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REVIEW ARTICLE

# Chemical Decellularization Methods and Its Effects on Extracellular Matrix

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**ABSTRACT**

**Background:** Extracellular matrix (ECM) produced by tissue decellularization processes as a biological scaffold due to its unique properties compared to other scaffolds for migration and implantation of stem cells have been used successfully in the field of tissue engineering and regenerative medicine in the last years. The objective of this manuscript was to provide an overview of the chemical decellularization methods, evaluation of decellularized ECM and the potential effect of the chemical decellularization agents on the biochemical composition. **Methods:** We searched in Google Scholar, PubMed, Scopus, and Science Direct. The literature search was done by using the following keywords: "ECM, biologic scaffold, decellularization, chemical methods, tissue engineering." We selected articles have been published from 2000 to 2016, and 15 full texts and 97 abstracts were reviewed. **Results:** Employing an optimization method to minimize damage to the ECM ultrastructure as for a result of the lack of reduction in mechanical properties and also the preservation of essential proteins such as laminin, fibronectin, Glycosaminoglycans (GAGs), growth factor is required. Various methods include chemical, physical and enzymatic technics were studied. However, on each of these methods can have undesirable effects on ECM. **Conclusion:** It is suggested that instead of the Sodium dodecyl sulfate (SDS) which have high strength degradation, we can use zwitterionic separately or in combination with SDS. Tributyl phosphate (TBP) due to its unique properties can be used in decellularization process.

## INTRODUCTION

Using extracellular matrices (ECM), as a bioscaffold for tissue regeneration has been welcomed in the past few years, therefore, in recent years (1-7), various methods from different tissues have been tested to produce the healthy ECM. So far, ECM from various tissues has been made, and efforts have been put to use some of these biological scaffolds in tissue engineering and regeneration of organs. As regards tissues that have been used to produce ECM, heart (8-11), blood vessels (12,13), skin (14), nerve (15), skeletal muscle (16), tendon (17), small intestine (18), oral mucosa (19-21), jawbone and teeth (22-24), sinusoidal mucosa (25), liver (26) and lung (27) can be noted. The unique structure of the ECM causes the cells with tissue repair properties, to multiply within the ECM. Using ECM reduces the immune responses, thereupon occasion enhances the success rate of tissue transplantation and lack of tissue rejection. Also, ECM contains factors for cell growth and proliferation, as well as containing non-collagenous proteins potentially, including laminin and fibronectin to increase the adhesion of cells to the scaffold (28) as well glycosaminoglycans (GAGs). Various methods, including physical, chemical and biological technics are used throughout the process of decellularization; however, the most effective decellularization process results from the combination of physical, chemical, and biological methods. Other important factors such as tissue type, density, and tissue thickness are also involved (5). The aim of this study was reviewing the chemical methods that are used in decellularization, evaluation of there, and effects of each chemical method on biochemical compounds, structural and mechanical behavior of ECM.

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## CHEMICAL METHODS

Using chemical methods facilitates decellularization which includes chemical agents, acid and bases, detergents, alcohols, hypotonic and hypertonic solutions.

### Acid and Base

Acids and bases cause nucleic acid decomposition and hydrolysis of cytoplasmic components (1,29). Acids and Bases that are used in decellularization protocols are acetic acid (30), peracetic acid (PAA), hydrochloric acid, sulfuric acid, ammonium hydroxide, calcium hydroxide, and sodium hydroxide (3).

PAA is commonly used throughout the process of decellularization (31). It can maintain growth factors such as growth factor β, VEGF, laminin and fibronectin, which are involved in adhesion of cells to the ECM (32-35) and have been established to have no adverse effect on the mechanical properties of ECM (36).

It also has been disinfecting, and microbial enzymes oxidizing properties (37,38). PAA concentration of 0.1% has been successfully used in decellularization of small intestine submucosa (SIS) and urinary bladder matrix (UBM) (39,40). One of the problems with using acids throughout the process of decellularization is the deletion GAG from the ECM (41).

Bases are mostly used to remove hair from skin samples before starting the process of decellularization (42,43); however, the damaging effects of bases on the ECM and its collagenous protein constituents, reduce the mechanical properties of ECM and removes the growth factors from the ECM (44).

### Hypotonic and Hypertonic Solutions

Hypotonic and hypertonic solutions cause cell lysis by inducing osmotic shock in the tissue and breakdown of the cell membrane in tissues or organs (44-48). Hypertonic solutions have the ability to separate protein from DNA (49). One of the reasons for using hypotonic solutions is a minimal change in the structure of the ECM (50). Moreover, hypotonic and hypertonic solutions are used for washing cell debris, but they are not able to clean and remove cell debris from the tissues entirely (51-53).

