The *in vitro* biocompatibility and calcification evaluation of Xenogeneic vascular graft

Nho Thuan Nguyen[2,3], My Thi Ngoc Nguyen[2,3], Hoang Minh Lam[1,3] and Ha Le Bao Tran[1,2,3*]
1. Laboratory of Tissue Engineering and Biomedical Materials, University of Science, Ho Chi Minh City, VIETNAM
2. Department of Physiology and Animal Biotechnology, Faculty of Biology and Biotechnology, University of Science, Ho Chi Minh City, VIETNAM
3. Vietnam National University, Ho Chi Minh City, VIETNAM
*tlbha@hcmus.edu.vn

Abstract
Xenogenic tissues, including pericardium and vascular vessels have been researched and used in the cardiovascular surgery. The Xenogenic tissues were treated with glutaraldehyde to reduce antigenicity and to improve strength and durability. Tissue derived extracellular matrix (ECM) has been shown to support host tissue remodeling and integration. However, calcification due to glutaraldehyde remains. Therefore, the field of cardiovascular patch fabricated from bovine pericardium is still being studied. In our previous research, the acellular bovine pericardium was treated with low glutaraldehyde concentration (so-called the modified pericardium). The modified pericardial membranes were investigated regarding in vitro biocompatibility toward endothelial cells and calcification effect.

It was demonstrated that the modified pericardium did not cause cell cytotoxicity and supported endothelial cell adhesion on the membrane surface. The in vitro and in vivo analysis presented a significantly low level of calcification, which was shown via a decrease in vitro calcium content accumulation and the absence of in vivo mineralized area. These results indicate the modified pericardium's potential for use as a cardiovascular graft.

Keywords: Xenogenic, glutaraldehyde, vascular graft, endothelial cells, biocompatibility, calcification, mineralization.

Introduction

Bovine pericardial tissue exhibits superior durability and thickness and is suitable for making surgical patches. The elongation strength of the pericardium can reach 13.86 ± 6.65 MPa and the average thickness is 350 ± 50 µm[6]. With abundant and readily available raw materials, patch products can be manufactured to satisfy the increasing demand for transplantation. The initial research used glutaraldehyde to treat bovine pericardial tissue by altering the xenogenic cell-related antigens[2,3].

Glutaraldehyde also promotes crosslinking between collagen fibers of the extracellular matrix, thus significantly improving the mechanical properties and stability of the graft in the bod[1,3,5]. Glutaraldehyde-treated bovine patches have been shown to have the ability to tighten sutures, thus reducing bleeding rates compared to synthetic patches[9].

Glutaraldehyde-treated pericardium products have been commercialized including Peri-Guard, Vascu-Guard (Baxter International Inc. Deerfield, Illinois, USA) and Tutomesh (RTI Surgical, USA). Glutaraldehyde (Glu) crosslinked bovine pericardium has been reported to cause graft calcification. This calcification morphology is mainly related to dead cells, cell debris and cell membrane phospholipid components in pericardial tissue and toxicity of Glu residue[8]. A potential approach was proposed: combining decellularization and reducing Glu concentration.

Decellularization of bovine pericardial tissue helps to remove the phospholipid component of the cell membrane and cell nucleus, leaving only the extracellular matrix component. This procedure allows both control of calcification risk and ensures graft biocompatibility. Decellularization and Glu treatment have significantly reduced calcium deposition in the sample compared to the bovine pericardium group crosslinked with 0.5% glutaraldehyde. The CardioCel patch (Admedus, Malaga, Western, Australia) is a commercial patch product made by decellularizing and glGlutaraldehyderosslinking bovine pericardium. The CardioCel patch demonstrated durable properties and significantly reduced calcification rates compared to the Peri-Guard patch group and 0.6% glutaraldehyde-treated human pericardium[5].

Our previous research has successfully obtained the modified pericardium[6] in which the bovine pericardium was collected, decellularized and treated with 0.1% glutaraldehyde. This concentration is considered optimal in improving the mechanical properties and sustainability of the membrane. The glutaraldehyde-treated acellular bovine pericardium is non-toxic to cultured cells and can support the relocalization and growth of mesenchymal stem cell lines and fibroblasts[6]. At the same time, due to the use of glutaraldehyde, the calcification properties of the membrane should be further evaluated. Additionally, the modified pericardial membranes were investigated for their effects on viability and endothelial adherence to the application in vascular surgery.

