UNIVERSITY OF MEDICINE AND PHARMACY "VICTOR BABES"
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PhD THESIS

SUMMARY

PHYSICO-CHEMICAL METHODS OPTIMIZATION FOR TISSUE DECELLULARIZATION TO OBTAIN BIOLOGICAL MATRICES

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SUMMARY

INTRODUCTION

In the last decades witnessing a spectacular development of life sciences in general and medicine in particular, with a significant impact in terms of increasing life. Medicine pathology specializes in elderly patients, even if it is not a new concept known a spectacular development in recent years.

Thus contemporary medicine is currently facing a chronic degenerative pathology increasingly frequently expressed in public, which requires finding new ways of replacement tissues and organs affected thereby imposing new trend in transplantology. Thanks to major advances made in biomedical sciences, technology was shaped bodies obtained in hard biomatrix decellularised and repopulated with autologous cells of the graft recipient. Technology biological organs obtained artificially is the newest therapeutic method appeared in transplantation medicine. "Tissue engineering" as it is called this technique, largely eliminates the drawbacks of organ transplant organs taken from live donor or cadaver and alternate electronic devices also disadvantages of organ function affected by pathological processes.

THE PhD THESIS OBJECTIVES

Closely following the studies published in the literature, needs medicine transplantation, and the concerns of staff Department of Physiology and Immunology, University of Medicine and Pharmacy "Victor Babes" in Power stations biotechnology, cell biology and molecular and regenerative medicine, this paper proposed study methods and experimental devices that can later be used to undertake other experiments to complete the development of technologies that provide new organs, fully functional to be used in transplantation medicine.

THE RESEARCH OBJECTIVES WERE:

1. Making a bibliographic documentation on the current level of knowledge in the taking of organs biomatrix by decellularization.
2. Develop, design and implementation of laboratory devices enabling decellularization electric field and mechanical vibration.
3. Testing of these devices and mathematical processing of the experimental data.

4. Develop an experimental model *in vivo* perfusion with hypertonic solutions to facilitate subsequent decellularization.
5. The study opportunities for microbiological decontamination of the devices tubing set and making an experimental device for this purpose.

The research results were published in a leading scientific journal stream - International Journal of Artificial Organs - IF 1.005, respectively Scientifical Papers Veterinary Medicine ISSN: -1221 - 5295 (BDI journals and B +) and Physiology SRSF.

Keywords: biotechnologies, decellularization, biomatrix, tissue regeneration.

1. STUDY 1:

DESIGNED AND DEVELOPED OF TWO EXPERIMENTAL DEVICES TIP LANGENDORFF FOR HEART DECELLULARIZATION IN RECTANGULAR ALTERNATING ELECTRIC FIELD.

1.1. PURPOSE OF STUDY

The aim of this study was to design and construct two experimental devices Laboratory on the principle Langendorff (one with pressure control and the other column of liquid fixed length - hydrostatic) allowing decellularization heart infused with a solution of the surfactant sodium dodecyl sulfate in the presence of an alternating electric field rectangular.

1.2. THE PRESSURE CONTROLLED DECELLULARIZATION DEVICE WITH HYDROELECTRIC PRESSURE TRANSDUCER.

The device provides for the perfusion pressure to the heart by means of a peristaltic pump whose operation is controlled continuously by a pressure transducer. Basically we can speak of a pressure regulating device according to the law "all or nothing", also known as fixed signal level.

The device consists of a vat of decellularization of the useful volume of 75 cm^3 placed on a magnetic stirrer. The stirring system is designed to continuously blend of decellularization solution to be homogenous.

The device is provided with a cannula adapted for rat aorta with a diameter of 1.5 mm (AD Instruments Company).

System pressure is continuously monitored and adjusted by hydro-electric transducer and can be read on the clinical use manometer placed in the hydraulic circuit of the device.
In decellularization tank we have an assembly of plan parallel electrodes through which rectangular alternating electric field is applied. These electrodes form an assembly type of electric capacitor.

