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BIOTECHNOLOGY

Development of equipment for decellularization using the perfusion method

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ABSTRACT. This study describes the development of equipment capable of transporting decellularization fluid through an organ by perfusion to create an *in vitro* acellular scaffold that maintains the three-dimensional architecture of the organ. The equipment was designed to be compatible with several existing decellularization protocols and can be used to modify the flow rate, perfusion pressure, and temperature in each decellularization protocol as well as the technique of cannulation of the organ in a controlled manner. The device was tested with chicken hearts and efficiently accomplished decellularization using the perfusion method. Further amendments can be made to improve the efficiency of the decellularization of diverse organs. It is expected that this equipment will aid in the characterization and improvement of the decellularization process and may have future applications in the process of recellularization of different organs.

Keywords: decellularization; cardiac bioengineering; biomaterials; perfusion process; heart decellularization

Desenvolvimento de equipamento para decelularização pelo método de perfusão

RESUMO. O presente estudo descreve o desenvolvimento de um equipamento capaz de transportar o fluido de decelularização através do órgão inteiro, por perfusão, para criar uma estrutura acelular *in vitro* que mantém a arquitetura tridimensional completa do órgão. O equipamento foi concebido ser compatível com vários protocolos de decelularização existentes e pode ser utilizado para modificar a vazão, a pressão e a temperatura de perfusão em cada protocolo, bem como a técnica de canulação do órgão de uma forma controlada. O dispositivo foi testado com corações de galinha e executou eficientemente o protocolo de decelularização, utilizando o método de perfusão. Outras alterações podem ser feitas para melhorar a eficiência da decelularização em diversos órgãos. Espera-se que este equipamento auxilie na caracterização e melhoria do processo de decelularização além de possibilitar futuras aplicações no processo de recelularização de diferentes órgãos.

Palavras-chave: decelularização; bioengenharia cardíaca; biomateriais; processo de perfusão; decelularização de coração.

Introduction

Heart disease is the main cause of death in developed countries (Roger & Go, 2012; Ptaszek, Mansour, Ruskin, & Chien, 2012). Native cardiomyocytes are not capable of restoring lost cells following myocardial infarction; because these cells are terminally differentiated, they do not possess reproductive capacity (Leor, Amsalem, & Cohen, 2005; Eschenhagen, Didié, Munzel, Shneiderbanger, & Zimmermann, 2002), and the myocardial tissue contains limited quantities of muscle stem cells (Jawad, Lyon, Harding, Ali, & Boccaccini, 2008). Thus, organ transplantation is the procedure most commonly used to treat end-stage heart failure. However, this treatment is limited by the shortage of donors and the antigenicity of transplanted organs, which may lead to rejection of the organs (Morimoto, Sunagawa, Fujita, & Hasengawa, 2010).

Therefore, the field of tissue engineering has great potential in identifying new transplant strategies to provide better quality of life (Angiolillo & Goto, 2010; Limbourg, Limbourg, & Drexler, 2009; Shlechta et al., 2010). The creation of scaffolds that can be used for the development of complex three-dimensional tissues or whole organs remains a serious challenge in tissue engineering (Gálvez-Mónton, Prat-Vidal, Roura, Soler-Botija, & Bayes-Genis, 2013). One solution would be to use the extracellular matrix (ECM) of the organ to be transplanted following decellularization; this process involves withdrawal and lysis of the cellular materials, creating a bioartificial scaffold (Gilbert, 2012).

Bioartificial scaffolds composed of ECM are commonly used for a variety of reconstructive surgical applications. These scaffolds are increasingly being employed in regenerative medicine, even for organ replacement. This type of scaffold is able to provide physical support for cells and has the structural and biochemical components needed to promote cell adhesion, migration, proliferation and differentiation (Lyons, Partap, & O'Brien, 2008; Grayson, Martens, Eng, Radisic, & Vunjak-Novakovic, 2009).

One challenge associated with the decellularization process is the design and construction of equipment capable of using perfusion to completely remove cellular debris from all regions of the organ without affecting the structural capacity of the ECM (Weymann et al., 2011).

