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Bioengineering of Vascular Conduits

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1. 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 host vessel. 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 bio fabricated 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.

Moreover, the main objective of tissue engineering is the restoration of function through the delivery of living elements which become integrated in the patient [8].Tissue engineering strategies have three basic components: firstly, the cells or source which must express the appropriate genes and maintain the appropriate phenotype in order to preserve the specific function of the tissue, secondly, the bioreactive agents or signals that induce cells to function, and thirdly, the scaffolds that house the cells and act as substitute for the damaged tissue [9, 10]. The source may either be embryonic stem cells (ESC) or adult stem cells(ASC) in origin, the scaffolds may be categorized as synthetic, biological, or composite, and the signals may include growth factors/cytokines, adhesion factors, and bioreactors [11]. Develop a bioengineered tissue is due to a precise clinical needs and different ways were searched to obtain the answer (Figure 1). So, joint to the ideal construct is also the research of the best tissue engineering approaches in terms of biocompatibility, feasibility and costs.

So from 1999 till now several studies, both in vitro than in vivo, were presented, particularly inherent on vessel scaffold, which a large number of information about cell sources, technology application, outcomes and future perspectives [12].

Until nowadays the most encouraging result are limited in basic and experimental research but the rapidly increasing of optimal result permit to access to numerously clinical trials. 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 induce regeneration and growth of a new vascular segment, biologic scaffold enhanced by stem cell seeding, decelularizzed native vessel with or without cell enhancement. In vitro ed 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 limitations applying 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.
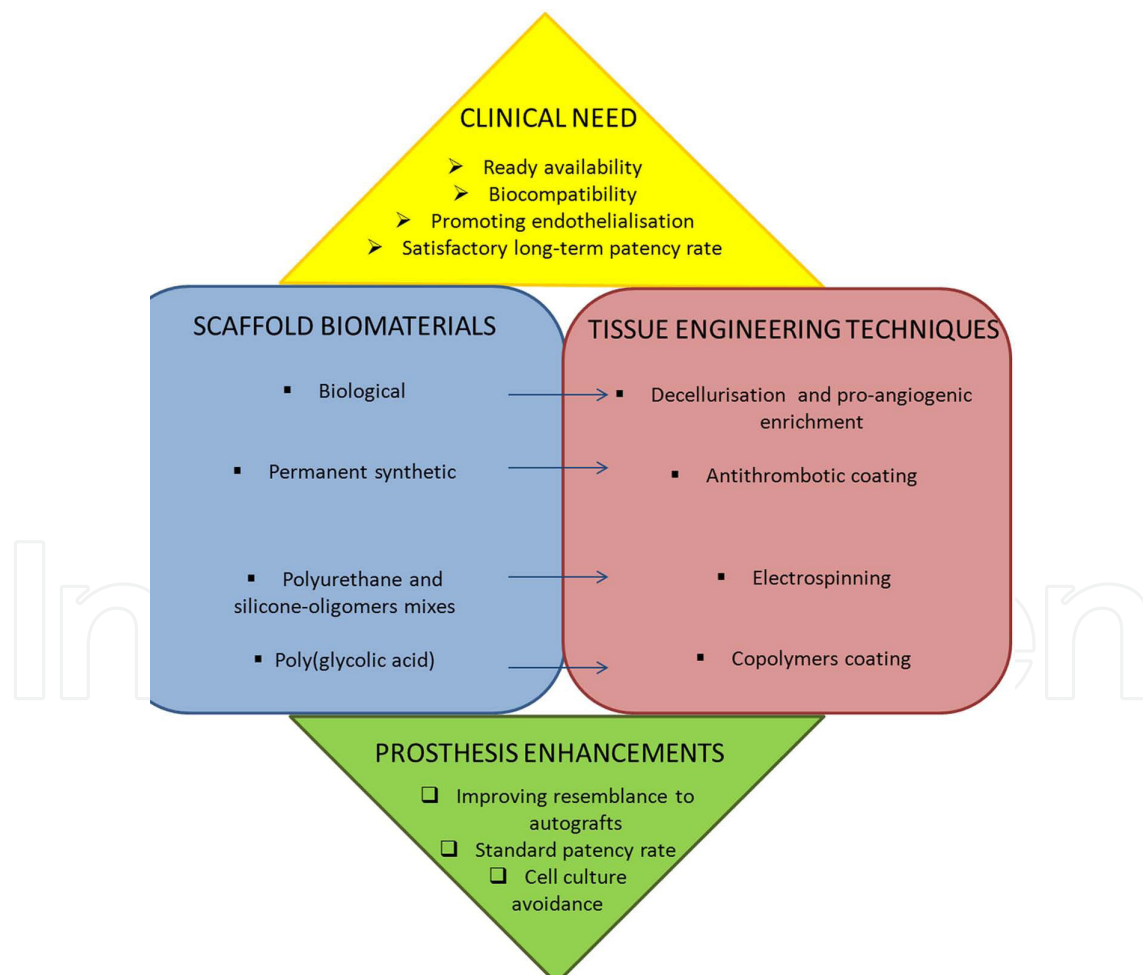

