



STUDY ON DECELLULARIZING PORCINE VESSEL FOR MAKING ARTIFICIAL VASCULAR GRAFT

Quan Minh To* and Ha Le Bao Tran

Department of Physiology and Animal Biotechnology, Faculty of Biology and Biotechnology, University of Science, Vietnam National University at Ho Chi Minh City, Vietnam.

***Author for Correspondence: Dr. Quan Minh To**

Department of Physiology and Animal Biotechnology, Faculty of Biology and Biotechnology, University of Science, Vietnam National University at Ho Chi Minh City, Vietnam.

Article Received on 04/02/2016

Article Revised on 25/02/2016

Article Accepted on 16/03/2016

ABSTRACT

In 2011, cardiovascular disease (CVD) still accounted for 31.3% of all deaths in America. One-third of the patients who need an arterial bypass procedure don't have suitable autologous vessels. Decellularization of allogeneic/xenogeneic vessels can bring new hope for patients. Porcine coronary arteries were bought from the market. They were treated with 1M NaOH for 6h or 0.1% Triton X100 for 24h or distilled water for 24h or 0.5% SDS for 24h or a combination of 0.5% SDS for 24 hours and distilled water for 24 hours. Decellularization efficiency was determined by HE staining and Trichrome staining. Acellular vascular grafts were disinfected by 0.15% glutaraldehyde for 1 hour. *In vitro* cytotoxicity of acellular vessels was tested by direct contact method according to ISO 10993-5. The umbilical cord blood-derived endothelial progenitor cells were seeded on lumen of acellular arterial pieces with concentration of 5×10^3 cells/piece and cultured for seven days. The growth of UCB EPC was tested by MTT assay, HE staining and SEM. Result: the combination of SDS 24h and distilled water 24h is the most efficient method in this study: cells were completely removed and extracellular matrix (ECM) was preserved. GA-treated acellular vessel was not toxic to fibroblast *in vitro*, according to ISO 10993-5. UCB-EPC grew on lumen of acellular arterial pieces from day 1 to day 7. Conclusion: decellularized vascular grafts were successfully made from porcine vessel by SDS and distilled water. GA can be used for disinfectant and not harmful *in vitro*. UCB EPC can grow on lumen of acellular vessels.

KEYWORDS: Decellularization, cardiovascular disease, porcine coronary artery, vascular tissue engineering, endothelial progenitor cell.

INTRODUCTION

In 2011, cardiovascular disease (CVD) still accounted for 31.3% (786,641) of all 2,515,458 deaths, about 1 of every 3 deaths in the United States. Coronary artery occlusion accounts for about 50% of deaths from cardiovascular diseases.^[12] In the early stage the fatty plaques are small, then they advances, occurs in many sites, many branches.^[15] In this case, bypass grafting surgery is the often preferred intervention. One or more autologous blood vessels (BV) such as saphenous vein, internal mammary artery, internal radial. are used to bypass the lesions.^[1,16] However, one-third of the patients didn't have adequate autologous BVs. Consequently, repeat surgery is often required within 10 years after bypass surgery.^[8]

Some synthetic materials such as Dacron, PTFE use as bypass grafts but poor clinical efficiency in small diameter vessel grafting (<6mm) is their drawback.^[13] Decellularization of BVs is considered as a new method for creating bypass grafts. Tissue or organ includes cells and extracellular matrix (ECM). ECM are products

secreted by resident cells in each tissue and organ such as collagen, hyaluronic acid, elastin.^[2] Cells are immunogenic potential which evoke immune response in xenogeneic/allogeneic transplants. Removing the cells from tissues/organs (decellularization) can reduce the immunogenicity. However, components of the ECM are generally homologous among species.^[3] Thus, ECM from xenogeneic/allogeneic can play a role as biological scaffolds for tissue engineering construct. Decellularized tissue/organ can overcome some current challenges in vascular tissue engineering such as: morphology, structure and structural, functional protein are similar to native tissue or organ^[1]. Its cost may be lower than other synthetic vascular grafts. Nowadays, many ECM grafts have been approved by FDA.^[3]

Characterization of decellularized tissue/organ depends on their structure, decellularization and disinfection/sterilization method. Removal of cells from the tissue/organ can alter component and structure of ECM. Some agents used for decellularizing cells such as: NaOH, Triton X100, distilled water, SDS (sodium

dodecyl sulfate). Each agent affects differently on ECM structure and cell removal.^[2] The most effective decellularization method is to remove all cells and to preserve structure, components of ECM. It usually includes a combination of many agents.^[3] Moreover, disinfection/sterilization of ECM scaffold is still a drawback because many recent disinfection/sterilization methods can disrupt ECM protein and harm patients.^[2] In this study, we researched on making acellular vascular grafts from porcine coronary arteries by decellularizing agents such as NaOH, Triton X100, distilled water, SDS and disinfectant as glutaraldehyde.

