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EVALUATION OF THE EFFICIENCY OF MECHANICAL AGITATION METHOD IN HEART DECELLULARIZATION PROCESS

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Abstract. *Decellularization has been successfully used in a variety of tissues for its application in regenerative medicine. This process can isolate the extracellular matrix from the organ without damaging it, while removing completely the cellular components. The aim of this study was to evaluate the efficiency of decellularization process using an alternative method of immersion with mechanical agitation to deliver the decellularization agents and compare with perfusion method proposed in the literature. Chicken hearts were immersed and mixed with Sodium Dodecil Sulfate (SDS) for 12 hours and every three or two hours this solution was replaced by Phosphated Buffer Saline (PBS) and mixed for 15 minutes. The final step included mixing with PBS for another 24 hours. After the process was completed, the atrial region and mainly blood vessels were shown whitish but the ventricular region remained with muscle tissue. The mechanical agitation method showed inefficient to deliver the decellularization agents into the heart at room temperature. The contact with the fluid and its movement didn´t promote the force required to remove the cell junctions. The role of a vascular network, the turbulence and the heating could be alternatives to increase the efficiency of the decellularization process in dense tissues.*

Keywords: *immersion, mechanical agitation, decellularization, extracellular matrix.*

1. INTRODUCTION

Heart diseases are one of the main reasons that lead people to death in the world (Hunt *et al*., 2009; Bristow and Lowes, 2004). Heart transplantation is the only alternative for final stage heart failure (Ott *et al*, 2008), but the donor shortage, the inability to monitor and control rejection, the lifelong treatment with immunosuppressive drugs and its side effects in the patient's body are limiting factors for the transplant (Murphy and Atala, 2013). The use of bioartificial engineering heart could be an alternative approach to resolve parameters as rejection rates by autologous stem cells. Thus, this bioartificial organ maybe useful in large scale production.

The ideal engineered tissue provides a scaffold with optimal structural, mechanical and electrophysiological properties, and capacity to interact and maintain viable transplanted cells (Wang et al., 2010). The synthetic scaffolds suffer from several limitations, such as ineffective nutrients delivery, poor cell proliferation and propagation (Mironov *et al*., 2009), potentially immunogenic, toxic and overpricing (Weymann *et al*., 2011). The extracellular matrix (ECM) is a great option to use as a scaffold for tissue engineering, because it is a natural basement that stimulates the attachment, proliferation, differentiation and propagation of cells (Methe *et al.*, 2014). The ECM is obtained through the decellularization process. The aim of decellularization process is to isolate the ECM from the organ without its loss, damage, or disruption, while completely removing the cellular components (Gilbert *et al.*, 2006; Crapo *et al*. 2011). The ECM can be used for subsequent repopulation with new contractile, vascular muscle tissue (Galvez-Monton C. *et al*., 2006*).*

Decellularized tissues have been successfully used in a variety of tissue for its application in regenerative medicine. More recently decellularized organs have been used as the first stages on organ engineering (Gilbert, 2012). There is a variety of methods and substances to decellularize organs and tissues. These methods can be mechanical (physical), chemical and enzymatic. The best method that can be used for decellularization depends of some tissue characteristics such as, organization, composition, cells density and thickness. Chemical substances, such as detergents, are able to interact with a cell membrane and DNA disrupting them, also removing cell residues. Enzymes can provide disruption in a very specific ligation, which is not sufficient to complete the decellularization without an additional method. Mechanical agents or methods can (1) promote mechanical abrasion; (2) improve the cellular lyses and (3) deliver decellularization agents (Crapo *et al.* 2011).