Figure 1. Clinical need and bioengineering of vascular conduit. Adapted from: Pontini et al. "Alternative Conduits for Microvascular Anastomoses" Surg Innov. 2013 Aug 20;21(3):277-282

2. Blood flow and functional implication for vascular replacement

Blood vessels are the conduit that allows the transportation in the human body to the organs and tissues of blood, oxygen, nutrients and catabolities removal. Small caliber arteries (< 6 mm), account for most of deaths in the United States every year [1] Atherosclerosis, due to numerously damage mechanism of *media and intima tunica* in vessel lumen, cause a progressive occlusion that could lead to a severe blood flow impairment and organ failure. Most common vascular disease based on critic vessel damage could be the cardiac infarction subsequent to a coronary artery occlusion, claudication or chronic ulcer because peripheral arterial disease, or stroke due to occlusion of carotid or cerebral arteries. Arterial replacement is widely considered a common treatment for vascular disease, accounting for more than 1.4 million arterial bypass operations performed annually in the United States [2] The "gold standard" treatment is based on autologous vessels employment such saphenous veins and mammary arteries but one-third of patients are lacking for suitable veins, often for general vascular disease or past harvesting in vascular procedures. Nevertheless autologous vein graft have shown high patency rate failure in the long term period principally due to intimal hyperplasia [13]. 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 are at least involved in a widely recognized need for an efficient, readily available 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 and low performance at anastomosis site have determined the progressive discharge of artificial conduits. [14]

The biological approach provided by tissue engineering was thought to allow a better performance, compatibility and host matching. [15]

3. Bioengineered vessel: definition and development

Vessel replacement is a clinical need in many vascular field. Blood vessel diseases, such as atherosclerosis and arteritis as reported from Ross in 1993 and Wilcox in 1996. The Chronic Venous Insufficiency (CVI) as described from Moriyama in 2011 and thrombosis remain globally the major vascular problems. Therapies for such diseases often require replacement of those vessels with vascular grafts. Autologous arteries or veins are the best substitutes for small-diameter (internal diameter < 6 mm) vessels as shown also from Tu and colleagues in 1997. However, in some cases of acute vascular disease, amputation or previous harvest, the implant of autologous vessels could be limited of the biodisponibility of patient. The most

important described application were the arterial occlusive disease such coronorapathy and ischemic periferical condition treated with artery by-pass [16]. Furthermore were described use also in thoracic surgery and for dialysis access [17, 18].

Literature review of the historical approach in vascular bioengineering shown multiple and different approach worldwide to obtain the best biocompatible and long lasting replacement. The research start from modification of synthetic material and was develop to biologic material assessment. 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 1998, 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 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.

In 1986, Weinberg and Bell generated what was widely regarded as the first tissue-engineered blood vessel substitute, consisting of cultures of bovine endothelial cells (ECs), smooth muscle cells (SMCs) and fibroblasts embedded in a collagen gel. However, the graft lacked sufficient strength and was unsuitable for implantation. This construct was evaluated in vivo as an arterial implant only after reinforcement with Dacron® as shown from Matsuda in 1995. Various methods of improving the mechanical properties of collagen gels (e.g., crosslinking agents such as glutaraldehyde) have been investigated, but none has proven to yield a structurally stable tissue-engineered vascular grafts (TEVG) as reported from Charulatha in 2003. As an alternative to collagen for natural ECM-based scaffolds, fibrin holds particular promise because of its ability to induce collagen and elastin synthesis and improved mechanical properties as shown from Swartz in 2005. Furthermore, encouraging result, even if in larger diameter vessel, was achieved by combining fibrin gels with biodegradable polymeric scaffolds followed by seeding of autologous arterial-derived cells, from the group of Tschoeke in 2009 as also endothelialized vessels have been successfully implanted in the carotid arteries of sheep from Koch and his team in 2010.

Decellularized tissue, often in the form of a Xenogenic, can serve as a naturally available scaffold. Examples of such scaffolds were 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 the process 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 neointimal 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 extracellular matrix. 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.

The purpose of this work was to evaluate the effects of using a decellularization protocol in samples of rabbit and human arteries and veins, involving mechanical processes and enzymatic reactions in order to obtain a scaffold suitable for the implantation in an organism recipient. Subsequently, a further purpose of this thesis is to obtain a new type of scaffold derived from skeletal muscle decellularization.

Resuming all the mentioned aspect we can confirm that autologous vessels and vascular allografts are the most reliable vessel source, but often their supply are insufficient for their widespread application [19]. For that reason we saw the use of synthetic prosthetic vessel, before tissue engineering onset. The principal vessel typology needed in a large number of

vascular substitution is represented by small caliber vessel (< 2 mm) and synthetic material failed because a low patency rate due to thrombosis [20].