### Alcohols

Glycerol is one of the most commonly used alcohols in decellularization which causes cell lysis, dehydration (54), and effectively removes lipids. It has more detergency and decomposing capabilities compared to lipase (42,55). Methanol along with chloroform are used as the other compounds for delipidation are (54). One of the problems of using methanol and ethanol to fix tissues is protein precipitation. Therefore, one should be cautious in using methanol and ethanol in the process of decellularization because it may damage the protein structure of ECM and affect its mechanical properties (43,56-60). Lipid occasion calcification, so alcohol is considered as an anti-calcifying agent (56,61).

### Detergents

Detergents are amphipathic molecules with a polar and aquaphobic head that can dissolve hydrophobic compounds in water. Detergents are used in 2-dimensional electrophoresis in addition to being used in the process of decellularization (62). Detergents are classified based on their polar head into three categories as follows: ionic, non-ionic and ion-dipole.

### Ionic Detergent

Ionic detergents are effectively used in removal and dissolution of membrane proteins and DNA associated proteins (63). Sodium dodecyl sulfate (SDS) is widely used in the process of decellularization (63). In comparison with other detergents, SDS has more effectiveness to the removal of the cell debris such as nuclear and cytoplasmic compounds from dense and thick tissues (e.g., heart) (9,64). Hence, it is the most commonly used detergent in decellularization (47,59,65). Although SDS cleaning power is stronger than many other detergents, it also has more destructive effects compared with other detergents (66-69). A disadvantage of using SDS as a detergent is reducing the amount of GAG and growth factors from the ECM (43). Due to the strong tendency of SDS toward protein, it can disrupt protein-protein interactions. Thus it may have a role in the loss of collagen integrity and change in the ECM structure (70-72). Using SDS in different concentrations leads to various injuries on ECM levels (5). Previous studies on the vein and gums were examined various concentrations of SDS that revealed optimized concentration with the highest level of decellularization (29,72).

The destruction level of a detergent depends on its type, detergent and fabric duration of exposure, type of tissue and age of the tissue donor (43,73). Sodium deoxycholate is an another ionic detergent which used in decellularization, but it has more destructive power compared to SDS, and it is typically used in combination with bipolar detergents (74,75). Triton X-200, similar to SDS and sodium deoxycholate is used as an ionic detergent in the process of decellularization (76-80).

### Non-ionic Detergents

Non-ionic detergents have lower destructive effects on the structure of ECM. However, detergents are widely used in the process of decellularization. Non-ionic detergents can break down lipid-lipid bonds, but cannot destroy the protein-protein bonds (81). Triton X-100 is used as a non-ionic detergent in decellularization. This detergent has been used in decellularization of tissues such as liver (82-84), pericardium (85), lung (7,86,87), gum (88) and skin (89).

This compound has biodegradable properties and a high ability to destroy and remove cells from the surface of ECM (90) and provide the conditions for the growth of cells. Some studies revealed the effects of Triton X-100 on decellularization of blood vessels, tendons, ligaments, myocardium and aortic walls that no success was seen, while it has been successfully used in decellularization of heart

valves (47,48,91). These researches show that the use of Triton X-100 in decellularization has both said results. Many studies regarding decellularization have demonstrated that Triton X-100 eliminates GAG from ECM surface (92). However, in a study on decellularization of anterior cruciate ligament (ACL), it was shown that this detergent does not have any harmful effects on GAG levels of ECM surface (47). Triton X-100 is known as the best detergent in the process of delipidation and compared to SDS, and other detergents such as sodium doxycycline lipase and even lipase have shown a much better performance (93,94).

**Bipolar Detergents**

Bipolar detergents have the properties of both ionic and non-ionic detergents that are mostly used in decellularization of thin tissues (95,96). Bipolar detergents such as CHAPS, sulfobetaine-10, and sulfobetaine-16 are used in the process of decellularization. For example, CHAPS were used in decellularization of blood vessels (48) and sulfobetaine-10, and 16 sulfobetaine were applied in decellularization of nerves (15).

**Chelates**

These compounds are tightly combined with an ion. EDTA and EGTA are examples of these chemical compounds that are used in the process of decellularization. These chemical's compositions destruct cell connections to collagen and fibronectin by separating ions such as $Ca^{2+}$ and $Mg^{2}$ (97-100). Chelating agents are usually used in combination with enzymes such as trypsin (9,52,101-103) or detergents (9,101) and do not have any application on its own (104). EDTA in the long term reduces the mechanical properties of the scaffold (105).

