Chemical decellularization: a promising approach for preparation of extracellular matrix

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Background. A biological scaffold from extracellular matrix can be produced by a variety of decellularization methods whose caveat consists in efficiently eliminating cells from the treated tissue. This scaffold can be used in diverse applications for tissue engineering and organ regeneration. Preservation of the extracellular matrix ultrastructure is highly desirable because of its unique architecture, contained growth factors and decreased immunological response. All of these properties provide attachment sites and adequate environment for cells colonizing this scaffold, reconstituting the decellularized organ. This review briefly describes chemical decellularization methods, evaluation of these protocols and the role of ECM in tissue engineering.

Conclusion. Chemical decellularization is an often used method for scaffold preparation and makes possible a well-preserved three dimensional structure of extracellular matrix.

Key words: chemical decellularization, detergent, extracellular matrix, cell lysis

INTRODUCTION

Decellularized tissues have become widely used in regenerative medicine and some of them are even commercially available, mostly in the USA (for example, human skin removed from cadavers which is supplied in dry form and commonly used in cases of third degree burn patients). The combination of physical, chemical and biological techniques provide the means for more efficient elimination of cellular and nuclear components, preservation of blood vessels' stroma and nerve pathways. The obtained decellularized matrix is complex in structure, functional protein composition, glycosaminoglycan content and growth factors. This extracellular matrix (ECM) can then be used as a scaffold, which can be colonized by cells with regenerative properties (e.g. pluripotent cells, embryonic stem cells, umbilical cord cells, mesenchymal cells etc.). The scaffold maintains its original graft size and shape after implantation. An added benefit of decellularization is the elimination of immunologically active molecules, therefore leading to reduced transplant rejection rates.

General decellularization protocols commonly begin with physical cell lysis, chemical or biological methods. Physical methods often involve changing temperature conditions that lead to intracellular ice crystal formation which disrupt the cell membrane. Other possible decellularization methods using physical conditions are high-pressure treatments. High pressure treated tissues undergo cell lysis and also eliminate viruses and bacteria. Physical methods are commonly used for tissue decellularization of tissues such as pericardium, larynx, bone marrow, cornea or blood vessels.

CHARACTERIZATION OF EXTRACELLULAR MATRIX

The extracellular matrix is structurally complex and diverse in its composition of functional proteins, glycoproteins and proteoglycans which are organized into a three-dimensional system. These characteristics provide elasticity, binding sites for cell surface receptors and signal factors to the ECM, which are essential for angiogenesis, cell migration and proliferation.

ECM properties are a result of its specific protein content. For instance, collagen is the most abundant protein within the ECM, and though over 20 different types of collagen have been reported, in ECM only types I, III, IV, VI and VII are commonly found. Collagen type I is a well conserved protein; therefore, it has become a good source for tissue engineering scaffolds. Other collagen types, such as collagen VI, are essential for mechanical and physical properties of collagen type I and the ECM. Hence, ECM is source of several collagen types that are able to provide proper environment for tissue reconstruction.

Fibronectin, another protein present in ECM although it is more commonly found in submucosal structures and basement membranes of tissues. This type of protein plays a major part in cell adhesion by ligand presentation. Fibronectin can be used as a coating protein in synthetic scaffold material. Laminin, which can also be found in the ECM, is typically localized to its basement membrane and its inherent properties confer good adhesion characteristics to the scaffold.

Growth factors present in the ECM are also necessary for successful tissue reconstruction. Decellularized
scaffolds contain diverse growth factors such as VEGF (important for vessel formation), bFGF (stimulates cell proliferation and has a crucial role in differentiation), KGF (necessary for epithelialization of wounds) among others, that are present in small but sufficient amounts for tissue regeneration.

The original concept of ECM passivity in tissue development was reconsidered with the discovery of dynamic reciprocity between the ECM and intracellular cytoskeletal and nuclear elements. The ECM has a major role in the differentiation of myoblast to myotubes\(^{15}\); also, the main structural component of ECM (collagen) is important for the development of salivary glands\(^ {16}\). This knowledge has become useful in tissue engineering, since it allowed the use of ECM as a scaffold for tissue reconstruction by applying cells with differentiation and proliferation potential.

ECM components are highly conserved and well tolerated among different species. This fact was confirmed when performing organ transplantation experiments; for example, heart valves\(^ {17,18}\), blood vessels\(^ {10,19}\), skin\(^ {20}\) and muscles\(^ {21}\).

