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Decellularization of lung tissue and analysis of its differentiative potential on bone marrow mesenchymal stem cells of rat

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Abstract

Background: Pulmonary diseases are one of the most common causes of mortality worldwide. Lung transplantation is the only curative treatment in some cases. Very few lungs are available for transplantation and the 5-year survival after lung transplantation is 50%. Transplant recipients require immunosuppressant therapy in that's time. Production of engineered lung tissue using stem cells is a promising approach. Extraction of natural ECM or decellularization of lung and application of it in tissue engineering is one of the most important strategies in this regard. It can preserve the natural characteristics of the ECM and would leads to the removal of the immunogen agents (MHC I, II) and allows reconstruction of graft.

Objective: we have decellularized rat lung and cultured bone marrow stem cells on it to evaluate its differentiative potential.

Methods: In this study along with extraction of rat lung, its femur and tibia bones were isolated for extraction of mesenchymal stem cells. Lung tissue was decellularized using SDS detergent. SEM and H&E staining used to assay decellularization. Mesenchymal stem cells were seed on the decellularized tissue sections and immunocytochemistry for CC10 and SPD performed on these cells after 21 days.

Results: H&E slides of decellularized tissue did not show any cells. In electron microscope images of decellularized tissue alveolar structure was well maintained. Immunocytochemistry assay showed differentiation of seeded mesenchymal cells toward lung tissue cells.

Conclusion: Decellularization take placed without any significant change in tissue structure. Decellularized tissue induced differentiation of bone marrow stem cells toward lung epithelial cells so natural ECM saved relatively.

Keywords: Decellularization, SDS, Differentiation, CC10, SPD

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Introduction

Pulmonary diseases are one the most common cause of mortality worldwide. Each year 7718 of Iranian and 400,000 of American people die due to lung disease. The incidence of lung diseases, especially COPD, has increased and it is expected to be the third leading cause of death in the world in 2020. For some lung diseases, including cystic fibrosis, emphysema, chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis, lung transplantation is the only curative treatment (1, 2).

Unfortunately, very few lungs are available for transplantation and also the 5-year survival after lung transplantation is only 50%. Furthermore, transplant recipients require immunosuppressive therapy during that time. Therefore, new therapeutic strategies are needed. Now the promising and growing approach is production of engineered lung tissue in the laboratory that can be transplanted into patients. Engineered lung can be achieved by using of biological derived matrixes or synthetic scaffolds seeded with stem cells. In addition to lung, tissue engineering is used for regeneration of other tissues such as skin, blood vessels, cartilage, bone, trachea and more complex organs such as the heart and liver (3-9). Engineering of a whole organ was not possible yet and use of decellularized tissues and organs invented in tissue engineering. In this method cellular elements removes from tissue but intact important extracellular matrix (ECM) proteins and three-dimensional architecture of it leaves (1). Intact extracellular matrix of a tissue or organ can differentiate stem cells into its cells. Therefore, decellularized tissue can be used as a normal scaffold and a natural inducer in tissue engineering. Furthermore, the immune system is less stimulated by decellularized tissue thus the success rate of tissue engineered transplants in this way is high.

There are several ways for decellularization including physical, enzymatic, and chemical methods. The chemical method is more efficient and common. In this method SDS, SDC and CHAPS are used (10-11). SDS is very effective to remove cellular components of the tissue, and compared to other detergents, SDS cause the complete elimination of nuclear debris and cytoplasmic proteins. It has been effective in decellularization of the host organs like kidney and liver. Usually, the duration of incubation with SDS is 24 hours at concentrations between 0.1% -1% to reduce side effects of its use (12-16).

In this study, we used 0.1% SDS in a new protocol for decellularization of rat lung and evaluated efficacy of this method and differentiative potential of decellulariezed lung tissue on bone marrow mesenchymal stem cells.

Material and Methods

Animals

5 male rats from Iranian Razzi Institute housed in controlled environment (humidity and temperature) on a 12/12 hours light/dark cycle with free access to standard food and water. On the body weight 200-250 gr animals used to isolation of mesenchymal stem cells and decellularization of lung. All efforts made to minimize animal suffering and to reduce the number of animals.

Tissue harvest

Tissue harvested by heart perfusion method. In this method PBS and other solutions perfused via heart into organ and whole body to remove blood. Animals sacrificed with ketamine and xylazine (100 mg/kg and 10 mg/kg). A vertical section made in midthorax to access to heart. By use of a catheter from right ventricle perfusion started with 250cc PBS supplemented with Heparin 5000u/ml and 1% penicillin and streptomycin. After perfusion, lungs and femur bones harvested. Before harvesting trachea and main vein and artery of each lung identify and fixed with catheters to use in decellularization process.