Material and Methods

Modified bovine pericardium fabrication: The modified bovine pericardium (called as MP) was fabricated according to our study[6]. Briefly, bovine pericardial tissues were

obtained and decellularized by Tris-HCl (Sigma, USA), followed by incubating in sodium dodecyl sulfate solution (SDS, Sigma, USA). For stabilization, decellularized samples were incubated in low concentrated glutaraldehyde. After that, the samples underwent a 24-48 hour rinse in phosphate buffer saline 1X (PBS, Gibco, USA). The modified bovine pericardial (MP) samples were freeze-dried and sterilized by gamma irradiation at 25 kGy.

***In vitro* cytotoxicity**

The direct-contact cytotoxicity assay and crystal violet staining: The MP samples were prepared in 0.5 cm × 0.5 cm sizes for the direct-contact cytotoxicity assay. Human endothelial cells (HUVEC) (ATCC, USA) were used in this research. 0.5 ml cell suspension was supplemented into each well, about $5x10^4$ cells per well. Cells were incubated at 37°C and 5% CO_2 for 24 hours. Secondly, the cultured medium was aspirated at about 0.4 ml and biomaterials were gently placed onto an 80% confluent layer of cells. Finally, cells were incubated with biomaterials at 37°C and 5% CO_2 for 24 hours. Each treatment was evaluated via crystal violet staining. In this experiment, culture medium and latex material were used as the negative and positive control respectively. After incubation with cells for 24 hours, biomaterials and cultured medium were removed and 0.5 ml crystal violet dye was added to each well and incubated for 30 minutes, protected from light. Finally, cellular morphology and response around the samples were observed via an inverted microscope (Olympus, Japan).

The liquid extract cytotoxicity and MTT assay: The membrane samples (1 cm x 1 cm) were incubated in cell culture medium at 37°C for 24 hours to collect its liquid extract. HUVECs were seeded into a 96-well plate (10^4 cells per well) and incubated for 24 hours. On the test day, the cultured medium was aspirated from the wells. 0.1 ml of liquid extract was added to each well and incubated for further 24 hours. In this experiment, culture medium and culture medium supplemented with 20% DMSO were used as negative and positive controls respectively. After 24-hour incubation, an MTT assay was conducted to determine the relative growth rate (RGR - %). Briefly, MTT solution (0.5 mg/ml) was added to each well and incubated at 37°C for 4 hours. The MTT solution was replaced with 0.1 ml DMSO/Ethanol mixture (ratio 1:1, v/v).

The resulting solutions were measured for OD value using a spectrophotometer at 570 nm. The %RGR was calculated following the formula: %RGR = OD570 nm (test group)/ OD570 nm (negative control) x 100%. If the value of %RGR is greater than 70%, the sample extract is considered non-cytotoxic[3].

Cell attachment evaluation: The modified bovine pericardium was cut into circular discs and placed into 96-well plates. HUVECs were seeded into 3×10^4 cells per well and incubated at 37°C and 5% CO_2 for 48 hours. Cell attachment on the sample discs was detected via calcein-AM assay. Regarding the calcein-AM assay, 0.1 ml Calcein-AM (1:1000) solution (Thermo Scientific, USA) was added into each sample and incubated at 37°C and 5% CO_2 for 35 minutes, protected from lights. Finally, the attachment of HUVECs on the sample was observed under a fluorescence microscope (Olympus, Japan).

Calcification assay: *In vitro* calcification was conducted to determine the ability of the modified pericardium to promote calcium deposition. Simulated body fluid (SBF) solution was used to mimic the physiological condition. The samples were freeze-dried and incubated in SBF solution (5 mL/cm^2) at 37 °C for 4 - 8 weeks. The SBF solution was refreshed every 3-4 days. At the indicated time points, the MP samples were harvested and gently rinsed with deionized water followed by freeze-drying. The calcium content in the MP samples was quantified using a calcium assay kit (Abcam, USA) and calculated using standard calcium concentrates. The raw pericardial tissues treated with 0.625% glutaraldehyde were used as a comparative control.