Prior research addressing the decellularization of whole organs has typically involved the use of equipment developed nearly exclusively for the decellularization protocol to be studied, and the equipment and its functions in the process are often not described. Guyette et al. (2014) showed the construction of the equipment used in the decellularization of small-animal experimental and clinically relevant models, using pressure-controlled perfusion. In the present paper, we prioritize description of the equipment and of the importance of each functionality for the decellularization process.

The aims of this study were to design and develop decellularization equipment and to determine the necessary conditions for this process. In addition to being able to provide different flow rates and perfusion temperatures, this equipment makes the process more autonomous, lowers the probability of failure, and provides a simpler and cheaper option compared with other methodology.

Methods

The equipment was designed to meet all of the needs of the whole-organ decellularization process. The main needs are control of the perfusion and control of the temperature during this process. Figure 1 shows the general scheme of the perfusion circuit and the positioning of each device.

Perfusion

To properly perform perfusion, decellularization equipment must provide the organ with a controlled flow of decellularization fluid. For this purpose, we utilize a peristaltic pump (5M6002; Travenol Laboratories; Citrus Heights, CA; USA; Figure 1b), which is the same pumping device as that used for cardiopulmonary bypass procedures. The pump was adapted to control the rotation speed by adding a module comprising an encoder disk (SG23FF; Kodenshi; Shinheung-dong, Iksan; Korea) and a microcontroller (PIC18F4550; Microchip Technology; Chandler, Arizona; USA). This module measures and controls the rotor rotation to provide the desired flow rate based on the diameter of the tubing used in the pump.

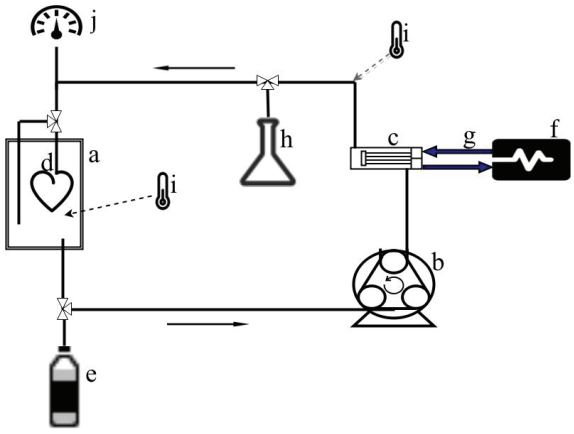

Figure1. The complete scheme of the equipment used in the decellularization process: a) The chamber for decellularization, b) the peristaltic pump, c) the heat exchanger, d) the heart cannulated through the aorta, e) the reservoir for the perfusate inlet, f) the thermal bath, g) the hot water at a controlled temperature, h) the reservoir for the perfusate outlet, i) the temperature gauge, and j) the pressure gauge.

In the perfusion circuit before the cannula, a pressure gauge (Figure 1j) was added to permit adjustments to the flow rate for each experiment. Moreover, the pressure gauge (EN 837-3; DFX; Rio de Janeiro, Rio de Janeiro State; Brazil) is used to help to identify and correct failures during the process; for example, the pressure gauge will detect an unexpected disconnection between the organ and the circuit or a pressure increase caused by an obstruction in the circuit.

The perfusion method used may vary between different organs. In this study, we used the equipment to perform chicken heart decellularization experiments. The retrograde perfusion technique presented by Langendorff (Langendorff, 1895) is advised for hearts because of its simplicity, low cost of implementation, and experimental reproducibility.

The equipment and organ are connected by the cannula. The cannula must have an outer diameter that is slightly larger than the internal diameter of the vessel to be cannulated. We use small barbs in the cannula to ensure attachment to the organ

(Figure 2), preventing slippage when the cannula is locked externally using a clamp or tied in place by a surgical cord. The heart may be protected by carefully using thin tweezers to place the cannula to avoid tearing or stretching the aortic wall. The cannula must be carefully inserted through the aorta to avoid causing obstructions in the coronary ostia or damaging the aortic valve.

