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Techniques of liver decellularization

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Abstract

Background: The growth of the number of people who need the liver transplant and the insufficiency of organ donors, have urged the advancement in bioengineering through the development of new therapeutic strategies which involve generation of functional artificial organs, obtained by the decellularization technology and creation of extracellular matrix and their subsequent recellularization.
Material and methods: Rat livers (n = 9) served as the object of this study which were subjected to decellularization with sodium dodecyl sulfate (SDS) solution 0.1 and 0.5% and the combination of SDS 0.1 to 0.5% and anticoagulant. Subsequently, the extraction of nucleic acids was performed according to the protocol QIAamp Blood Mini Kit (2003). The histological analysis was performed with haematoxylin-eosin (H-E) and the quantification of hydroxyproline via spectrophotometric method.
Results: After the liver tissue decellularization we obtained the matrix of decellularized liver. The genetical, biochemical and histological analysis revealed a better decellularization by the combined method versus the method with SDS solution only.
Conclusions: The quantification of nucleic acids content, hydroxyproline and the histological analysis of decellularized matrix with anticoagulant and detergent SDS method, suggested a more efficient decellularization of liver tissue segments and we achieved a decellularized bioconstruction for recellularization.

Key words: decellularization, recellularization, liver matrix.

Introduction

Liver pathologies with viral or alcohol abuse etiology often results in the need for liver transplantation. The insufficiency of donors and high costs are limiting factors in the ability of the medical system to cure many cases of these diseases [3, 24]. Thus, in order to improve the existing situation, a new field of research is emerging, such as tissue engineering, which presents a promising strategy to reduce the transplanted organ deficiency [5, 23, 25, 29]. Tissue engineering is an interdisciplinary field that allows the production of 3D biological scaffolds with could be used for restoring, maintaining or improving the function of a tissue or organ by combining the principles of tissue engineering and biological sciences. 3D scaffolds obtained by tissue engineering techniques can be oriented in two directions: as matrices for supporting *in vitro* seeded cells to produce a functional tissue which will subsequently be used for transplantation and as a growth factor-releasing device [7]. An important aspect in the strategy based on functional tissue engineering is the technology for obtaining 3D biological matrices by decellularization process with detergents and tissues solubilization. This technology has a specific priority that consists in preservation of the biochemical and structural components of the native extracellular matrix [20]. The native extracellular matrix (ECM) presents the architectural basis of the organs and tissues, which supports the cell types specific to the organs and allows their normal functioning [12]. According to the literature, the extracellular matrices obtained by decellularization are derived from their home tissue, thus serving as a substrate for the cultivation of specific tissue cells, proliferation and efficient differentiation thereof [17].

Also, another strong point in favor of using extracellular matrixes obtained by decellularization is that they are non-immunogenic and prevent organ rejection after transplantation [28].

Decellularized liver scaffolds are capable to maintain cellular function, which is a positive indicator in liver tissue regeneration, only a decellularization method should be found which would allow for the preservation of the native extracellular matrix and would not disrupt the conjuctive liver architecture. The methods for decellularization may be of a physical, chemical or enzymatic nature and could influence tissue ultrastructure, biochemical composition and mechanical behavior of the extracellular matrix [8, 10].

The decellularization methods used should maintain the physical properties and integrity of extracellular matrices, but also remove cellular elements as efficiently as possible: DNA, mitochondria, membrane lipids and cytosolic proteins to prevent the occurrence of an adverse inflammatory response and inhibit constructive remodeling [15, 16, 9]. An important aspect in the technology of obtaining of extracellular matrix of the liver is to maintain the integrity and mechanical properties of the microvascular network to ensure the nutrient intake and the exchange of substances in the cells with which the scaffolds will be repopulated [1, 27].

The aim of our study is to obtain by decellularization process the extracellular matrix with integrity of the vascular network.

