TITLE :

Experimental model of bioengineered small caliber vessel trough decellularization of human artery and vein

Author : Alex Pontini

Department of Neurosensorial Specialties, Clinic

of Plastic Reconstructive Surgery and Burn Unit –

Padova University Hospital

Tel : +390498212713

Fax: +390498218199

Address:

Plastic Surgery Clinic,

Vth Floor Padova University Hospital –

Via Giustiniani, 2 – 35100 Padova – Ialy

e-mail : alex.pontini@sanita.padova.it

SUMMARY

Introduction

Synthetic prostheses could offer to vascular microsurgery a possible support to the need for ready-to-use and simply-to-manage small diameter vessels but inherent thrombogenicity and compliance mismatch could represent their drawbacks. Decellularized vessels, indeed, preserving the extracellular matrix and its collagenic and elastic components, could be the more suitable solution in terms of biocompatibility and satisfactory patency rates in the long term. The objects of this study were to develop a new type of biological scaffold after the decellularization of human arteries and veins and to evaluate their integration capability in a rabbit model.

Materials and Methods

The human blood vessels scaffolds were obtained through a detergent-enzymatic decellularization protocol involving the use of Trypsin and Triton X-100 solution. Morphological (hematoxylin and eosin, azan Mallory, Van Gieson and DAPI staining) and ultrastructural (Transmission Electron Microscopy, TEM) analyses, together with DNA quantification, were performed both in native vessels and in decellularized samples. The human artery and vein scaffolds were implanted in a rabbit and maintained for two weeks. Perfusion analyses of the implanted scaffolds were tested during the implant and also before the explants to supervise possible thrombogenic events. After sacrifice of the animal, the implant was analyzed by histological stainings mentioned above.

Results

Hematoxylin and eosin staining showed the effectiveness of decellularization process resulting in biologic scaffolds mechanically suitable for surgical sutures and free from nuclear materials, as confirmed also by DAPI staining and by quantitative evaluation of DNA. The collagenic fiber components of the artery and the vein were not altered by the use of detergents and enzymatic solutions, preserving the native three-dimensional organization around the vascular channels, as demonstred by azan Mallory staining and TEM analyses.

Van Gieson staining demonstrated the preservation of the elastic fibers of the intimae lamina after decellularization. After the implant in a rabbit, the human artery and vein scaffolds resulted completely biointegrated and recellularized by the host cells after two weeks. In particular, the lumen of the scaffolds that remained open also after the implant, was colonized by the endothelial cells. No rejection or inflammation were observed. By clamping the extremities of the implanted matrices, the blood perfusion appeared to be satisfactory confirming the absence of thrombotic events.

Discussion and Conclusions

New biological vascular scaffolds resulting from artery and vein decellularization could be useful for regenerative medicine. The availability of small caliber vascular substitutes could be helpful in such case where autologous vessels are not sufficient or synthetic polymers cause thrombosis. Surgical results in rabbit showed the efficiency of these vascular constructs to restore, regenerate and repair vascular tissue deficiency.

BODY OF THE MANUSCRIPT

Introduction

Tissue engineering research was applied in the last few years to vascular conduit field, aiming to obtain a suitable and ready to use substitute for vessel replacement. Based on the possibilities to obtain *in vitro* a biocompatible structure it is established that is theoretically and experimentally possible to provide vessels that can be employed to replace both diseased than damaged native blood vessels overcoming the massive worldwide clinical need and the poor supply of natural graft and, the same time, offering a better long term performance than the artificial conduits. The challenges reported in literature about the approach of tissue engineering for replacing blood vessels are continuously increasing. It has been reported natural vessel like structure, with similar elastic wall properties that are necessary for the cyclic blood flow loading with similar native vascular diameter to allow a perfect match with the hostvessel. Fundamental are also the result obtained in term of antithrombotic lumen [1, 2]. Particularly important achievement were reached for cardiovascular application but also the potential range of application could easily been expanded to all microsurgical and vascular applications [3]. Tissue engineering has been projected as an alternative treatment to these problems by replacing the damaged tissue or organ function with constructs which are biofabricated based on the required tissue or organ features [4]. In particular, cardiovascular tissue engineering is more valuable and relevant compared to other fields of tissue engineering mainly because it increases life expectancy, preserve the extremities, and provide solutions to a large number of disease [5]. Tissue engineering could at least been see as an interdisciplinary field that applies the principles of engineering and life sciences towards the development of functional substitutes for damaged tissues. It is strictly related to the fundamental concept of utilizing the body's natural biological response to tissue damage in conjunction with engineering principles [6, 7]. Besides, tissue engineering is planned to produce biomimetic constructs, which resemble normal tissues. Biomaterials technologies for vascular replacement must obtain an ideal graft that could overcome the needing of autologous vessel, that often it's not applicable, but, at the same time, providing similar properties.

