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Introduction

Each year, more than 100,000 vascular bypass graft procedures are performed, creating an important demand for vascular grafts (Williams, 2000). Currently, autogenous saphenous veins, synthetic grafts or acellular human umbilical veins are used as grafting conduit. However, the results were far from satisfactory. In the future, tissue engineered arteries may serve as an alternate source of vascular grafts for the patients. In last two decades, there are several artificial blood vessel under investigate such as (1) synthetic grafts (2) Matrix template or acceluar tubes of ectracellular matrix (3) tissue-engineered blood vessels (4) artificial arteries derived from peritoneal granulation tissue in body "bioreactor" (Thomas et al., 2003). The successful artificial blood vessel must meet following requirements. (1) It has to be biocompatible and biodegradable. (2) It has to be leak-proof and thromboresistant, but with adequate porosity for healing and angiogenesis. (3) It has to have appropriate mechanical properties such as strength, kink resistance, and compliance. (4) It has to possess appropriate vasoactive physiological properties such as contrict or relax (Anita et al., 2003).

Porcine are popular for experimental study, as they have similar anatomy and physiology to that of humans (Rashid et al., 2004). Porcine models have been used to assess the durability and functionality of tissue engineering conduits. Niklason and co-worker developed vascular grafts using biodegradable polyglycolic acid (PGA) scaffolds chemically modified by sodium hydroxide. Onto this were seeded bovine SMCs and ECs before these constructs were pulsed in a flow circuit within bioreactors for 8 weeks. On implantation in a porcine model these grafts showed patency after 24 days by angiography (Niklason et al., 1999).

The objective of this study was to evaluate the patency of biological blood vessel equivalent in animal model. Porcine smooth muscle cells and endothelial cells were seeded on biodegradable polymer and amniotic membrane base matrix. The conduits will be implanted into animal to assess the practical usage in vivo.

Material and methods

1. Animals

Porcine was used in this study to provide AM, EC and SMC. Animal care and use were performed in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996.

2. Preparation of Amniotic membranes

The amniotic membranes were obtained from full-term pregnant female porcine. After cesarean section, AM were collected and washed with PBS containing antibiotics (100U/ml penicillin and 100ug /ml streptomycin). The AM, a thin sheet consisting of the epithelium, basement membrane and some underlying compact stroma, were separated from the bulk of the tissue and stored in DMEM containing 50% glycerol at -80℃.

3. Preparation of endothelium and smooth muscle cells from Aorta

Endothelial cells were isolated according to the procedureof Sharma and Davis with modification. Freshsegments of thoracic aorta from 7-month-old pig was collected. A thin layer was gently scraped from each arteriallumen by sterile surgical blades and transferred into a sterile flask to incubate with 1 mg/ml collagenase (typeII) at 37 °C for 10 min to release the endothelial cells. The mixture was centrifuged and the supernatant discarded. The pellets were washed twice in sterile basal medium with additional antibiotics and plated in sterile tissue culture dishes with culture medium (basal medium supplemented with 10% FBS) in a humidified atmosphere of 5% CO2 and 95% air at 37°C. The primary culture of PAECs was passed and maintained routinely in culture medium at 37°C and the culture medium was changed every 2–3 days until the cells reached confluence. PAECs were identified by a Dil-AcLDL uptake assay and subsequently examined by fluorescent microscopy. Microscopic examination was used to observe cell morphology and monitor cell growth. The 10th through 12th passages of PAEC primary cultures were used for construction of artificial blood vessel..

After removing endothelium cells, trypsin was added into the thoracic aorta matrix and incubated for 5 5- 10 mins. A thin layer of matrix membrane was gently scraped from arterial lumen by sterile surgical blades. The artery was dissected into segments of approximately 0.5cm^2. The segments were placed intimal side down in Petri dishes and maintained in CO_2 incubator for 5 to 10 days. The smooth muscle cells migrated off the segments and become established in two-dimensional culture.

4. Evaluation of grafts rejection:

(1) Analysis of lymphocyte proliferation by MTT assay

The lymphocyte proliferation is assayed by incubating lymphocytes with mitomycin C-treated transplanted cells (EC, or SMC). After 3 days, the proliferation of lymphocytes will be measure by MTT method (Mosmann, 1983). This method is base on the cleavage of yellow tetrazolium salt MTT by mitochrondrial dyhydrogenases of metabolically active cells to purple formazan crystals, which are then solublized and sepectrophotometrically quantified.

