REVIEW

Tissue Engineering Heart Valves – a Review of More than Two Decades into Preclinical and Clinical Testing for Obtaining the Next Generation of Heart Valve Substitutes

Dan SIMIONESCU[1,3], Marius Mihai HARPA[1], Agneta SIMIONESCU[1,3], Codrut OPRITA[2], Ionela MOVILEANU[1]

ABSTRACT

Well documented shortcomings of current heart valve substitutes – biological and mechanical prostheses make them imperfect choices for patients diagnosed with heart valve disease, in need for a cardiac valve replacement. Regenerative Medicine and Tissue Engineering represent the research grounds of the next generation of valvular prostheses – Tissue Engineering Heart Valves (TEHV). Mimicking the structure and function of the native valves, TEHVs are three dimensional structures obtained in laboratories encompassing scaffolds (natural and synthetic), cells (stem cells and differentiated cells) and bioreactors. The literature stipulates two major heart valve regeneration paradigms, differing in the manner of autologous cells repopulation of the scaffolds; *in vitro,* or *in vivo,* respectively. During the past two decades, multidisciplinary both *in vitro* and *in vitro* research work was performed and published. *In vivo* experience comprises preclinical tests in experimental animal model and cautious limited clinical translation in patients. Despite initial encouraging results, translation of their usage in large clinical scenarios represents the most important challenge that needs to be overcome. This review purpose is to outline the most remarkable preclinical and clinical results of TEHV evaluation along with the lessons learnt from all this experience.

Keywords: Heart valve regeneration, scaffold, heart valve prosthesis, tissue engineering.

REZUMAT

Neajunsurile bine știute ale actualelor proteze valvulare cardiace – biologice și mecanice le fac pe acestea să fie niște alegeri imperfecte pentru pacienții diagnosticați cu boală valvulară care prezintă indicație de înlocuire valvulară. Cercetarea în domeniul medicinii regenerative și ingineriei tisulare reprezintă fundamentul următoarei generații de proteze valvulare – valvele cardiace obținute prin tehnici de bio-inginerie tisulară (TEHV). Imitând structura și funcția valvelor native, TEHV-urile sunt structuri tridimensionale obținute în laboratoare ce încorporează scffold-uri - schele (naturale și sintetice), celule (celule stem și celule diferențiate) și bioreactoare. Literatura de specialitate prezintă două paradigme de lucru pentru regenerarea valvelor cardiace, care diferă prin modul de repopulare cu celulelor autologe a scaffold-urilor; *in vitro,* respectiv *in vivo*. În ultimele două decenii, au fost efectuate și publicate lucrări de cercetare multidisciplinare atât *in vitro,* cât și *in vitro*. Experiența *in vivo* cuprinde testări preclinice la modelul animal experimental și o translație clinică limitată și prudentă la pacienți. În ciuda rezultatelor încurajatoare inițiale, utilizarea lor în scenarii clinice de mare anvergură reprezintă cea mai importantă provocare. Scopul acestui review este prezentarea celor mai remarcabile rezultate preclinice și clinice ale evaluării TEHV, împreună cu lecțiile învățate din toată această experiență.

Cuvinte cheie: regenerare de valve cardiace, scaffold, proteză valvulară, inginerie tisulară

[1] Laboratory of Regenerative Medicine, „George Emil Palade" University of Medicine and Pharmacy, Science and Technology, Targu Mures, Romania

[2] Tissue Engineering and Regenerative Medicine Club, „George Emil Palade" University of Medicine and Pharmacy, Science and Technology, Targu Mures, Romania

[3] Tissue Engineering Laboratory, Department of Bioengineering, Clemson University, Clemson, South Carolina, USA

Contact address:

Marius HARPA, Laboratory of Regenerative Medicine, „George Emil Palade" University of Medicine and Pharmacy, Science and Technology, Targu Mures, Romania.

E-mail: marius_harpa@yahoo.com

INTRODUCTION

Regenerative Medicine and Tissue Engineering target to obtain living tissues and organs in order to replace damaged and dysfunctional ones. Regarding heart valve disease (HVD), it represents an important burden of the healthcare system, affecting patients worldwide with no regard to the development status of countries[1]. It is appreciated that by 2030 over 4.5 million people will be diagnosed with degenerative aortic disease[2].

