Obtaining liver tridimensional scaffold through the decellularization of rabbit whole liver in 24 hours

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Abstract. In the present work, we development a new protocol for liver decellularization in which the hole decellularization was reached over 24 h.

Introduction: the availability of transplantable livers is not sufficient to fulfill the current demand for grafts, with the search for therapeutic alternatives having generated different lines of research, one of which is the use of decellularized three-dimensional biological matrices and subsequent cell seeding to obtain a functional organ.

Objective: to produce a decellularization protocol from rabbit liver to generate a three-dimensional matrix in which the time period involved didn’t pass 24 h.

Methods: The decellularization is obtained through the use of water and SDS (0.1-0.3 %), after freezing at -80 degrees, is the best alternative of different physical and/or chemical mechanisms to break down organ cells and leave only the extracellular matrix. After 24 h of retrograde perfusion, a decellularized translucent matrix was generated. To evaluate if the decellularization protocol was successful, with the extracellular matrix being preserved, we carried out histological (light microscopy) and biochemical (DNA quantification) studies.

Results: the decellularization process was verified by macroscopic observation of the organ using microscopic observation corroborated the macroscopic results, with the hematoxylin-eosin and Masson staining showing no cells or nuclear material. In addition, the DNA quantification was less than 10 % in the decellularized liver compared to control. Finally, the time taken to develop the decellularization protocol was less than 24 hours.

Introduction
Liver transplantation is currently the only therapeutic alternative for acute liver failure, terminal liver disease or metabolic disorders originated in the liver. The complexity of the function of the liver makes it impossible to use artificial systems to provide temporary hepatic support as for example in patients with renal insufficiency [1, 2]. Biologic scaffolds composed of extracellular matrix (ECM) are commonly used in reconstructive surgery and it is advancing its development for application in regenerative medicine to replace tissues and organs. The ECM represent the secreted products of resident cells of each tissue and organ, and provide the molecular keys necessary for cellular mechanisms of adhesion, migration and proliferation [3]. The ECM is currently used to create scaffolds of many tissues including skin [4], intestinal submucosa [5], urinary urethra [6], blood vessels [7] and heart valves [8], among others.

Recently the decellularization of organs such as heart [9], [10] and lung [11, 12], generated acellular natural three-dimensional scaffold which can be subsequently seeded with functional parenchymal cells or progenitor cells of these populations to generate a complete organ suitable for transplant. The decellularization is obtained through the use of physical and/or chemical agents that disrupt by different mechanisms the organ cells leaving only the ECM, which then provide support to posterior cell seeding [13]. The techniques and materials used for this process are constantly being reviewed in order to achieve a greater cell lysis, which preserves the ECM and decreases the time required to carry it out. To date, liver decellularization protocols have involved perfusion of a combination of detergents: Triton X-100 and SDS were used in the portal or caval vein. Shupe and his colleagues [14] used increasing concentrations of Triton X-100 followed by SDS 0.1 % in a rat liver, while Uygun et al. [15] perfused 0.1 % SDS. Moreover, Soto-Gutierrez et al. [16] first used an enzymatic agent: trypsin and then 3 % Triton X-100. In our group, we were able decellularize one rabbit liver in approximately 72 hours with a combination of physical (freeze-thaw) and chemicals agents [17]. The new protocol described in the present investigation involved an initial treatment with trypsin, which allowed a better detergent transference to the tissue, then perfusion was carried out with 1% SDS and finally with Triton X-100.

In this work the decellularization technique previously published by our group [17] was improved. He recently began to discuss the process of decellularization; this is because the proper preservation of the matrix and its conservation are essential to prevent failure of the recellularizationsubsequent step [18, 19]. While the protocol described [17] was verified by optical microscopy and scanning electron microscopy, vasculature, biliary ducts and Glisson capsule preserved; it was considered necessary to reduce the time required for complete organ decellularization.