Mouse subcutaneous implantation: The experiments were conducted following the Animal Care and Use Committee (Registration No. 706B/KHTN-ACUCUS) for the animal experiment. Mice, Mus musculus var. albino, 6–8 weeks old, 25–30 g, were used in this experiment. The MP samples were prepared in 0.5 × 0.5 cm^2 size and implanted subcutaneously. Aseptic conditions were maintained during the transplantation. After four weeks, the animals were sacrificed. The implanted samples were obtained and fixed in 10% formalin for Von Kossa staining (Abcam, USA) to detect calcification.

Statistical analysis: All quantitative data were presented as mean value ± standard deviation. Statistical analysis was conducted using GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA). Student's t-tests determined differences between the groups. Statistical significance was defined as a P value of less than 0.05.

Results

The effect of modified bovine pericardium towards HUVEC viability: In the contact cytotoxicity assay, there was a significant difference between the positive control (latex material, figure 1 C, F) and the two other samples, the negative control (HUVECs cultured medium (Figure 1 A, D) and modified bovine pericardium sample (Figure 1 B, E). Regarding the positive control, the result showed that cells could not spread and proliferate. Regarding the pictures shown, at the sample site, the density of cells decreased significantly and almost no cells were found on the culture surface due to the toxic reaction of the latex material causing the cells to shrink, deform and peel during staining.

In contrast, in terms of the negative control, HUVECs still adhered and preserved their morphology, which could be detected by the cells staining with crystal violet dye. When comparing between the negative controls and the

experimental samples, no cell deformation was found. The majority of HUVECs were adherent with normal appearance.

In liquid extract cytotoxicity, DMSO 20% in the complete medium was used as the positive control (Figure 1 J) and the appearance of HUVECs adhering to the culture surface was no longer visible after performing the MTT experiment due to the toxic effect. Meanwhile, in the negative control (Figure 1 H) and the liquid extract sample (Figure 1 I), the formation of formazan crystals was clearly observed, showing a high density of cell viability. In terms of the %RGR value of each test group, the %RGR value of DMSO 20% solution as the positive control showed the lowest value as 1.642% ± 0.436% therefore proving that DMSO 20% solution caused a severe cytotoxic to the cells. The %RGR value of the complete medium as the negative control was about 100% ± 4.181%. The %RGR value of the experimental extract was 87.98% ± 1.364% which illustrated that the liquid extract of the sample was safe for the cells.