As listed before, the thrombogenic surface of many engineered materials becomes particularly relevant in microvascular grafts.

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.

Laube et al. reported a patency rate of 90% in 27 months for ePTFE prostheses used in coronary bypass, after additional incubation with endothelial cells which allowed them to adhere to the material [23]. The major limitation of this method is the need for cell cultures and withdrawal of tissue from the patient, and in any case it remains a two-step procedure. A tubular structure of ePTFE is also left in place, with the risk of later infection. Constructs composed entirely of cells (Tissue Engineered Blood Vessels: TEBV) have been studied to overcome these complications [24]. Although the method promises amazing results, it is time-consuming and very expensive (Fig. 2).

To avoid the cost of cell cultures, many researchers have tried to improve endothelial coverage of prostheses by coating them with endothelial-friendly compounds with good haemocompatibility. E-PTFE prostheses have been coated with perlecan [6] and endothelial-specific adhesion proteins such as fibrin–and hirudin [25, 26].

Fibronectin coating seems to be a successful method, apart from loss of lining at high flow rates. This is why a functional ligand for fibronectin was used, with covalent binding of short peptide sequences (Arg-Gly-Asp, RGD) to improve cell adhesion. Instead of coating prostheses with the above substances, another possibility would be to use absorbable, already biocompatible biomaterials, to make entire prostheses.

In spite of all these experiments, endothelialisation in various types of vascular prostheses has been shown in animals but never satisfactorily in humans.

The type of material is not the only essential point to allow the endothelialisation but it's fundamentally correlate with the physiopathology of endothelialisation, which takes place in three main ways: trans-anastomotic endothelialisation; transmural, and due to 'fall-out' of circulating pluripotent cells. Therefore, trying to enhance endothelialisation means acting on each of these three modalites of cell growth.

Trans-anastomotic endothelialisation (TAE) appears to be very difficult in humans. Early studies on synthetic prostheses report that they cannot be longer than 0.5 cm, even after prolonged implantation. In spite of a long period of observation, internal endothelialisation has not been observed in humans, except in sites of anastomosis [27].

Several factors have been observed to influence this, such as species, senescence, anatomic dimensions of the vessel, and prosthetic materials, but even in animals TAE is limited [28].

Study of endothelial cells, both human and canine, compared in vitro, suggest that human cells have a greater potential for migration but a lower capacity for adhesion, which may explain the lack of re-endothelialisation in vivo, when blood flow may obstruct cell adhesion [29].

Instead, the transmural pathway seems to enhance rapid endothelialisation, according to recent studies on materials with sufficient porosity. Pore size takes on importance in these studies, since the prosthesis must be sufficiently large to allow cell growth, but not too large to cause loss of intercellular adhesion [30].

Materials with differently sized pores inside and outside the conduit have even been experimented, in order to obtain an biocompatible surface internally and a colonizable one externally.

Pore size also alters the haemocompatibility of biomaterials, as well as their compliance and degradation time. An optimal pore size for vascular engineering has been hypothesized, ranging from 30 to 50 microns. It appears that smaller pores would not allow growth of endothelial cells, and larger ones would cause excessive leakage of blood.

Pores in the walls of prosthetic materials can also avoid intimal hyperplasia.

It has been hypothesized that a thrombus initially deposited on the walls of the prothesis later organizes itself into muscle-like tissue, which then gives rise to intimal hyperplasia.

The precocious growth of endothelial tissue would avoid thrombosis and thus the consequent cascade of events leading to intimal hyperplasia. Increased pore size causes increased failure compliance of the material.

Several studies have shown that vascular implants with fibers organized in a circular fashion do not cause dilation. Even surgical technique could determine an influence in local hyperplasia at the anastomotic site [31].