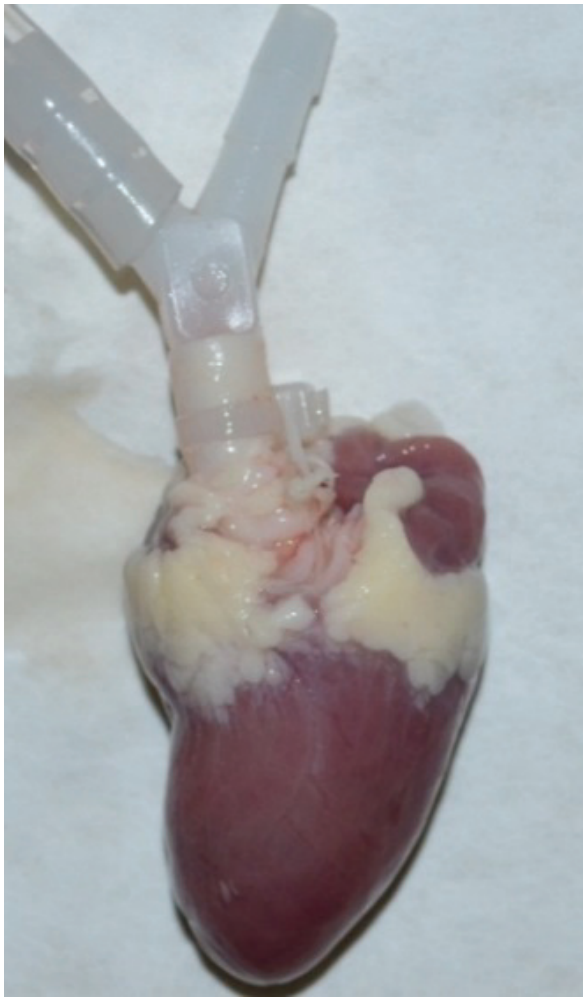

Figure 2. The cannula used to perform retrograde Langendorff perfusion.

After the cannula is fixed, a ligature is quickly strapped around the aorta, holding it between the cannula barbs. The cannula must be connected to the perfusion set and fixed in the decellularization chamber cover, and then the decellularization process should be initiated as quickly as possible.

The drainage of the perfusate from the right side of the heart, which occurs naturally through the pulmonary artery, must not be prevented. A small incision could be performed at the base of the pulmonary artery to ensure proper drainage of the perfusate (Sutherland & Hearse, 2000).

To facilitate the decellularization process, a decellularization chamber was specifically designed to avoid contamination, reduce the need for consumables, and improve standardization. The chamber is constructed of acrylic due to this material's transparency, thermal insulation capacity, and ease of assembly (both to machine and to paste) (Figure 3).

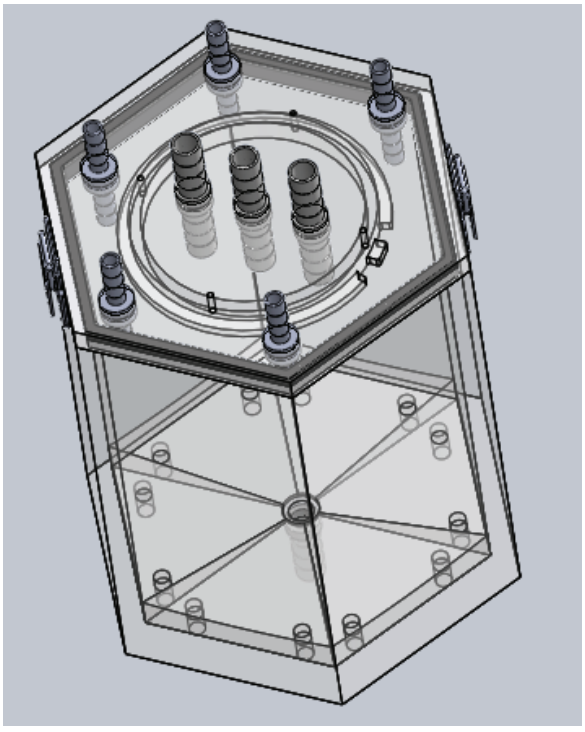

Figure 3. The decellularization chamber design with all inputs and outputs, allowing performance of the process without internal manipulation.

Input and output connectors were added to the perfusate reservoir to facilitate the decellularization process, simplify the exchanges during the protocol, and maintain cleanliness by avoiding contact with or manipulation of the organ. We included several three-way valves in the circuit, which can change the flow direction if arranged adequately. The valves allow the easy and quick exchange of priming solution and can be used to purge air from the system (Figure 1).