In fact the ideal bioengineered vascular substitute must be not thrombogenic, overall in small caliber vessel, and also when a long graft is needed. Thrombosis mechanism into vascular substitutes, especially in artificial one, is the main cause of obliteration and subsequent failure of most microvascular prostheses. Autologous native vessel, are the most currently used material for small-diameter arterial replacement. Immune acceptance is a major advantage offered by this technique but the time of dissecting, harvesting and preparing autologous graft limited the microvascular emergency surgery and, in elective surgery, it could be possible that no one suitable vessel could be harvested. For that reason, the tissue engineering was applied to improved prosthetic performance at the blood biomaterial interface. Different approach were described to optimize vascular bioengineered conduits as completely bio-resorbeable vascular prostheses with the capacity for induceregeneration and growth of a new vascular segment, biologic scaffold enhanced by stem cellseeding, decelularizzed native vessel with or without cell enhancement. In vitro and in vivo study of all these different approaches shown the possibility to overcome the limitations of the artificial prostheses that are nonviable and based on allogenic materials lacking the capacity of growth, repair, and remodeling. The use of bio engineered vascular conduit are fundamental in small caliber vessel where the artificial replacement is affected by a very low patency rate meanwhile the possibility to obtain bioengineered large vessel replacement is actually less important due to the satisfactory result and still less expensive use of artificial or homograft conduit. Synthetic prostheses offer to microsurgery a possible solution for microvascular need of a ready to use and simple to manage small diameter vessel. Availability in multiple different diameters and lengths, uncomplicated storage associated with easy handling are some of main advantages of such grafts; nevertheless, inherent thrombogenicity and compliance mismatch could represent their drawbacks. The research aim is to obtain and ideal prostheses, particularly in term of biocompatibility and satisfactory patency rate in long period of time. In fact, similar outcomes as large vessel replacement were not achieved in microvascular surgery. Best performance was obtained when the blood flow is high and the resistance is low, because those conditions allowed to overcome possible thrombogenic events that occur in large part in small-diameter prostheses. Multiple strategies were studied to overcome these limitationsapplying tissue engineering techniques.

Ideally artificial conduit ready to use should be composed of viable tissue, able to contract in response to hemodynamic forces and chemical stimulation, and secrete physiological blood vessel substances. Anastomoses using artificial prostheses should also allow complete healing without immunologic reaction, remodeling according to surroundings environment, and even have the ability to grow when placed in children. For all that reasons our aim was to employ for the first time decellularized human small caliber vessel to replace artery and vein in vivo in an animal model to asses their biocompatibility, stress response, recellularization and adaptation in order to provide ready to use small vessel for microsurgical purpose.

MATERIALS AND METHODS:

Four pieces (two artery and two vein) from the hand of two different patient envolved in crush injuries were collected. Such anatomical components do not have any macroscopic damage and were present in structure not necessary for reconstructive purpose (Fig. 1).

Both the artery than the vein were submitted to a decellularization protocol as purposed by Sheridan in 2012 through a detergent-enzymatic decellularization protocol involving the use of Trypsin and Triton X-100 solution. Morphological (hematoxylin and eosin, azan Mallory, Van Gieson and DAPI staining) and ultrastructural (Transmission Electron Microscopy, TEM) analyses, together with DNA quantification, were performed both in native vessels and in decellularized samples. (8). The decellularized vessel were implanted in vivo in a rabbit model.

According to the Helsinki protocol for animal studies, two male adult rabbit ranging from 3,5 to 4,5 kg were submitted under general anaesthesia to dissection of their femoral vessel (diameter < 1 mm) and a portion of femoral vein were retained. Under microscope magnification the decellularized vein were grafted with prolene 10/0 sutures. After the clamp release the blood flow and the anastomoses patency were assessed by the milking text at the time 0 and after 10 and 20 minutes. The same procedure were realized for the two arterial implants(Fig. 2). The rabbits receveid both antibiothic than analgesic drugs with regularly veterinary supervision. After two weeks the animals were sacrified after the control of patency in vivo and the implant were collected and submitted to ematossilin-eosin and Azan Mallory stain followed by electronic microscope evaluation and DNA quantification.