(2) Leukocyte adhesion to ECs cultured on AM.

In order to determine the adhesive interaction between leukocytes and ECs cultured on AM,

with LPS and without LPS, leukocytes were isolated from fresh porcine blood with Histopaque 1119 (Sigma-Aldrich, USA) and spread on ECs in 6-well confluent culture dishes (2×10^5 cells/well in M199 plus 10% FBS) with or without LPS treatment for 20 h. After incubation under static adhesion assay conditions at 37 °C for 15 min, non-adherent leukocytes were removed by washing three times with PBS. Cells were fixed with 4% paraformaldehyde at room temperature for 30 min and stained with DAPI. For each experiment, the numbers of leukocytes from three fields per well were counted under a fluorescence microscope and summed. All experiments were performed in 6 to 8 times.

5. Construction of blood vessel equivalent

Three different blood vessel equivalents were constructed and subjected to the animal experiments.

(1) blood vessel equivalent construction 1

Decellularized AM was placed on the PLGA (Johnson & Johnson, USA). Endothelial cells were seed on the left hand side of AM (10^5 cells/cm^2) and smooth muscle cells were seed on the other side of AM. The cells were maintained in 37℃ CO^2 incubator for 5 days. The AM with cells was rolled around a Teflon-coated stainless steel support tube (Figure 7).

(2) blood vessel equivalent construction 2

Decellularized AM was placed on the poly- ε -caprolectone membrane(PCL), and then AM side was face down on the Petri dish. Smooth muscle cells were mixed with fibrin gel (25mg/ml; Sigma, USA). The final cell number of the mixture was 10^6 cells/ml. The mixture was filled the pores in the PCL membrane. After 5 days incubation, turn the AM side up, then cultured the endothelial cells on the AM (10^5 cells/cm^2). After 5 days incubation, the membrane was sewing to form a tube (Figure 8).

(3) blood vessel equivalent construction 3

Ten to 15 layers of decellularized AM were rolled on Teflon-coated stainless steel supported tube. The multiple layers of AM were dried by air in laminar flow. The 0.1% Formaldehyde solution was used to cross-link the multiple layers of AM. The endothelial cells were then cultured in the inner layer of the tube for at least 5 days (Figure 9).

6. Animal experiments

To assess the practical utility of these cultured arteries in vivo, we were undertaken initial implantation in porcine. Animal care and use will be performed in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996. All animals will be medicated with a daily asprin (30mg/kg) beginning 3 days before surgery. The pigs were anesthetized with ketamine (10 mg/kg) induction and isofluorane maintenance. The carotid arteries were exposed through lateral neck incisions (two carotid arteries each). When the common carotid arteries are identified, the animals are heparinized (100 u/kg). A piece of studied vessel, 4 cm in length, was used to create a carotid-carotid jump graft on the common carotid artery in an end-to-end fashion (Figure 4). The neck wounds will be closed in layers with 3-0 Nylone sutures. The animals were administrated the Aspirin and injected Dexamethasone (0.4mg/kg) daily. The patency of implanted grafts will be monitored at regular

intervals with color Doppler ultrasonography. The harvested grafts was fixed in 4% paraformaldehyde in PBS and embedded in paraffin for hematoxylene-eosin stain (Sigma, USA).

Results and discussion

1. **Preparation of AM**

AM was prepared from full-term pregnant female porcine. Before using, the epithelium cells have to be removed. To prevent the contamination, AM was sterilized by UV illumination. Endothelium cells and smooth muscle cells were grown on AM firmly. This result indicates that AM is a good matrix for endothelium and smooth muscle cells.

2. **Isolation of endothelium and smooth muscle**

Endothelial cells were isolated from porcine thoracic aorta and the primary culture of PAECs was used as experimental material in this study (Figure 1). Results from the uptakeof Dil-AcLDL showed that PAECs were brilliantly fluorescent, while the fluorescent intensities of other celltypes (rat L8 myoblasts and porcine smooth muscle cells) were near background level (data not shown). In addition, expression of factor VIII was examined by RT-PCR. The result showed that factor VIII expressed in PAECs (Figure 2).