In figure 1.2. observed schematic diagram of the device:

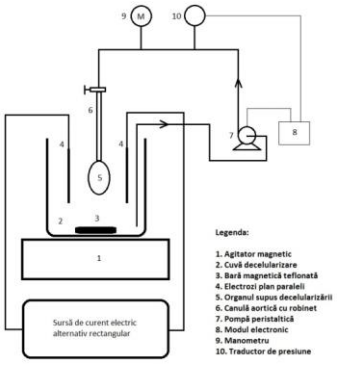

Fig. 1.2. The decellularization device with pressure control transducer. Schematic**.** (O*riginal*)

1.3. HYDROSTATIC DECELLULARIZATION DEVICE - LIQUID COLUMN.

The device perfusion type "column of liquid with constant length" which is a device hydrostatic pressure, recirculating solution decellularization, which allow for determination of kinetic biochemical taking of evidence, the volume of solution can be considered constant during an experiment .

This device is easier to do in terms of having fewer technological control electronics. It is closest to the classical model of Langendorff device.

In figure 1.3.a. observed schematic diagram of the device:

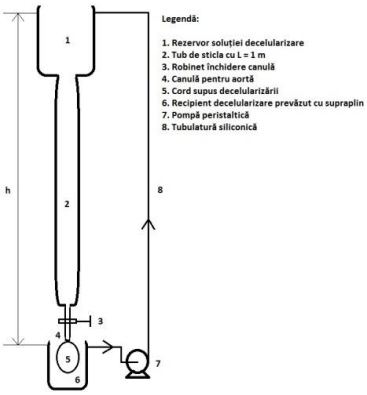

Fig. 1.3.a. Principle schematic of hydrostatic pressure decellularization device. Type Langendorff. (O*riginal)*)

Application rectangular alternating electric field is at the lower container through two metal electrodes.

The electrodes are arranged in a pattern "plan parallel " exactly the same as the previous device. Organ under decellularization is placed between two electrodes, so the geometrically it is in this area homogeneous electric field (Figure 1.3. b.) If both experimental devices presented.

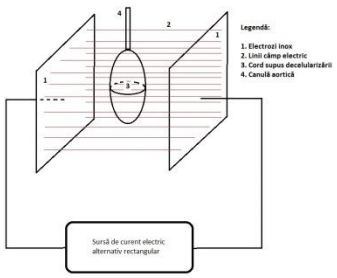

Fig. 1.3.b. Decellularization plan parallel electrodes asembly. Schematics. (*Original*)

Evaluating the results of the study suggest a method for kinetic analysis of the release of nucleic acids and proteins in organs that are being decellularised, showing that when concentrations become constant, practically no longer release new quantities of molecules, so decellularization may be considered completed.

It was tested a set of eight experimental protocols on both devices built for decellularization purpose. Each experiment was performed in triplicate.

Table 1: The parameters of these protocols.

Table 1.

	SDS Concentration (%)	EDTA-Na_2 Concentration (%)	Alternating rectangular electric field 20 KHz
Experiment 1 (E1)	1,5	-	No
Experiment 2 (E2)	1,5	0,5	No
Experiment 3 (E3)	1,5	-	Yes
Experiment 4 (E4)	1,5	0,5	Yes
Experiment 5 (E5)	0,5	-	No
Experiment 6 (E6)	0,5	0,5	No
Experiment 7 (E7)	0,5	-	Yes
Experiment 8 (E8)	0,5	0,5	Yes

The duration of each experiment was 600 minutes by taking samples for of nucleic acids and proteins determination for each 30 minutes. In all cases it was determined the concentration of nucleic acids and proteins by UV spectrophotometry.

1.4. REZULTS

Determine the effectiveness of each protocol decellularization by plotting the moment it is considered complete decellularization, taking as reference the results obtained by applying the experimental protocol decellularization E5 where conditions were the less aggressive (SDS solution concentration - 0.5% disodium EDTA absence and the absence of alternating electric field). For this experiment we will consider during the decellularization as 100%. In the experiments E3, E4, E5 and E6 was observed differences when reaching the plateau value for the two analytes follow the nucleic acids or proteins. To determine the effectiveness of decelullarization we will consider the maximum observed in all cases for the protein concentration (Figure 1.4.). If decellularization is achieved only with sodium dodecyl sulfate solution, then higher efficiency is obtained if the solution is more concentrated (1.5% vs 0.5%); the addition of ethylenediaminetetraacetic acid disodium salt (0.5%) with 10% increase the effectiveness of decellularization, the solution is more concentrated SDS (1.5%) and only 5% if it is diluted (0.5%), the value borderline significance.