Therapies for heart valve disease are represented by interventional and surgical approaches, since pharmacological agents only address symptoms management. Using surgery, the options are represented by valve repair or valve replacement. Although valve repair has several advantages when compared to replacement in terms of hemorrhages (secondary to the anticoagulant therapy), lower operatory-related mortality and preserved systolic function of the left ventricle (appreciated by evaluation of the ejection fraction)[3-5], studies revealed an increased number of valve replacements[6]. Current heart valve substitutes are represented by biological and mechanical prostheses, each of them presenting significant improvements since their first usage but their inherit drawbacks make them far from ideal options. Mechanical prostheses require lifelong anticoagulation therapy whereas the biological ones are characterized by an increased rate of re-intervention secondary to prosthesis deterioration and secondary dysfunction[7].

Resembling the histological architecture and the function of a native heart valve, Tissue Engineering of Heart Valves (TEHV) focuses on manufacturing a viable and functional heart valve, which doesn't require anticoagulation therapy, is durable, biocompatible and doesn't trigger an immune response[8]. Being a living tissue, TEHVs own their metabolisms with absence of degeneration, calcification and with ability to grow with the human body, targeted especially for the pediatric patients' group, characterized by the requirement for multiple reoperations due to their growing organism[9].Mimicking the native valve extracellular matrix (ECM) by using a temporary scaffold, cells, growth factors and bioreactor preconditioning[10] TEHV represents the next valvular substitute generation.

Up to the present, research in this domain encompasses different stages of significance with accountable and valuable victories in their in laboratory (*in vitro*) testing[11-13] and animal model (*in vivo*)[14-15] evaluations, but the TEHVs translation towards large clinical use remains a tremendous challenge. Having this goal, a more detailed evaluation of cells and scaffold interactions along with tissue functionality apprehension could reveal the key answers for this long-waited patient's and clinician's needs.

The concept – replicating the native valve

The premises are to recreate a heart valve prosthesis, mimicking the structure, function and durability of the native valves. Stipulated by one of the pioneering personalities in the heart valve surgery field, a series of principles should be achieved by an ideal cardiac valve substitute: encompassing non-immunogenicity, non-thrombogenicity, capacity to self-repair and dynamic adjustment to the circulation environment[16]. The heart valve's role is to ensure unidirectional blood flow through heart's chambers, systemic and pulmonary circulations.

Consisting of 3 to 5 mm three layered structures, the leaflets endure recurrent bending[17] processes along with exposure to oscillating pressures up to over 100 mmHg. Histologically, the cusps are a three-dimensional assembly of ECM containing an important proportion of collagen fibers and cells – interstitial and endothelial cells. The heart valve could be regarded as an organ itself, having its own metabolism and growth capacity during lifetime[18].

Taking into consideration the fact that brisk research of literature returns extensive and multidisciplinary results on heart valve regeneration research area, this review's goal is to confer an overview of fundamental concepts and its most important results in the in vivo pre-clinical and clinical testing.

The Tissue Engineered Heart Valve

Resembling the architecture of a native valve, the laboratory obtained TEHV represents a manufactured and human tailored tissue incorporating a temporary mechanical cell support and the receiver autologous cells isolated and propagated in cultures, preconditioned in a bioreactor[19]. Multidisciplinary teams joined work, aspire to design and produced a ready to use TEHV, overcoming standard stipulated testing phases. Exploring and analyzing different sources and types of the used cells, types of scaffolds and protocols of preconditioning and implantation, current research has overcome many challenges but yet their usage did not reach the safe translational phase.

A) Scaffolds

Functioning as a temporary support for cells, heart valve scaffold mandatory characteristics are: to ensure

a mechanical support, to allow cell attachment permitting their subsequent development and to mimic the valve architecture and structure[20]. Various scaffold types were obtained and analyzed, using different sources and fabrication methods, dividing the scaffolds in three main categories: natural, synthetic and hybrid – a mixture of both synthetic and natural origins.