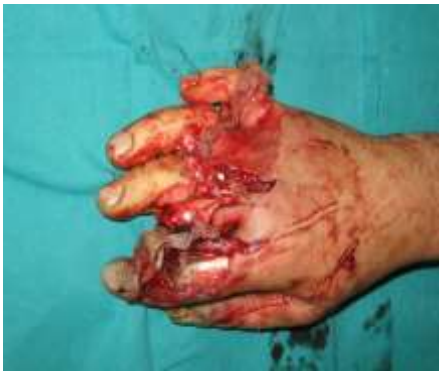
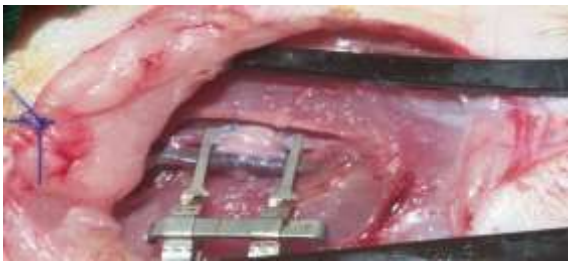
A)  B) 

Fig 1 : A) Crush injuries with not microsurgical indication B) Portion of not damaged digital artery retained from avulsed part.

A) 

B) 

C) 

Fig 2 : A) Decellularized human arterty before implantation B) Microsurgical implant as artery graft on femoral rabbit artery C) Sacrifice after two weeks. Biointegration of the artery graft.

RESULTS :

Hematoxylin and eosin staining showed the effectiveness of decellularization process resulting in biologic scaffolds mechanically suitable for surgical sutures and free from nuclear materials, as confirmed also by DAPI staining and by quantitative evaluation of DNA (Fig.3) The collagenic fiber components of the artery and the vein were not altered by the use of detergents and enzymatic solutions, preserving the native three-dimensional organization around the vascular channels, as demonstred by azan Mallory staining and TEM analyses.

Van Gieson staining demonstrated the preservation of the elastic fibers of the intimae lamina after decellularization (Fig. 4) All the animals survived to the microsurgical procedures, only one case showed a superficial infection without affecting the anastomoses site. The sacrifice were performed under general anaeshesia showing the patency of the graft after two weeks at the milking test. At the TEM evaluation both the human artery than the vein scaffolds resulted completely biointegrated and recellularized by the host cells In particular, the lumen of the scaffolds showed a complete patency and an initial colonization by the endothelial cells. No rejection or inflammation were observed (Fig. 5).

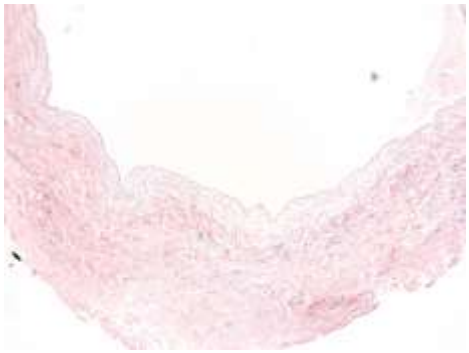


Fig 3 : Decellularized artery. Hematossilin- eosin stain. 20x. Decellularized artery observed at TEM. No cells are present with complete preservation of EC and lumen.

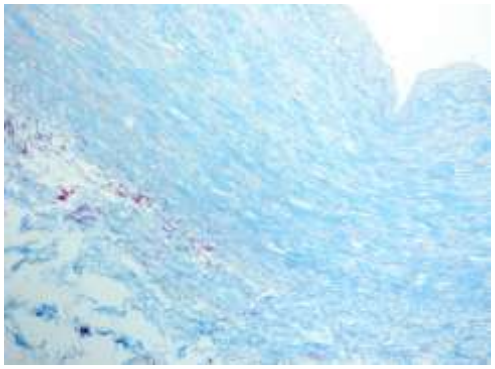


Fig. 4 : Decellularized artery. Van Gieson stain at 20x. Complete preservation of the elastic fiber and the fundamental steructure of the endothelium, necessary for cell infiltration and recellularization

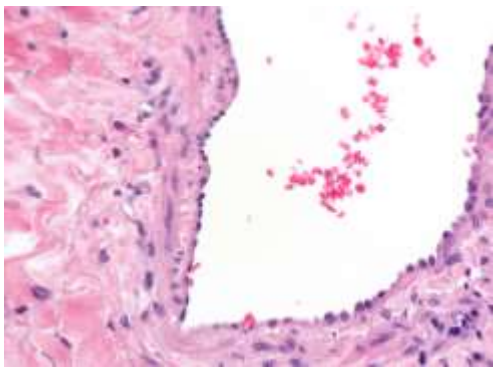


Fig 5 : Recellularized artery. Presence of viable cell and correct recellularization of the lumen. Not inflammation cells. Not trombosis phenomenon and good wide lumen.