MATERIALS AND METHODS

Decellularization method

Coronary arteries (CAs) were obtained from healthy porcine in the market. They were transferred to laboratory in cool PBS (Phosphate Buffer Saline) containing Penicillin (400 U/ml), Streptomycin (0.4 mg/ml) and Amphotericin (1µg/ml). Muscle and fat tissue were removed in biological safe cabinet.

CAs were divided to 5 groups: (1) were treated with 1M NaOH for 6 hours, (2) were treated with distilled water for 24 hours, (3) were treated with 0,1% Triton X100 for 24 hours, (4) were treated with 0,5% SDS for 24 hours, (5) were treated with 0,5% SDS for 24 hours and distilled water for 24 hours. Decellularized CAs (DCAs) were stained with HE (Haematoxyline and Eosin) and Trichrome to determine the efficiency of cell removal and collagen preservation.

Disinfection

DCAs were immersed in 0.15% glutaraldehyde for 1 hour. Then, they were washed with distilled PBS for 24 hours. They were used for cytotoxicity test and EPC (Endothelial Progenitor Cell) seeding.

cytotoxicity Test

Cytotoxicity was conducted according to ISO 10993-5.^[18] DCAs were cut into 0.3x0.3 cm² pieces. Fibroblast was seeded on 4-well dishes with concentration of 3x10⁴ cells/well. After 1 day, the pieces were placed on top of cell layer, 6 pieces/6 wells. After 1 day, the pieces were removed. Cells were observed by microscope, stained with HE staining (3 wells/6 wells).

Cell viability was measured by MTT assay. MTT at concentration of 5 mg/ml, volumetric of 0.02 ml was added to medium and incubated for 3 hours. After 3

hours, MTT was discarded. DMSO/ethanol (0.2 ml) was added and incubated for 3 hours. Then, OD of solution was measured at wave length of 545 nm.

Growth of endothelial progenitor cell (EPC) on DCAs

EPCs from umbilical cord blood were obtained as previous study and Laura E. Mead.^[9] EPCs were cultured in EBM-2 medium supplemented with 5% FBS, incubated at 37°C, 5% CO₂. UCB EPCs at passage 4 were used to seed on lumen of DCAs.

DCAs were cut into many pieces of 0.4x0.4 cm². These pieces were placed in 4-well dish in way such that lumen is upward (3 pieces/well). EPCs were detached from flask surface by Trypsin/EDTA (0.25%:0.02%), centrifuged at 3000 rpm in 5 minutes and adjusted to 3x10⁵ cell/ml. EPCs were seeded on lumen (10 µl/piece), incubated at 37°C for 30 minutes. After 30 minutes, medium was added (200 µl/well) and cultured at 37°C, 5% CO₂. Medium was changed every 2 days.

MTT assay for EPCs growth

After cell culturing for 1, 3, 5, 7, 9 day periods, the proliferation of EPCs were measured by MTT assay. At one point of time, three sample pieces and three control pieces (pieces weren't seeded with EPCs) were tested by MTT assay. The EPCs-seeded pieces were transferred to 96-well dish (1 piece/well). Medium was added to well (0.1 ml/well). MTT (concentration of 5 mg/ml, volumetric of 10 µl) was added to well, incubated for 3h at 37°C, 5% CO₂. After 3h, DMSO/ethanol was added to solubilize MTT crystals and OD was measured at wave concentration of 545 nm. Moreover, EPCs growth was determined by HE staining and scan electron microscope (SEM).

RESULTS

Decellularization

After removing mussel and fat tissue, CAs were red, 6-7 cm length, 3-4 mm external diameter, 2-3 mm internal diameter (Fig. 1). CAs was divided to 3 layers: tunica adventia, tunica intermedia, tunica intima. The tunica intima is the thinnest layer consisting of one layer of endothelial cells lying on basement membrane and a subendothelial layer. The tunica intermedia is the thickest layer consisting of circularly arranged smooth muscle cells and collagen. The tunica adventia is the outer layer consisting of connective cells with low concentration (Fig.).



Figure 1. Porcine coronary artery. A: Native vessel. B: Decellularized vessel.

