell Progenitor Marker Test on Bovine Pericardium Scaffold as ADMSC Growth Media

Fibroblasts synthesize an extracellular matrix consisting of type I and III collagen, fibronectin and proteoglycans. The gene encoding the filament-associated calcium-binding protein, fibroblast-specific protein 1 (FSP1), is highly specific for fibroblasts and is associated with the conversion of epithelial cells to a fibroblast phenotype. FSP1 is specifically expressed in fibroblasts but at very low levels in epithelium and MSCs.[13] Fibroblasts can continue to grow for 14 weeks, forming thick, strong multilayer cell sheets and secreting abundant ECM proteins and proteoglycans.[14] In this experiment, it was found that FSP1 expression on scaffolds decellularized with ASB-16 was lower than the control. This can be caused by the culture being carried out for less than 14 weeks, so that the fibroblasts have not grown optimally. In addition, the decellularization process may result in the depletion of key ECM proteins that can play a role in cell growth and differentiation.[13]

Vimentin Fibroblast Cell Marker Test on Bovine Pericardium Scaffolds as ADMSC Growth Media

Vimentin is a biomarker of epithelial to mesenchymal transition (EMT) and an intermediate filament protein that functions during cell migration to maintain structure and motility.[15] Vimentin has also been reported to be involved in cell migration by regulating actomyosin contraction forces, interactions with the extracellular matrix, and also in the ability of cells to move their nuclei forward. Vimentin-deficient cells also show defects in cell motility and directionality as well as reduced wound healing capacity.[16] Vimentin is normally located in the cytosol and is removed by decellularization. Immunohistochemical staining confirmed removal of vimentin from decellularized rhesus monkey kidney scaffolds. Meanwhile rhesus macaque scaffolds implanted with late second/early third trimester fetal kidney cells for 5 days showed two distinct phenotypes, either as dense clusters of cells positive for vimentin and cytokeratin, or as individual vimentin-positive/cytokeratin-negative cells. However, immunohistochemical analysis revealed only decellularization with that resulted in complete removal of the intracellular cytoskeletal protein, vimentin, compared with Triton X-100 or the organic solvent, tributyl phosphate.[10] In this study, scaffolds decellularized with ASB-16 and implanted with ADMSCs showed Vimentin marker activity. This study showed that Vimentin marker activity was higher than the control, but in an insignificant amount. This is possible because the planting process is only carried out 3x24 hours. In this study, it was concluded that the scaffold and control media had the same ability to accommodate fibroblast cell differentiation.

CONCLUSION

In summary, the bovine pericardium scaffold can be used as a growth medium for ADMSC to be able to proliferate and differentiate into mesothelial as evidenced by the presence of WT1 and Calretinin markers, and into fibroblasts as evidenced by the presence of FSP1 and Vimentin markers. The resulting scaffold that has been seeded using ADMSC can be used for tissue engineering.

Ethıcal Clearance

A certificate of ethical clearance (ethical clearance) was given by the Health Research Ethics Committee (KEPK) Dr. RSUD. Soetotomo Surabaya with certificate number 0306/KEPK/XI/2021.

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None.

Conflict of Interest

Nothing to declare.

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