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CURRENT TRENDS IN THE CREATION OF CELL-FREE ALLO- AND XENOTISSUES FOR RECONSTRUCTION OF HEART STRUCTURES

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Tissue engineering has significant potential for solving the problems of durability of biological tissues when used in cardiac and vascular reconstructive surgery. A decellularization technology has been proposed for obtaining a biomaterial, morphologically and functionally similar to the damaged human heart tissue. This review discusses various aspects and models of biological tissue decellularization, including the modern technology of using supercritical carbon dioxide as the most eco-friendly and promising method.

Keywords: heart valve, tissue engineering, decellularization, supercritical carbon dioxide.

Biological prostheses have been used in cardiovascular surgery since the early 1960s, when D. Ross and B. Barratt-Boyes transplanted a cadaveric aortic valve into an orthotopic position in 1962. In 1968, A. Carpentier began using glutaraldehyde for chemical treatment of biological tissue, performing aortic valve replacement with a stented bioprosthesis. Today, porcine aortic and bovine pericardial prostheses are widely used all over the world, even without a stent. About 275,000 artificial heart valves are implanted worldwide every year, of which about half are mechanical and half are biological. This indicates the increasing popularity of bioprostheses in recent decades. However, mechanical and biological prostheses come with some limitations, such as infection, risk of thromboembolism, need for lifelong anticoagulation (mechanical), or limited durability (biological). Allograft is an alternative to mechanical or biological prostheses and has several advantages over existing valves. Homovital (taken from a living heart) and cryopreserved allografts consist of viable tissue relatively resistant to infection and has excellent hemodynamic properties. On the other hand, viability of foreign cells induces immune response, possibly leading to a later, but still, degeneration of the valve. At the same time, antibiotic-sterilized human allograft valves have limited durability due to the lack of living cells inside the matrix [9].

Glutaraldehyde is most commonly used to reduce immune response and xenograft rejection [4, 5, 6, 15, 25]. Although tissue processing reduces its immunogenicity, cytotoxicity and calcification remain the main undesirable components. Calcification plays a major role in degenerative dysfunction of bioprosthetic heart valves, which in turn is initiated mainly by residual dead cells due to glutaraldehyde treatment. The mechanism involves reaction of calcium-containing extracellular fluid with membrane-bound phosphorus, resulting in the formation of mineral deposits of calcium phosphate. In addition, calcification is accelerated by known factors such as young age of the recipient and increased mechanical stress of the bioprosthetic valve cusps. The valve leaflets undergo repeated opening and closing cycles about a billion times during their lifetime. Thus, the structural changes in the native valve occurring with age in the form of sagging of the leaflets, leading to insufficiency or calcification of the leaflets, which causes stenosis, are explainable.

Biological tissues composed of extracellular matrix are used in reconstructive surgery and increasingly find application in regenerative medicine for organ and tissue replacement. Bioengineered valves obtained from acellular xenotissues or decellularized native valve tissue can become the best alternative to mechanical and classical bioprostheses – in experiments they provide repopulation by the recipient's own cells with the possibility of tissue growth and repair. In addition, repopulation valves are considered less prone to calcification and provide ideal hemodynamic parameters. In cardiac surgery, the sources of such materials are allogeneic or xenogeneic tissues for heart valve replacement, creation of "patches" and conduits. However, to date, complete autologous recellularization of implanted acellular heart valves has not been achieved [25]. Valve resellularization is limited only by formation of endothelial recellularization on the leaflet surface. This scenario is much better than cryopreserved valves, which sometimes undergo degeneration and leukocyte infiltration of the entire valve. The fact that repopulation of acellular valves is limited only to the leaflet surface reveals a problem, since it is the leaflet

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tissue that is the main site of degeneration process of the cryopreserved prosthesis [25]. It is expected that without a viable cell population capable of replicating within the valve leaflet, acellular valves will suffer the same fate as cryopreserved valves.

Long-term studies of allo- and xenotissues in cardiac surgery have shown that cellular components of grafts can promote calcification or immune reactions [15, 19, 20, 24, 25]. In order to obtain a biomaterial that is structurally and functionally close to the damaged structure of the human heart and at the same time immunologically safe, decellularization of tissues and organs from humans or animals has been proposed. The goal of all existing protocols is to remove all viable cells while maintaining the integrity of the extracellular matrix. Decellularization methods thus include osmotic, chemical, enzymatic and mechanical. The main problems of decellularization remain to a greater or lesser extent severe disorders in the extracellular matrix structure, increased immunogenicity and thrombogenicity of decellularized biotissue.

The decellularization process is primarily aimed at ensuring immunological inertness and preserving the main structural and functional components of biological tissue, such as proteins, collagen and glycosaminoglycans [5, 6, 10, 25].