Only immersion has been sufficient to decellularize some cardiac tissues (Eitan *et al.*, 2010; Yu *et al.*, 2013; Grauss *et al.*, 2005; Akhyari *et al.*, 2010; Courtman, 1994) and other tissues slices (Daly *et al.*, 2012) but when the purpose is to generate a three dimensional structure such as a whole heart, this process is not efficient. Agitation and perfusion, two different types of mechanical methods, facilitate transportation of decellularization solutions and take out cellular debris from the tissue (Gilbert, 2012) induced by the fluid movement. They can be an alternative to improve the decellularization process with three dimensional scaffolds and multi-layers organs. An efficient decellularization protocol is determinant to supply a clean ECM and to build a viable artificial organ. Studies have been reporting great results from decellularized rat and porcine whole heart. However, in none of them was observed the complete removal of remaining cellular content (Akhyari *et al.*, 2011; Weymann *et al.*, 2011; Wainwright *et al.*, 2010; Ott, *et al.* 2008).

Thus, the aim of this study was to evaluate the efficiency of decellularization process using an alternative method to immerse with mechanical agitation to deliver the decellularization agents and compare with perfusion method proposed in the literature.

2. MATERIALS AND METHODS

2.1. Heart decellularization by mechanical agitation

The chemical method of decellularization was proposed by Weymann *et al.* (2011) and used phosphate-buffered solution (PBS) 1X (pH 7,4) and 4% w/v sodium dodecyl sulfate (SDS) in PBS 1X delivered by perfusion at 37ºC. This protocol was tested with modifications: (1) room temperature; (2) mechanical agitation was used to delivery decellularization solutions and (3) increased number of washes.

Cold chicken heart was used in a decellularization assay. They were obtained from a slaughter house and transported in a cooler with ice to the laboratory to maintain the low temperature and prevent cell lysis and matrix damage. Hearts were weighed in a precision balance (Marte®, model AY220) after removal of excess fat. They were measured with a ruler, fixed on a support through the aorta and immersed in the solutions. The structure was placed on a magnetic stirrer (Biomixer, model 78HW-1) (Fig. 1A) to mix with decellularization solutions (Fig. 1B). At the end of treatment, the hearts were weighed and measured again. All hearts submitted to decellularization process were called treated hearts.

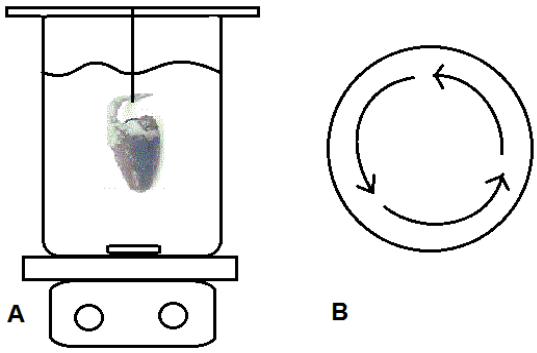

Figure 1. Schematic representation to mechanical agitation method. (A) The chicken heart was tied in a glass stem to remain immersed in the solution, and it was mixed by a magnetic stirrer. (B) Fluid movement in the beaker due to magnetic stirring.

2.1.1. Pilot assay

Chicken hearts were immersed and agitated with PBS to remove the coagulated blood and tissue debris for 15 minutes. Then, the organ was immersed and agitated with SDS solution for 12 hours. During this process, every three hours, the hearts were immersed and agitated with PBS for 15 minutes in order to remove residual substances. The final washing step after SDS treatment included immersion and agitation in with PBS for another 24 hours to remove remnant detergents and cell debris, changing every 12 hours. This assay was performed at room temperature and the stirring speed remained constant. According to the results observed in the pilot assay, it was decided to remove the excess of fat and connective tissue from the heart and increase the number of washes with PBS and SDS on the next assays.

2.1.2. Whole and cut heart decellularization

Basically, the method was similar to the one used in a pilot assay. The structure, the total time to immerse and agitate the heart, the concentration of chemical solutions and ambient conditions was maintained, but the SDS was replaced every two hours instead of three. The assay was performed separately with a whole and a cut heart. For this last one, the left and right ventricles were cut lengthwise to expose the inner of the heart chambers.