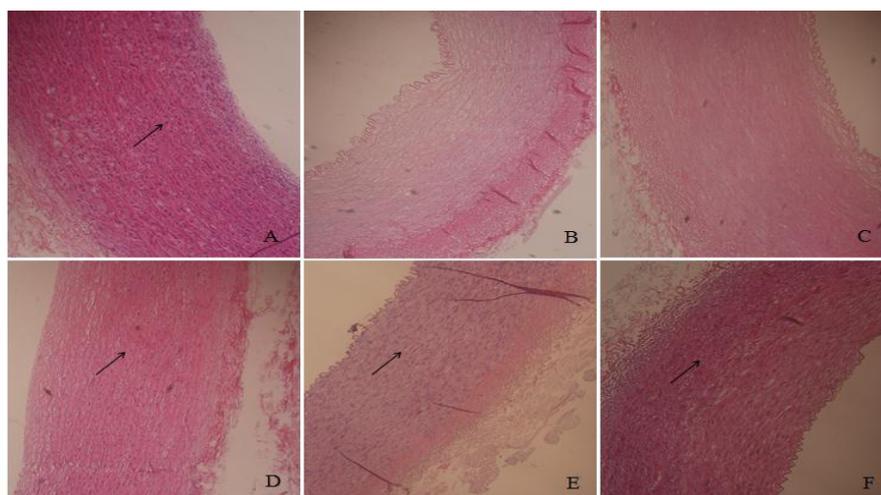


Figure 2. The result of HE staining of decellularized CA (x40). A: Control group. B: 0.5% SDS 24h + H₂O 24h. C: 0.5% SDS 24h, D: 0.1% Triton X100 24h, E: 1M NaOH 6h, F: H₂O 24h. Arrow: cell.

Result of HE staining showed that NaOH/H₂O/Triton X100/SDS-treated vessels had 3 layers and contained a lot of cells. Based on retaining cell number in HE stained sample, efficiency of decellularizing agents ranged from SDS 0.5% 24h (the best), Triton X100 0.1% 24h, NaOH 1M 24h and distilled water for 24h (the worst). However, SDS didn't remove all of the cells in the vessels. The combined method between SDS and H₂O (the combination) gave a better result that all cells were removed on from the vessels (Fig. 2).

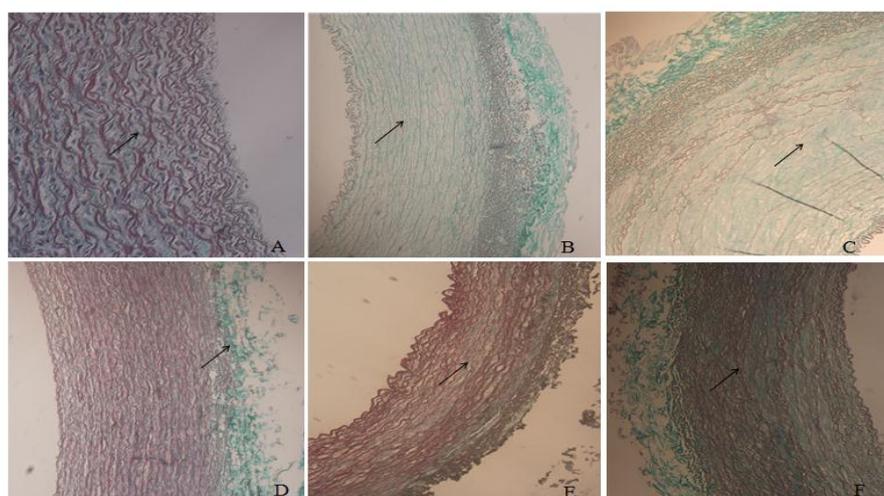


Figure 2. The result of Trichrome of decellularized CA (x40). A: Control group. B: 0.5% SDS 24h + H₂O 24h. C: 0.5% SDS 24h, D: 0.1% Triton X100 24h, E: 1M NaOH 6h, F: H₂O 24h. Arrow: collagen (green).

Result of Trichrome staining showed that collagen was differently preserved in different samples. Efficiency of collagen preservation of these agents ranged from distilled water for 24h (the best), Triton X100 0.1% 24h, SDS 0.5% 24h and 1M NaOH 6h (the worst). In NaOH-treated vessels, layer borders weren't clear and collagen was less than others. Distilled water preserved collagen better than others. The combination method between SDS and H₂O preserved collagen as well as Triton X100, SDS.

Table 1. Summary of decellularization efficiency.

Method	Cell removal	Collagen preservation
NaOH 1M in 6h	++	**
Distilled water in 24h	+	*****
Triton X100 0,1% in 24h	++	***
SDS 0,5% in 24h	++++	***
SDS 0,5% in 24h and distilled water in 24h	+++++	***

(+++++: remove all of cells; *****: collagen in native coronary artery).