Temperature control

We constructed a coiled-tube heat exchanger using stainless steel to control the perfusate temperature (Figure 4). The perfusate flows inside the stainless steel tube and exchanges heat with the external fluid, which has its temperature precisely controlled to enable the regulation of the perfusate temperature.

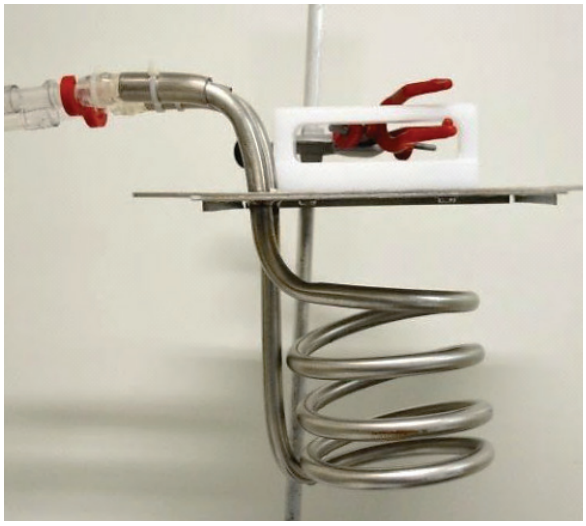

Figure 4. The coiled-tube heat exchanger used in the equipment.

An Ultra Thermostatic Bath (ECO RE 630; Lauda; Lauda-Königshofen; Germany) is used to provide the temperature-controlled fluid. The circuit has two points for temperature measurements (Figure 1i). The first point uses the PT100 sensor of the Ultra Thermostatic Bath, which is located in the heat exchanger outlet. This temperature value can be used as a control variable to improve the temperature control efficiency. The second temperature-measuring point is located within the decellularization chamber and enters the cover through a digital thermometer (TM879; Equiterm; Porto Alegre, Rio Grande do Sul State; Brazil). This temperature measurement can be used as a redundancy check for the temperature control and indicates the closest value of the perfusion temperature within the organ.

Chicken heart decellularization testing

We tested the decellularization device using chicken hearts and a modified decellularization protocol described by Weymann et al. (2011). For all of the experiments, the hearts were obtained from a slaughterhouse. Following standard slaughterhouse procedure, chickens were slaughtered between the ages of 42 and 49 days if weighing between 2150 and 2550 g (Young, Northcutt, Buhr, Lyon, & Ware, 2001). After the usual procedures of removal and cleaning were performed, the hearts were cooled for a maximum of 24 hours. On the following day, the hearts were weighed and cannulated for retrograde perfusion through the aorta, and the apertures of the subclavian vessels were sutured. After connecting the organs to the equipment, the hearts were first perfused with phosphate-buffered solution (PBS) for 15 minutes to remove excess blood.

The hearts were then perfused with a constant flow, which provided an average pressure of approximately 50 mbar. The solution, consisting of 4% sodium dodecyl sulfate (SDS) in PBS, was heated to 37.6°C. The hearts were perfused for 12 hours. During this process, the hearts were washed with PBS for 15 minutes every 2 hours to remove residual substances. The final washing step after SDS treatment included perfusion with PBS for another 24 hours to remove remnant detergents and cell debris. At the end of decellularization, the hearts were removed from the equipment and weighed after removing the excess perfusate. The ECM of the hearts was then dissected and analyzed macroscopically.

Histological analysis

The hearts were fixed in 10% formalin, dehydrated in increasing concentrations of ethanol, embedded in paraffin and sectioned following standard protocols (Ott et al., 2008). The tissues were cut into 5 μm sections and stained with hematoxylin and eosin (H&E) stain according to the manufacturer's instructions (Histokit; EasyPath; Indaiatuba, São Paulo State; Brazil) to determine if remnant nuclear structures could be observed. All analyses were conducted using a light microscope (BX41; Olympus; Tokyo; Japan), and the micrographs were photographed with a microscope (BX40; Olympus; Tokyo; Japan) coupled to a digital camera (Moticam 2500; Motic; Xiamen, PR China).