Regarding the natural – biological scaffolds, significant interest is focused on natural polymers and decellularized valvular tissues. The most used natural polymers utilized in TEHV scaffold fabrication are: collagen[21], fibrinogen[22], gelatin and alginate[23]. Their usage is constricted by a series of flaws such as: structure modification secondary to exposure to treatment agents, large batch-to-batch inter-variability and insufficient mechanical properties[24,25]; but they are advantageous because they own sites for cells adhesion and surfaces are biomimetic[25].

By exposing fresh tissue to a mix of chemical, biological, mechanical and physical agents, all having a combined synergic action, resident cells are removed from the extracellular matrix. Gradually evolving from simple structures such as tissue sheets[26] to whole valve decellularization[27-29], decellularization protocols target to obtain a non-immunogenic scaffold replicating the valve extracellular matrix (Figure 1). The strengths of these methods are represented by the facts that the resulting acellular structure resembles the one of the native valve conferring proper biological conditions for cell seeding and ulterior expanding.

The downsides are represented mostly by consequences of decellularization procedure – affecting the extracellular matrix integrity secondary to various agents' harsh action whereas an incomplete decellularization procedure, leaving remaining cells may activate the immune system[25].

The most delicate aspect of this manufacturing procedure is represented by maintaining a secure balance between the active cell removal action and preserving the extracellular matrix structure unaltered. Although the last years presented various tissues and methods of decellularization, a standardization of the results has not been settled up to date. The literature offers a list of stipulated proprieties (nucleic material detection and histological assessments) that should be encountered in a decellularized tissue: fewer than 200 base pair per DNA fragment, under 50 ng of double chained DNA per milligram of extracellular matrix and absence of visible nucleic material when histologically examined[30]. Multiple classes of chemical agents are involved in this cell removal procedure such as: anionic and non-anionic detergents[31,32], enzymatic agents – trypsin[33], deoxyribonuclease, ribonuclease[34] and physical agents – osmotic shock[35] and applied pressure gradients[36].

Secondary to cell removal protocol, some research groups aimed to improve following cells adherence and expansion. By chemically conjugation of the structure, the scaffolds were coated with extracellular specific proteins with role in cells adhesion and

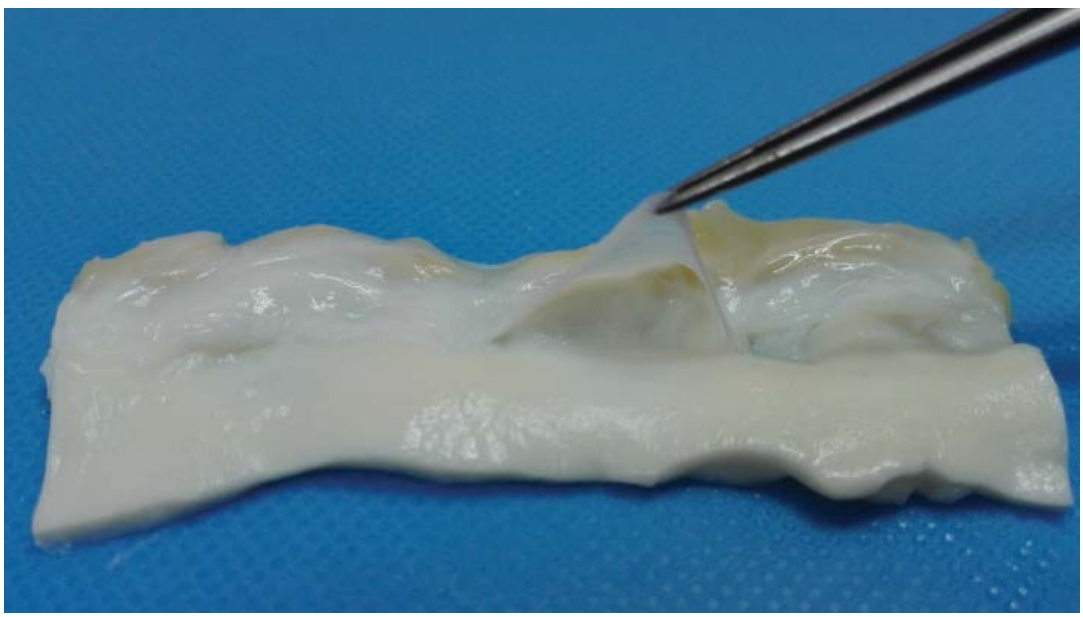

Figure 1. Decellularized porcine pulmonary valve.

multiplication[37] or antibodies[38]. Their exposure to circulatory hemodynamics demonstrated increased cell repopulation onto the valve's surfaces yet incomplete cell infiltration due to their lack at the interstitial level.