HE staining and Trichrome staining showed that the combination of 0.5% SDS for 24 hours and distilled water for 24 hours was the best efficient decellularization method. This method was used to create DCAs for the next experiments.

cytotoxicity test *in vitro*

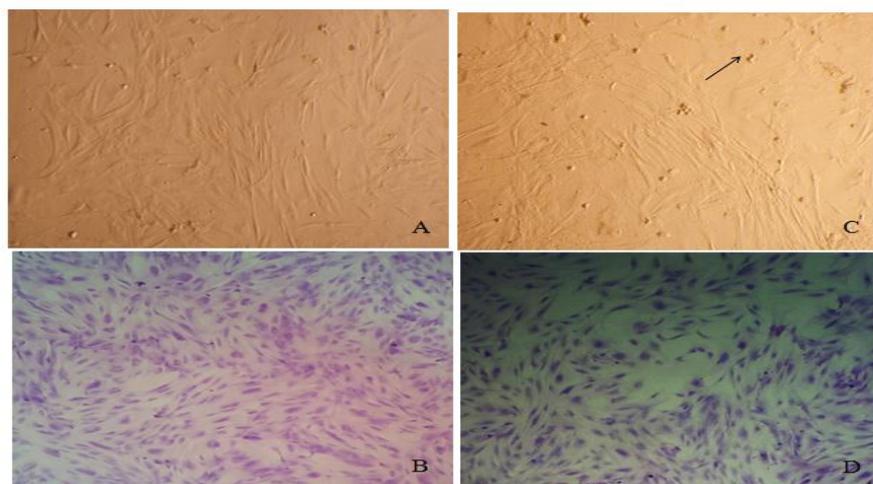


Figure 3. Morphology of fibroblasts after cytotoxicity test (x100). A, B: control; C, D: sample. A, C: observed by microscope. B, D: stained by HE.

Toxicity was assessed by direct contact method according to ISO 10993-5. Results showed that only few cells below the pieces died and detached from the culturing surface. The other cells retained their morphology and grew normally as a control group (Fig. 3).

Ratio of OD sample (0.226) to OD control (0.239) is 94.6%. Based on ISO 10993-5, its cytotoxicity is level 1, GA-treated DCAs aren't poisonous for fibroblast *in vitro*.

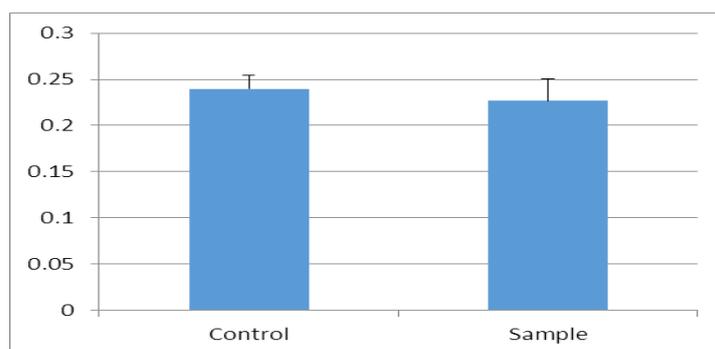


Figure 4. The result of MTT assay for determining cytotoxicity *in vitro* of GA-treated DCA.

endothelial progenitor cell attachment

EPCs from umbilical cord blood were obtained as previously described. UCB EPCs is cobblestone-like morphology, diameter of 50-60 μm with large nucleus in center (Fig. 5). UCB EPCs express some endothelial markers as CD105, KDR, Thrombomodulin, vWF, Ve-cadherin, CD146 and did not express some haematocyte markers as CD14, CD45 (Fig. 11).

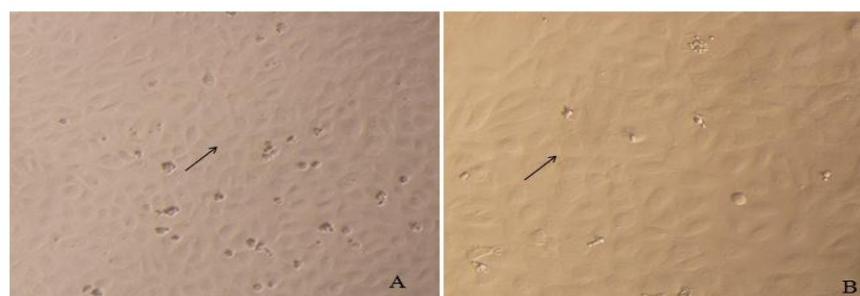


Figure 5. Endothelial progenitor cell at passage 4 on surface of cell culture dish. A: x100, B: x200.