Results

The equipment efficiently performed the whole-organ decellularization procedure. The perfusion model was excellent and constantly maintained flow control. The flow capacity ranged from 30 to 8000 mL $min.^{-1}$ The decellularization chamber (Figure 5a) allowed the experiment to be observed as it progressed and created a myocardial scaffold. The total volume of the circuit was reduced and optimized for the organs studied (300 mL), which reduced the cost of the consumables needed foreach decellularization. The output and input ports of the circuit (Figures 5d and f) facilitated the exchange of the decellularization solutions without the need to handle the organs. The equipment was developed using devices commonly implemented for surgery, which makes it easier to handle, inexpensive, and safe. The final assembly of the equipment is shown in Figure 5.

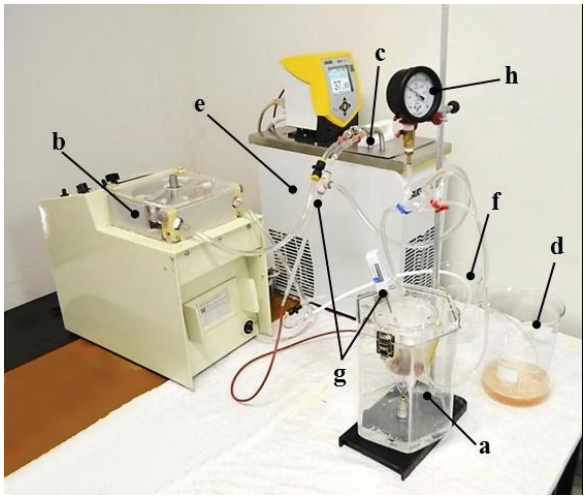

Figure 5. The equipment used during the chicken heart decellularization process: a) The chamber for decellularization, b) the peristaltic pump, c) the coiled-tube heat exchanger, d) the reservoir for the perfusate outlet, e) the thermal bath, f) the reservoir for the perfusate inlet, g) the temperature gauge, and h) the pressure gauge.

The equipment was able to heat and cool the perfusate (Figure 5e) to temperatures ranging from -30 to 200°C and maintained control, with a fast response and a stability of ± 0.02 K. The equipment was also capable of pumping a temperature-controlled fluid to stabilize the temperature at other points of the circuit.

During the process, the chicken hearts continuously lost their natural color and became translucent and yellowish. The color loss began at the right ventricle, followed by the atriums and finally the entire left ventricle (Figure 6). After decellularization, the average weight loss was 35.8% relative to the initial mass.

The histological analysis (Figure 7) showed that the process of decellularization transformed the tissue into a biological scaffold that was nearly acellular. In the control tissue (Figure 7A), it was possible to view the longitudinal arrangement of the muscle fibers, colored pinkish, and the nucleus of the fibers, colored purple. In contrast, in the decellularized tissue (Figure 7B), it was not possible to observe the conventional arrangement of the muscle fibers. Beyond an absence of cellular structures, remnants of the nuclei, which were rich in proteins or amines, were present, as were collagen fibers.

Histological analysis also revealed the structural permanence of vessels in the tissues, which showed well-preserved tunica (intima, media and adventitia); this feature contributed to the continuity of decellularization and was important for future repopulation. All of the analyzed samples presented normal cardiac morphology, without any visually perceptible defects, after the decellularization.