Smooth muscle cells were isolated from aorta and smooth muscle specific marker, SM22α, was used to identify the cells. The morphology of smooth muscle cells is different from PAECs (Figure 3). Expression of SM22α was detected in these cells (Figure 4).

3. Analysis of lymphocyte proliferation by MTT assay

To investigate the effect of blood vessel equivalents on the host immune response, the lymphocyte proliferation was measured by incubating lymphocytes with mitomycin C-treated transplanted cells (endothelial cells). the proliferation of lymphocytes was determined by MTT method. The result showed that the proliferation of lymphocyte was stimulated by the transplanted cells (Figure 5).

4. Reduction of leukocytes adherence in AM-based EC culture

To further examine the anti-inflammatory properties of AM, we assayed the adherence of leukocyte to endothelial monolayer in both plastic control and AM-based cultures, with or without LPS stimulation. In the absence of LPS, the leukocyte adherence to ECs appeared to be mildly attenuated in AM-based culture (Figure 3). However, the enhanced leukocyte adherence to ECs resulting from LPS challenge was significantly reduced in AM-based culture (Figure 6). The results were consistent with the lowered CAM expressions in ECs cultured on AM, and suggest the potential anti-inflammatory properties of AM in the future BVE construction (Tsai et al 2007).

5. Animal experiments

(1) blood vessel equivalent construction 1

The blood vessel equivalent construction was implanted into animal (Figure 10A). Three weeks later, the graft was harvested. The result of histochemical stain showed a server thrombosis was formed in the blood vessel equivalent (Figure 10B).

(2) blood vessel equivalent construction 2

Due to the degradation rate of PLGA, PCL was provided by Department of Chemical engineering in Tunghai University. The pore size of PCL is 100 um. blood vessel equivalent construction 2 was implanted into animal (Figure 11A, B). Four weeks latter, the result from Doppler ultrasound

imaging showed that the blood flow was normal in implanted graft (Figure 11C). The implanted graft was collected after 5 weeks implantation. The histology demonstrated that the thrombosis was formed in the implanted blood vessel equivalent (Figure 11D)

(3) blood vessel equivalent construction 3

Due to the low compability of artificial polymer and AM, a multiple layers of AM was constructed to replace the artificial scaffold (Figure 12A, B). L'Heureux et al (2007) reported that after implantation of graft, the smooth muscle cells will migrate to the outer layer of blood vessel equivalent. Therefore, blood vessel equivalent 3 did not included smooth muscle cells. After 4 weeks of implantation, migration of smooth muscle cells in the inner layer was observed (Figure 12D). In addition, endothelial cells were washed by blood stream within 24 hours.

Conclusion:

AM is a good matrix for growth of endothelial cells. In addition, AM reduced the inflammatory response in endothelial cells. In animal model, thrombosis is the major problem in the blood vessel equivalents. Increase the incubation time for the blood vessel equivalent in bioreactor can help increase the ability of endothelial cells to against the blood shear force.

References

1. Darwich, L., Balasch, M., Plana-Durán, J., Segalés, J., Domigo, M., Mateu, E. (2003) Cytokine profiles of peripheral blood mononuclear cells from pigs with postweaning multisystemic wasting syndrome in response to mitogen, superantigen or recall viral antigens. J Gen Viol 84: 3453-3457.

2. L'Heureux, N., Dusserre, N., Konig, G., Victor, B., Keire, P., Wight, T. N., Chronos, N. A., Kyles, A. E., Gregory, C. R., Hoyt, G., Robbins, R. C., Mcallister, T. N. (2006) Human tissue-engineered blood vessels for adult arterial revascularization. Nat. Med. 12:361-365.

3. L'Heureux, N., Dusserre, N., Marini, A., Garrido, S., de la Fuente, L., Mcaalster, T. (2007) Technology insight : the evolution of tissue-engineered vascular grafts-form research to clincal practice. Nat. Cli. Pract. Card. Med. 4:389-395.

4. Mitchell, C. K., Aeschlimann, S. E., Korcarz, C. E. (2004) Carotid Intima-media thickness testing: Technical considerations. J. Am. Soc. Echo.17:690-692.