**METHODS OF DECELLULARIZATION**

A commonly used technique in decellularization involves the combination of chemical and physical methods. The most effective decellularization processes include a combination of physical, chemical and enzymatic agents. However, the final result is dependent on factors such as tissue cellularity, density, lipid content and so on\(^ {22}\).

**Alkaline and acid treatments**

Acid and basic treatments cause the hydrolysis of cytoplasmic components and the degradation of nucleic acids; however, it could have adverse effects on glycosaminoglycan content. Peracetic acid is a frequently used acid agents; however, it could have adverse effects on glycosaminoglycan content. Peroxidase treatment results in decreased ECM mechanical properties\(^ {25,33}\). SDS, as a decellularization agent, seems to be more effective than Triton X-100 since its effectiveness is higher, especially with dense organs such as kidney, liver or temporomandibular joints\(^ {30-32}\). On the other hand, SDS has deleterious effects upon the scaffold, which are comparable with those caused by treatments with Triton X-100 or hypertonic solutions.

**Ionic detergents**

SDS (sodium dodecyl sulfate) solubilizes cell membranes, but tends to denature proteins by disrupting their reciprocal interactions\(^ {25}\). SDS, as a decellularization agent, seems to be more effective than Triton X-100 since its effectiveness is higher, especially with dense organs such as kidney, liver or temporomandibular joints\(^ {30-32}\). On the other hand, SDS has deleterious effects upon the amount of GAG in the ECM. This, in itself, decreases the mechanical stability of the scaffold\(^ {25,33}\).

SDS has a different incubation time from Triton X-100, being more effective in 24 h incubation periods at 0.1-1% concentration\(^ {20,34,35}\).

**Zwitterionic detergents**

CHAPS or 3-[\{(3-cholamidopropyl) dimethylammonio]-l-propanesulfonate combines the properties of non-ionic and ionic detergents. As confirmed by histological analyses, CHAPS is ideal for thin tissues such as peripheral nerves\(^ {16,17}\), blood vessels\(^ {39}\) or lungs\(^ {39}\). It also has proved to be non-damaging to collagen and elastin structures. However, it also promotes decreased mechanical properties to the scaffold, which are comparable with those caused by treatments with Triton X-100 or hypertonic solutions.

**Alcohols**

Alcohols remove lipids from tissue very effectively and cause tissue dehydration leading to cell lysis. However, the use of either methanol or ethanol promotes protein precipitation; therefore, their use is limited to the elimination of phospholipids from tissue, since the preservation of phospholipids in the tissue could cause calcification\(^ {40,41}\);
usually, a combination of methanol with chloroform is used. It is worth keeping in mind that any residual chemical agents present in the ECM after decellularization could have cytotoxic effects on colonizing cells. In order to achieve a complete removal of these residues, several washing cycles with PBS are used.

**Biological decellularization**

Biological agents such as proteolytic enzymes, are used in combination with chelating agents and nucleases in decellularization methods. Trypsin proteolysis is widely used in decellularization of heart valves or dermis. However, the stability of extracellular components is limited against trypsin and its use could induce undesired alterations in collagen, elastin or GAG content. It is therefore recommended to utilize it for short periods of time to prevent damage against the basic components of ECM. On the other hand, the harsh characteristics of trypsin can be used for membrane disruption, increasing the efficiency of subsequent decellularization treatments.

**DECELLULARIZATION TECHNIQUES**

Each kind of tissue has specific structure and different composition, so it is not possible to use only one standard protocol for each type of tissue (Table 1). The choice of decellularization agent to be applied depends on the type of tissue to be treated as well as of its composition (thickness, density of tissue). Apparently, the most effective method is the use of immersion/agitation and whole-organ perfusion processes.

**Agitation and immersion**

Principle of decellularization by means of agitation/immersion is a two-step process: the sample tissue is immersed in decellularization solution (e.g. SDS) followed by its agitation in an orbital shaker, allowing the solution to permeate through the tissue. Thinner tissues (such as urinary bladder) can be effectively decellularized after a short incubation with peracetic acid, which disrupts its surface tissue without the need for agitation.

In addition, agitation can also be used for rinsing residual detergents, by means of PBS washing cycles. It may be used for heart valves, muscles, peripheral nerves, trachea and dermis.