Disodium EDTA complexing metal ions favors by decellularization (especially Ca2 +) cell membrane destabilization and lysis of it easier.

The effect of the application of electric field appears to be dependent on the concentration of the surfactant and the presence of the chelating agent. When using more concentrated solution of sodium dodecyl sulfate (1.5%) both in the presence and absence of EDTA disodium, the effect is insignificant. But if it is used diluted solution of SDS (0.5% - E7 Protocol) is the maximum effect (45% reduction time reference to decellularization). If we add the disodium EDTA (0.5%) when efficiency is reduced (30% reduction in the duration of decellularization from baseline) but still significantly improved over E6 protocol that uses both SDS (0.5%) and EDTA disodium (0.5%) during the decellularization which is reduced by only 5% over the reference.

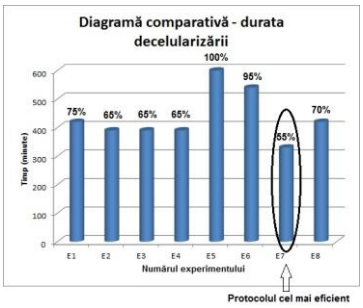

Fig. 1.4. Efficiency of heart decellularization. Experiments E1-E8.
(*Original*)

The presence of disodium EDTA, decrease the efficiency of decellularization, most likely through the mechanism of competition to transport electrical charges: some of the current is carried by EDTA that has no direct decellularization effect.

2. STUDY 2:

AN EXPERIMENTAL DEVICE TYPE LANGENDORFF FOR THE HEART DECELLULARIZATION WITH OSCILLATION PERFUSION PRESSURE.

2.1. PURPOSE OF STUDY

The aim of this study was to design and construct a device Langendorff type hydrostatic liquid column provided with the opportunity to overlap the pressure perfusion pressure oscillatory tens of hertz. Basically it generates a column of liquid that vibrates vertically. The decellularization solution is sodium dodecyl sulfate in this case.

2.2. AN EXPERIMENTAL DEVICE FOR HEART DECELLULARIZATION TYPE LANGENDORFF IN THE PRESENCE OSCILLATORY PERFUSION PRESSURE

In the laboratory of Imunofiziology and Biotechnology, Department of Physiology of "Victor Babes" University of Medicine and Pharmacy, was designed and built an experimental laboratory device for the study of heart decellularization using a surfactant (sodium dodecyl sulfate) with or without a chelating agent (sodium salt of ethylenediaminetetraacetic acid) in the presence of a mechanical factor: a perfusion pressure fluctuating (oscillating pressure). Figure 2.2. It is a schematic diagram of the device assembly and construction details that generate electromagnetic oscillations pressure.

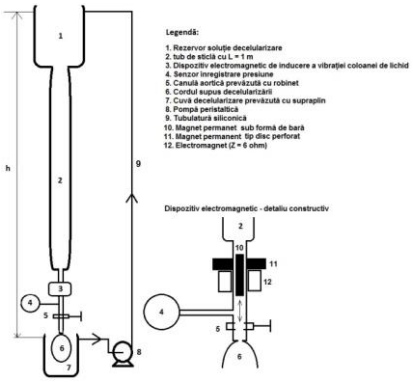

Fig. 2.2. Oscillatory pressure experimental device for heart decellularization.
Schematics. (*Original*)

The experimental device is similar to that presented in the previous chapter, with the same useful volume. The differences in construction are: the introduction of a electromagnetic setup, the cannula which carried the oscillations of a column of liquid of decellularization.

Electromagnetic assembly consists of a perforated disc-like permanent magnet fixed to an electromagnet, which also has a central hole of the same diameter of the magnet superimposed on it. Through this hole passes a glass tube which is continuous with the aortic cannula of the device. In the glass tube there is another bar-type permanent magnet with a diameter lower than the internal diameter of the tube (to allow the passage of liquid through the space so formed), which in the absence of current in the electromagnet is kept in equilibrium by the magnetic field of the disk. When the solenoid is energized with alternating current magnetic field generated by it are added up or subtracted cyclically one of the permanent magnet type disc and thus induce the oscillation of the magnet rod tube, oscillation obviously passed the column of liquid perfused heart.