Material and methods

As object of study were used the livers from Wistar line rats (n=9) weighing 200 - 230g. Prior to isolation of the rat liver, was performed the euthanasia in the carbon dioxide chamber respecting the requirements of the Ethic Committee. After the rats were fixed on the work table, by the median incision the abdominal cavity was opened. Was identified the portal vein, ligated to the proximal end and cannulated with a butterfly catheter with a needle 2 cm length. The excised liver was mounted to the perfusion system and washed with 1-1.5 liters of distilled water and subsequently decellularized by two methods: with decellularization agent – 0.25% or 0.5% sodium dodecyl sulfate (SDS) [20] and the second method, in which we provided perfusion of the liver with anticuagulant solution (citrate phosphate dextrose) before decellularization and then – decellularization with 0.25% or 0.5% SDS. Subsequently, the liver was perfused with 1-1.5 L of 1% PBS solution. The segments of the intact and decelularized liver were fixed in 10% formalin and subsequently was performed histological samples preparation and H-E staining. The extraction of nucleic acids was performed according to the QIAamp Blood Mini Kit extraction protocol (2003). Each sample was quantified by a spectrophotometer (Nano Drop 200 C Thermo Scientific). The hydroxyproline content was determined on a spectrophotometer based on the oxidation of hydroxyproline in pyrrole under the action of chloramine B.

Results

The macroscopic evaluation of the liver at the experiment beginning, showed that after its washing with distilled water (first method) and with distilled water and citrate dextrose phosphate (second method) in the second one the liver became to be more pale in comparision to the first one (fig. 1, 2). However, visual differences in perfusion liquid color were observed: the perfusion liquid obtained after

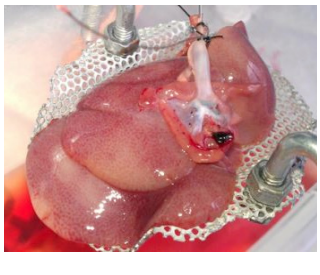

Fig. 1. Liver perfusion with distilled water.

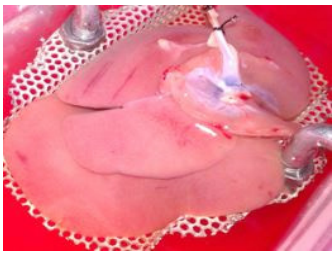

Fig. 2. Perfuzed liver with distilled water and anticoagulant solution.

washing using the distilled and anticoagulant mixture has more intense brown color, which is explained by the more efficient removal of the blood clots.

Also, macroscopic results after decellularization showed a uniform liver decellularization and a whitish color in the second experimental lot (fig. 3 A) compared to the first lot where liver was washed without anticoagulant solution, the yellow color indicating the presence of the cellular residue (fig. 3 B).

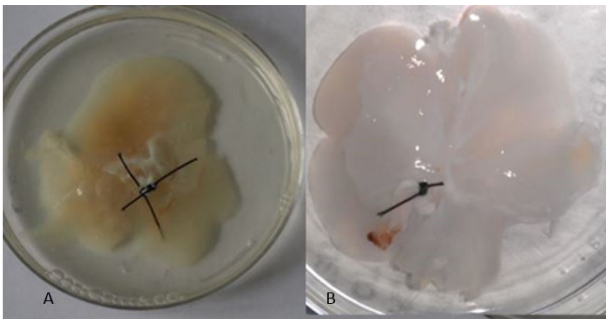

Fig. 3. A – extracellular matrix decellularized by the SDS without anticoagulant washing and B – extracellular matrix obtained by decellularization with SDS and anticoagulant washing.

In the histological analysis of intact liver samples no changes were observed. We have visualized in the inverted with phase contrast microscope KZD the centrolobular and perilobular blood vessels and the hepatocytes which correspond to the native liver (fig. 4).

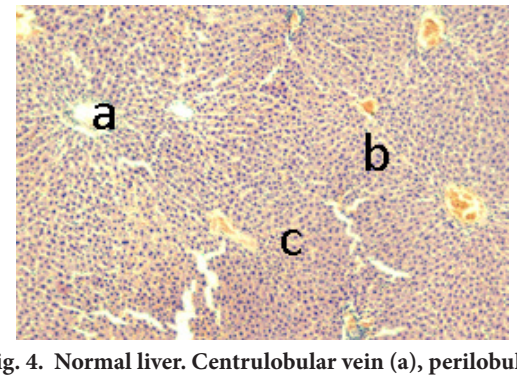

Fig. 4. Normal liver. Centrulobular vein (a), perilobular congested hepatic artery (b), contoured hepatocytes. H-E, x 90.