5. McCullough, K.C., Schaffner, R., Fraefel, W., Kihm, U. (1993) The relative density of CD44-positive porcine monocytic cell populations varies between isolations and upon culture and influences susceptibility to infection by African swine fever virus. Immunol Lett 37: 83-90.

6. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immuno. Method. 65:55-63.

7. Nichols, W. W., O'Rourke, M. F. (1998) McDonald's blood flow in arteries. 4th ed. London: Arnold, E.

8. Niklason, L. E., Gao, J., Abbott, W. M., Hirschi, K. K., Hourser, S., Marini, R., et al., (1999) Functional arteries grown in vitro. Science 284:489-493.

9. O'Rourke, M. F., Mancia, G.. (1999) Arterial stiffness. J Hypertens. 17:1-4.

10. Raymond, C. R., Wilkie, B. N. Th-1/Th-2 type cytokine profile of pig T-cells cultures with antigen-treated monocyte-derived dendritic cells. Vaccine 22:1016-1023, 2004.

11. Rashid, S. T., Salacinski, H. J., Hamilton, G., and Seifalian, A. M. (2004) The use of animal models in developing the discipline of cardiovascular tissue engineering: a review. Biomaterials 25: 1627-1637.

12. Ronald, M. A. et al. (2003) Arterial stiffness increases with deteriorating glucose tolerance status. The Hoorn Study. Circulation. 107:2089-2095.

13. Thomas, A. C., Campbell, G. R., Campbell, J. H. (2003) Advances in vascular tissue engineering. Cardi. Path. 12:271-276.

14. Tsai, S. H., Liu, Y. W., Tang W. C., Zhou, Z. W., Hwang, C. Y., Ou, B. R., Hu, C. P., Yang, V. C. And Chen, J. K. (2007) Characterization of porcine arterial endothelial cells cultured on amniotic membrane, a potential matrix for vascular tissue engineering. Biochemical and Biophysical Research Communication. 357:984-990.

15. William, S. K. (2000) Tissue engineered vascular grafts: from bench to clinical use. FASEB J. 14:A305.

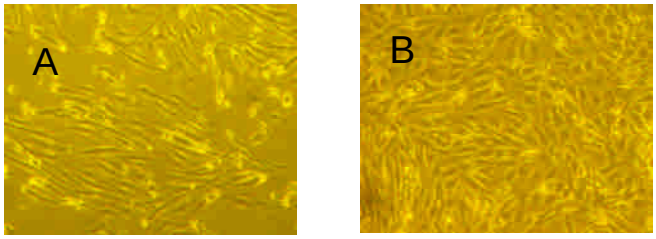

Figure 1. PAEC isolated from porcine.
(A): 8th passage; (B): 19th passage

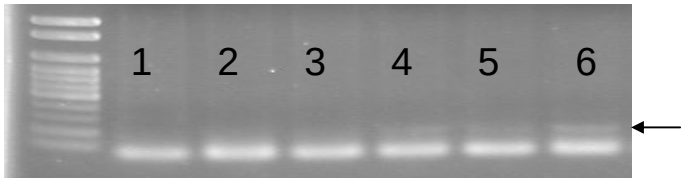

Figure 2. Expression of Factor VIII in PAECs.

Lane 1, 2, 3: smooth muscle; Lane 4, 5, 6:PAEC

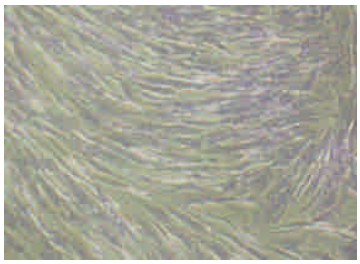

Figure 3. Smooth muscle cells isolated from porcine.