Anyway, this is local, since cells undergo mechanical stress and are thus conditioned in their spatial orientation. ''Fall-out healing'' leads to the formation of endothelial islands, with no connection with the formation of trans-anastomotic or transmural tissue. This is a late phenomenon that appears to be the mechanism for repairing small vascular lesions however, recent studies show how this mechanism may be enhanced, by attracting EPC cells to participate [32] One of the last procedures presented to improve the patency rate is constituted from nano modification of the vascular lumen with heparin addition to obtain a very low trombogenic vessel The employment and the wide possibility offered in the vascular field from the use of nanotechnology was also investigated [33].
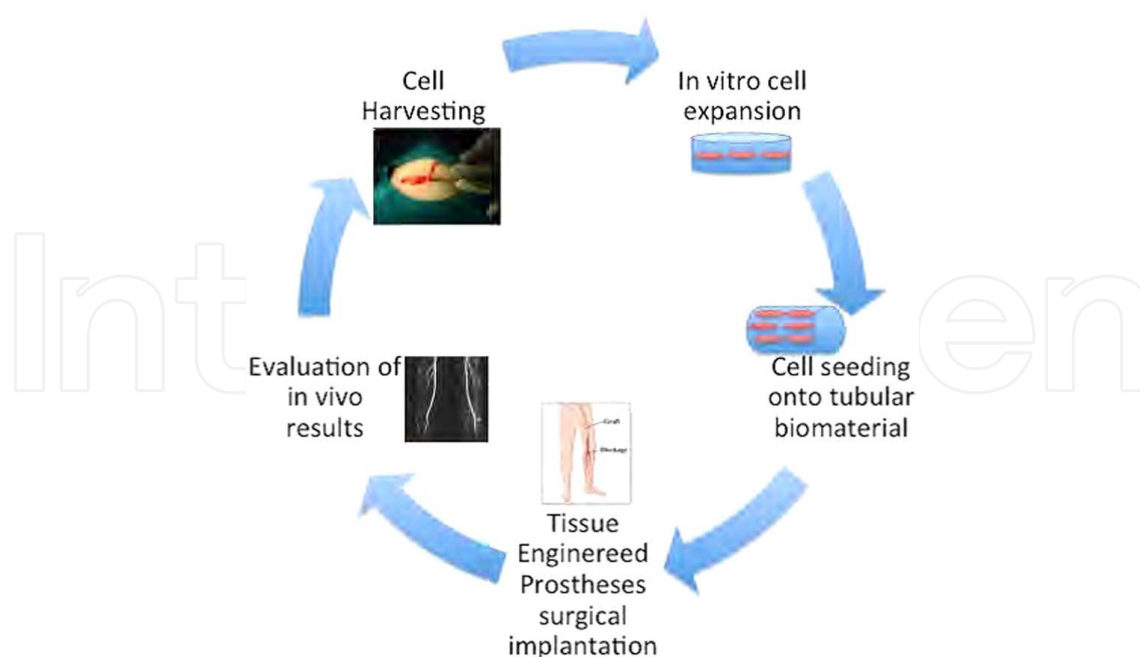
Figure 2. The in vivo –in vitro production of a tissue engineered prostheses. Adapted from: Pontini et al. "Alternative Conduits for Microvascular Anastomoses" Surg Innov. 2013 Aug 20;21(3):277-282

4. Methods and result in vascular tissue engineering

Many research groups have approached the problem of developing the ideal prosthesis in a variety of ways. Their main activities ranges around the triad scaffold-cells-growth factor. Scaffolds are ideal biomaterials for conduits, and cells can be seeded and cultivated on them, after preconditioning with various growth factors. To date, despite numerous scaffolds that have been manufactured trough varied forms of tissue engineering techniques, the construction of an entirely biomimetic blood vessels is still underway. To achieve a successful clinical application of tissue-engineered blood vessels, the bio fabrication of vascular grafts necessitates a vigorous yet time-efficient biotechnological process [34].

Several tissue engineering strategies have emerged to address biological flaws at the blood-material interface of the synthetic scaffolds, hence, paving the way to vascular cell seeding and design of bioactive polymers for *in situ* regeneration.

Moreover, advances in biomaterial design have been directed towards the generation of suitable materials that does not only mimic the native vascular tissue's mechanical properties but also promote cell growth, inhibit thrombogenicity, and facilitate extracellularr matrix production. In addition, an important characteristic of artificial scaffolds in advanced biomaterial vessels substitutes is not just the tolerance of the cells but the capacity to mimic the natural ECM in order to regulate extent and strength of cell adhesion, growth activity, cel ldifferentiation, and maturation to the desired phenotype [35, 36].

The extracellular matrix proteins such as collagen, elastin, fibronectin, vitronectin, and laminins which mediate cell-material adhesion have been thoroughly assessed in an earlier review [37].Materials for vascular replacements should be biomimetic in such a way that they should be resistant not only to thrombosis, but also to inflammation, and neointimal proliferation, and for all intents and purposes, they should resemble the native vessels [37]. For these reasons, it is necessary to investigate the physical, chemical, and biological properties and modifications of materials to further understand the molecular mechanism of the cell material interaction [37].

The lack of endothelial cells on the luminal surface of the artificial grafts contributes to synthetic graft thrombogenicity and promotes intimal proliferation within the graft. Endothelial cell (EC) seeding on the synthetic grafts has been attempted to mitigate these problems. The first group to perform endothelial cell (EC) isolation and their subsequent transplantation into vascular graft were Herring and his group [38]. Current researches indicate the significance of such process in vascular tissue engineering. The polymer surfaces which have been formerly investigated for endothelial attachment, proliferation, and function had been listed in an earlier review [39]. On the other hand, the synthetic polymers for reconstructing blood vessels for clinical practice which are based on polyethylene terephthalate (PET) or polytetrafluoroethylene (PTFE) had been previously reviewed [37].