2.2. Heart decellularization by perfusion

For comparison, was performed a perfusion. The aorta was connected to perfusion machine with a thin cateter (3 mm) and the wash solutions were pumped by a roller pump through a silicon tube with output ranging around 350 ml/min and rotational speed of 26rpm. The chemical protocol used for perfusion is the same to that used for whole and cut heart decellularization established after a pilot assay.

At the end of all experiments the native hearts (controls) and treated hearts were preserved in 10% formalin for subsequent histological analysis.

2.2. Histological analysis

Atria and ventricles of control and treated hearts were dissected. They were fixed, dehydrated in increasing concentrations of ethanol, embedded in paraffin, and sectioned (5μm) by microtome. After being rehydrated, were stained with hematoxilin and eosin (H&E) to determine remaining cellular structures (Rajabi-Zeleti *et al.*, 2014). Samples were assessed under a light microscope and compared.

3. RESULTS

3.1. Heart decellularization by mechanical agitation method

In a pilot assay was observed that decellularization started on a whole heart in arteries after 2 hours. After four hours, the aorta became whitish, the pulmonary artery was translucent, and the decellularization begun in the atrium. During this time, the heart was clearer. At half time of SDS treatment, the atrial region and mainly blood vessels appeared whitish and translucent. In the mechanical agitation method the decellularization was more pronounced and faster in the upper region of the heart where the tissue is thin. The main difference in the heart's color occurred at the last step with PBS 1X. The heart lost its superficial redbrown coloration. After 12 hours shaking the solution was very

turbid and the heart was more whitish. The ventricular region became clearer than natural color but there were remaining muscle tissue after 37 hours.

In the whole heart experiment with modifications relative to pilot assay the heart's surface was more exposed because the excess fat was removed and the number of washes was greater, however there were no large differences against the pilot assay. Compared to control (Fig. 2A), the hearts were not completely decellularized, only aorta, atria and pulmonary arteries were whitish (Fig. 2B) and it was possible to see a dense muscle tissue in ventricles (Fig. 2C). The whole heart reduced its weight when compared to initial condition. In a first assay the heart's weight was 7,83g before decellularization process and 6,49g after decellularization process. In a second assay the heart's weight was 9,76g before decellularization process and 7,9g after decellularization process. They were on average, 1,6g less than in the beginning of the treatment but it lenght was not modified.