Decellularization of lung tissue

The SDS 0.1% for 48 hours at 4 C was perfused from the trachea, veins and arteries with a handmade device while lung was flouted in the SDS 0.1%. Then 0.1% Triton X100 in PBS similarly perfused in 72 hours. Finally samples were perfused with PBS for 24 h and keep in PBS at 4C prior to use. All procedures performed in a sterile condition.

Hematoxillin and eosin staining

Hematoxillin and eosin staining protocol (H & E) used to tissue assay. Lung tissue (before and after decellularization) fixed in paraformaldehyde 4%, after fixation transferred to tissue processor device to dehydration and clearing. Finally embedded in paraffin and sectioned in 5 micron diameter. Sections on glass lam stained with hematoxillin and eosin and observed in a light microscope.

Morphology observation

Morphology of decellularized lung ECM was assayed by a scanning electron microscope. Some sections with 2-5 mm thickness fixed in glutaraldehyde 2.5% and post fixed in osmium tetroxide 1% then dehydrated with alcohol and frieze dried for 4 hours. Finally sections observed with a scaning electron microscope.

Isolation of bone marrow stem cells

Bone marrow stem cells isolated from femurs of rats that their lungs used for decellularization. The femurs of animals removed by surgery and transferred into laminar flow hood. At a sterile condition two ends of each bones cut and bone marrow extracted by flashing of 5 ml DMEM from one end. Bone marrow cultured in DMEM supplemented with 10% FBS for 24 hours. After 24 hours and then every 48 h the culture medium exchanged (17). The morphology of isolated cells examined under a phase-contrast microscope. The cells in third passage used to seeding in decellularized lung.

Cell Seeding

A suspension of bone marrow stem cells in DMEM (105 in 100µl) add to decellularized lung sections in culture plate. 24 hours later and every 48 h medium exchanged.

Immunohistochemistry

21 days after cell seeding, immunohistochemistry done for CC10 and SPD proteins. Cultured BMSCs with decellularized lung sections fixed with paraformaldehyde 4% and washed 3 times with PBS. After permeablization for 30 min at room temperature with 0.25% Triton X-100 (Sigma), and blocking by applying 0.3% BSA (Sigma) with 5% NGS, sections were incubated for an overnight at 48C with primary antibodies that were diluted in 0.3% BSA/TBS. Then sections rinsed thrice with TBS prior to secondary antibody application. Secondary antibody was diluted 1:500 in 0.3%

BSA/TBS and applied to cells for 3 hr at room temperature in the dark. Tissue was subsequently washed in TBS, and nuclei were counterstained with lg/ml DAPI (Sigma-Aldrich) in TBS. The sections were preserved with glycerol buffer under coverslips.

Results

Tissue Harvesting

Use of perfusion method for tissue harvesting caused to remove of blood from tissue so tissue color after perfusion turned to white.

Hematoxillin and eosin staining

After decellularization by mentioned method, sections in 5 µm stained with H&E. In figure 1, panel A show normal long tissue and panel B show decellularized lung tissue. The nucleuses of the cells clearly see in the A but there is not any nucleus in B. After decellularization cytoplasm and nucleus destroyed and removed as showed in fig B. The result of H&E present decellularization was effective.

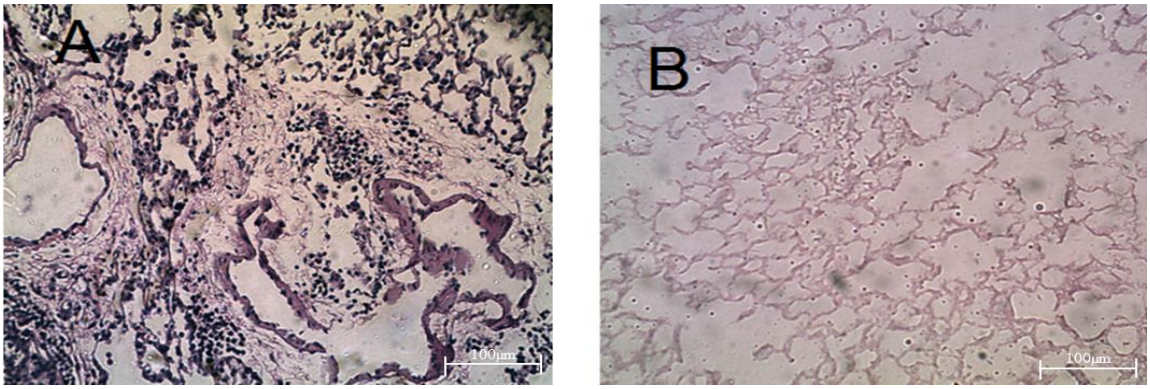

Figure 1: H&E staining of lung tissue. A: Before decellularization, B: After decellularization. Cell nucleuses stained dark.