Synthetic scaffolds are advantageous due to the large extent of control in their manufacturing process regarding their mechanics, durability and geometry. Their needs to be biocompatible and biodegradable are highlighted by desideratum of subsequent cell mediated scaffold resorption. A large variety of materials and techniques were investigated: polymers – polylactic, polyglycolic acid orpolyhydroxyalkanoate polymers[39,40], composites investigated and usage approved by the Food and Drugs Administration Agency[41]. Although conferring an adequate initial mechanical support the most crucial downside is represented by the low pH environment created by their hydrolytic degradation impacting cells viability[42].

The various shapes and sizes of the polymeric structures are obtained through different preparing techniques such as: electrospinning, freeze drying, solid free form and solvent casting[12]. Each one of these polymeric scaffolds manufacturing techniques are used in order to obtain a specific type of scaffold characterized by standard proprieties. The electrospinning method generates high porosity scaffolds along with a decreased volume to area ratio being disadvantageous in terms of production times and lesser mechanical strength[44]. The freeze-drying method produces structures in which the control in pores dimensions are controlled by physical elements and solution's concentration[12].

Usage synthetic origin scaffolds as templates for the new to be secreted ECM by the seeded cells is an advantageous option considering the high degree of control in their production, exiting the opportunity of various combination in order to increase their mechanical characteristics. Additionally, they can be reproduced in any wanted quantities and sized replicating the entire micro-architectural aspects, representing the „of the shelf" idea of scaffolds for heart valve regeneration processes. Their main stipulated drawbacks are the eventual toxicity of degradation residues towards the repopulating cells and presence of fragmentation when *in vivo* tested[10].

B) The cells

The central piece of the regeneration process, they enliven the scaffolds having the desiderate of its resorption followed by extracellular matrix elements secretion as replacement. There are two main paradigms of tissue regeneration differentiated by the cell repopulation manner (Figure 2). The in vivo strategy is based on the implantation of acellular scaffolds with secondary invasion and attachment of autologous cells whereas the in vitro scenario implies bioengineering techniques of cells seeding[45]. Several advantages and disadvantages characterize each of these approaches