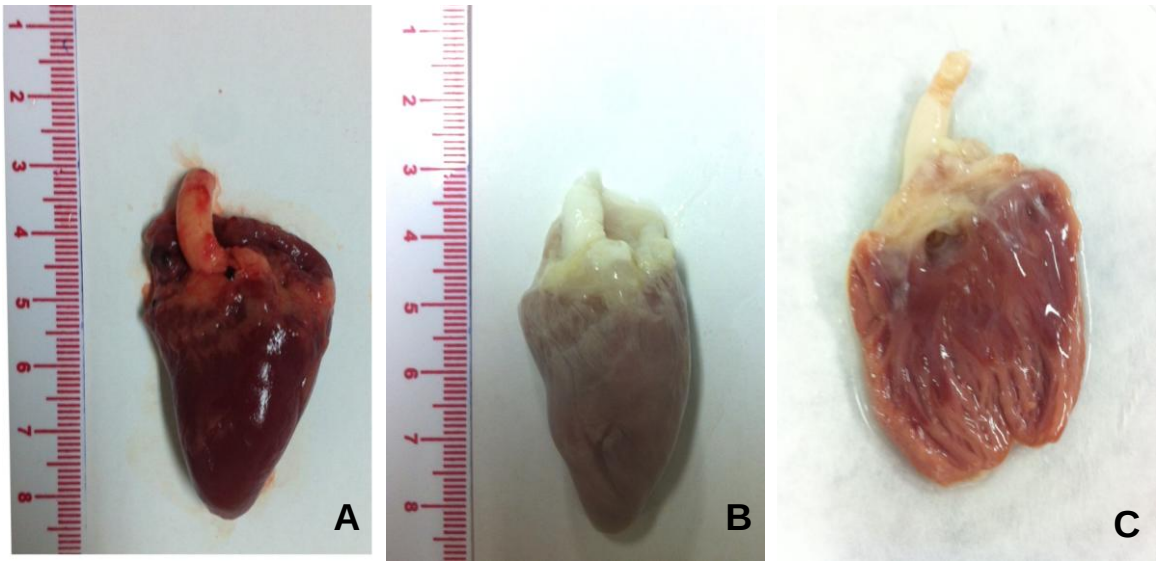

Figure 2. Representative images of chicken hearts before and after the decellularization process by mechanical agitation. (A) Before decellularization process. (B) After decellularization process. Aorta and atria were whitish but remain a dense muscular tissue in the ventricles. (C) Left ventricle cut after decellularization process.

The decellularization process using mechanical agitation on a cut heart was faster than the whole heart and its weight reduction was more accentuated. The cut heart's weight was 10,55g before decellularization process and 8,07g after decellularization process (2,48g less). However, the decelullarization was still inefficient. The atria were more translucent after four hours to SDS treatment and the right ventricle became translucent after 8 hours of SDS treatment. After 37 hours, the upper region and the right ventricle were whitish, translucent and very flaccid. In the end, the left ventricle was slightly modified, only the thin layer of external and internal surface was more whitish, the middle layers of the ventricle wall remained dense and with redbrown color. The aorta in the last decellularization step was very whitish and supported the heart's weight during all experiments without disrupt (data not shown). Therefore, the chemical plus mechanical agitation methods proved inefficient to deliver the decellularization agents and removed cellular content mainly on dense tissues and complex organs such as a heart.

3.2. Heart decellularization by perfusion

Perfusion decellularization of chicken hearts was more efficient than mechanical agitation methods. After 2 hours of SDS treatment the ventricles already exhibited spots more whitish than others, the same was observed in aorta and pulmonary artery. The time to decellularize the atrium wasn't very different of that observed in agitation method results. The last step was determinant to remove the cells. During the wash with PBS 1X for 24 hours, the heart was very whitish, soft and translucent with few regions in the ventricle that remained a visible muscular tissue (Fig. 3). The aorta decellularization results were similar to those obtained by mechanical agitation experiments. The heart's weight was 9,0g before decellularization process and 6,05g after decellularization process. It reduced 2,95g at the final of perfusion.

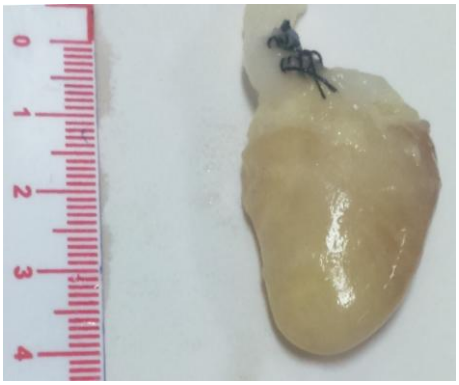

Figure 3. Representative image of chicken heart after perfusion. The whole extension was more whitish than the heart obtained after mechanical agitation method, but there were still a few remnants of muscle tissue. Scale bar: 1 cm.

3.3. Histological analysis

Histological evaluation of control and treated chicken heart revealed that perfusion (Fig. 4D) and mechanical agitation method (Fig. 4B and 4C) were not efficient for removing completely the cells and maintain only ECM. Remaining muscle cells nuclei stained violet were visible in atria (data not shown) and ventricles for all treated hearts analyzed but there was a decrease in cell volume when compared with control. Control ventricle histology showed a dense muscle tissue with typical intercalated disks link the cells together (Fig. 4A). The ventricle of cut heart (Fig. 4C) was more decellularized than ventricle of whole heart (Fig. 4B) when they were treated with mechanical agitation because there were fewer cells and they were more disorganized. Perfused hearts histology (Fig. 4D) revealed absence of nuclei in largely tissue which indicated more cell removal than mechanical agitation method using cut and whole hearts.