Furthermore, blood vessel stem cells have been studied in combination with recent and alternative types of scaffolds/polymers. Parallel to this, in scaffold-based blood vessel engineering, bioreactors and pulsatile flow systems, designed by many scientists, have been found to progress the mechanical property of the engineered blood vessels by augmenting the deposition and remodeling of extracellular matrix as well as the maturation and differentiation of self-assembled micro tissues [40].

Bioreactors, which were originally designed for industrial use, have high degree of reproducibility, control, and automation for specific experimental bioprocesses and these have been the reasons for their transfer to large scale applications including vascular tissue engineering. The bioreactors allow scientists to manipulate the environment and the parameters such as pH, temperature, pressure, nutrient supply, and waste removal in order mimic the *in vivo* physiological condition and allow biological or biochemical processes to occur and subsequently develop the desired tissue [41].

Taken together, the formation of a microvasculature within a tissue-engineered organ or tissue will depend on multiple factors: the biochemical environment, EC type, the micro-architecture presented by the scaffold material, and mechanical signals [42]. Due to the goal of developing biomimetic blood vessel scaffolds, many groups have designed such biomaterials.

The polymers used in scaffold fabrication for tissue engineered blood vessels started from polyglycolic (PGA) to varied types such as polyglycolic acid-poly-L-lactic acid (PGAPLLA), Collagen/Elastin, chitosan, Poly (glycerol sebacate) (PGS), and very recently polyglycolide knitted fiber, and an L-lactide and ε-caprolactone copolymer sponge crosslinked to amniotic fluid. Furthermore, amniotic membranes have been used as scaffolds which signify that

scaffold based tissue-engineered blood vessels can be fabricated from autologous cells at a reduced manufacturing period.

Resuming the most important that have been used as scaffolds, we could generally subdivided them into four main categories: permanent material included polyurethane material, resorbeable material and biological ones as allografts, xenografts, and derived products.

4.1. Permanent materials

Synthetic polymers such as e-PTFE and Dacron have not provided a satisfactory results in small diameter vessels (<6 mm). In literature are present a wide number of works on such implants as also the problems linked to the permanent synthetic polymers which are often difficult to overcome. Many works report variable results in terms of long-term patency. These materials have been implanted in humans, but do not develop an endothelialised surface, thus causing platelet adhesion and the development of a fibrin layer which may lead to thrombosis. Later failure may also be due to thrombosis after stenotic occlusion of the vessel consequent upon the development of endothelial hyperplasia. Several methods have been applied in the past to reduce thrombosis risk by application of tissue engineering procedure as application of anti-thrombotic drugs in their surface or surface ligands [43]. Bordenave et al illustrates how this procedure, in time, has moved from one-stage to two-stage techniques although, in spite of discouraging results, not much space was devoted to clinical practice, mainly because of its three most serious limitations: the impossibility of executing these techniques in emergencies; the need for prior withdrawal of cells; and the need for a GLP laboratory to treat human cells [15].

4.2. Polyurethane materials

These polymers are biocompatible and highly versatile, since their tensile strength and radial compliance vary according to segment composition, stiff segments being responsible for tensile strength and soft segments for elasticity [31] Originally produced as permanent biomaterials, they do deteriorate in vivo, due to oxidation and enzymatic and cell-mediated degradation, with the result that their biostability is under revision.

The differing composition of PU segments may lead to products with various degrees of biostability. PU have been combined with highly crystalline segments such as polycarbonates and silicon oligomers to increase their stability [34].

The most relevant development of small diameter vascular prostheses composed by PU revealed a total of 22 articles on polyurethanes, 14 in vitro, 4 on production of material and its medical properties, and only 4 in vivo.

The cellular compatibility of several PU (associated with other substances) has also been studied according to method of preparation, e.g., the use of porous structures. Electrospinning has been applied to other materials in the field of vascular engineering, and produces small diameter fibers with good tensile strength on the final material.

As regards chemical composition, PU has been combined with silk fibroin, showing better histocompatiblity of pure PU after implant in rat muscular tissue [44]. Many experiments have also been made on the mixed-composition PU PDMS (polydimethylsiloxane), a silicon-based polymer. In this case, PDMS not only increased biostability but also increased haemocompatibility and immunocompatibility. In vivo studies show encouraging long-term viability: in one, a PEUU/PDMS polymer was created with the spray phase inversion technique in a tubular form with two-phase porosity [45].

It showed good re-endothelialisation 24 months after implant. Another in vivo study in this series used poly(ester urethane)urea (PEUU) combined with a thrombogenic polymer not similar to a phospholipid, poly(2-methacryloyloxyethyl phosphorylcholine-co-methacryloyloxyethyl butylurethane) (PMBU), to create a fibrillar scaffold by electrospinning, with good tensile strength and compliance. The association with PMBU made the PU less prone to platelet deposition and hypertrophy of muscle cells. The in vivo patency of 1.3-mm conduits implanted in rat aorta after 8 weeks varied from 40% for pure PU to 67% for PU PMBU [46].