The electromagnet is powered by a modified audio amplifier for low frequency playback. The signal generator is used in experiments in the previous study. It works at a frequency of 15 Hz where the preliminary determination was that the pressure variation is set high and uniform (to achieve the resonance condition of the device).

In this study four experimental protocols were applied. Each experiment was performed in triplicate.

Table 2: The parameters of these protocols.

Table 2.

	SDS Concentration (%)	EDTA-Na_2 Concentration (%)	15 Hz Oscillating pressure
Experiment 1 (E1)	0,5	-	No
Experiment 2 (E2)	0,5	0,5	No
Experiment 3 (E3)	0,5	-	Yes
Experiment 4 (E4)	0,5	0,5	Yes

The duration of each experiment was 600 minutes by taking samples for of nucleic acids and proteins determination for each 30 minutes. In all cases it was determined the concentration of nucleic acids and proteins by UV spectrophotometry.

2.3. RESULTS

It was determine the effectiveness of each protocol decellularization by plotting the moment it is considered complete decellularization, taking as reference the results obtained by applying the experimental protocol E1 decellularization where conditions were the less aggressive (SDS solution concentration - 0.5% absence of disodium EDTA and nonoscilatory constant hydrostatic pressure). For this experiment we will consider during the decellularization as 100%. In the experiments E2 and E3 were observed difference between the time to achieve the set value for the two analytes follow the nucleic acids or proteins. To determine the effectiveness of decellularization will consider the maximum time observed in this case for the protein concentration. (Figure 2.3.a.).

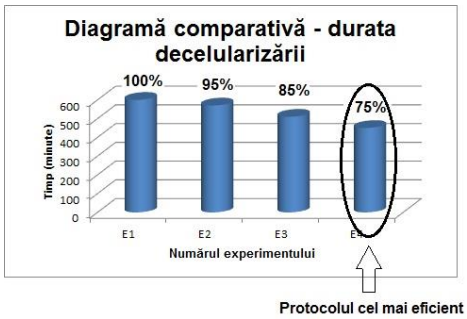

Fig. 2.3.a. Efficiency of heart decellularization. Experiments E1-E4.
(*Original*)

It is observed that exposure to hydrostatic pressure fluctuating with the cumulative effect of disodium EDTA. It favors decellularization by complexing metal ions (especially Ca2 +) cell membrane destabilization and lysis of it easier. The addition of disodium EDTA 0.5% during the decellularization reduced by 5%, of borderline significance.

The application of fluid pressure with disodium EDTA oscillatory reduced by 15% during decelluralization, and with the addition of EDTA to achieve a reduction in the time required by 25%.

It tried strictly macroscopic evaluation of coronary system from biomatrix obtained with this experimental device, without claiming a study completed by injecting through the aortic cannula to a 1% blue Evens dye penetration and tracking in the coronary system. For relevant results histological studies would be needed. Figure 2.3.b. observed the progression of dye in the coronary system.

Fig. 2.3.b. Biomatrix coronary system. Blue Evans dye 1%. (*Original)*

3. STUDY 3:

DEVELOPMENT OF A COMPLEX EXPERIMENTAL PROTOCOL FOR ASSESSING EFFECTS ON HYPEROSMOLAR STATUS INDUCED IN VIVO, SUBSEQUENT CARDIAC DECELLULARIZATION

3.1. PURPOSE OF STUDY

The aim of this study was to assess the appropriateness induce a severe hyperosmolar state animal undergoing general anesthesia before sampling the heart for future decellularization. The administration is slowly, at a rate of 10 ml / h by means of an automatic syringe, intraarterial, a concentrated (45%) of sucrose heparinized (100 IU/ml) until cardiac arrest onset. The heart will remove and is subject for decellularization.