Materials and methods

2.1. Animals: Livers of male New Zealand rabbits were used 2,100 ± 120 g. Total hepatectomy was performed with preservation of the vasculature. Subsequently, the extracted livers are decellularized by the following protocol. As a control were used rabbit livers which not were decellularized. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, as approved by the Animal Care and Use Committee of the Universidad Nacional de Córdoba, Argentina, and efforts were made to minimize animal suffering and the number of animals used.

2.2. Surgical technique: Anesthesia was performed by injecting with 35 mg/kg ketamine and 5 mg/kg Xylazine intramuscularly (IM) as a single dose. The rabbits were placed in the supine position with their legs fixed. Was performed median incision which was later extended to the right hemithorax. Mesenteric vein was identified and immediately 1000 IU of heparin was injected intravenously. Identification of the portal triad was performed and attached by a ligature, identification and dissection of the infra-hepatic inferior caval vein was carried out. The adhesions of the liver were removed and the incision was extending into the thorax. Identification, dissection, ligature and cutting of the supra-hepatic inferior caval vein were performed along with a total hepatectomy. In order to
start with the protocol decellularization it was introduced a catheter or Teflon cannula in the suprahepatic or infra-hepatic inferior caval vein respectively, which was fixed with a prolene 4/0 stitch. It was perfused using a syringe with a PBS wash solution and then perfused with a peristaltic pump (15-20 ml / min) for about three hours with deionized water; subsequently it is frozen at -77 °C.

2.3. Decellularization protocol: as already described after hepatectomy, the liver be infused for about three hours with deionized water and then frozen at -77 °C. After 24 h at -77 °C the liver was thawed at room temperature to aid cell lysis. The catheter or teflon cannula inserted into the inferior caval vein was connected to a peristaltic pump and retrograde perfusion cycles was started at a rate of between 15 and 20 ml/min at 4°C as follows: it perfused with 0.3% SDS in deionized water, alternating with deionized water. Once the process of decellularization (which is checked macroscopically) completed, successive washes were performed with deionized water and PBS to drag cell debris and detergent (1h of deionized water, 2 washes 1 h with PBS, 30 min of deionized water, 2 washes of 30 min with PBS). Later, the organ was perfused for 1 hour with 4 % ethanol in PBS to disinfect the organ and finally, 1 h perfused with 10,000 units / ml penicillin-10,000 μg / ml streptomycin in PBS. Macroscopically decellularization evidenced by a MEC translucent.Staining of the biliary tree on two rabbits was performed with methylene blue through the gallbladder puncture, which revealed the existence of the bile ducts. A mixture of methylene blue with agarose was perfused through the inferior caval vein in two other rabbits and a good vascular staining was observed.

2.4. Microscopic morphological evaluation: for evaluation of the effectiveness of the decellularization, the two histological staining techniques, hematoxylin-eosin (HE), and Masson's trichrome staining were used. These stainings were performed on the decellularized and control livers.

2.5. DNA quantification: The amount of DNA was quantified using a method described by Laird et al. [20]. Briefly, 25 mg of tissue from the control and decellularized liver were incubated with a solution containing 2 % SDS, 5 mM EDTA, 200 mMNaCl and 100 mM TRIS-HCl, pH 8.5 for 48 h at 55 °C. The DNA extraction was carried out in isopropanol and later dissolved in a solution of 10 mMTris-HCl, 0.1 mM EDTA, pH 7.5. The amount of DNA was determined spectrophotometrically at 260 nm.