4.3. Bioresorbable materials

These materials may be synthetic or biopolymers already constituting the extracellular matrix. The most common absorbable biomaterials are polyesters. This category contains poly(α-hydroxylester poly(L-lactic acid) (PLLA), poly(-glycolic acid) (PGA), polylactone polyorthoesters (POE) and polycarbonates. When these materials are implanted in vivo, their polymeric structure is subject to a hydrolysis process and metabolization of the resulting products, such as lactic and glycolic acids.

Their safety and biocompatibility are now established.

However, the mechanical resistance of these products does not reach the desired levels – an anticipated outcome, as PGA was originally in the form of a non-woven fabric, and thus does not have measurable tensile strength [28]. Most studies have therefore concentrated on preconditioning methods to increase resistance, e.g., use of pulsatile flow bireactors, alternative techniques of cell culture, and administration of various growth factors [47.]Another substantial problem with PGA is its stiffness, which does not confer the elastic properties of native arterial tissues. In this case too, the use of copolymers has improved results. A fibrillar scaffold based on polyglycolic or polylactic acid coated with a 50:50 L-lactate or L-caprolactone (PCLA/PGA or PCLA/PLA) copolymer has been specifically tested for vascular repair, resulting in compliance closer to that of the original vessel with better surgical handling [48].

One study showed how PGA-based matrices have greater cellularity and production of proteins of extracellular matrices based on PHAV and P4HB. The authors explained this phenomenon as due to the higher porosity of PGA (> 90%), yielding a contact surface greater than that of cells.

To support the remodeling process in vivo, a biomaterial that functions only as a temporary absorbable guide, similar to an in vivo "Artery-Bioregeneration Assist Tube" (ABAT), which can promote the sequential and complete regeneration of vascular structures at the implantation site, entirely made of Hyaluronic Acid was used in different in vivo experimental model [49].

Other example of bioresorbeable material used for vascular purposed could be represented from collagen, which the author have experienced in small caliber vessel replacement in vivo experimental studies (Fig. 3)

Figure 3. Example of pre-in vivo experimental implant of a biocompatible artificial collagen based artery scaffold. Protocol study of the author.

4.4. Biological materials

These biomaterials are widely available and they are of course excellent substrates for cell adhesion. In addition, the processing method can retain all their advantageous mechanical properties (tensile strength, elasticity) [50]. As the main disadvantages are possible residual antigenicity and infection after implant, techniques for their decellularization and sterilization have been refined [51]. Most recent article published on biological graft mainly deal with materials already naturally present as tubular structures in the body (arteries, veins, urethers) and submitted to decellularization. They are often studied as allo-or xenografts, and enriched with cells, bFGF, heparin and VEGF to improve patency in the long term. Of special interest for the physiopathology of tissue healing after implant is one study reporting trends after implants of decellularised porcine arteries in rat, concluding that the initial inflammation due to integration in tissues does not interfere with long-term modelling. One in vitro study examines the creation of a biotube produced by reaction to a foreign body1-3. These studies show the good mechanical properties of this biomaterial, but also the poor long-term patency of conduits [52, 53].

Decellularization have so represented one of the most reliable procedure to obtain an ideal scaffold for vascular replacement, in particular for its peculiar property to retain native ECM that is the fundamental aspect for cell seeding and cell host colonization (Fig.4).

Figure 4. Example of pre-in vivo experimental implant of a decellularized artery. Protocol study of the authors.

There is not a unique decellularization protocol but every tissue need specific reactions and solutions. Protocols usually require a combination of physical, chemical and enzyme processes: the first phases are dedicated to rupture the cell membranes in order to release the intracellular components, which are then separated, dispersed, and degraded by enzymatic detergents and solutions. The last phases are directed to the elimination of cellular debris, that remained within the matrix, and of the reagents, that could interfere with the subsequent recellularization of scaffold or cause adverse reactions into recipient organism [54].

Some examples of physical treatment are the fast cycle of freeze/thaw that induce effective rupture of cell membranes, or the application of mechanical forces or the agitation of the material in combination with chemical detergents to promote the solubilization of homogenous membranous components [55].

As regards chemical treatments, the commonly solutions are usually composed by acid or alkaline solutions which are used for decellularization of thin layers of tissue samples as the bladder submucosa [56]. This type of treatment is effective in removing cell debris and, at the same time, has an antibacterial and antifungal activity [57]. Despite this, it has been demonstrated that the use of chemical solvents for prolonged times may alter the structure of the matrix, causing the detachment of glycosaminoglycans (GAGs) from collagen fibers [58]. Ionic detergents are another category of chemical agents usually employed during decellularization and they are involvede, with effectiveness, in removing the nuclear and cytoplasmic fragments. Their disadvantage could be the possible denaturation of ECM proteins.

In the decellularization protocols are also used chelating agents such as EDTA, whose function is to bind and ions, which are physiologicaly essential for covalent bonds between cells and matrix components, such as collagen fibers and fibronectin. In this way their use facilitate the loss of interconnections of the cells, resulting in disintegration and removal of cellular material [59].