DISCUSSION

Need for vascular grafts are also important in reconstructive surgery, vascular trauma, organ transplantation, so a large number of vascular conduit are needed in clinical daily practice.Besides are must to be considered that a significant morbidity and high economical costs are associated with autologous vessel preparation. Multiple factor area at least involved in a widely recognized need for an efficient, readilyavailable and simple to manage small-diameter vascular graft. The first step on not autologous vessel replacement was constituted by artificial vessel based on different permanent material as polyurethane, polyethylene terephthalate and polytetrafluoroethylene (ePTFE). All these prosthetic materials have proved to be inferior to autologous conduits, especially for small caliber. Low patency rate outcome with important thrombosis risk, infection andlow performance at anastomosis site have determined the progressive discharged of artificial conduits. [9] The biological approach provided by tissue engineering was thought to allow a better performance, compatibility and host matching. [10]There have been so many attempts to develop a small-diameter vascular graft made of synthetic or natural polymers. The synthetic polymeric materials include polyethylene terephthalate and expanded polytetrafluoroethylene (ePTFE) as described from Teebken in 2002. Although these polymeric vascular grafts have been successfully employed to replace blood vessels above 6 mm in ID, these polymeric grafts cannot be used for treatment of small diameter vascular diseases due to thrombus formation as demonstrated from Veith in 1986 and then from Chard in 1987. Coating of the intimal side with antithrombogenic materials, such as heparin was the approach for example of Devine in 2001, polyethylene oxide was the attempt of Kidane in 1999, or, previously, with endothelial cells as described from James in 1997, has been applied to solve this problem as we also reported below. Unfortunately these approaches still remaining doubtfull in vivo and in long-term and are considered unsuccessful. For that reason tissue engineered blood vessels (TEBV) arising as a a promising approach to address the shortcoming of such problems. Many design criteria have been proposed for the development of blood vessels scaffold as it's possible to read in the works of Conte et al. in 1998; Mitchell et al in 2003 and Teebken et al. also in 2003. Scaffold must be biocompatible, i.e. non thrombogenic, non-immunogenic, and resistant to infection, all of which are associated with a confluent, quiescent, non-activated endothelium.

Furthermore, it must induce an acceptable healing response that does not result in inflammation, hyperplasia, or fibrous capsule formation, and, ideally, leads to the integration of the graft into the body such that it eventually becomes indistinguishable from a native vessel. It must possess appropriate mechanical properties, which include physiological compliance, the ability to withstand long-term hemodynamic stress without failure, and no susceptibility to permanent creep that can lead to aneurysm formation. Scaffold must have an appropriate permeability to water, solutes, and cells and must exhibit physiological properties, such as vasoconstriction/relaxation responses. Finally, easy handling and suturability are crucial for such vessels to be viable from a surgical standpoint. These design criteria are quite challenging given the demanding mechanical environment of the cardiovascular system. Although different approaches attempt to meet these criteria in different ways, it is widely held that 3 components are necessary for these criteria to be met: a biocompatible component with high tensile strength to provide mechanical support (collagen fibers or their analogue);a biocompatible elastic component to provide recoil and prevent aneurysm formation(elastin fibers or their analogue); a non-activated, confluent endothelium to prevent thrombosis.Decellularized tissue, often in the form of a xenogeneic, can serve as a naturally available scaffold. Examples of such scaffolds can be realized by Lantz in 1993, who used the small intestinal submucosa (SIS) as a vascular implant. The SIS was decellularized and then implanted in aorta, carotid and femoral arteries of dogs. The grafts resulted completely endothelialized at 28 days post-implantation. At 90 days, the grafts were histologically similar to normal arteries and veins and contained a smooth muscle media and a dense fibrous connective tissue adventitia. Follow-up periods of up to 5 years found no evidence of infection, intimal hyperplasia, or aneurysmal dilation. One infection-challenge study suggested that SIS may be infection resistant, possibly because of early capillary penetration of the SIS (2 to 4 days after implantation) and delivery of body defenses to the local site. Kaushal, in 2001, has employed a decellularized porcine iliac arteries, seeded them with endothelial progenitor cells (EPCs), and implanted the constructs into ovine carotid arteries. These TEVG constructs remained patent out to 130 days and were remodeled into neovessel, whereas the unseeded control group occluded within 15 days. These results indicate that decellularized vascular scaffolds are susceptible to early failure unless first undergoing endothelialisation or additional modification.