1. Results and discussion

The objective of this work was reduce the time to obtain a rabbit decellularized liver [17, 19]. We assayed different chemical combinations, different detergent concentrations and other chemical agents. Also it was changed the order that these agents were used. It was established that concentrations of SDS bellow 0.3% were the ones which obtained best results. On the other hand, for good detergent penetration, it was necessary (this is the step was shown to be determinant), perfusing the fresh organ with cold water before being frozen. The water was perfused until the liquid perfused stopped go out colored. This perfusion was fundamental so then the detergents can penetrate the organ. Generally, the water was circulated for a period of about three hours. It was evident that is complicated establish duration time and perfusion volume, each organ has different weight, morphology and anatomy. In some cases, we can observe internal striations in the lobes that difficult the decellularization because they produced an asymmetric perfusion of the detergents. Finally, the organ was perfused while submerged. We tried organ perfusion with a container mesh, suspending and submerging the organ; also alternating suspension, submerging and the mesh. Finally, we can observe a better preservation of the elasticity and the macroscopic aspect of the MEC, when the organ was submerged during the decellularization and in the mesh during the washes. Frequently, the organ suspension causes more
difficult for detergents working because when we suspend the organ, the perfusion diverts to the lobe that has more cells. But the suspension produces seriously damages to the vasculature and Glisson’s capsule, and this affects the reproducibility of the process and the protocol success. The decellularized mesh of the organ is very frail, which obstructs to suture it in cases of vasculature breaking. Moreover, when MEC obtained with Nari et al. [17] protocol was incubated with epithelial cells to study the citotoxicity, we observed bacterial contamination, and also shelling of the MEC. Although they were preserved the tract biles and the tract blood; those structures did not support incubation at 37 °C during 72 hours. To try to improve the culture resistance at 37°C, we worked with at 4°C with different procedures of washes and we aggregated different antibiotics. With the improvements introduced in the protocol, we have reduced the decellularization time and increased the reproducibility and success of the protocol. In the next scheme, we show a summary of the new protocol of liver decellularization:

In the figure 1, we can observe the process of the liver decellularization, over the course of 24 hours while the organ is perfused. In the pictures, we can see that the MEC obtained is totally translucent and also we can see that the vasculature of the organ is preserved. The perfusion rate is 15-20 ml/min.
Figura 1: Macroscopic observation of decellularization procedure. A) Liver thawed (perfused with water, before freezing to -70°C); B) Liver perfused after 8 hours; C) y D) MEC obtained after 24 hours of perfusion.

Once the decellularization protocol is established, its efficiency was verified by microscopic techniques. For this, we take tissues cuts of control liver and of the MEC obtained and fixed it in glutaraldehyde at 4% in PBS, that then we process for perform the histologic stains of Hematoxylin-Eosin and Trichromic staining of Masson. In the figure 2, with the Hematoxylin-Eosin stain, we can observe de totally absence of cores or nuclear debris in the MEC. Washing is a critical step of the process of decellularization, because the heavier debris is more difficult to eliminate of the tissue. The microscopic techniques showed that there was not nuclear debris. In the figure 3, with the Masson trichromic staining, we verified the presence of collagen fibers and elastic fibers. This staining uses three dyes: hematoxylin, fuchsin and green light. This staining is very useful to show the collagen fibers and the connective tissue.
Figura 2: Hematoxylin-Eosin staining. A) and B) are histological sections of control’s livers. C) and D) are histological sections of MEC. The pictures are representative of respective groups. (control: n=3, MEC: n=4).
**Figura 3:** Masson trichromatic staining of histological cuts. Left: correspond to control’s liver. Right: correspond to MEC. The pictures are representative of the respective groups (Control: \(n=2\); MEC: \(n=4\)).

On the other hand, DNA amount was 100 times lower (two orders of differences of magnitude) in the tissue from the MEC, than in the tissue from the control liver (Figure 4). These results are accord with the optical microscopic studies, where we showed the absence of cores and nuclear debris.

**Figure 4: Quantification of DNA.** Bars represent mean ± S.E.M. Control Group: 10350 ± 1750 ng DNA/mg of tissue (\(n=3\)), MEC group: 264 ± 37 ng DNA/mg of tissue (\(n=6\)).

**Conclusion**

The decellularization protocol allowed to obtain a preserved and translucent matrix without remains of cells and DNA after 24 hours. The protocol was developed at 4 °C and involved an initial perfusion of water during 3 hours, freezing the organ to -77 °C, liver defrost at ambience temperature and a perfusion of 24 hours with 0.3 % SDS intercalated with washing water at a rate of 15-20 ml / min.
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