**Organic Solvents**

Tributyl phosphate (TBP) is one of the most widely used organic solvents in the process of decellularization. TBP performs as a destructor of the protein structure. These chemical's compositions also have anti-viral properties. Using organic solvents such as TBP is also a suitable alternative for ionic and non-ionic detergents for thick tissues. One of the advantages of TBP compared to others is the minimal damage that may cause to the mechanical properties of ECM. It is used in decellularization of dense tissues such as tendons and ligaments. Therefore, further studies are required in this field (17,47).

**Effects of Decellularization on ECM**

The process of decellularization may have some adverse effects on the ECM, including reduction of collagenous and non-collagenous proteins such as laminin, fibronectin, glycosaminoglycans. It also removes water molecules attached to it and thereby reduces the elasticity of tissue. Removal of collagenous proteins as well decreases the mechanical properties of ECM (17,47).

**Evaluation of Decellularization Process**

For standard assessment of a method of tissue decellularization, histological analysis, determination of the amount of DNA, GAG, biomechanical analysis, continuity of collagenous scaffolds, non-collagenous proteins are required.

Several methods are available to identify the effects of decellularization on the removal of cellular materials and on the ECM itself, for instance, hematoxylin-eosin staining to the investigation of histological analysis and Movat's Pentachrome staining for detecting the presence of cytoplasmic and ECM molecules.

A specific quantitative amount of cellular materials has not been determined in the process of decellularization although it is possible to overcome the problem that based on laboratory research findings as well as in vivo responses to the reconstructed models. For instance, the amount of DNA in every mg of ECM should not be less than 50ng.

The amount of DNA fragments should be less than 200 bp, and nuclear materials should also stay undetected in 4', 6-diamidino-2-phenylindole (DAPI) or hematoxylin-eosin staining because DNA directly causes an immune response in the host body (1,106,107). DNA density can also be identified by using fluorometric stainings such as Hoechst (108-110). DNA is present everywhere in the tissue and could be easily detected and used as an overall indicator for measuring the elimination of cell debris from the surface of the ECM (111). Immunohistochemistry is used as a staining for detecting the presence of non-collagenous proteins such as collagen, laminin, fibronectin, glucose, GAG, actin, and myosin (47). Pyrroline, a major amino acid in the ECM structure is used as a standard indicator in assessing the amount of protein degradations such as collagen and elastin (109,112). Evaluation of the effects of decellularization on the mechanical properties of ECM and cell debris on the ECM after the process has always been one of the main issues in this process. In previous studies, the effects of different detergents on the ECM were assessed, and it was demonstrated that these detergents cause damage to the ECM collagen and therefore, reduce the mechanical properties.

However, there are also exceptions, in studies on the use of Triton X-100 in the process of decellularization of ACL, it was revealed that Triton X-100 have not any destructive effects on ECM collagen (17)

## CONCLUSION

The use of ECM as a biological scaffold in the past few years has been studied. Decellularization of the trachea and successful deployment of its ECM in tracheal reconstruction has shown an excellent prospect in the field of tissue engineering by using bio-scaffolds. In order to obtain a healthy ECM, it is necessary to implement an optimized method with the lowest level of damage to the structure of ECM and lack of decrease in its mechanical properties as well as preserving important proteins such as laminin, fibronectin, GAGs and growth factors. A suitable protocol should also be able to clear nuclear materials and cell debris from the ECM. The presence of these materials in terms of in-vivo conditions leads to host's im-

mune response. However, no protocol has been recorded with 100% efficiency. This efficiency depends on some factors, including the type of tissue, type of decellularization method as well as the composition of these methods. As mentioned earlier, a complete decellularization requires a combination of all three biological, chemical and physical methods. In the process of decellularization, detergents such as SDS play a key role. However, these detergents cause damage to collagenous and non-collagenous proteins, GAGs and growth factors. It also causes irreparable damage to the ECM structure. For this reason, it can be suggested to use di-ionic detergents (bipolar) that are less destructive compared to SDS. Even bipolar detergents could be used in combination with a low concentration of SDS for increased cleaning power of this cellular detergent. Hence, this review can helps clinicians to select the appropriate method in decellularization processing for achieving ECM as a scaffold in organ transplantation processes.

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Amir Hossein Akbari Zahmati, Reza Alipoor, Arash rezaei shahmirzadi, Vahid Khori and Mohammad Mahdi Abolhasani gathering data and Amir Hossein Akbari Zahmati Analyzed Data, wrote primary draft, revised and submitted it.

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