The development of decellularization of xenogeneic tissues began in the 1980s. Several enzymes and detergents have been tested to eliminate interstitial cells, but most of these treatments have proved ineffective. The first tissue-engineered porcine heart valve Synergraft® (Cryolife Inc., USA) was developed as an alternative to conventional biological valves. Porcine aortic valve, composite aortic grafts (model 500) or whole pulmonary valve roots (model 700) were made cell-free using the patented Synergraft® technology. However, the SynerGraft technology, using a combination of DNase and RNase enzymes, decellularization, cryopreservation and radiation, has proved ineffective. Already the first results showed that they cannot be used clinically. P. Simon et al. [9] in 2001 reported the results of implantation of Synergraft® prostheses in the right ventricular outflow tract in four male children (age 2.5–11 years). Two patients underwent the Ross procedure and two had homograft replacement. Three children died shortly thereafter. Among the three dead children, one died on day 7 after surgery due to a sudden valve rupture, the second and third died on 6 weeks and 1 year after implantation. The fourth prosthesis was explanted at day 2 after implantation prophylactically. The used method of xenograft decellularization, apparently, did not ensure antigen elimination. Implantation of decellularized allografts gives mixed results. Conventional (cellular) cryopreserved valve allografts cause increased levels of reactive human leukocyte antigens class I and II [15]. Sayk et al. [16] reported macrophage infiltration of a decellularized SynerGraft pulmonary valve allograft as early as week 5 after implantation. Increased levels of donor-specific antibodies against leukocyte antigens class I and II was also found in adult patients who were implanted with an allograft that was decellularized using an ionic detergent sodium dodecyl sulfate [17].

Freezing is one of the physical methods used in tissue decellularization, which includes direct pressure, sonication, and agitation. Rapid freezing of tissue produces intracellular ice crystals that destroy cell membranes and cause cell lysis. The rate of temperature change must be carefully controlled so that ice formation does not disrupt the cell scaffold itself. Although freezing can be an effective method of cell lysis, it can only be used in combination with other methods of removing cellular material from tissue [5].

To remove cellular components from bovine xenopericardium, D.W. Courtman et al. [4] in 1994 described a stepwise process of using detergent and enzymatic extraction to create a cell-free matrix, which represented a promising approach to biomaterials for the reconstruction of cardiovascular structures. Tissue treatment included the use of hypotonic and hypertonic solutions, detergents (octylphenoxy polyethoxyethanol and sodium dodecyl sulfate), as well as DNase and RNAse, which, by inhibiting autolysis, removed all cells from tissues along with lipids [4]. The process resulted in a material composed mainly of elastin, insoluble collagen, and closely bound glycosaminoglycans. Light and electron microscopy confirmed that almost all cellular components were removed without ultrastructural signs of damage to the fibrous components. Biochemical analysis revealed preservation of collagen and elastin and some differential extraction of glycosaminoglycans. Tests for elastic-strength properties have shown that the mechanical properties of the tissues were practically unchanged.

Hypo- and hypertonic solutions effectively remove intact cellular elements. However, numerous studies on antibodies to the major histocompatibility complex (MHC) have found a positive reaction correlating with the intensity of matrix infiltration by T cells in vivo. Obviously, aqueous hypo- and hypertonic solutions are unable to eliminate membrane-bound MHC antigens after osmotic cell lysis. However, such a relatively mild decellularization technique is characterized by a more complete preservation of acellular matrix structures [2].

In order to reduce the risk of tissue matrix damage, Akatov et al. [1] developed a method that was based on the use of EDTA and digitonin. This method induced rapid death of donor cells in grafts, but did not remove dead cells from the matrix. Digitonin is a non-polar detergent, and, by binding to the plasma membrane cholesterol, it disrupts its integrity. EDTA is a chelator of calcium, magnesium and a number of other metal ions and was used by the authors to inhibit accumulation of

calcium and phosphate in mitochondria during cell death. However, this method of processing aortic xenografts did not eliminate immune response, which led to matrix reorganization and damage [1].

Additional tissue treatment with trypsin or nucleases has been suggested. Short-term enzymatic action on the basement membrane cleavage by trypsin has proven to be an effective method for removing the cellular barrier to cell invasion [12]. S. Cebotari et al. washed aortic and pulmonary artery allografts twice with phosphate-buffered saline and incubated them with constant shaking in trypsin/EDTA (0.5% trypsin and 0.2% EDTA) at 37 °C for 48 hours [9]. The decellularized valves were then washed to remove residues and stored in fresh phosphate-buffered saline at 4 °C. Further studies by I. Tudorache et al. comparing the treatment of the pulmonary artery trunk with 1% sodium deoxycholate, 1% sodium dodecyl sulfate or 0.05% trypsin/0.02% EDTA, showed that all methods resulted in complete decellularization of the valve tissue, but only sodium dodecyl sulfate and deoxycholate allowed complete removal of all cells from the pulmonary artery wall and valve [8]. The morphological integrity and safety of the scaffold proteins were significantly higher in the groups treated with the detergent. Enzymatic treatment, on the other hand, led to the destruction of the basement membrane and deterioration of the wall longitudinal extension parameters (stiffness, elasticity, ultimate force, stress and deformation) in the trypsin/EDTA group ($p < 0.05$). All these methods reduce inflammatory and immunological response after implantation and at the same time provide a matrix that is structurally similar to the native valve. However, further studies have shown that sodium dodecyl sulfate can also lead to structural changes in the matrix, changing mechanical properties such as elasticity and extensibility [11].