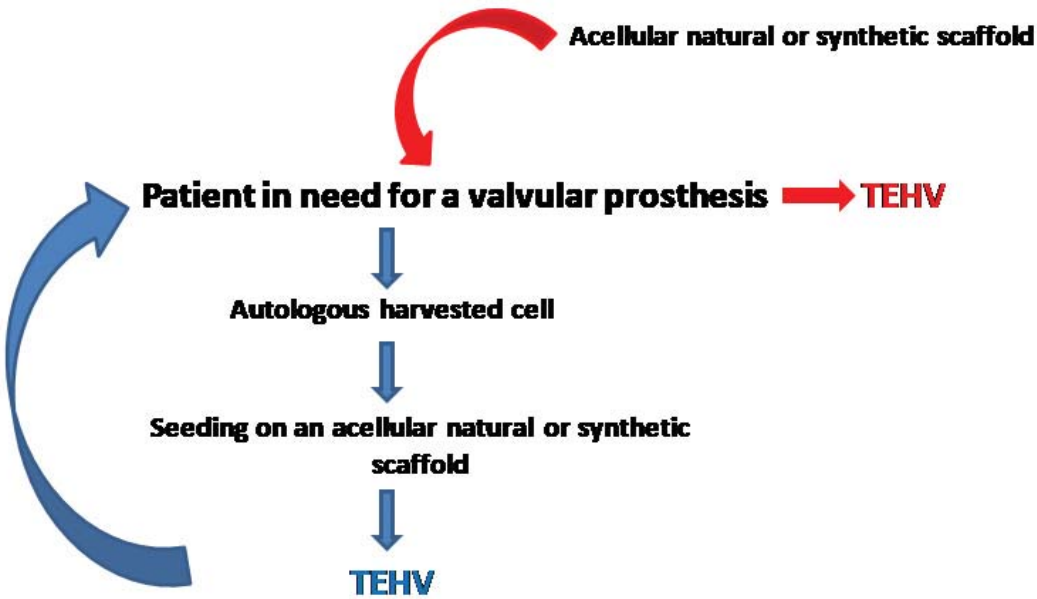

Figure 2. TEHV manufacturing concepts. The *in vivo* repopulation marked in red, the *in vitro* strategy presented in blue.

aiming for a common result: the in vitro seeding is regarded as more time and resources consuming while offering a better control of the process whereas the in vivo repopulation requires a structure capable to withstand the mechanical challenges simultaneously with the ECM resorption process.

Resident cells of heart valves are represented by endothelial cells and valvular interstitial cells, elements that ensure valve's remodeling capacity[46]. Preservation of ECM architecture and structure secondary to fatigue is conferred by these cells. In cardiac valves regeneration, the used cell sources are classified based on their origin as autologous (the donor coincides with the recipient), allogenic (the donor has the same species as the recipient) and xenogenic (the donor belongs to another species)[47].

A large variety of cells are investigated in this research work, in different stages of their development, from stem cells[48] to already specialized cells[49,50]. Stem cells are defined by their self renewal capacity along with the ability to differentiate towards specialized mature cells. Based on their plasticity, stem cells are categorized as omnipotent, pluripotent, multipotent and unipotent[51]. Recently research added anew cell type to this stem cells classes – the genetic reprogrammed induced pluripotency stem cells (iPS)[52]. Based on their origin, stem cells are divided into four categories: embryonic stem cells, fetal stem cells, adult stem cells and iPS[53]. Embryonic stem cells are isolated from the inner mass of the blastocyst form of the embryo, found in the 5-6 days after fertilization[54]. Usage of these cells is strictly controlled and limited due to ethical concern[55].

Both animal and human cell origin are presented in the current literature as potential sources for TEHV manufacture. Based on the above classification, the choice between these two is made upon their usage – for the preclinical steps of research the animal origin is preferred due to cost efficiency, facile procurement and lessened ethical implications[56].

Providing a large assortment of cell types and harvesting places, the mesenchymal stem cells represent one of the most frequent cell types used in cardiac valve regeneration, having fibroblast-like behavior[57]. They can be isolated from a large tissue range: bone, peripheral blood, adipose tissue, placenta and umbilical cord, being characterized by a brisk and extensive multiplication in cultures[58].

Usage of autologous endothelial cell seeded scaffold in preclinical[59] and clinical[60] scenarios revealed good hemodynamic function and presence of a monolayer endothelium. The *in vitro* repopulation of cells is additionally limited by the inhomogeneous adherence to the scaffold surface[61].

In order to anticipate and to evaluate the behavior of TEHV, considerable attention is given to biomechanics using dedicated bioreactors and functioning models in laboratories[62-64], but the imminent challenges to overcome are their *in vivo* translation.

TEHV in experimental animal models

Large scale experimental animals are used for the TEHV in vivo performance and behavior evaluations. Implanted through techniques used in human medicine, either surgically – with open heart surgery[65] either interventional – transcatheter[66], the procedures replicate the ones currently used in clinics. Surgically, valves are positioned orthotropic (in the same position of the cardiac valve being removed) or extra-anatomic (by-passing the native valve that was purposely previously occluded)[67,68]. Heterogeneous animal species were involved in animal studies investigating the TEHV such as: pigs[69], lambs[70], dogs[71], and non-human primates[72]. Concluding from all completed research works, in terms of cardiac valve replacement in preclinical studies, the sheep is pointed to represent the gold standard animal model[73].

Regarding the cardiac valves research, it was observed that a preliminary placement of them in the pulmonary position is approached due to the undemanding pulmonary circulation hemodynamic when compared with the left situated heart chambers.