3.2. EXPERIMENTAL PROTOCOLS

In this study were used 6 rats divided into two groups:

- Group A (n = 3) "CONTROL" were administered intraarterially of a isotonic heparinized saline solution (100 IU/mL). The administered volume was 20 cm^3 over a period of two hours.

- Group B (n = 3) "HYPERTONIC" which was administered hypertonic sucrose solution The heparinized (100 IU/mL) until installation of the cardiac arrest. Cardiac arrest appear after about 90 to 100 minutes (corresponding to a volume of hypertonic solution of 15 to 17 cm^3).

After concluding intra-arterial infusion / installation cardiac arrest cord detaches according to the techniques described in previous chapters and apply a decellularization protocol is identical for both groups, with a solution of sodium dodecyl sulfate 0.5% in distilled water. It will use the following notations:

- E1 decellularization experiments in sample A "CONTROL" (average)
- E2 decellularization experiments in sample B "HYPERTONIC" (average)

It was used, a test device hydrostatic - the column of liquid of the type used in the earlier study, which was removed from the electromagnetic module. The duration of each experiment was 600 minutes. The samples was taking for each 30 minutes. In all cases it was determined the concentration of nucleic acids and proteins by UV spectrophotometry.

3.3. RESULTS

Determine the effectiveness of each protocol decellularization by plotting the moment it is considered complete decellularization, taking as reference the results obtained by applying the experimental protocol corresponding batch A - E1. To determine the effectiveness of decellularization we will consider the maximum observed in this case the for protein concentration (Figure 3.3.).

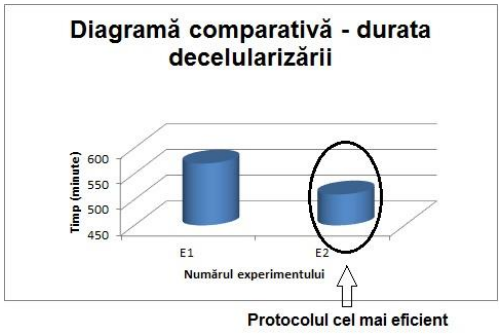

Fig. 3.3. Efficiency of heart decellularization. Experiments E1-E2.
(*Original*)

4. STUDY 4:

MAKING AN ELECTROCHEMICAL MICROREACTOR WITH ASYMMETRIC CURRENT DENSITY FOR IN SITU SANITIZING TUBING SET OF DECELLULARIZATION DEVICES

4.1. PURPOSE OF STUDY

In our laboratory it was built electrochemical microreactor with asymmetric current densities which generates a controlled amounts of free chlorine and hypochlorite ions respectively which can be connected directly to both devices decellularization suggested. Thus these devices can be sterilized tubing in order to investigate further the biomatrix recellularisation obtained.

4.2. DESCRIPTION OF THE EXPERIMENTAL DEVICE

We chose electrochemical reactor with asymmetrical current densities as compared to conventional reactor equal electrode surface, it favors the desired electrochemical reactions and prevents the diaphragm which has many disadvantages (expensive material, low mechanical strength, high ohmic resistance).

From the constructive point of view of the electrochemical reactor consists of a vat of cylindrical shape plaxiglas the useful volume of 100 cm^3. At the bottom is a nozzle filling / emptying the device can connect to the peristaltic pump device decellularization. The reaction mass is continuously stirred by a mechanical stirrer driven by an electric micro-motor provided with a speed reduction unit (Figure 4.2).

Electrodes (anodes eight of the assembly of the graphite and the cathode of stainless steel (stainless steel food), and the motor drive system are mounted in reactor cover. The resulting gases are discharged from the electrolysis of a small compressor outside the workspace. Also on the cover is a port for sampling during operation of the device to physical and chemical analyzes.

The reactor is connected to a reference calomel electrode (electrochemical couple: Hg / calomel / KCl - saturated) which continuously measure been to pick potential anode with a high input impedance voltmeter.

The electrolyte solution used is 0.9% sodium chloride (saline) suffering a series of anodic oxidation processes with the formation of hypochlorite ions and small amounts of chlorine free (physically dissolved) with strong sterilizing effect. Cathodic processes are greatly hampered by the construction of the reactor.