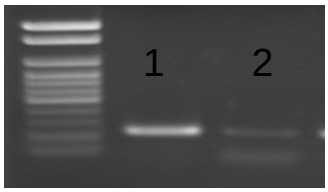

Figure 4. Expression of SM22 α in smooth muscle cells. Lane 1:Smooth muscle cells; Lane 2: PAEC

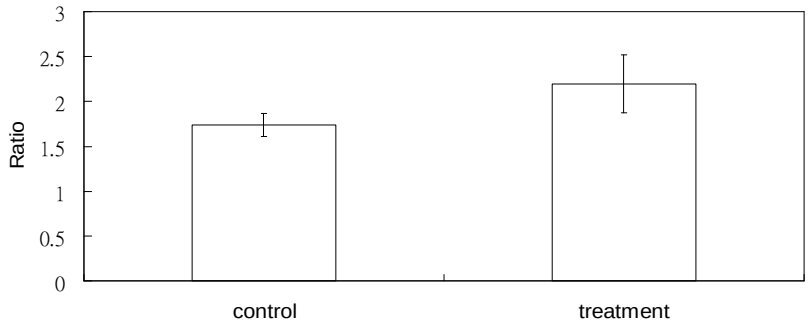

Figure 5. Analysis of lymphocyte proliferation by MTT assay. Control: lymphocyte only; treatment: lymphocyte with mitomycin C-treated endothelial cells.

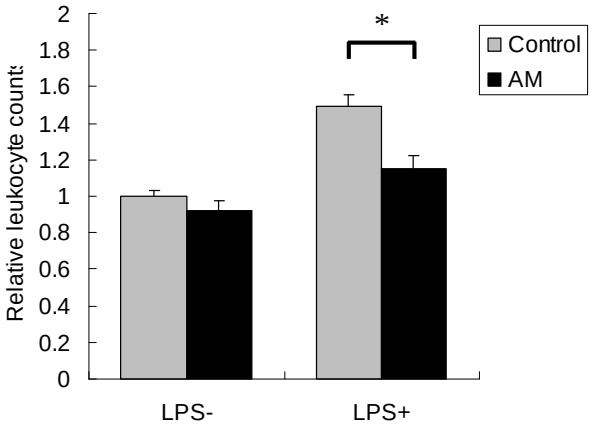

Figure 6. Reduction of leukocytes adherence in AM-based EC culture.