The ovine experimental animal model

Pre-clinical research results using the ovine animal model had their pioneering years early in the '90, starting with the simplest structure of a valve – the leaflet. Although represented by a limited study, it represented the promising ground of following research. On a synthetic scaffold, autologous (n=3) respective allogenic (n=4) fibroblasts, smooth muscle cells and endothelial cells were seeded, and next incubated in vitro conditions for two weeks followed by surgical implantation in lambs. One month later, when explanted the allogenic group showed shrinkage and degradation, processes absent in the autologous group[74].

Regarding the in vitro repopulation strategy, acellular both synthetic and biological (with either allogenic or xenogenic origin) scaffolds were investigated. The synthetic scaffold are represented by bio-polymeric structures, biocompatible and characterized by an environment dictated resorption. Their main advantages are represented by thoroughly control of their

mechanic and durability proprieties, being tailored in any sizes and forms.

The *in vivo* repopulation strategy

Having the debuting years in 1999, implantation of five decellularized aortic valves in sheep presented with good performance along with fibroblast infiltration of valves without any signs of calcification at the end of the five months long follow-up[75]. Further, the decellularized biological scaffolds were repopulated with endothelial cells[76] respectively endothelial and myo-fibroblasts[77] with autologous origins. The results pointed infiltration with endothelial and interstitial cells in both cases with no signs of calcium deposits in their structures after a follow-up period of five respectively six months.

Multiple pre-clinical investigations regarded the behavior of decellularized porcine aortic and pulmonary valves surgically implanted in sheep. n=4 aortic valves were followed for a maximum of 4 months, pointing re-cellularization with host's interstitial cells and good valvular function in the early post-implantation period[78]. A five years later conducted study, placing decellularized porcine aortic valves in pulmonary position in sheep, presented at the end of the five months follow-up sufficient hemodynamic function of valves along with re-cellularization with collagen-secreting interstitial cells and endothelial cells[79]. Regarding the decellularized porcine pulmonary valves, a study investigating seven valves implanted orthotopically revealed when examined during three to six months, presence of endothelialization and newly secreted collagen by infiltrated fibroblasts[80].

A distinct decellularized scaffold evaluated for TEHV is represented by processed porcine small intestine submucosa; two studies performed five years apart reveal their performances in pulmonary position when used in heart valve bio-engineering. The first one, that took place in 2015 pointed out enlargement of valve diameter and microscopically, host cell invasion[81]; five years after n=20 animals were implanted with unfavorable results, animals developing heart failure and valve stenosis. The outcomes were interpreted as secondary to an insufficient decellularization[82].

Polymeric based scaffold was used in pre-clinical studies for both aortic and pulmonary replacement. For the pulmonary valve replacement, implantations were performed surgically of via catheterization. By using the minimally invasive technique of implantation, the valves proved good acute hemodynamic performances but with leaflets remodeling and secondary regurgitation at four respective eight weeks[83-85].

TEHV in humans

Translation to large mass access for patients needing a heart valve prosthesis represents the ultimate desiderate of this entire work. Multiple challenges had been outreached yet numerous improvements need to be addressed prior to their large-scale clinical usage. Initial clinical phase of the research work is documented in the early 2000th. The first bioengineered heart valve implanted in human was performed in 2000, consisting in a pulmonary allograft decellularized with deoxycholic acid implanted in a 43 year old patient. Further follow-up revealed good hemodynamic performances along with absence of major valvular dysfunction[86].

Gradually becoming a certainty, heart valve replacement with TEHV initiated with several case reports focused on the immediate post-procedure behavior of the prosthesis. Due to non-demanding hemodynamic condition of the right heart, the pulmonary position was the preferred site for implantation site. In 2003, a study enrolling n=4 children aged between 2.5 and 11 months implanted with decellularized porcine pulmonary valves revealed good initial performances with subsequently major valve dysfunction (valve rupture and degeneration) and sudden deaths in three cases. Microscopy examinations pointed a non-complete decellularization procedure of the xenograft[87].