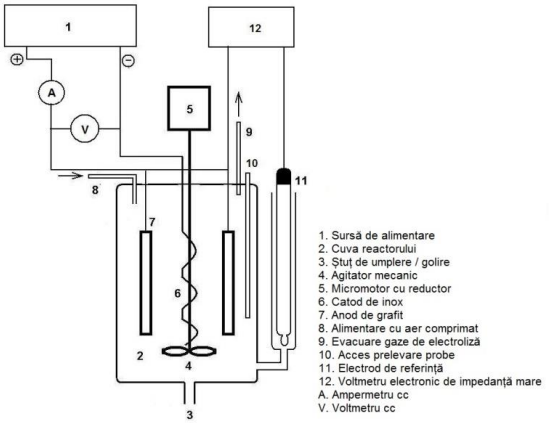

Fig. 4.2. The electrochemical microreactor with assymetric current densities.
Schematic. (*Burian*)

4.3. REZULTS

To determine the effectiveness of sterilization was used Staphylococcus aureus strain to strain (ATCC 25923) and the concentration of free chlorine by titrimetric with methylorange in sulfuric acid medium.

Under the experimental conditions presented it shows that the first three minutes of operation of the pilot plant, microbial load is reduced from 9.48 to 0 for a log of the active species concentration of free chlorine below 3 mg / dL.

FINAL CONCLUSION

The aim of this work was to evaluate some physicochemical methods used to obtain biomatrix rat heart by heart decellularization. Research objectives outlined in the introduction

to this paper have been met entirely within the four experimental studies and whose final results allow the following conclusions:

1. Testing Langendorff type experimental devices with pressure control, versus column of fluid revealed no significant differences in terms of length decelularizării efficiency of the two devices may be considered identical.

2. Kinetics decellularization process is significantly influenced by the following factors:

- The concentration of SDS solution: 1.5% SDS solution is better than the 0.5% solution with respect to time of decellularization, but more concentrated solution has an adverse effect on the integrity of the three-dimensional biomatricii obtained.

- The addition of disodium EDTA to the solution of SDS in order complexation of Ca2 +, followed by destabilization of cell membranes, the decellularization favor the use of more concentrated solution of SDS (1.5%) and to a lesser extent in the case of diluted solutions (0.5%) - of borderline significance.

- Applying alternating electric field (20kHz) significantly increases the efficiency decelularizării (45% reduction from baseline decellularization time) but only dilute solutions of SDS (0.5%); for concentrated solutions (1.5%) this effect is greatly reduced. The addition of disodium EDTA in the presence of the electric field does not favor any solutions of SDS decellularization diluted nor for the concentrated, probably through a competition mechanism to transport electrical charges between dodecyl sulfate ions and ion ethylenediaminetetraacetate.

3. An effective way to sterilize the piping used for decellularization Langendorff device type is the generation of reactive species such as free chlorine physically dissolved and the hypochlorite ions to a solution of 0.9% sodium chloride solution subjected to the electrolysis anode graphite in a microreactor asymmetric electrochemical current densities coupled with Langendorff device. A running time of three minutes is sufficient to destroy the bacterial load 3x108 bacteria / ml (S. Aureus strain ATCC 25923) at a volume of 100 cm^3 solution.

ORIGINAL CONTRIBUTIONS

1. Design, implementation and testing of three laboratory devices used decellularization type Langendorff rat heart: a controlled pressure Langendorff device type, device type Langendorff liquid column and another device but also with liquid column provided with a mode which provides a hydrostatic pressure electromagnetic oscillations with a frequency of 15 Hz. For all these devices have been developed experimental protocols validated by which their operation.
2. Design, implementation and testing of an anesthetic ventilator for small laboratory animals used in experiments *in vivo*.
3. Development of a Protocol to induce intracellular dehydration condition of the heart in vivo by administration of a concentrated solution of sucrose in the animal undergoing general anesthesia. Taken heart after cardiac arrest has undergone installing decelularizării. This sequence intracellular dehydration followed by intravital organ decellularization is original.
4. Design, implementation and testing of an electrochemical microreactor with asymmetrical current densities to generate reactive species hypochlorite ion type devices used to sterilize tubing Langendorff.

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