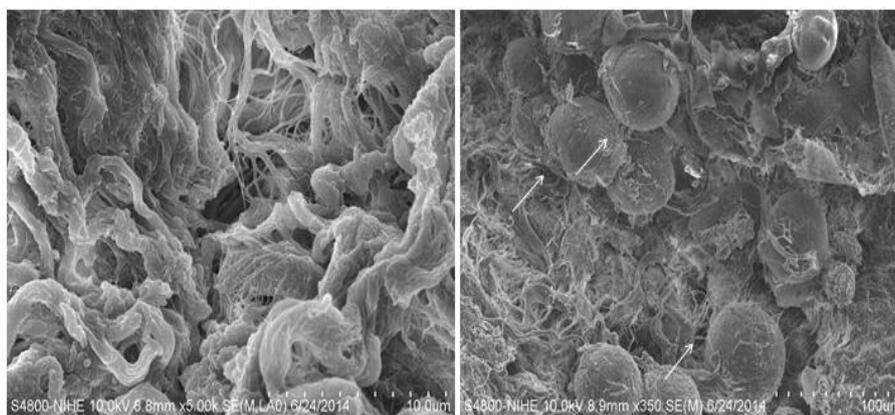


Figure 6. SEM image of EPC-seed DCAs after 2 days. A: Control (don't have EPC), B: sample.

Curve growth was determined by MTT assay. Mean value of OD increased sharply from day 1 to day 5, increased steady from day 5 to day 7 (the highest value) and decreased from day 7 to day 9 (Fig. 8). This result demonstrated EPCs can grow on the lumen of acellular vessels. This result was supported by HE staining and SEM. HE staining and SEM showed that EPCs attached and grew on the lumen of acellular vessels from day 1 to day 7 (Fig. 6, 7).

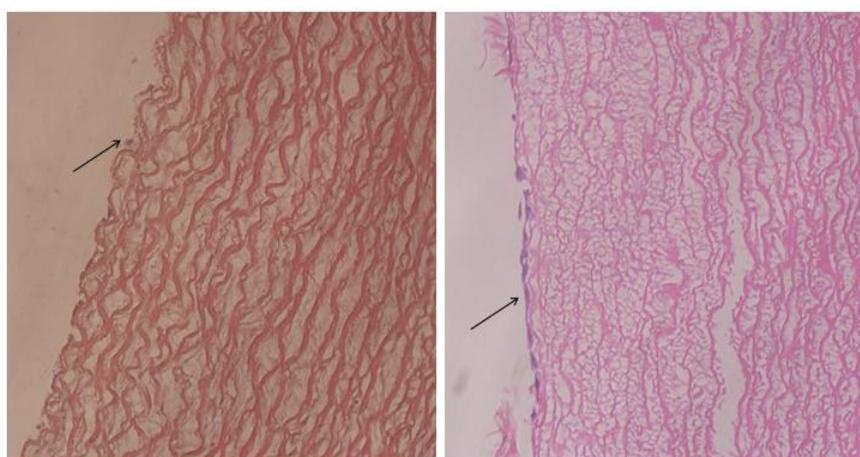


Figure 7. HE staining of EPC-seed DCA (x100). A; After 5 days, B: after 7 days.

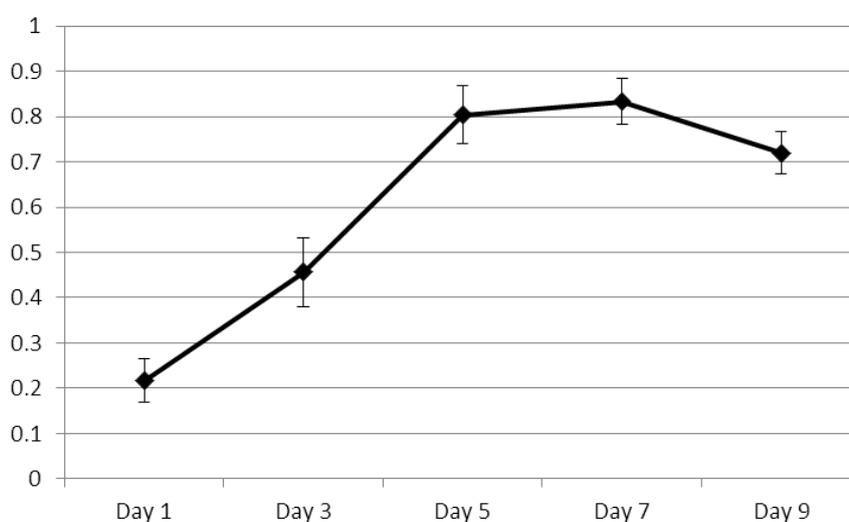


Figure 8. Growth curve of EPCs on lumen of DCA after 9-day culture.