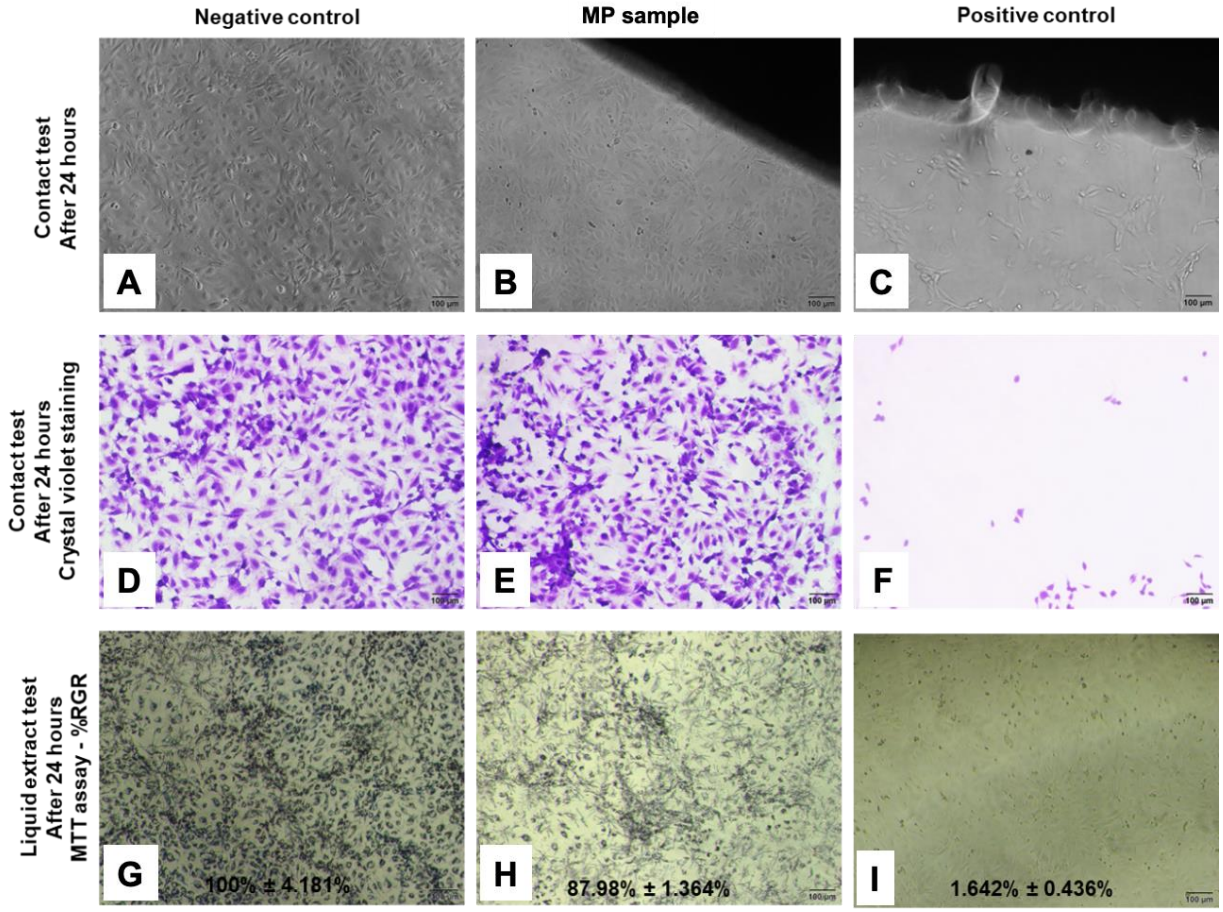

Figure 1: *In vitro* cytotoxicity assessment of modified bovine pericardium toward human endothelial (HUVEC)

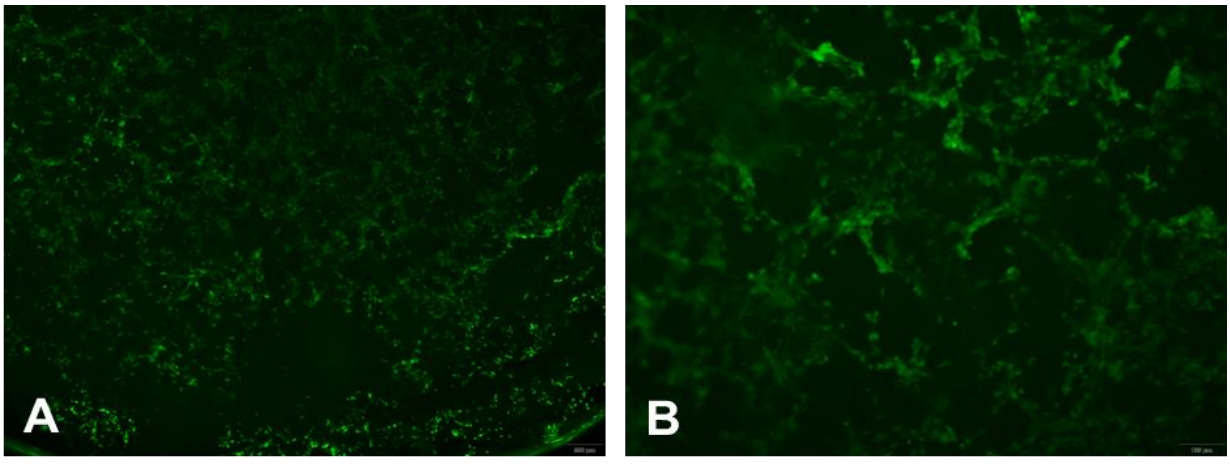

Figure 2: Calcein staining of modified bovine pericardium after HUVEC seeding. A – 4X magnification. Scale bar represents 200 µm. B – 10X magnification, scale bar represents 100 µm.