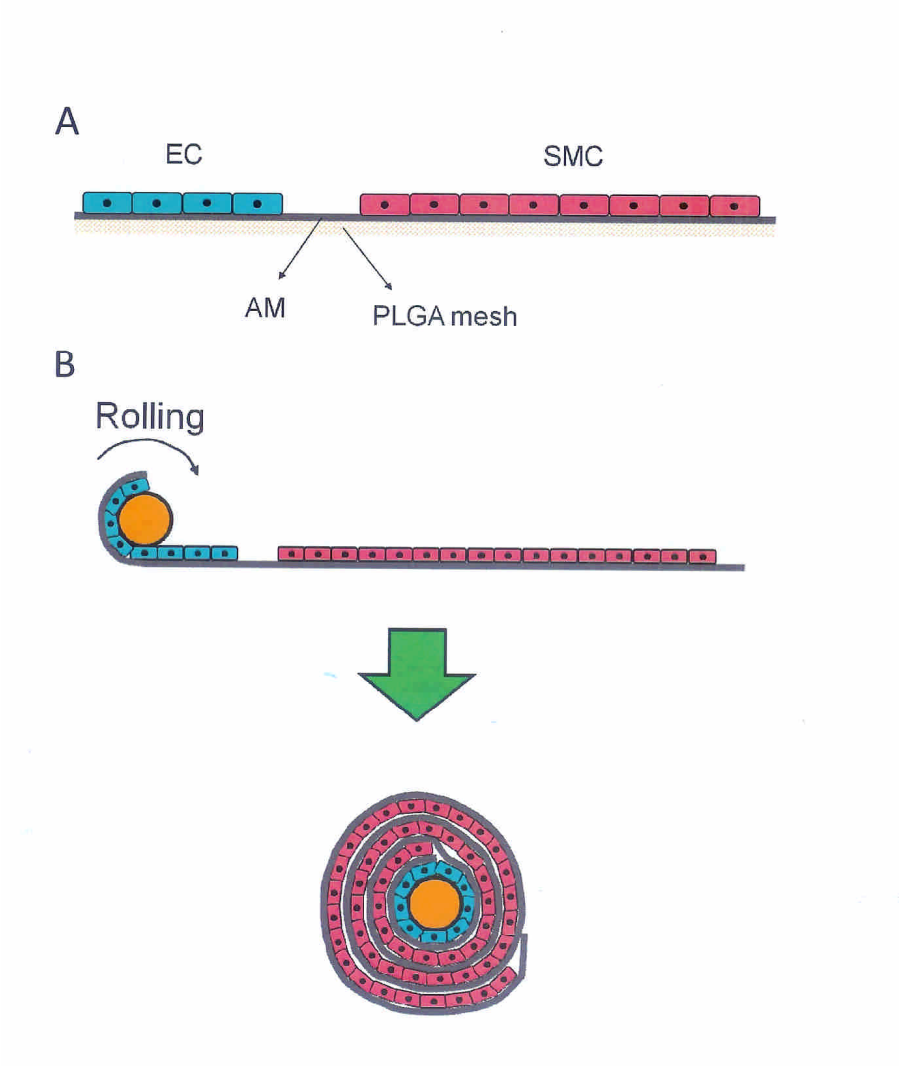

Figure 7. Construction of blood vessel equivalent 1. (A) endothelial cells and smooth muscle cells were seeded on the decellularized AM. (B) The AM with cells were rolled to form a tube..

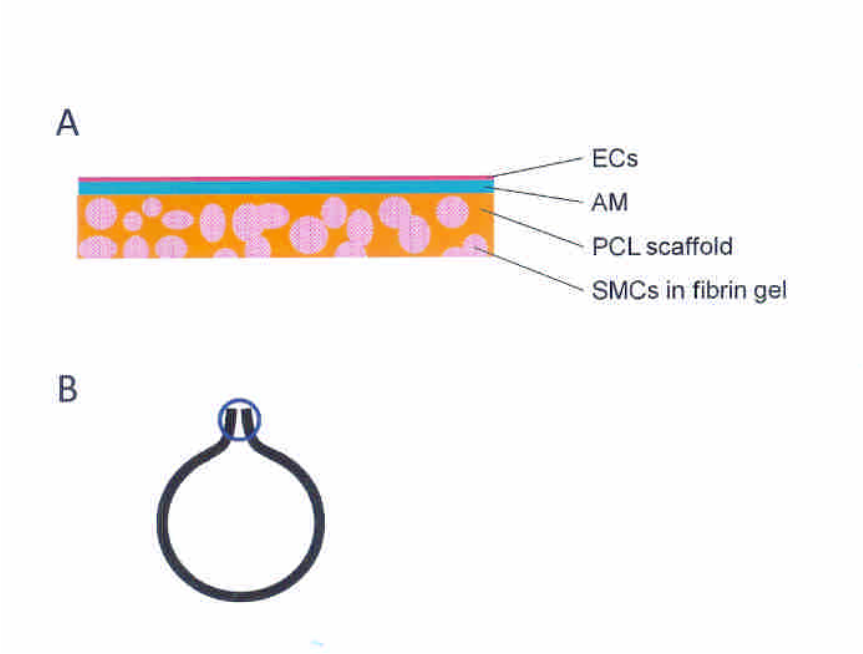

Figure 8. Construction of blood vessel equivalent 2.