Morphology assessment

SEM can present three dimensional structure of a scaffold or tissue so we done it for normal and decellularized lung tissue. Our result showed in figure 2.There was no significant changes in microstructure of tissue after decellularization. Consequently our method of decellularization can save microstructure of normal tissue.

Bone marrow stem cell isolation

Bone marrow stem cells (BMSCs) attached in flask during 24h. Debris removed by medium exchanges. After tree passage they had a spindle shape. (Figure 3)

Immunohistochemistry

BMSCs seeded in decellularized lung tissue assayed for differentiation and expression of CC10 and SPD markers 7, 14 and 21 days after seeding. Our result of Immunohistochemistry showed expression of these markers after 21days. Figure 4 showed this result.CC10 and SPD expressed in the BMSCs that seeded in decellularized lung tissue.

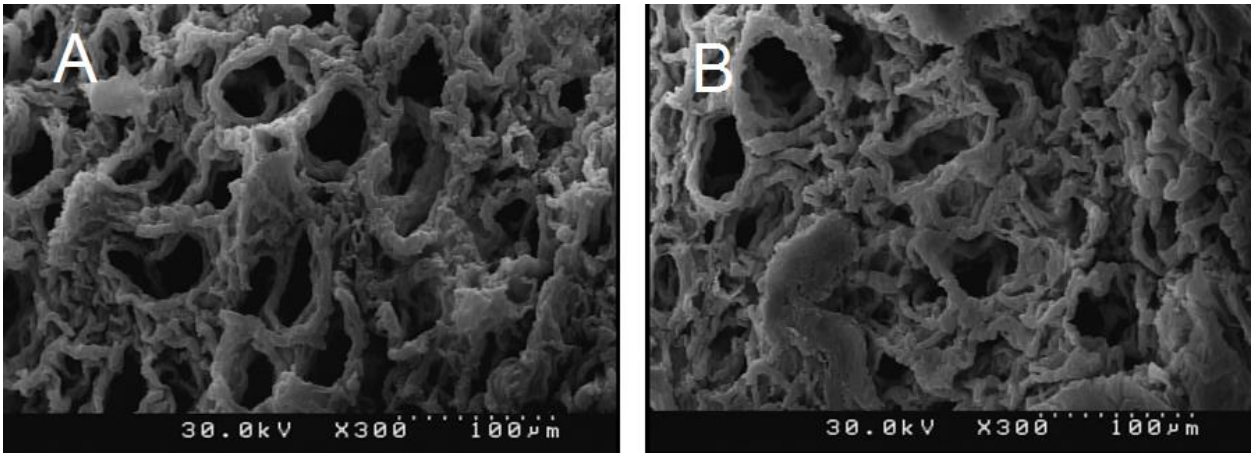

Figure 2: SEM graphs of lung tissue. A: Before decellularization, B: After decellularization

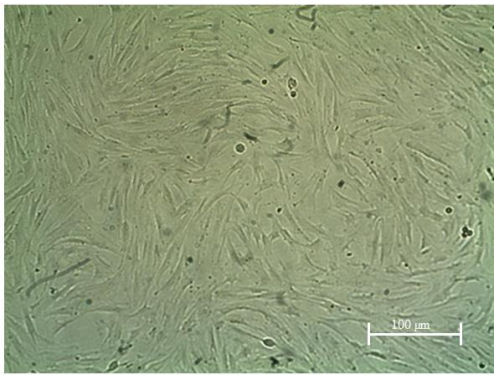

Figure 3: Bone marrow mesenchymal stem cells in passage 3.