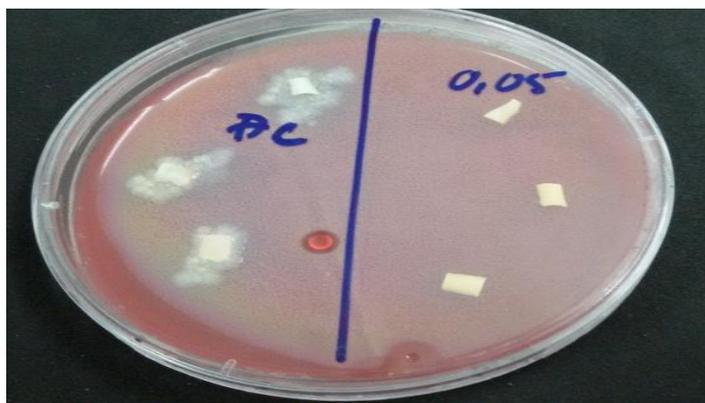


Figure 9. DCAs were treated with GA on blood agar plate after 10-day test.

Disinfection/sterilization is an important step in making acellular bioscaffold. To minimizing the risk of contamination, all decellularization steps were operated in biosafety cabinet by sterilized tools and solutions. Glutaraldehyde (GA) is a simple, cheap, efficient disinfectant. However, GA can alter ECM and free GA can harm the host. Disinfection efficiency and toxicity of GA belong to its concentration and processing time.^[4] So, we test many concentrations of GA (0.1; 0.15; 0.5; 1%) and many sterilizing times (30, 60, 90 minutes and 1 day) and the result showed that GA at concentration of 0.15% in a period of 1 hour can be used as a good disinfection method (data not shown) (Fig. 9). After disinfection, the structure of DCAs was tested again by HE and Trichrome staining. Results showed that structure of GA-treated DCAs is similar to DCAs (Fig. 10).

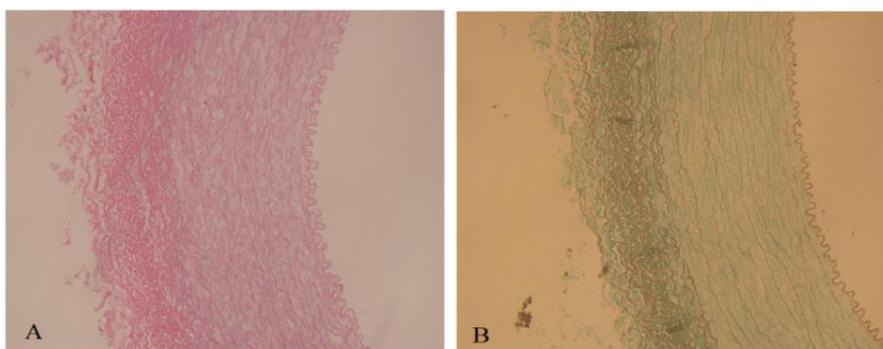
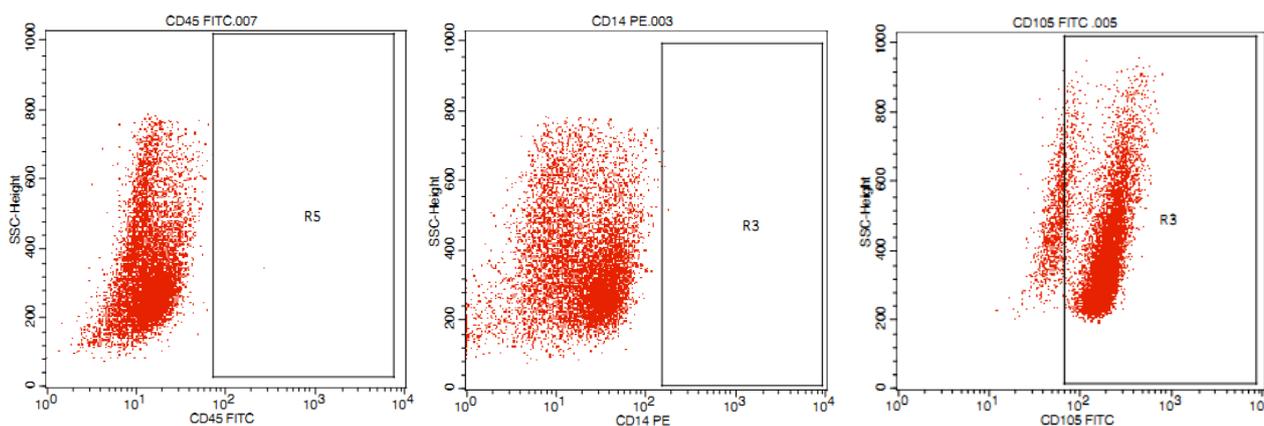


Figure 10. Histological staining of GA-treated DCAs (x10). A: HE staining, B: Trichrome staining.