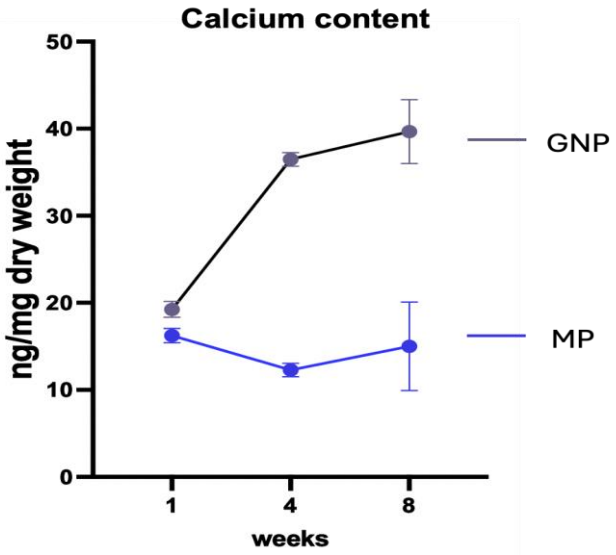

Figure 3: Calcium measurements for samples treated in stimulated body fluid

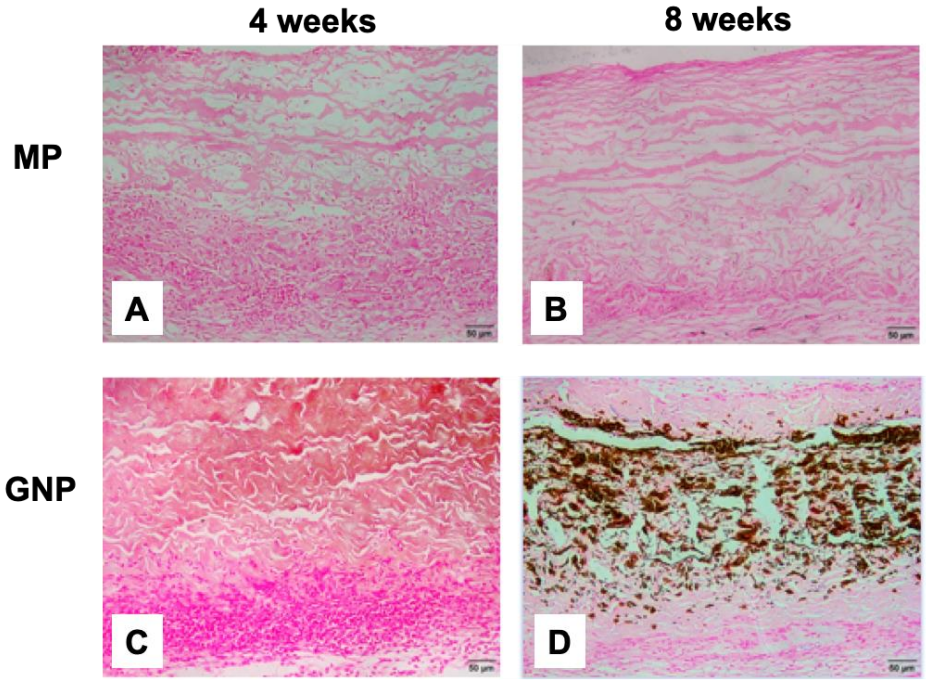

Figure 4: Von Kossa staining following subcutaneous implant after 4 and 8 weeks

The effect of modified bovine pericardium towards HUVEC attachment: HUVEC was used to examine the scaffold's ability to support cell attachment. HUVECs were seeded onto modified bovine pericardium and visualized by calcein staining (Figure 2). Fluorescence images showed that HUVECs could adhere with high density on the scaffold surface after 24-hour incubation, as shown in figure 2 A.

***In vitro* calcification assay:** Calcium content values related to dry weight of GA-treated raw pericardium (GNP) raised significantly from 0.235 ± 0.017 µg/mg dry weight after 4 weeks and increased further in 8 weeks (as 0.428 ± 0.007 µg/mg dry weight). In contrast, the MP samples exhibited significantly low calcium content (0.168 ± 0.008 µg/mg dry weight) after 4 weeks and decreased further in 8 weeks (0.124 ± 0.025 µg/mg dry weight) (Figure 3).

***In vivo* calcification detection:** After 4 and 8 weeks, tissue implants were collected and evaluated for calcification by Von Kossa staining (Figure 4). Staining samples with Von Kossa silver molecules reacting with calcium in the sample resulted in distinctive black dots. The GluBP group showed a significant calcification, observed via the positive staining area with the tissue. In the modified pericardium, no calcification was observed on the Von Kossa examination. This result confirms that the modified pericardium, which was applied to the decellularization process, helps to minimize the calcification.