In the case of decellularized liver scaffolds without using the anticoagulant solution, we observed that there are cellular conglomerates, necrotic detritus and autolysed cells, conjunctival liver architecture is disorganized, the vascular wall is in some portions with the contracted contour and perivascular autolysed cellular elements (fig. 5).

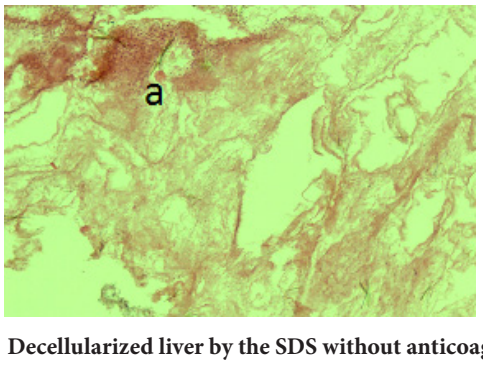

Fig. 5. Decellularized liver by the SDS without anticoagulant washing. Conglomerate of autolysed cells with cariolysis and plasmolysis (a), the disorganization of the connective architecture of the liver (b). H-E, x 90.

The decellularized liver scaffolds with SDS and initially washed with anticoagulant showed histologically: the thickened contour of the centralobular perilobular vascular wall,

the retained conjunctival architecture. The thickened contour of the vascular wall is clarified by the presence of collagen fibers which is a favorable condition for the mattrices used for recellularization (fig. 6).

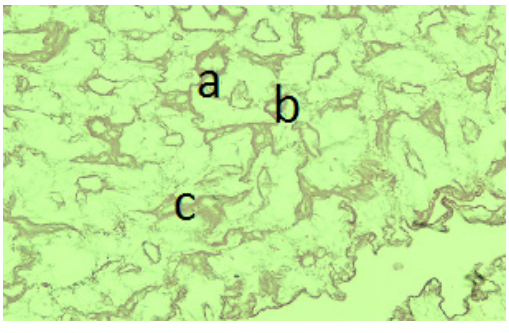

Fig. 6. Decellularized liver by the SDS with anticoagulant washing. Centrolobular vein with thickened contour (a), the thickened liver triad casing (b), preserved liver conjunctive architecture (c). H-E, x 90.

The comparative histological study of decellularized liver scaffolds demonstrated a greater efficacy of decellularization by the previous washing by anticoagulant (phosphate citrate dextrose) with good collagen architecture preserved.

If we compare the DNA concentration obtained from the biologic samples from the liver subjected to decellularization by both methods with versus the DNA concentration obtained from intact liver tissue we notice that it is lower (tab. 1). In samples obtained by decellularization with SDS, which were washed with anticoagulant solution, the DNA concentration was 1.04 ± 0,25 ng/µl, which constituted 5 times less cellular debris than in decellularized SDS samples and was previously washed only with distilled water at which the concentration is 5.2 ± 1,266 ng/µl. This can be explained by the fact that the anticoagulant used allowed more efficient removal of blood clots, thus allowing the ionic decellularization agent to pass through the entire surface of the liver to produce cell lysis and eliminate it.

As the preservation of the extracellular scaffolds after decellularization is a necessary condition for subsequent *in vitro* cellular repopulation, we determined the free hydroxyproline content that is directly proportional to the collagen content in the analyzed biological material. Therefore, we obtained an amount of hydroxyproline of 0.256 ± 0.019 mM/g of dried tissue from intact biological material which is much lower compared to the amounts of hydroxyproline in decellularized samples that are 2.035 ± 1.255 mM per g of dried tissue by the method in which was not used phosphate citrate dextrose and 4.62 ± 0.33 by the method in which was used anticoagulant. The lower amount of hydroxyproline in intact tissue is explained by the presence of cells, that is to say, the same amount of material taken for the research of intact tissue contains cellular components as well as the connective tissue component, but in the extracellular scaffolds tissues predominate the elements of the conjunctive tissues. The fact that the method of using anticoagulant contains higher amount of hydroxyproline results in a more efficient decellularization and the presence of a lower amount of cellular residue.