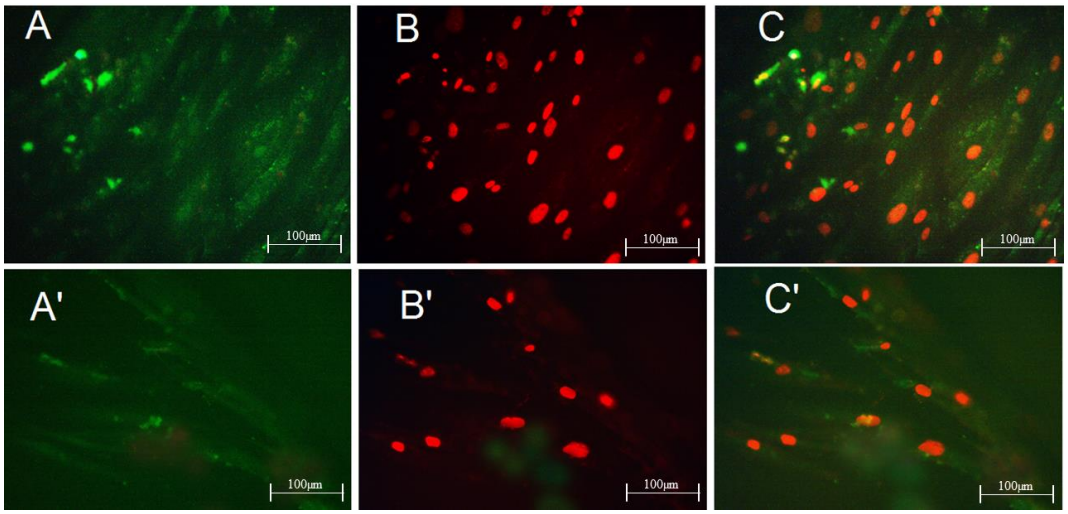

Figure 4: Immunohistochemistry of cells seeded on decellularized lung tissue. A, B & C for CC10 and A', B' & C' for SPD. Left column related to markers, middle column related to nucleus and right column is merged of them.

Discussion

Lung diseases are one of the leading causes of death in the world. In some cases lung transplantation is the only treatment. Lung transplantation such as other organ transplantation has its own limitations (1, 2). Therefore scientists are trying to engineer lung tissue. One promising technique in organ tissue engineering is use of decellularized organ in which intact important extracellular matrix (ECM) proteins and three-dimensional architecture of it leaves (1). Intact extracellular matrix of a tissue or organ can differentiate stem cells into its cells.

Bone marrow mesenchymal stem cells can be easily obtain, store and survive for long time. More importantly, they can be used in autologous transplantation (18). The influence of normal cellular scaffolds prepared from the decellularized lung on the differentiation of MSC cells has not been studied. Thus the purpose of this study was to evaluate the effect of extracellular matrix scaffold considering its spatial form and key proteins on the differentiation of human mesenchymal stem cells. The lung cell scaffolds were prepared by two methods: sectional and whole organ decellularization. The MSC of bone marrow cells were seeded on scaffolds. The results showed that the ECM obtained from decellularized lung tissue can cause the differentiation of MSCs into cells expressing the CC10 and SPD, the markers of Clara epithelial cells and type II lung pneumocytes, respectively. This ECM obtained by SDS decellularization protocol. SDS, SDC and CHAPS were used in several studies (19).

Detergents such as SDS led to the dissolution of the nuclear membrane and cell cytoplasm. It also desire to denature proteins by disrupting interactions between them. It also has a detrimental effect on glycosoaminoglycans (GAG) in the extracellular matrix, which reduces the mechanical stability of the scaffold (20, 21). Furthermore, SDS is very effective to remove cellular components of the tissue, and compared to other detergents, SDS cause the complete elimination of nuclear debris and cytoplasmic proteins (22). The SDS has been effective in decellularization of the host organs like kidney and liver (23). Usually, the duration of incubation with SDS is 24 hours at concentrations between 0.1% -1% (24, 25).

In a comparative studies between chemical detergents commonly used in decellularization, including (SDS, SDC, CHAPS), CHAPS has been introduced as a mild detergent with minimal tissue damage and better collagen and elastin protection and tensile strength of the scaffold. However, this detergent is effective only in the elimination of nuclear proteins and compared with SDS it lacks the ability of removing cytoplasmic proteins (26, 27).

In this study, we used 0.1% SDS, due to its high efficiency in the removal of cells and cell debris. It is the easiest and most accessible material for decellularization. This study showed that SDS as a strong detergent in tissue decellularization has the ability to maintain tissue proteins that provide cellular connectivity. This result is consistent with other studies comparing the effects of detergents on decellularization of tissue. The SDS is weaker than other detergents in maintaining the protein structure.

To check the result of decellularized context, the following tests are used:

1. The tissue photos before and after decellularization to show the preserved macroscopic properties (28).
2. Injecting (perfusing) a dye such as trypan blue after decellularization, to demonstrate the absence of any obstructions or restrictions on reagent or cell release after decellularization (29).