Then, GA-treated DCAs were washed with sterilized PBS many times in one day to remove free GA and SDS. After that, cytotoxicity of GA-treated DCAs was assessed *in vitro* by ISO 10993-5.^[18] HE staining and MTT assay showed that these pieces were not poisonous *in vitro*. However, GA is a cross-linker which can link between –COOH and –OH group in ECM so that strength of DCAs can increase.^[11] Tensile of strength of GA-treated DCAs is higher than DCA and higher than native vessels (CA) (data not shown). These results demonstrated GA can be used in disinfection of decellularized vessels at concentration of 0.15% and treating time of 1 hour.



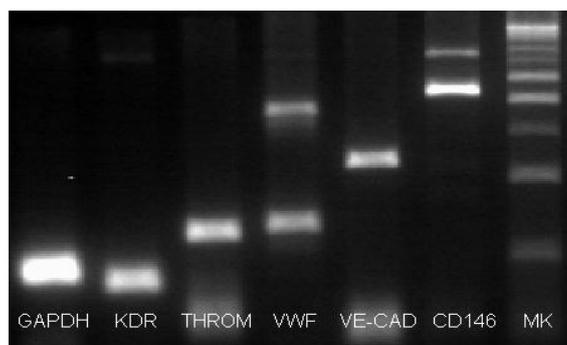


Figure 11. Characteristic of EPC at passage 4. A: flow cytometry, B: Reverse transcription polymerase chain reaction (RT-PCR).

DISCUSSION

In the United States, more than 2150 Americans die of CVD each day. Adequate autologous vessels is lacking in many patients who suffer advanced CVD and need the arterial bypass procedure. Xenogeneic and allogeneic vascular graft is also another choice for vascular bypass grafts. They can be largely isolated but they can elicit host immune responses. Cells are immunogenic potential. Removal of cells from the tissue or organ can reduce immunogenicity of xenografts/allografts, so decellularized xenografts/allografts can be used as vascular grafts in arterial bypass procedure.^[10]

The decellularizing agents can be divided to 3 classes: physical, chemical, enzyme. Physical agents disrupt cell walls by mechanical force, include: freezing, direct pressure, sonication, agitation. Chemical agents can solubilize cell membrane, cytoplasmic components, nucleic acid., including alkaline/acid, non-ionic detergent, ionic detergent, hypotonic/hypertonic solution. Enzymatic agents are trypsin (protease), endonucleases, exonucleases.^[2, 3] NaOH, Triton X100, H₂O, SDS are the most frequently agents which usually are used for the decellularization method. NaOH is an alkaline which can solubilize cytoplasmic components, remove glycosaminoglycan (GAG) and decrease mechanical strength.^[14] Triton X100 is a non-ionic detergent which disrupts lipid-lipid, lipid-protein.^[7] SDS is an ionic detergent which can solubilize cytoplasmic, nuclear membrane and denature protein.^[6] Distilled water is a hypotonic solution in which cells are swollen and disrupted.^[14] Decellularization efficiency of each agent depends on concentration, the processing time and tissue/organ origin. So, we study on efficiency of each NaOH, Triton X100, H₂O and SDS in decellularization of porcine vessel. Cells and structure of DCAs were determined by HE staining. Collagen is the most abundant protein in vessels, so collagen preservation is an important factor for determining efficiency of decellularization method. The result is summarized in Table. 1. SDS is the most efficiency decellularizing agent in removing cell among these factors but SDS did not remove all cells (about 10% cells were retained in treated vessels). H₂O is the most efficiency agent in preserving ECM but H₂O only disrupt few cells. NaOH and Triton X100 are not efficient in removing cells. So,

we try to combine SDS and H₂O to make a better solution. Result showed that cells were completely removed from the vessel and ECM was retained. This method was used for the next research. In vascular tissue engineering, the low patency and thrombosis limit clinical application of vascular grafts. Some cells having several antithrombotic properties such as endothelial cell (EC), endothelial progenitor cell (EPC) can be useful.^[13, 17] However, ECs lie on the lumen of vascular, so it is difficult to isolate autologous ECs of patients. EPCs can differentiate to EC, express thrombomodulin (antithrombotic marker) and be isolated from peripheral blood.^[5] In this study, UCB-EPCs were cultured in EBM-2 supplemented with 5% FBS. At passage 4, EPCs express some endothelial markers as CD105, KDR, Thrombomodulin, vWF, Ve-cadherin, CD146 and did not express some haematocyte markers as CD14, CD45. UCB-EPCs were seeded on lumen of DCAs (Fig. 11). UCB-EPCs attached on surface in day 2 and some cells got under surface of lumen (SEM). UCB-EPCs grew on the lumen from day 1 to day 7 (MTT assay). On 7th day, UCB-EPCs covered most likely the whole surface of the lumen but they did not cover all the surface (HE staining). These results demonstrated UCB-EPCs can grow on the lumen of surface of DCAs but they didn't cover all lumen's surface. It demonstrated that static culture is not the best condition for cell growth in bioscaffold.