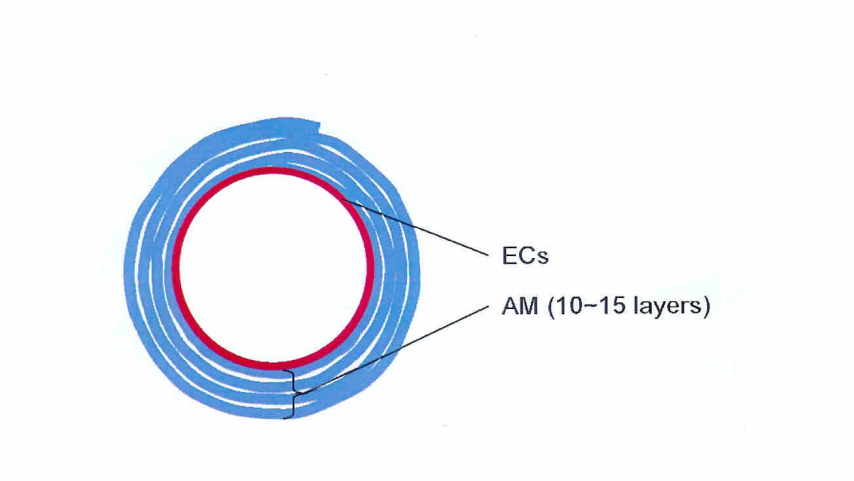

Figure 9. Construction of blood vessel equivalent 3

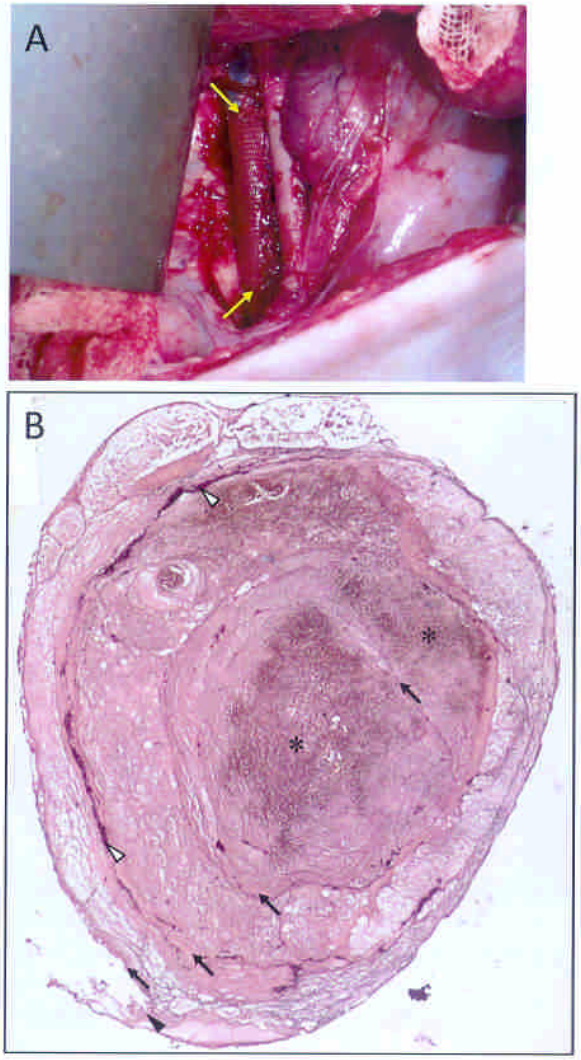

Figure 10. Blood vessel equivalent 1anastomosed as end-to-end interpositional femoral graft. (A) Immediately after removal of the cross-clamps. (B) Thrombosis formation in blood vessel equivalent.