Seven years later, in 2010, n=16 patients – children and young adults were similarly implanted with porcine pulmonary valves. 38% of them became dysfunctional at ten months secondary to valve stenosis and obstruction. The process was caused by tissue infiltration with inflammatory cells and presence of calcification[88]. Chronologically, further studies were conducted in the following years of 2011, 2012, 2013 and 2014[89-92]. The earlier one, including n=61 patients, children and adults reported need for re-operation in four cases due to valve failure. TEHVs proved good valvular function, and function in congenital patients[89]. N=93 pediatric patients (with an average age of 20 months) were implanted with decellularized porcine pulmonary valves in a study developed in 2012 – results pointing a 29% valvular dysfunction associated with a 35.5% failure rate secondary to valve dilatation or presence of valvular stenosis[90]. During the next year, in 2013, n=26 patients were also implanted with decellularized porcine pulmonary valves through both surgical and transcatheter approaches. The valves proved moderate to severe insufficient associating

stenosis, facts translated by a 52% need of reoperation[91]. N=21 adults underwent valve replacement with decellularized porcine pulmonary valves in a research performed in 2014, resulting in major valve failure due to stenosis of the valve secondary to inflammatory cells infiltration and tissue necrosis[92].

More recent, in 2019, a larger clinical research work, including n=492 patients aged 57±11 years surgically implanted with decellularized porcine pulmonary xenografts revealed an important rate of valvular dysfunction along with right heart failure, associating a need of re-intervention of 30.5 % from the total number of patients[93].

Regarding the *in vitro* seeding strategy, autologous endothelial pre-seeded on cryo-preserved allogenic decellularized pulmonary valves, n=11 valves were implanted in adults aged 44 ±13.7 years surgically in pulmonary position, revealing good functionality of the TEHV and presence of recellularization processes[94]. These initial results were four years later validated by the ESCORE trial in 2011 - the ten years follow-up results showed good valvular function along with absence of calcification evidence in the TEHV[95].

At this moment, two major clinical trials involving decellularized allogenic pulmonary and aortic valves are in the follow-up stage of their study. Implanted through a minimally invasive technique, using catheterization, their preliminary results appear encouraging when compared to current valvular substitutes alternatives. The ESPOIR trial which enrolled n=121 patients aged 21.3 ± 14.4 years compared fresh decellularized allogenic pulmonary valves with the Contegra™ conduits and cryo-preserved homografts. Preliminary results pointed the TEHV to be safe with good valvular performance and trivial insufficiency and decreased need for explantation[96].

The ARISE trial implanted using the same trans-catheter technique, decellularized allogenic aortic valves in aortic position in young adults aged 19.7±14.6 years. Initial results present the TEHV with good functionality, without any systemic hemodynamic conditions towards the valve structure. TEHV presented trivial regurgitation and preserved annular dimensions, with no signs of secondary dilatation[97].

A series of conclusions and lessons could be withdrawn from these initial clinical experiences. Regarding the usage of TEHV with xenogenic origin, important valvular dysfunction and calcifications were observed secondary to an incomplete decellularization procedure and to the host immune response. The cells remaining triggered and consecutively activated the immune system causing the TEHV infiltration with immune cells representing the grounds of the thereafter dysfunction. Clinical results of allogenic decellularized scaffolds show good valvular function in vivo along with presence of cell infiltration when microscopically examined.

Further studies need to focus on improving the decellularization procedures and strategies to lower the antigenic load of the scaffolds without usage of chemical fixation agents that interfere with cells infiltration, adhesion and infiltration.

CONCLUSIONS

Prior to extensive clinical trials implantation, TEHV behavior should be carefully assessed. The most important aspect is represented by the balance between scaffold desorption and the new tissue formation, equilibrating between the tissue loss and acquired strength. Additionally, an extensive and detailed investigation of the thrombogenicity and immunogenicity should precede their large-scale usage.

Until reaching the status of being an approved treatment option, TEHV have a series of tremendous challenges to overcome from this initial state of a promising pioneering multidisciplinary research field.

Compliance with ethics requirements:

The authors declare no conflict of interest regarding this article. The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law. Informed consent was obtained from all the patients included in the study.

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