CONCLUSION

This research is a first step on creating suitable decellularized vessels for vascular tissue engineering. A lot of research needs to be taken place to test safety and remodel of decellularized vessels *in vivo*. However, we conclude that: decellularized vessels can be made by combining SDS 0.15% in 24 hours and distilled water in 24 hours; GA can be used as disinfectant at concentration of 0.15%, a period of 1 hour and UCB-EPCs can grow on the lumen of acellular vessel graft.

REFERENCE

1. Catto V, Farè S, Freddi G, Tanzi MC. (Vascular Tissue Engineering: Recent Advances in Small Diameter Blood Vessel Regeneration). ISRN Vascular Medicine, 2014; 2014: 1-27.

2. Crapo PM, Gilbert TW, Badylak SF. (An overview of tissue and whole organ decellularization processes). *Biomaterials*, 2011; 32(12): 3233-3243.
3. Gilbert TW, Sellaro TL, Badylak SF. (Decellularization of tissues and organs). *Biomaterials*, 2006; 27(19): 3675-3683.
4. Herruzo-Cabrera R, Uriarte MC, Rey-Calero J. (Antimicrobial effectiveness of 2% glutaraldehyde versus other disinfectants for hospital equipment, in an *in vitro* test based on germ-carriers with a high microbial contamination). *Rev Stomatol Chir Maxillofac*, 1999; 100(6): 299-305.
5. Ingram DA, Mead LE, Tanaka H, Meade V. (Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood). *Blood*, 2004; 104(9): 2752-2760.
6. Kasimir MT, Rieder E, Seebacher G, Silberhumer G. (Comparison of different decellularization procedures of porcine heart valves). *Int J Artif Organs*, 2003; 26(5): 421-427.
7. Koley D, Bard AJ. (Triton X100 concentration effects on membrane permeability of a single HeLa cell by scanning electrochemical microscope (SECM)). *Proceedings of National Accademic of Sciences*, 2010; 107(39): 16783-16787.
8. Li S, Sengupta D, Chien S. (Vascular tissue engineering: from *in vitro* to *in situ*). *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*, 2014; 6(1): 61-76.
9. Mead LE, Prater D, Yoder MC, Ingram DA. (Isolation and characterization of endothelial progenitor cells from human blood). *Curr Protoc Stem Cell Biol*, 2008; Chapter 2(Unit 2C 1).
10. Meyer SR, Nagendran J, Desai LS, Rayat GR. (Decellularization reduces the immune response to aortic valve allografts in the rat). *J Thorac Cardiovasc Surg*, 2005; 130(2): 469-476.
11. Migneault I, Dartiguenave C, Bertrand MJ, Waldron KC. (Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking). *BioTechniques*, 2004; 37: 790-802.
12. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK. (Heart disease and stroke statistics--2015 update: a report from the American Heart Association). *Circulation*, 2015; 131(4): e29-322.
13. Quint C, Kondo Y, Manson RJ, Lawson JH. (Decellularized tissue-engineered blood vessel as an arterial conduit). *Proceedings of National Accademic of Sciences*, 2011; 108(22): 9214-9219.
14. Reing JE, Brown BN, Daly KA, Freund JM. (The effects of processing methods upon mechanical and biologic properties of porcine dermal extracellular matrix scaffolds). *Biomaterials*, 2010; 31(33): 8626-8633.
15. Ross R. (Atherosclerosis - An inflammatory disease). *Mechanism of Disease*, 1999; 340(2): 115-126.
16. Seifu DG, Purnama A, Mequanint K, Mantovani D. (Small-diameter vascular tissue engineering). *Nat Rev Cardiol*, 2013; 10(7): 410-421.
17. Shirota T, Yasui H, Shimokawa H, Matsuda T. (Fabrication of endothelial progenitor cell (EPC)-seeded intravascular stent devices and *in vitro* endothelialization on hybrid vascular tissue). *Biomaterials*, 2003; 24(13): 2295-2302.
18. Standard I-. (Biological evaluation of medical devices - Part 5: Tests for *in vitro* cytotoxicity). 2009.