To improve the effectiveness of the decellularization process, it is necessary the use of enzyme solutions, mainly consisting of trypsin, which specifically cleaves the protein bonds and exerts its maximum activity at the temperature of 37°C and pH 8. In addition to trypsin, is also frequently the use of the endonucleases, which include the deoxyribonuclease and ribonu-

clease. These enzymes catalyze the hydrolysis of covalent bonds of, respectively, DNA and RNA. However, it is recommended to avoid the exceeding exposure of samples with the action of the enzyme solutions because they could damage in the structure of the extracellular matrix. All decellularization solutions are usually implemented with antibiotic or antifungal agents to prevent any bacterial or fungal contamination.

To obtain an effectively decellularized scaffolds, it is important to remove the antigenic components, such as cell surface receptors, cytoplasmic proteins and nucleic acids that, due to their immunogenic properties, could trigger a defensive reaction of the immune system of the recipient. In contrast, components of the extracellular matrix, such as collagen and elastic fibers, are widely conserved among individuals of the same or different species and, therefore, they usually do not evoke the immunogenic reaction of the host. A low intensity inflammatory response of the receiving was observed, at least, at histological level and there have been reports of significant rejection reactions, as described by Kasimir and colleagues, after implant of porcine valve prosthesis in pediatric patients [60].

4.5. Clinical relevance and rationale for the use of ECM as a biologic scaffold

The use of ECM derived from decellularized tissue is increasingly frequent in regenerative medicine and tissue engineering strategies, with recent applications including the use of three-dimensional ECM scaffolds prepared by whole organ decellularization [61, 62]. Clinical products such as surgical mesh materials composed of ECM are harvested from a variety of allogenic or xenogeneic tissue sources, including dermis, urinary bladder, small intestine, mesothelium, pericardium, and heart valves, and from several different species. The potential advantage of tissue specificity for maintaining selected cell functions and phenotype has been suggested by studies of cells and ECM isolated from tissues and organs such as the liver [63], respiratory tract, nerve [64], adipose [65], and mammary gland [66]. The ECM has been shown to influence cell mitogenesis and chemotaxis, direct cell differentiation [67-71], and induce constructive host tissue remodelling responses [72-74]. It is likely that the three-dimensional ultrastructure, surface topology, and composition of the ECM all contribute to these effects. There is also evidence that residual cellular material attenuates or fully negates the constructive tissue remodelling advantages of biologic scaffold materials *in vivo* [75]. Therefore, tissue processing methods, including decellularization, are critical determinants of clinical success [76]. It should be understood that every cell removal agent and method will alter ECM composition and cause some degree of ultrastructure disruption. Minimization of these undesirable effects rather than complete avoidance is the objective of decellularization

4.6. Scaffold Free Method

An alternative method based on a scaffold free technique was also described and provide the advancement in the cell technology field. The application of such procedure depends from the necessity to improve the bioengineered vessel in terms to overcome process like the chronic inflammation, thrombosis, rejection, and poor mechanical properties of allogeneic or xenogeneic and synthetic vessels that as previously reported have impaired their clinical applications [77]. In addition it has emerged due to the failure cell to cell interaction and the assembly and

alignment of ECM components, and the complex host response to scaffolds, [78]. In scaffold-free tissue engineering approach, the fabrication of the tissue construct is anchored in the crucial capability of the cells to manufacture their own extracellular matrix [39]. In 1998, the first scaffold-free tissue-engineered human blood vessel was established by L' Heureux and has been replicated for further preclinical evaluation using rat and mice models in 2006 [77]. Years later, groups of scientists reported a fully biological self-assembly approaches by implementing rapid prototyping bioprinting method and stimulation via bioreactors for scaffold-free small diameter vascular reconstruction [80].

Similar to scaffold-based technique, in tissue engineering for scaffold-free blood vessels, the bioreactors were also used to provide specific biochemical and physical signals to regulate cell differentiation, ECM production, and tissue assembly by using chemical, mechanical, or electromagnetic stimulation techniques to produce *de novo* tissue with properties comparable to the damaged or desired tissues [81, 82]. There are many types of launched bioreactors however, in engineering the vascular tissues, designs of various bioreactors have been based on the expansion and recoil properties of blood vessels, and so the combinations of stress, strain, and perfusion stimulation in biomimetic bioreactors have successfully developed vascular tissues [83, 84]. In case of cell senescence problem, lifespan extension via telomerase expression in vascular cells (smooth muscle cells and endothelial cells) from elderly patients has been found as an effective strategy for engineering autologous blood vessels and eventually provides bypass conduit for atherosclerotic diseases. Human telomerase, composed of an RNA component and a reverse transcriptase (hTERT), maintains the telomere length at the ends of the chromosomes [85, 86].Absence of hTERT expression in mature somatic cells induces lack of telomerase activity thus its ectopic expression has been shown to restore telomerase activity, arrest telomere shortening and senescence in some cells [87]. While high cell population is essential in cell-based vessel biofabrication and the expansion process is lengthy, cell-based therapies are more promising in terms of efficacy despite the fact that they are more complex and costly than scaffold-based techniques. Therefore, many researchers have focused on this approach and the representative studies are presented in. Among the well-studied scaffold-free techniques are the coculture system, sheet-based engineering, decellularization, direct cell injection, bioprinting, and bio fabrication in a bioreactor system.