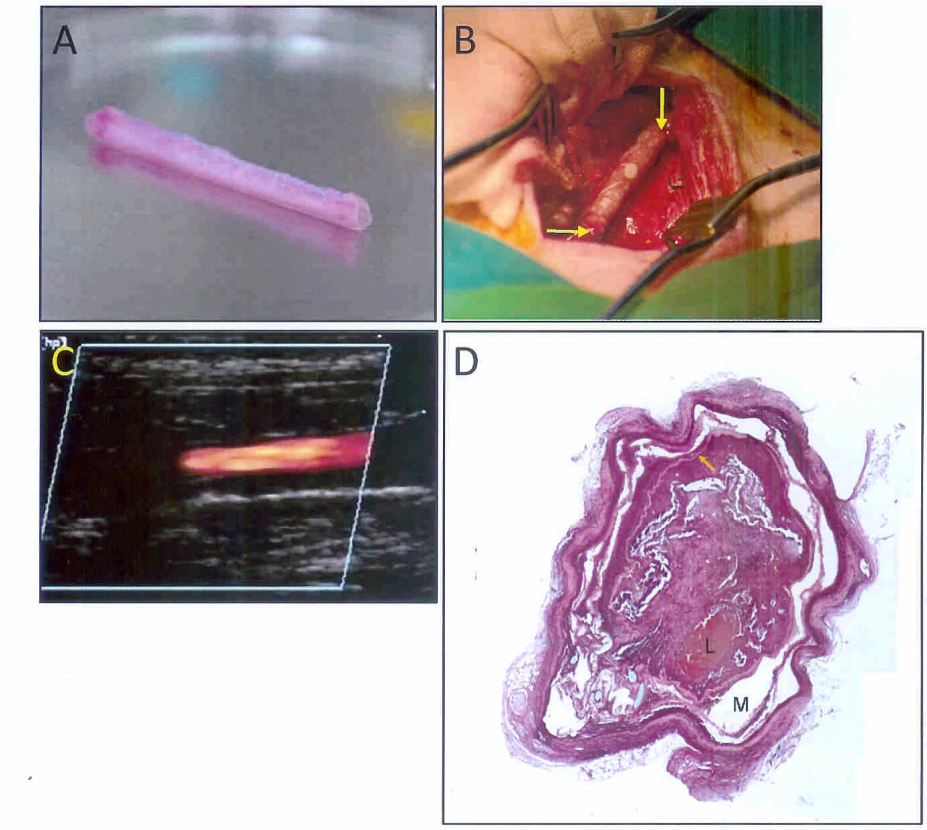

Figure 11. (A) blood vessel equivalent 2 (B) immediately after removal of the cross-clamps (C) Doppler-ultrasound imaging aat 4 weeks. (D) Thrombosis formation in blood vessel equivalent 2

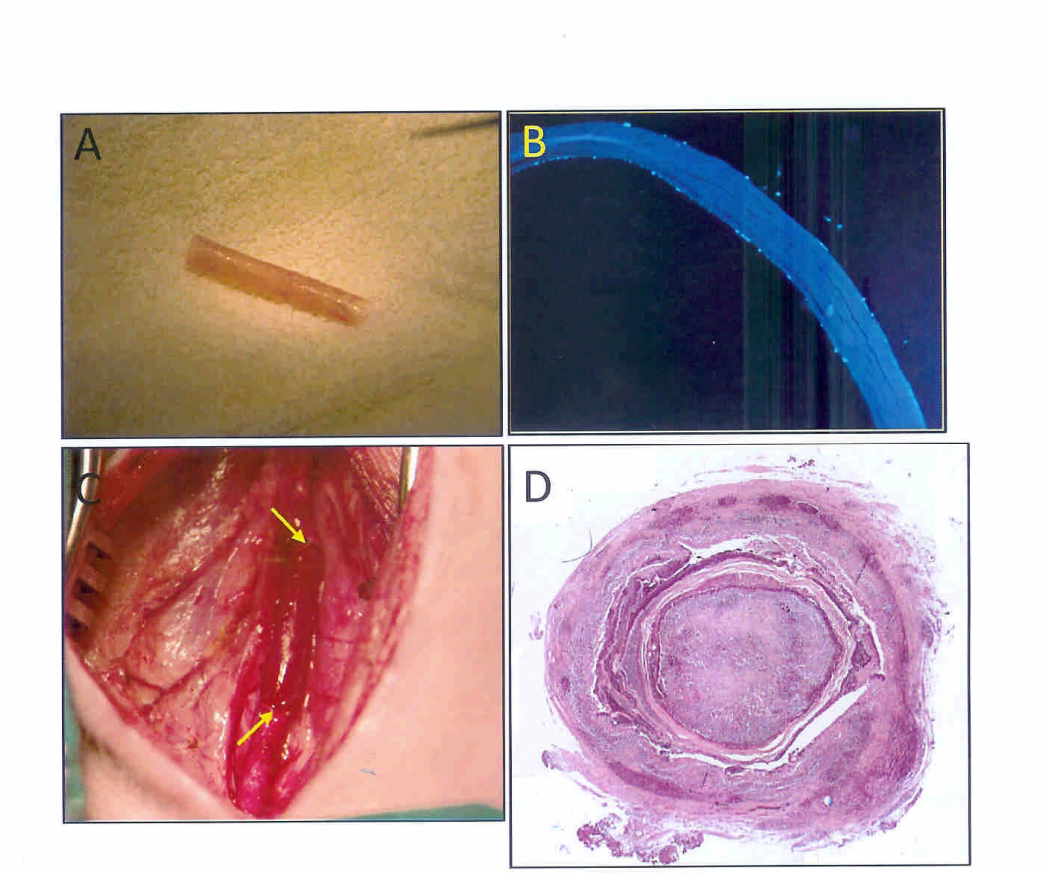

Figure 12. (A) blood vessel equivalent 3 (B) cross section of multiple layers of AM (C) immediately after removal of cross-clamps (D) thrombosis formation in blood vessel equivalent 3