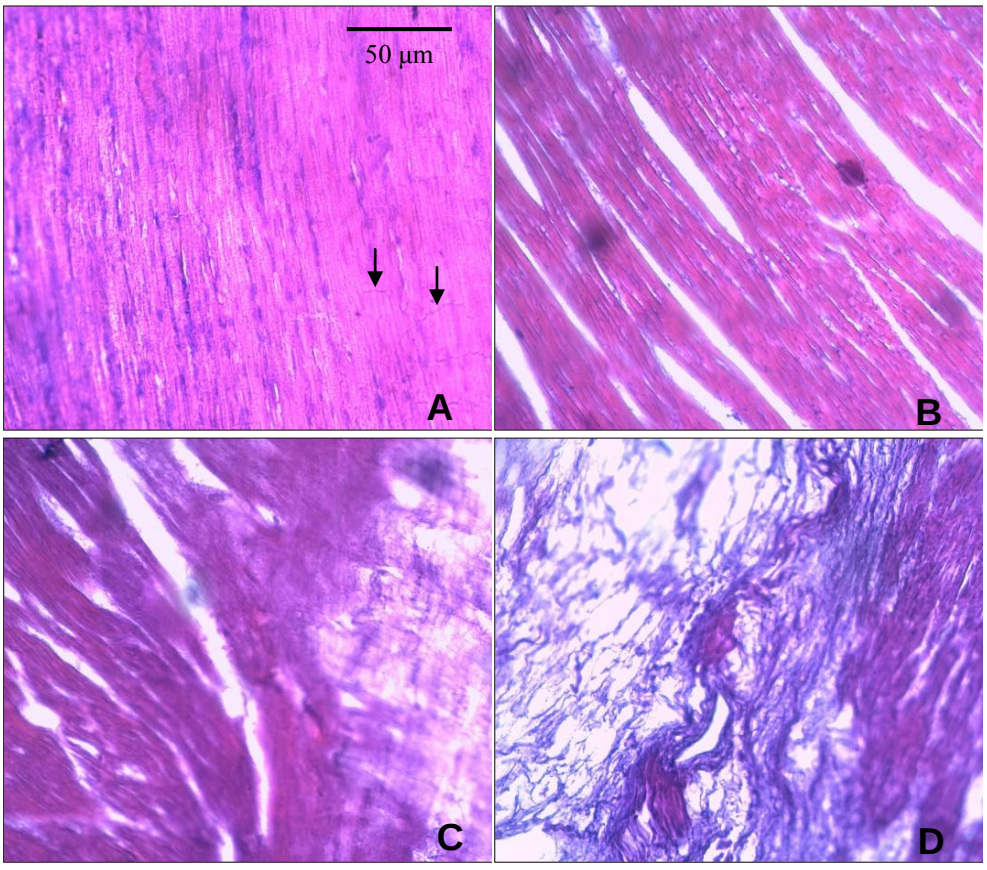

Figure 4. Representative H&E-stained samples of control and treated ventricular tissues. (A) Control. Arrows indicates intercalated disks. (B) Whole heart decellularized by mechanical agitation method. (C) Cut heart decellularized by mechanical agitation method. (D) Perfused heart. Magnification: 200X. Scale bars: 50 μm

4. DISCUSSION

The decellularization is a promising method for engineering bioartificial organs. Jungebluth et al. (2011) described a recellularized trachea implanted in patients, as well as, Ott *et al.* (2008) and Carvalho *et al.* (2011) that showed it is possible to recellularize a complex organ, like a heart, with stem cells. The ECM was able to induce a differentiation and cell proliferation to endothelial and muscular cells with contractile properties (Ott *et al.*, 2008). The maintenance of integrity to the ECM is very important because fibrillar collagen, elastin and proteoglycans are critical elements for biomechanical profile in the body, mainly in the cardiovascular system (Fomovsky, *et al.*, 2009).