In fact, Simon in 2003, shown as elements of the ECM are exposed to physical and chemical stresses during theprocess of decellularization, which can adversely affect the biomechanical properties of the ECM. This deterioration might ultimately lead to degenerative structural graft failure. Additional drawbacks of decellularized materials included the inability to modify the ECM content and architecture, the variability among donor sources, and the risk of viral transmission from animal tissue.In 2011 Quint has developed a unique method of developing decellularized tissue for small diameter arterial grafts using biodegradable polymers. They developed a different approach to arterial tissue engineering that can substantially reduce the waiting time for a graft. Tissue engineered vessels (TEVs) were grown from banked porcine smooth muscle cells that were allogenic to the intended recipient, using a biomimetic perfusion system. The engineered vessels were then decellularized, leaving behind the mechanically robust extracellular matrix of the graft wall. The acellular grafts were then seeded with cells that were derived from the intended recipient, EPC or EC, on the graft lumen. TEV were then implanted as end-to-side grafts in the porcine carotid artery, which is a rigorous test-bed due to its tendency for graft occlusion. The EPC-and EC-seeded TEV all remained patent for 3D in this study, whereas the contralateral control vein grafts were patent in only 3/8 implants. Going along with the improved patency, the cell-seeded TEV demonstrated less neo intimal hyperplasia and fewer proliferating cells than did the vein grafts. Proteins in the mammalian target of rapamycin signaling pathway tended to be decreased in TEV compared with vein grafts, implicating this pathway in the TEV's resistance to occlusion from intimal hyperplasia. These results indicate that a readily available, decellularized tissue-engineered vessel can be seeded with autologous endothelial progenitor cells to provide a biological vascular graft that resists both clotting and intimal hyperplasia. Decellularized xenografts have been identified as potential scaffolds for small-diameter vascular substitutes. Xiong, for example, in 2013 shown a work that has aimed to develop and investigate a biomechanically functional and biocompatible acellular conduit using decellularized porcine saphenous arteries (DPSAs), through a modified decellularization process using Triton X-100/ solution and serum-containing medium. Histological and biochemical analysis indicated a high degree of cellular removal and preservation of the extracellularmatrix. Bursting pressure tests showed that the DPSAs could withstand a pressure of 1854 ± 164 mm Hg. Assessment of in vitro cell adhesion and biocompatibility showed that porcine pulmonary artery endothelial cells were able to adhere and proliferate on DPSAs in static and rotational culture. After interposition into rabbit carotid arteries in vivo, DPSAs showed patency rates of 60% at 1 month and 50% at 3 months.

No aneurysm and intimal hyperplasia were observed in any DPSAs. All patent grafts showed regeneration of vascular elements, and thrombotic occlusion was found to be the main cause of graft failure, probably due to remaining xenoantigens.Patency properties of vascular grafts could be considered the key point to obtain a conduit with a relevant chance of stable replacement of damaged vessel. In fact, thrombosis is the main mechanism of obliteration and subsequent failure of most microvascular anastomoses using artificial conduits. Various methods have been recorded to avoid it, such as coatings with antithrombotic drugs, as heparin, hirudin, aspirin, or tissue factor pathway inhibitor [21]. There have been attempts to emulate the endothelial cellular surface which, coated with heparan sulphate proteoglycan, produces a negative surface charge which helps to prevent platelet adherence. Some prostheses are therefore coated internally with heparin sulphate, which is quickly degraded, and some materials with an electronegative surface have been created, with uncertain results [22]. So far, many researchers have described seeding endothelial cells in conduits.

APPLICATION FIELD OF HUMAN DECELLULARIZED VESSEL

All the tissue engineer attempts to obtain the ideal vascular graft, particularly in the field of microsurgery , show high costs and are mostly time consuming. Instead the employ of human decellularized tissue provide at the same time a native vessel with maintain the capability of biointegration, cells reconstitution, particularly in the very small vessels where the endothelisation process seems to be faster and better than in the large vessel. We realized a model of inverse xenograft where an animal recipient receveid a scaffold from an human donor. We also firstly demonstrate by a preliminary report an alternative solution that seem simple, easy to apply, fast and satisfactory in term of good microsurgical handling, pressure resistance, patency and recellularization induction. Further study are needed to evaluate the endothelization and patency rate in a long period of time, blood flow assesing by eco doppler imaging and stress resistance by mechanical testing. At that time the application field of such construct seems to be wide ranging from ready to use artery graft in Hand surgery with more resistance and no phenomenon of intimal hyperplasia as the vein graft or to obtain a longer flap pedicle in reconstructive surgery.

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