Discussion

Tissue regeneration membranes are crucial in vascular grafts. Safety and biocompatibility are all essential considerations for an implant. Our previous studies[5,6] showed that the modified pericardium satisfied the requirements for acellularity, with residual DNA less than 50ng/mg dry sample and tensile strength. Furthermore, the extracellular matrix structure has not altered substantially during treatment. This study was carried out with *in vitro* biocompatibility and calcification analysis to confirm its potential application as a vascular graft. The implant's in vitro safety has been verified by tests conducted following ISO 10993-5, via effect on endothelial cell viability. Additionally, endothelial cells lining the inside of blood vessels help to stabilize and maintain physiology, which is clearly demonstrated by the function of preventing thrombosis.

For materials that interact directly with the bloodstream, the formation of an endothelial lining on the surface can ensure long-term anti-coagulation properties of the material. The ability to support cell adhesion of the pericardium surface was mentioned by Shklover and colleagues[7] after the first 6 and 24 hours of culturing human venous endothelial cells. As in the survey results of Liu et al[4], it was also noted that the surface of decellularized bovine pericardium effectively supported mesenchymal stem cells to adhere and proliferate. Thus, our recorded result of the adhesion of human endothelial cells on the surface of the modified pericardium was confirmed and exhibited a similarity to related studies.

In the calcification assessment, after 4-8 weeks, both *in vitro* and *in vivo* results showed that the modified pericardial membranes did not accelerate the calcium accumulation compared to the GNP control group. Thus, it was implied that our proposed fabrication procedure could limit the risk of calcification, indicating the stability of the membrane upon *in vivo* transplantation.

Conclusion

In this study, the Xenogenic vascular grafts fabricated from bovine pericardial tissue were evaluated for their effects on endothelial cell viability and attachment. The grafts also presented a low level of *in vitro* calcium accumulation. Upon *in vivo* condition, there was no calcification effect, which provided information to predict the stability and potential application as vascular grafts.

Acknowledgement

This research was funded by the University of Science, VNU-HCM, under grant number T2023-134.

References

1. Botes L., Laker L., Dohmen P., Van den Heever J., Jordaan C., Lewies A. and Smit F., Advantages of decellularized bovine pericardial scaffolds compared to glutaraldehyde fixed bovine pericardial patches demonstrated in a 180-day implant ovine study, Cell Tissue Banking, 1-15 **(2022)**

2. Hülsmann J., Grün K., El Amouri S., Barth M., Hornung K., Holzfuß C., Lichtenberg A. and Akhyari P., Transplantation material bovine pericardium: biomechanical and immunogenic characteristics after decellularization vs. glutaraldehyde-fixing, *Xenotransplantation*, **19(5)**, 286-297 **(2012)**

3. ISO, 10993-5: Biological evaluation of medical devices, Tests for in vitro cytotoxicity **(1999)**

4. Liu Z.Z., Wong M.L. and Griffiths L.G., Effect of bovine pericardial extracellular matrix scaffold niche on seeded human mesenchymal stem cell function, *Scientific Reports*, **6(1)**, 37089 **(2016)**

5. Nguyen M.T.N., Doan V.N. and Tran H.L.B., *In vitro* study on chondrogenic differentiation of human adipose-derived stem cells on treated bovine pericardium, *Turkish Journal of Biology*, **43(6)**, 360-370 **(2019)**

6. Nguyen M.T.N. and Tran H.L.B., Effect of modified bovine pericardium on human gingival fibroblasts *in vitro*, *Cells Tissues Organs*, **206(6)**, 296-307 **(2019)**

7. Shklover J., McMasters J., Alfonso-Garcia A., Higuita M.L., Panitch A., Marcu L. and Griffiths L., Bovine pericardial extracellular matrix niche modulates human aortic endothelial cell phenotype and function, *Scientific Reports*, **9(1)**, 16688 **(2019)**

8. Sinha P., Zurakowski D., Kumar T.S., He D., Rossi C. and Jonas R.A., Effects of glutaraldehyde concentration, pretreatment time and type of tissue (porcine versus bovine) on postimplantation calcification, *The Journal of Thoracic Cardiovascular Surgery*, **143(1)**, 224-227 **(2012)**

9. Texakalidis P., Giannopoulos S., Charisis N., Giannopoulos S., Karasavvidis T., Koullias G. and Jabbour P., A meta-analysis of randomized trials comparing bovine pericardium and other patch materials for carotid endarterectomy, *Journal of Vascular Surgery*, **68(4)**, 1241-1256 **(2018)**.

(Received 31st July 2024, accepted 07th September 2024)