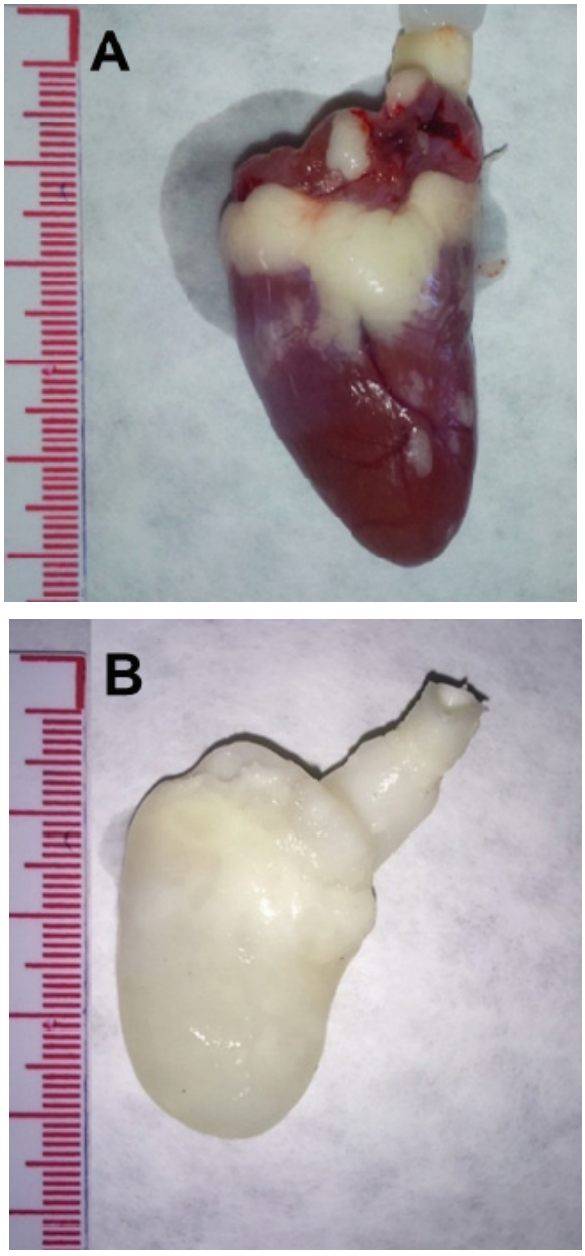

Figure 6. Representative images of the macroscopic appearance of a chicken heart treated with perfusion decellularization. (A) Before decellularization and (B) after 12 hours of decellularization with 4% SDS.

Discussion

In this study, we report on the development and construction of equipment for conducting whole-organ decellularization using the perfusion method. The machine controls the perfusion flow and perfusion temperature and has inputs and outputs that promote rapid perfusate exchange while avoiding external contamination. The use of a decellularization chamber facilitates the process by providing shelter for the organ during the process and allowing its fixation, which reduces losses and

failures. Decellularization tests with chicken hearts demonstrated the proper functioning of the equipment and allowed visual monitoring to ensure complete organ decellularization.

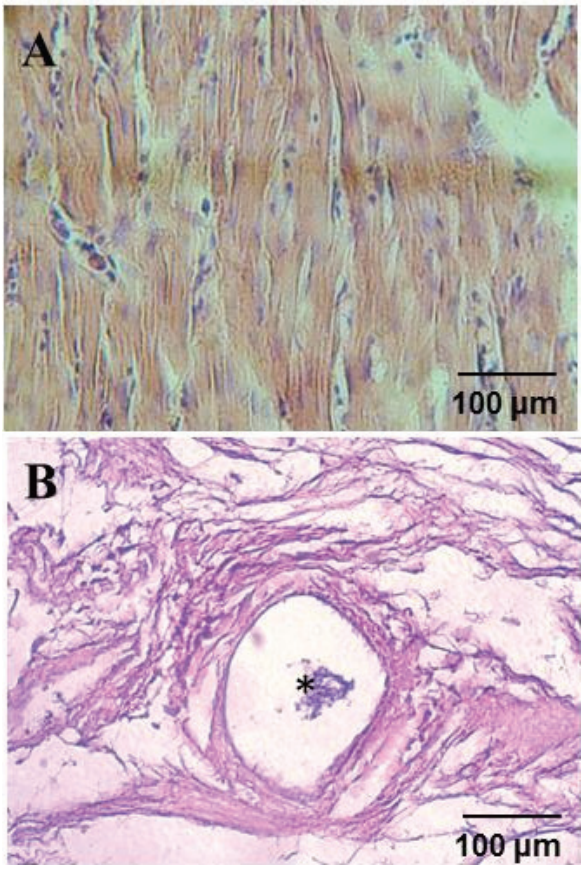

Figure 7. Representative H&E-stained samples of ventricular tissue with an absence of nuclear staining after decellularization. (A) Native left ventricle. (B) Decellularized left ventricle. Purple: nuclei or proteins/amines.

Pinkish: collagen fibers. * Example of the permanence of vessels (arteries) in the tissue after decellularization. Scale bars, 100 µm.