To see the hepatic vessels integrity the intravascular injection of the blue tripan through the portal vein was done, and it was distributed through all the liver vessels, which revealed that the vessels are intact and that the ionic detergent did not cause their mechanical destruction (fig. 7).

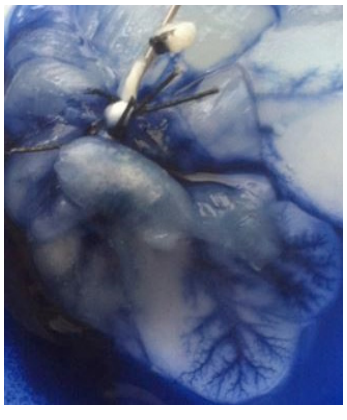

Fig. 7. Contrast with blue trypan of hepatic vessels.

Discussion

A good preservation of the extracellular scaffold would allow future differentiation, proliferation and maintenance of cellular functionality after *in vitro* repopulation, that is a priority in organ bioengineering [6, 18].

In extracellular matrixes obtained from animal organs by decellularisation, which is ideal to completely decode, a mandatory step to estimate the degree of decellularization is needed, which can be done based on the spectrophotometric quantification of total genomic DNA in the residual cell after the step decellularization [26]. The results obtained in our research have shown that the received three-dimensional matrices contain a small amount of residual cellular material, so the matrices are less immunogenic, which would not favor posttransplant rejection. Joery De Kock et

Table 1

The content of DNA and hydroxyproline in intact and decellularized liver

Type of biological material	Hepatic native tissue	Residual liver tissue after decellularization by SDS method without anticoagulant solution washing	Residual liver tissue after decelularization by SDS method and previous anticoagulant solution washing
DNA Concentration (ng/ µl)	93.3 ± 0.41	5.2 ±1.266	1.04 ± 0.25
Hydroxyproline content mM to g of dried tissue	0.256 ± 0.019	2.035 ± 1.255	4.62 ± 0.33

Note: statistically significant difference with the control group * p <0.05.

al. show that extracellular matrices in their research retain their structural components such as collagen and laminin [14], and in our researches the hydroxyproline content revealed the preservation of architecture of the obtained matrix. Also detergents used in the decellularization process cause cell lysis and cell elimination, but their time of action and concentration should be monitorised for the preservation of extracellular matrix proteins [13].

We have obtained extracellular liver matrices using SDS ionic detergent that has cell membrane solubilization potential and complete cell removal, but according to literature it may be used to obtain a matrix with improved chemical, physical, biological properties and combinations of these agents [1]. The concept of organs bioengineering involves not only obtaining a three-dimensional scaffold after removing cells with immunogenic potential, but also preserving vascular network structures for efficient supply of oxygen and nutrients [2, 21, 22]. According to experimental studies of Basak E. et al. the preserving of the microvascular structure of the extracellular matrix after decellularization is essential for its functionality in case of its transplantation [4].

The introduction of the blue tripan solution highlighted the intact structure of the blood vessels of the liver, indicating an eficiency of the used method and favorable conditions for the subsequent repopulation of the obtained extracellular matrices. The extracellular matrices obtained by decellularization can be used not only for transplantation but also as a three-dimensional *in vitro* model for cytotoxicity testing of some drugs because the liver is the main metabolizing organ [11].

Conclusions

1. The comparative evaluating of the DNA tissues content after decellularization by those two methods in relation to intact tissue proves that the method of decellularization of the liver with SDS and washing with phosphate citrate dextrose shows better results.

2. The maintaining of connective architecture and collagen fibers, the higher content of hydroxyproline in ECM obtained by our method demonstrated their high feasibility for the subsequent use as bioengineering structure for recellularization process.

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