**Whole organ perfusion**

Perfusion seems to be a more effective decellularization method, since this method able to preserve tissue vasculature; also, it’s more efficient in the delivery of decellularization agents inside the tissue and able to wash out cellular debris. Whole organ perfusion is used for tissue such as heart, liver, lungs or bladder. The organs are previously cannulated to ease the access of decellularization solutions. A previous study described liver decellularization by means of whole organ perfusion with SDS, proving to be an effective method since the tissue was completely decellularized and the ECM’s composition and structure remained intact. Subsequently, the tissue was recellularized with hepatocytes, thus providing evidence on the preservation of ECM components able to support cell colonization.

**DECELLULARIZATION EFFECT ON THE ECM**

Any decellularization process can affect the 3D structure of the extracellular matrix; for instance, glycosaminoglycans could be depleted, losing any water molecules bound to it and leading to decreased tissue elasticity. Some detergents can induce alterations in the collagen fibers resulting in decreased mechanical stability of the tissue.

**EVALUATION OF DECELLULARIZED ECM**

Standard evaluation methods for decellularized ECM include histological analyses, immunohistochemistry, biochemical analyses, DNA/GAG quantification, hydroxyproline assay, 2D electrophoresis among others. The concept of “decellularization” has not yet been described in terms of quantitative metrics; however, it can be said (upon the findings of other studies) that decellularized tissue could be defined as follows: it should contain no...
more than 50 ng of DNA per mg of extracellular matrix, any present DNA should be in fragments less than 200bp in length and the ECM should lack any visible nuclear material as confirmed by standard hematoxylin or DAPI staining\(^2\).

Hematoxylin-eosin staining is the standard technique for basic histological inspection and analysis. Although alternative staining methods can be used, such as Masson’s trichrome or Movat’s pentachrome, to examine the presence of cytoplasmic or extracellular molecules. The presence of DNA molecules can be detected by DAPI staining or BrdU assay.

Immunohistochemistry is also used to determine the presence or absence of extracellular proteins e.g. laminin, fibronectin, collagen or GAG. We could also use Western blot for this purpose since it has similar principle.

DNA quantification can be determined by spectrophotometric analysis, measuring DNA concentration by means of fluorometric stains, e.g. ethidium bromide or Hoechst, which are incorporated into the structure of DNA. Stained DNA is then exposed to a specific UV radiation wavelength (\(\lambda\_{260}\text{nm}\)): the resulting emitted radiation rate is directly proportional to the concentration of nucleic acids in the sample.

Proline is an essential amino acid that can be found in hydroxylated form in tissues; its quantification is commonly used as a standard reference for protein degradation measurement, e.g. collagen or elastin. Quantification of hydroxyproline is carried out by lysing the samples with hydrochloric acid, which then will react with p-dimethylaminobenzaldehyde, and later analysed by spectrophotometry. Color intensity determines the concentration of hydroxyproline present in the analysed sample. The results of this test indicate if the decellularization process was too harsh on either collagen or elastin structure.

Electrophoresis is a basic separative method for proteins, using the distribution of molecules on a synthetic matrix based on their molecular weight and charge in electrical field as an analysis tool. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is the most commonly used gel electrophoresis technique used for proteins. One study, focused on decellularization of skin\(^1\), SDS-PAGE was used as an evaluation tool and the results showed that decellularization was effective since collagen did not show any alterations.

CONCLUSION

Extracellular matrix (ECM), as a biologic scaffold derived from a process of decellularization, can be used in regenerative medicine. The main objective of decellularization is the preservation of ECM’s three-dimensional structure which consists of structural and functional proteins, glycosaminoglycans, growth factors, etc. ECM is an immunologically inert scaffold that preserves the native vascular and neural network of the source material and can be utilized in tissue engineering. The efficiency of the decellularization process is dependent upon several factors such as tissue density and organization, decellularization method, biologic components and targeted clinical applications. The combination of physical, chemical and biological methods provides a better outcome regarding cell removal. The development of an optimal decellularization protocol is a major concern and one still persisting in tissue engineering and regenerative medicine.

ABBREVIATION

BrdU, 5-bromo-2′-deoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; ECM, Extracellular matrix; GAG, Glycosaminoglycans; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS, Sodium dodecyl sulphate.
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