While developing the equipment, we considered the possibility of its adaptation for use in various published protocols (Akhyari et al., 2011; Momtahan, Sukavaneshvar, Roeder, & Cook, 2014; Gilbert, Sellaro, & Badylak, 2006; Ott et al., 2008). To achieve perfusion, it is essential to assess which method best fits the organ utilized. It is also best to use the organ vasculature to reach all regions and create a three-dimensional scaffold (Ott et al., 2008). In most cases, the organ will have one or more main arteries through which fluid may be perfused and one or more outputs from which the perfusate can be drained after perfusing the organ. In the case of the heart, the retrograde perfusion method is recommended in the literature (Robert, Mihaela, & Derek, 2011).

In the retrograde perfusion method, fluid is perfused through the aorta with sufficient pressure to also reach the coronary vessels and to perfuse the whole heart using its own vascular network. The perfusate enters the heart through a cannula positioned in the aorta, which is in the opposite direction of the natural physiological flow. The aortic valve closes under the pressure, and the coronary vessels are filled by the two coronary ostia (right and left ostia) in their respective cavities situated in the aortic root. The perfusate then passes through the vascular bed before exiting through the coronary veins into the coronary sinus in the right atrium. One key feature of this method is the direct cannulation of the aorta, rather than direct cannulation of the coronary artery, which allows the circulation of decellularization fluid throughout the heart (Langendorff, 1895).

Positive-displacement roller pumps are frequently used for the perfusion of both organs and patients because these pumps are simple and because there is no direct contact between any of the parts of the pump and the fluid. Other advantages include the accuracy relative to the flow, the durability, the versatility, the absence of contamination (because the fluid remains inside the hose throughout the pump circuit), and the ability to provide continuous flow with low vibration. Constant pressure control was used in previous studies (Weymann et al., 2011; Sierad et al., 2015) and can be achieved by adding a controller and a pressure transducer to the system. Several recent publications (Sierad et al., 2015; Momtahan et al., 2014) evaluated the advantages of performing constant-pressure perfusions for decellularization. However, the present study used constant flow control, and the pressure was monitored by a pressure gauge without controlling the rotation as a function of the pressure. This approach is a less accurate solution but is simple and inexpensive, and our tests confirm that the system functions properly in most cases because the perfusion pressure remains stable at a specific flow rate.

The decellularization chamber is able to eliminate the bubbles in the circuit during the exchange of perfusate via a secondary path in the circuit that reaches the chamber (Figure 1). However, depending on the physiological characteristics of the organ and the perfusate employed, it may be necessary to add a bubble-trap circuit before the cannula to ensure the removal of bubbles and to prevent embolisms in the organ. In that case, the volume used inside the trap bubbles must be consistent with the total volume used for the perfusion, or the cost may significantly increase.

The temperature, which is a critical aspect of the decellularization process, is very well controlled and stable and quickly responds to changes. The temperature control system allows the process to be optimized and used in various decellularization protocols that require enzymes in the decellularization fluid. A study by Akhyari et al. (2011) compared different decellularization protocols and demonstrated the importance of temperature control for different protocols and perfusates with different active principles.

Our tests showed results compatible with those of similar protocols found in the literature (Weymann et al., 2011). The decellularization performed in the present study, which used SDS, resulted in a biological matrix with a very small perceptible presence of cellular material. Visual inspection showed that the decellularization occurred evenly throughout the heart, indicating the functionality of the perfusion device. The loss of approximately one-third of the mass also indicated cell loss. The blood vessels were well preserved, and the collagen fibers supported the preservation of the scaffold during the decellularization process. Additional parameters should be studied to evaluate the decellularization quality and the resulting ECM. The scaffold could be tested using DNA quantification, histological analyses, and mechanical testing (Wang et al., 2010). Furthermore, future studies should be conducted to improve the decellularization protocol because currently, changes may involve modifications in the following aspects: the decellularization fluid (Wainwright et al., 2009), process time, flow rate, perfusion pressure, and temperature.

Conclusion

The results show that the equipment described here is a useful and simple tool with excellent functionality in producing three-dimensional bioartificial scaffolds, performing control of the perfusion flow and perfusion temperature and rapid perfusate exchange and reducing losses and failures. Additional modifications may be made to adjust the model for the decellularization of other organs or tissues.

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