The quality of the tissue decellularization depends on numerous factors such as the tissue size and thickness, the incubation protocol (mechanical method, temperature) and the species of origin (Oberwallner *et al.*, 2014). In this study it was chosen to reproduce with some modifications the protocol established by Weymann *et al.* (2011) because it maintains the ECM mechanical integrity and didn't use enzyme and other solution that could disrupt the ECM. Merna *et al*. (2013) showed that trypsin weakened the cardiac ECM when compared to Triton treatment.

Decellularization protocols vary widely in the literature (Nomtahan, *et al.*, 2014). The immersion with mechanical agitation methods were used for decellularize mainly heart valves (Yu, *et al.*, 2013) or tissue slices such as myocardium (Oberwallner *et al*., 2014; Wang *et al*.,), pericardium (Rajabi-Zeleti *et al.*, 2014) and left ventricle (Brás *et al*, 2013). All these studies showed that decellularization process was successfully performed and the ECM obtained was viable to use on engineering tissues. Unlike the protocol used in this work, the methods reported by them required more decellularization agents such as enzymes and/or Triton and the time to complete process were higher.

The pilot assay was modified in order to improve the decellularization by mechanical agitation. In order to increase the contact surface with the detergent, the hearts were cleaned, the excess of fat and connective tissue were removed. Thus, to enhance the removal of cell debris and intensify the decellularization process, the number of washes with SDS and PBS was increased but maintaining the total time of immersion not to cause any damage on the ECM. Anyway, the results indicate, under the conditions tested, that mechanical agitation method was inefficient to deliver the decellularization agents in a whole heart when compared to perfusion described by Weymann *et al.*, probably because the decellularization solutions had large difficulty to diffuse in the ventricle heart and didn't reach the internal layers. The ventricular thickness is greater than diffusion barrier (approximately 100μm) which difficult the inner cell removal (Sarig and Machluf, 2011; Kaully *et al.*, 2009).

Hearts obtained after mechanical agitation experiments exhibited an external layer more clear than the inner. Therefore, it was hypothesized that mechanical agitation method is able to decellularize external layers caused by friction and shear stress due to laminar flow while the major efficiency in perfusion method was also determined by the fluid movement in the vasculature network that could cause turbulent shear flows and the small (Kolmogorov scale) turbulent eddies were increasing cell lysis as it occur in the hemolysis process (Kameneva *et al.*, 2004).

The perfusion results observed in this study showed that this process was more efficient than agitation but it still wasn't enough to remove the major or complete heart tissue, remaining visible spots of muscular tissues in assays performed at room temperature. So, probably the temperature influences decellularization efficiency, higher temperatures, such as 37ºC, used by Weymann *et al.* increase the speed of this process when compared to assays performed at lower temperatures. This was proved by Oberwallner *et al*. (2014) that showed that the protocol performed at 4ºC was slower than another performed at room temperature.

During all experiments the aorta was a great support of the heart in the agitation and perfusion because it didn't disrupt during both process. Robertson *et al.* (2014) showed that arterial vessels have a great resistance because they are subjected to a greater shear stress all the time.

So, the next steps to work on will be to test more parameters such as heating, different speed stirring and turbulence devices to improve the agitation and perfusion methods without using enzymes or other chemical substances and to make more analysis to measure the amount of DNA, ECM composition and mechanical properties.

5. CONCLUSION

The results obtained until now, showed that the mechanical agitation method was not enough to perform a complete decellularization of the heart. Therefore, the use of a vascular network for the fluid movement in the organ, the turbulence and the heating could be alternatives to increase the efficiency of the decellularization process in complex tissues. The knowledge about the parameters that could influence the efficiency of decellularization process in different tissues is essential to establish a specific protocol to be used to create each organ.

6. ACKNOWLEDGEMENTS

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