Qi Xing et al. [13] compared three biological tissue decellularization methods: high concentration (0.5 wt%) of sodium dodecyl sulfate, low concentration (0.05 wt%), and freeze-thaw method. Preservation of the extracellular matrix, mechanical properties, ability to respond in vitro and ability to repopulate cells were assessed. The results showed that treatment with high concentration of sodium dodecyl sulfate removed up to 90% of the DNA, but significantly reduced the mechanical strength of the cell-free matrix. The modulus of elasticity and viscosity decreased by about 80% and 62%, respectively. The freeze-thaw method maintained the structure and mechanical strength of the cell-free matrix, but retained a large amount of cellular components in the scaffold (about 88% DNA). With all three methods, in vitro tests did not induce a significant immune response and were able to maintain cell repopulation in vitro.

The use of decellularization techniques to reduce the immunological potential of xenogeneic tissues and organs is based on the assumption that the cellular component of a xenograft is the only factor contributing to its antigenicity. Approaches for evaluating acellularity of the scaffold after decellularization have mainly involved histological evaluation of residual nuclei [18, 19], although this information does not provide knowledge about the removal of known xenogeneic antigens such as galactose-alpha-1,3-galactose (alpha-gal) and major histocompatibility complex class 1 (MHC I) – in the form of transmembrane glycoproteins contained on the surface of all nucleated cells. Gonçalves A. et al. studied the degree of decellularization of biological matrices from bovine pericardium [18]. The pericardium was subjected to standard decellularization, consisting of hypotonic lysis and DNase/RNase treatment. Additionally, the tissue was treated for 24 hours with solutions: 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, alpha-galactosidase (5 IU/mL) or phospholipase A2 (150 IU/mL). The tissues were then washed for 96 hours with gentle agitation at 27 °C, and then evaluated using light microscopy. It turned out that standard processing resulted in only partial removal of histological cellularity and persistence of alpha-gal, MHC I and alpha-actin. The addition of deoxycholate treatment resulted in clear acellularity, but preserved xenogeneic antigens. Sodium dodecyl sulfate provided complete acellularity and removal of xenogeneic antigens, with alpha-galactosidase treatment selectively removing alpha-gal from bovine pericardium.

Sodium dodecyl sulfate and sodium deoxycholate are ionic detergents and are effective in dissolving both cytoplasmic and nuclear cell membranes, but tend to denature proteins by disrupting protein-protein interactions. Triton X-100 is the most widely studied non-ionic detergent for decellularization protocols and can be an effective method of decellularization, although its effectiveness largely depends on other methods with which it is combined in a particular protocol [5].

Further studies to more finely assess matrix acellularity included DNA quantification [20], analysis of residual DNA fragment length [21, 22], and assessment of residual cellular structural proteins (without proven antigenicity) [15, 23]. However, there is currently no standard for decellularization success criteria. Thus, the reliability of a cell-free scaffold as a measure for assessing residual antigenicity also requires careful study [15].

The most effective agents for decellularizing each tissue and organ depend on many factors, including the cellular composition of tissue (e.g. liver versus tendon), density (skin or adipose tissue), fat content (brain or bladder), and thickness (skin or pericardium). Importantly, each agent and method used to remove cells will still alter the composition of the extracellular matrix and cause some degree of destruction to its ultrastructure. Minimizing these undesirable effects, rather than preven-

ting them completely, is the goal of any decellularization method [5].

One relatively understudied method worthy of attention at present is the use of supercritical carbon dioxide ($scCO_2$) and ethanol as a medium for cell extraction. A number of researchers have reported that the high permeability and high transfer rate of the supercritical fluid makes this method very effective.

J. Won Lee et al. [27] analyzed lipid extraction with supercritical CO_2 and concluded that supercritical carbon dioxide ($scCO_2$) is an eco-friendly supercritical fluid that is chemically inert, non-toxic, non-flammable and non-polluting. As a green material, $scCO_2$ has desirable properties such as high density, low viscosity and high diffusivity that make it suitable for use as a solvent in decellularization. The growing concern surrounding environmental pollution has triggered the development of green analysis methods based on the use of $scCO_2$ in various laboratories and industries. $ScCO_2$ is becoming an effective alternative to conventional organic solvents.