3. H & E slides preparation from normal and decellularized tissues to show effective removal of cells and to show healthy scaffolding (29).

4. SEM images of the tissue before and after decellularization, to study the micro-structure of the alveoli (29).

5. DAPI staining which is a fluorescent dye to check the presence of DNA after decellularization (23).

6. Different types of tissue staining such as MassonsTrichomePicrosirius; gomoritrichrom (to assess collagen) and Pentachrom (to assess collagen and elastin); Alcian Blue (to assess glycosaminoglycans).

The important point in decellularization is maintaining the structure of cell scaffolds and key proteins of cell matrix such as collagen, elastin and fibronectin. Our results of decellularization using H & E slides and electron microscopy SEM images showed that no cells were observed in H & E slides and the cell scaffold was maintained. Electron microscopy images showed that the structure of the alveoli and airways and blood vessels are intact and the overall texture is preserved. Also, comparing the normal and decellularized lung showed that the macroscopic structure of the tissue is preserved during the process. Our strong point compared with other studies is in using a strong detergent with high efficiency in the removal of cells and cellular debris than other detergents but with more tissue damage, the ability to manage it in order to minimize tissue damage using different concentrations of SDS and the decellularization times.

The use of cell therapy using stem cells in a clinical setting is facing with obstructions such as concerns due to the lack of knowledge of the fate of transplanted stem cells in the damaged lung, and the regulation mechanisms of activation of resident progenitor cells in the lungs. However, information obtained from the experimental research on the safety of stem cell therapies in the treatment of chronic lung disease, showed promising results (30).The unique feature of mesenchymal stem cells compared to other stem cells, makes them the main candidates for therapeutic applications in lung disease. MSC can be useful in lung bioengineering strategies. They have several desirable features including support, adjustment and repair of the texture (30).

For example, sheep MSCs showed promoting the growth of epithelial cells in co-cultures, transplantations and provisional matrix synthesis on the decellularized lung scaffolds which causes improved lung tissue in sheep model of emphysema. Similarly, the mouse MSCs in the elastase-induced damage model (32).

In a study of type II alveolar cell co-cultured with lung MSCs it causes the expression of CK18, CK19, and SPC in the MSCs, showing the in vitro ability of stromal cells in the lung to differentiate into alveolar cells (33). In a study the differentiation ability of bone marrow mesenchymal cells into the type II alveolar epithelial cells through BMSC co-culture with MRC-5 cells in SAGM modified media was shown (34).

In another study the differentiation of lung stem cells into lung epithelial cells using growth factors such as HGF and FGF in the decellularized lung scaffold was designed with the help of a bioreactor (35). Another study uses the growth medium containing growth factor (FGF1) and (FGF7), epidermal growth factor, hepatocyte growth factor and insulin, for MSC differentiation into type II alveolar epithelial cells (36).

According to the studies mentioned and the success of mesenchymal stem cells to differentiate into lung

tissue, in the study of mesenchymal stem cells derived from femur and tibia were used to differentiate into lung epithelial cells. But unlike the mentioned studies for the differentiation of these cells into the lung stem cells, the growth factors or co-cultures were not used, but the difference is only performed by adjacent the stem cells with ECM. This distinction will facilitate the progress towards achieving the goal of engineered tissue in the treatment of lung disease. The markers of CC10 (Clara cell secretory protein in the cytosolic and alveolar) and SPD (the secreted surfactant in type II alveolar epithelial cells) was used to study the differentiation of the cells of the lung. In other studies on the differentiation of stem cells into lung epithelial cells, the Clara cell secretory protein (CC10) and lung secreted surfactants (SPC, SPD) has been used (23,29). In this study, MSC extracted from rat bone marrow was seeded on the cellular scaffolds prepared from lung tissue. We found that some of the cells express CC10 and some cells express SPD. Differentiation markers were examined by immunocytochemistry test.

In this study in order to investigate the effect of lung proteins on MSC differentiation, regardless of the effect of the cell scaffold, the supernatant of decellularized and homogenized tissue was also used in the process of mesenchymal cells culture, which showed a weak expression of CC10 and SPD markers. Therefore it can be concluded that the scaffold spatial shape will play an important part in the process differentiation.

The MSCs seeded on the decellularized tissue of the whole organ results in cell death after 24 hours; which may be due to the complexity of the whole lung tissue in detoxification and removal of SDS debris after decellularization. This study showed that the microscopic structure of the extracellular matrix of decellularized lung has been well maintained and this matrix can act as a cell substrate, not only to survive seeded cells but also to provide differentiation potential of stem cells to the lungs.

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