Application of supercritical fluids for complete decellularization of porcine cartilage

V I Sevastianov¹, E Nemets¹, A Lazhko², Yu Basok¹, L Kirsanova¹ and A Kirillova¹

¹ V I Shumakov National medical research center of transplantology and artificial organs, Ministry of healthcare of the Russian Federation, 123182, Moscow, Russia
² Federal research center of crystallography and photonics of the Russian Academy of Science, Moscow, Russia

E-mail: viksev@yandex.ru

Abstract. Porcine cartilage was ground by cryomill (Retch, Germany) at –196°C. A fraction of cartilage microparticles (CMP) of size 100-250 µm was isolated. CMP was decellularized at room temperature with periodic mixing in 3 shifts of 0.1% sodium dodecyl sulfate buffer solution, containing an increasing concentration (1, 2 and 3%) of Triton X100. CMP treatment in a supercritical CO₂ (sc-CO₂) atmosphere was carried out at a pressure of 300 bar, T = 35°C, with a flow rate of sc-CO₂ of 2.5±0.5 ml/min for 8-24 hours using RESS-SAS equipment (Waters Corporation, USA). Ethanol (96%) at a concentration of 10% was used as a polarity modifier. The degree of decellularization of CMP was assessed by histological methods (stained by hematoxylin and eosin) and by detection of the residual amount of DNA in samples using DNA-binding fluorescent dye DAPI. In the case of treatment with the detergents only and detergents after sc-CO₂, the required degree of decellularization of CMP was not achieved. Histological analysis of the samples has shown that only a partial release of chondrocytes occurs. CMP treatment by detergents followed by sc-CO₂ was more effective. Complete removal of cells can be achieved if the cartilage is first treated with surfactant, and then CO₂. When ethanol was added as a polarity modifier, histological studies confirm that non-disrupted cells were almost completely absent and study with the DAPI dye has shown that more than 90% of CMP samples were completely free of DNA or contained only single whole cells. To achieve the highest possible degree of decellularization, the treatment of cartilage microparticles should be carried out first with detergent solutions followed by exposure to sc-CO₂. The introduction of a polarity modifier (ethanol) at a concentration of 10% has a positive effect on the degree of decellularization and in combination with lengthy treatment time allows to reach complete decellularization of cartilage tissue.

1. Introduction
The creation of injectable forms of cell-engineered structures for the stimulation of regeneration processes in damaged organs is based on the cultivation of cells on matrices consisting of microparticles among which tissue-specific matrices obtained from decellularized tissues are of particular interest and provide a microenvironment for cells similar to the native extracellular matrix.

To date, it is known that the decellularization method is used for the formation of tissue-specific scaffolds of blood vessels, heart, or separate heart valves, cartilage, liver, lungs, adipose tissue, skin, kidney, tendons, nerves, and pancreas [1].
The choice of decellularization procedure depends on the structure or characteristics of a particular tissue or organ [2]. In addition, it is clear that decellularization of the whole organ by perfusing solutions into the bloodstream and processing fragments of organs and tissues by immersion in the solution require completely different conditions [3].

A wide range of methods are applied for decellularization: treatment with acids and alkalis [4], cyclic freezing and thawing [5] hypo- and hypertonic solutions [6,7], numerical and protein-free solutions [6,8] but among the existing methods of decellularization ionic detergents are the most effective [9]. However, the decellularization of dense cartilage tissue under the influence of detergents occurs only to a small extent [10], thus it is necessary to develop a combination of methods that provide complete cartilage decellularization.

The aim of this work was to prove the possibility of obtainment of a tissue-specific completely decellularized matrix from microparticles of pig cartilage by consecutive treatment of CMP by detergents and supercritical CO$_2$ and EtOH as a polarity modifier.

2. Materials and methods

2.1. Grinding procedure
Porcine cartilage was ground by cryomill (Retch, Germany) in a grinding jar cooled with liquid nitrogen (−196°C), at 25 Hz for 2 min. A fraction of microparticles of 100-250 µm was isolated by sieving through a set of screens with appropriate pore size.

2.2. Decellularization by surface active compounds
Decellularization of cartilage microparticles (CMP) was performed in three changes of phosphate buffer solution (PBS, pH=7.4), containing sodium dodecyl sulfate (SDS, Sigma-Aldrich, United States) and increasing concentrations of Triton X100 (Sigma-Aldrich, United States):
- A. 1-1.5 g of CMP + 400 ml of PBS containing 1% Triton X-100 and 0.1% SDS.
- B. 1-1.5 g of CMP + 400 ml of PBS containing 2% Triton X-100 and 0.1% SDS.
- C. 1-1.5 g of CMP + 400 ml of PBS containing 3% Triton X-100 and 0.1% SDS.

Sample was treated by each solution with periodical mixing by magnetic stirrer (200 rpm, 1 hr, 3 times a day) during one day. At the end of the decellularization process samples were rinsed by 400 ml FBS.