In addition, $scCO_2$ biotissue treatment cycle can be short, lasting a few hours instead of days required when using other detergents. Eliminating the use of detergents such as sodium dodecyl sulfate will also help reduce damage to the extracellular matrix and decrease cytotoxicity due to detergent residues [3].

In 2008, Sawada et al. [3] presented a study on supercritical decellularization with carbon monoxide. The authors reported adequate removal of DNA and cells, but at the same time found intense tissue dehydration, which caused the tissue to harden, while making it more brittle, potentially threatening the use of the material and creating a major obstacle to progress in cellular technology.

Tissue dehydration is an important parameter determining the suitability of tissue as an implant, and it has been suggested that low water content impairs the mechanical properties of biological tissue, although the minimum degree of hydration to maintain its functional properties remains unknown [9].

Supercritical carbon dioxide containing a small amount of entrainer was an adequate medium for extraction of cell nuclei and cell membranes from biological tissue [5]. Under mild extraction conditions (15 MPa, 37 °C), cell nuclei were completely eliminated within one hour. However, the efficiency of phospholipid removal largely depended on the rate of carbon dioxide transfer into the tissue. In this case, mechanical strength did not decrease even with prolonged treatment. Thus, the authors believe that decellularized tissue can be prepared quickly enough and obtained in a completely dry state, which is beneficial in terms of long-term storage without rotting or contamination.

Taking into account the experience of previous researchers, D.M. Casalia et al. [26] presented a new decellularization technique that preserves the hydration state of the matrix and its mechanical properties. A porcine aortic wall was used for the study, from which all adipose tissues were carefully removed. It was then cut into thin rectangles (approximately 3 cm × 2 cm) and stored in phosphate-buffered saline at 4 °C for at least 48 hours prior to use. Each tissue sample was dried for 15 minutes in a light vacuum using filter paper and a Büchner funnel. Intensive drying in a vacuum oven (37 °C, vacuum 38.1 cmHg) was used as a control. Changes in tissue weight were recorded after 1, 2, 3, 6 and 24 hours. To prevent water extraction from the aortic tissue and to avoid critical dehydration, full thermodynamic equilibrium (i.e. full saturation) between $scCO_2$ and water was first achieved. This equilibrium between $scCO_2$ and water was achieved at a liquid CO_2 flow rate of 5 mL/min or lower. With an increase in the flow rate, the equilibrium could not be maintained. The hydrated $scCO_2$ was then used to treat the matrix. The treatment factor (i.e. total mass of CO_2 per unit mass of hydrated material) and other conditions used (including temperature, pressure and depressurization rate) were set to be similar to those used by Sawada et al. for comparison.

Until the tissue was examined for decellularization, it was stored at –20 °C, prewashed in phosphate-buffered saline and cut into circular slices about 1 cm wide.

Despite high water content (more than 97%) in the aortic wall, complete decellularization was not achieved with $scCO_2$ alone. The researchers included four different additional components in the pre-saturation chamber to determine if they improved decellularization: water, water + Dehypon Ls-54 surfactant (BASF America, Florham Park, NJ), pure ethanol, and a mixture of water and ethanol. After treatment, the tissues were fixed in 10% formalin for at least 24 hours and immersed in paraffin. After slicing and deparaffinization, they were stained with hematoxylin and eosin, or Masson's trichrome stain was used. DNA quantification was performed using DNyzol reagent (Invitrogen, Carlsbad, Calif.). DNA concentration was calculated based on absorbance and initial tissue mass measurements.

The authors concluded that the presented new hybrid method of using $scCO_2$ combines a short processing period and complete decellularization, which was confirmed by histology and DNA quantification (<0.04 μg DNA/mg tissue), while maintaining tissue structure and mechanical properties.

R.S. Hennessy et al. [28] analysed the use of supercritical carbon dioxide for sterilization of decellularized valves. They found that this method is superior to others (gamma irradiation, hydrogen peroxide, ethanol) and may be promising, despite the residual content of peracetic acid in the tissue after treatment, which is one of the ingredients in the sterilization method and helps to maintain tissue sterility for a long time. In the study, in vivo implantation in animals showed no side effects due to the

presence of acid in the heart valve tissue. But research is ongoing to produce completely sterile decellularized heart valves without the presence of peracetic acid.

In conclusion, it must be said that by optimizing decellularization processes, it is possible to obtain valves and other tissues taken from humans and animals that will minimize the donor and patient specificity that is necessary to ensure compatible grafts. However, in order to truly reduce the differences between donors and patients in need of transplantation, it is necessary to develop new tissue engineering methods, since current methods do not provide absolute durability and functionality of decellularized tissues. In addition, recellularization techniques need to be improved to evenly distribute the desired cell types throughout the tissue and ensure sufficient delivery of nutrients and oxygen for optimal cell viability.

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