5. Consideration on cost/effectiveness

Nevertheless systematic studies about tissue engineering costs and clinical benefit are not still available, due to many differences in procedure, techniques and materials, is well known that is a medicine promising but expensive tool. In fact, since now most of the result are due to high cost basic research with low effect on daily clinical practice and, in most cases, not widely available. The scientific surgeon community agree on necessity to use the best achievement in cell tecnologies to obtain an ideal and easy way to replace damaged tissue but also the cost to obtain it must be controlled. If the industry employ large sum of money to obtain a product its cost could be not widely available and so a novel promising technology could have a low impact worldwide.

More than 70 tissue engineering related start-up companies spent more than $600 million/year, with only two FDA-approved tissue-engineered products [88]. Given the modest performance in clinically approved organs, tissue engineering still remain a promising field. Often is a lacking in experimental model that avoid a perfect matching with human clinical situation. The community of bioengineering technology is advocating the application of clinically driven methodologies in large animal models enabling clinical translation.

The employment of sophisticated tecnologies in cell treatment as in decellularization process, cell isolation and in vitro expansion, costs of growth factor and bioreactor and so on are the most principles obstacle to a low cost wide available bioengineered tissue.

The huge clinical needing and the necessity of the low risk application of engineered substitute still represent a limitation in prevision of clinical application about most of the promising experimental result. At the same time such encouraging result provide the base for further development and spending limitation.

Since now all in vitro advancement in vessel replacement technology must consider its success not only in term of ideal vessel production but also in terms of feasibility, economic and time saving procedure.

6. Conclusion

Critical reading of researches in the field of microvascular tissue engineering gave the general impression of progress in the search for an ideal replacement for small diameter vessels but the goal its aimed to is still lacking. In fact, even if several studies seems to be promising they must be completely proved in vivo in human clinical situation and in long term period as also they must obtain a therapeutic result within an acceptable cost for the community.

Tissue Engineering in the context of Regenerative Medicine has been hailed for many years as one of the most important topics in medicine in the twenty-first century. While the first clinically relevant efforts were mainly concerned with the generation of bioengineered skin substitutes, subsequently tissue engineering applications have been continuously extended to a wide variety of tissues and organs.

The advent of either embryonic or mesenchymal adult stem-cell technology has fostered many of the efforts to combine this promising tool with tissue engineering approaches and has merged the field into the term Regenerative Medicine. As a typical example in translational medicine, the discovery of a new type of cells called *telocytes* that have been described in many organs and have been detected by electron microscopy opens another gate to regenerative medicine.

Besides cell-therapy strategies, the application of gene therapy combined with tissue engineering has been investigated to generate tissues and organs. The vascularization of constructs plays a crucial role besides the matrix and cell substitutes.

Therefore, novel in vivo models of vascularization have evolved allowing axial vascularization with subsequent transplantation of constructs.

This article is intended to give an overview over some of the most recent developments and possible applications in regenerative medicine through the perspective of tissue engineering achievements and cellular research. The synthesis of bioengineering with innovative methods of molecular biology and stem-cell technology appears to be very promising.

Most studies indicate the use of absorbable biomaterials, in view of their good integration, with the hope of developing autogenous vessels to replace prostheses. However, not one of these products has yet been approved for clinical experimentation. Degradability is one of the characteristics which tend to dissuade surgeons at the crucial moment of implant. In addition, synthetic not degradable material could not offer adequate surface to maintain and adequate patency in long period. There are many gaps in the examined articles. The first problem, already examined by many authors, is variability in animal models, which hinders direct comparison of results. Homogeneous studies on mechanical studies are also lacking, since so many of them focus on tensile strength, and neglect compliance, which is an essential feature of vessels.

An effective model of an artificial vessel is very far from being achieved, particularly considering the field of microvascular graft. So its development must take into account the context in which it could be applied. Experimental models have already been super-ceded, if we think that the application of a bio-absorbable prosthesis means that cells must be able to reconstruct a new artery and that, in clinical microsurgery practice, this must be achieved in already damaged arteries

However, the procedures are time-consuming and very expensive, requiring dedicated laboratories able to guarantee sterility and suitability for in vivo re-implantation of cell cultures.

As regards urgent procedures, such as revascularisation of all types, the cell culture step should be avoided. The ideal choice would be ready-to-use materials, that actually are needing their improvement.

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