2.3. Treatment by supercritical fluids
CMP treatment in a supercritical CO$_2$ (sc-CO$_2$) atmosphere was carried out at a pressure of 300 bar, $T = 35^\circ$C, with a flow rate of sc-CO$_2$ of 2.5±0.5 ml/min for 8-24 hours using RESS-SAS equipment (Waters Corporation, USA). Ethanol (96%) at a concentration of 10% was used as a polarity modifier.

2.4. Study of degree of decellularization
The MP degree of decellularization was assessed by histological methods and by detection of the residual amount of DNA in samples using DNA-binding fluorescent dye DAPI (Sigma-Aldrich, United States).

2.4.1. Histological examination
Decellularized samples were fixed in 10% formalin solution in PBS, washed for 15 min in running water and dehydrated in alcohols of increasing concentration (70%, 80%, 96%; for 5 min in each change of alcohol solution), and kept for 5-7 min in an ethanol and chloroform mixture, then in pure chloroform and were embedded in paraffin.

Sections 4-5 microns thick obtained using the microtome Leica RM3255 were deparaffinized, rehydrated, and stained with hematoxylin and eosin dye (Sigma-Aldrich, United States). The analysis and photographing of the preparations obtained was performed using a Nikon eclipse microscope equipped with a digital camera.
2.4.2. Residual DNA testing

The degree of CMP decellularization was assessed using a modified method [11]. Microparticles were stained with a solution (1 µg/ml) of DNA-binding fluorescent dye DAPI (λ_{ex} = 358 nm, λ_{em} = 461 nm). Using a fluorescent microscope Nikon Ti (Nikon Corporation, Japan), the amount of particles in each sample was calculated and divided into three groups according to the degree of decellularization (figure 1).

![Figure 1. Cartilage microparticles: 1 — completely decellularized particles; 2 — partially non-decellularized particles, containing rare separate cells; 3 — non-decellularized, cell-rich particles. Stained by DAPI.](image)

3. Results and discussion

The application of the standard procedure of washing by buffered solutions of surface active substances, which allows effective decellularization of human liver fragments [12] is not accompanied by any noticeable decellularization of fine particles of pig cartilage (figure 2): the cells remain in the gaps, they are not destroyed.

![Initial vs Decellularized](image)

**Figure 2.** The influence of treatment by the detergents only on CMP decellularization. Stained by hematoxylin and eosin.

The effect of the order of sequence of detergents and sc-CO₂ on the effectiveness of decellularization of pig MDC cartilage was studied. In the case of treatment with detergents after sc-CO₂, the required degree of decellularization of CMP was not achieved (figure 3).
A. sc-CO$_2$, 8 h  
B. sc-CO$_2$ + EtOH (10%), 8 h  
C. sc-CO$_2$, 24 h  
D. sc-CO$_2$ + EtOH (10%), 24 h

Figure 3. The influence of treatment by detergents after sc-CO$_2$ (300 bar, 35°C) and 10% of EtOH addition on CMP decellularization. Stained by hematoxylin and eosin.

Histological analysis of the samples has shown that only a partial release of chondrocytes from the lacunae (structural formations in the cartilage tissue) occurs, as a result of which in some microgranules, areas free from cells are found, but the cell integrity remains. An increase in processing time from 8 to 24 hours is accompanied by an increase in the number and size of CMP areas free from cells due to their exit from the lacunae, but even in this case the destruction of chondrocytes does not occur. The introduction of 10% ethanol as a polarity modifier leads to the destruction of some of the chondrocytes directly in the gaps, but the number of lysed cells is insignificant, so additional research with the DAPI dye was considered impractical. The treatment of CMP by detergents followed by sc-CO$_2$ increased the effectiveness of cell removal (figure 4).
The influence of treatment by sc-CO₂ (300 bar, 35°C) after detergents and 10% of EtOH addition on CMP decellularization. Stained by hematoxylin and eosin.

Histological preparation demonstrates that there were much less non-disrupted cells in CMP treated first by sc-CO₂ than by detergents, compared to corresponding samples (see figure 4) and the introduction of 10% ethanol as a polarity modifier leads to the destruction of most of the chondrocytes. After 24 hr whole cells are almost completely absent.

A study with the DAPI dye has shown (figure 5) that depending on the choice of treatment mode from 42 to 92% of MDC samples of cartilage were completely free of DNA or contained only single whole cells. The introduction of ethanol enhances the degree of CMP decellularization.

The influence of treatment by sc-CO₂ (300 bar, 35°C) after detergents on DNA removal: A — initial; B — detergents only; C — detergents and sc-CO₂, 8 h; D — detergents and sc-CO₂, 24 h; E — detergents and sc-CO₂ + EtOH (10%), 8 h; F — detergents and sc-CO₂ + EtOH (10%), 24 h.
4. Conclusion

To achieve the highest possible degree of decellularization, the treatment of cartilage microparticles should be carried out first with detergent solutions followed by exposure to sc-CO₂. The introduction of a polarity modifier (ethanol) at a concentration of 10% has a positive effect on the degree of decellularization and in combination with lengthy treatment time allows to reach complete decellularization of cartilage tissue.

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