Decellularization and recellularization of porcine livers and design and construction of an automatic machine setup for liver perfusion decellularization

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Abstract: Liver transplantation remains to date, the only option for patients with end-stage liver disease (ESLD). Several bioengineering approaches have been developed over the years to increase the number of options for these patients, mostly failing due to lack of a vascular network. This work aimed to recellularize the liver vasculature in decellularized porcine livers in order to solve the aforementioned problem. Porcine livers were decellularized through detergent perfusion at constant pressure or flow rate, with results that show the possibility of decellularization without using strong detergents such as sodium dodecyl sulfate (SDS), while seemingly conserving the microarchitecture of the liver extracellular matrix (ECM). The results obtained also suggest that efforts to stabilize the liver in the decellularization process are rewarded with better results. The decelularized liver scaffold (DLS) were then recellularized with human mesenchymal stem cells (hMSCs) and human umbilical vein endothelial cells (hUVECs), in a perfusion setup with pressure control. Recellularization results were lacking and, as such, no conclusions were taken at the moment as further testing is needed. In an effort to circumvent some issues related with the decellularization protocol, the automatization of said process was started and a decellularization machine is being constructed. As it is still unfinished, no decellularization was performed in this setup and no results were collected at the present stage.

1 INTRODUCTION

In the past years, organ bioengineering has flourished and several techniques have proved to be suitable candidates for the job at hand. Most strategies have relied on scaffolds with increasing complexity in order to better replicate the liver microarchitecture and niche, but there is also some work done in scaffold-free organogenesis focused approaches. More recently, there has been an alternative to the approaches mentioned above, in which instead of trying to produce liver tissue or liver niche cell cultures and co-cultures, there has been a shift towards the more complex alternative of bioengineering whole livers or physiologically relevant liver structures like liver lobes, liver buds or liver vasculature and ducts.

1.1 Decellularized scaffolds

So far, the most widely described technique for liver bioengineering is the use of decellularized livers as scaffold for liver regeneration. In this technique the rationale is that if the ECM remains in a good condition after decellularization, then the different components of the ECM will guide the different cell types into their proper positions and serve as anchors for them to attach, aiding not only in the actual attachment but also in the formation of the various structures that characterize the liver. This kind of approach is based on a two-step process, the decellularization step and the recellularization step. For the first step, the decellularization, as the objective is to remove all cells and cellular material, protocols are based on the combination of various cell-damaging factors such as freezing/thawing cycles, isotonic stress, enzymes, shear stress and chemical action being that the chemical action of detergent solutions is the most widely described for dense non-hollow organs such as the liver. These solutions are always perfused throughout the organ vasculature in order to detach the cellular material from the ECM so that only the structured ECM remains. As far as these solutions go, there is a tendency for the use of detergent solutions such as Triton X-100 and ECM (Baptista et al., 2011; Sabetkish et al., 2015; Wang et al., 2015; Bühler et al., 2015; Struecker et al., 2015), but there have been also various other papers using solutions ranging from enzymes such as trypsin to chelating agents such as EDTA or EGTA (Soto-Gutierrez et al., 2011; Nari et al., 2013).

Additionally there have been successful attempts while using as inlet the vena cava (VC) (Shupe et al., 2010), the portal vein (PV) (Uygun et al., 2011) and the hepatic artery (HA) (Struecker et al., 2015), as well

as using fixed flow (Wang et al., 2015), fixed pressure (Bühler et al., 2015) or even oscillating pressure conditions (Struecker et al., 2015; Hillebrandt et al., 2015), even existing video articles on the subject(Hillebrandt et al., 2015).

1.2 Recellularization of scaffolds

Excluding the artificial bioprinted scaffolds (which are seeded in the fabrication process), both the natural and the artificial scaffolds must then be seeded. This process is called recellularization in the case of the DLS seeding, as said scaffolds are effectively being returned cells to substitute those that were removed.
In order to recellularize a tissue, one must repopulate it either with all the cell types that naturally populate said tissue or repopulate it with progenitors of said cells and then induce some degree of expansion and differentiation in order to replicate as close as possible the natural tissue. The liver is composed of a large amount of cell types, organized in a very structured environment, in which one can clearly distinguish various different defined structures, with different cell types. As such, through perfusion, hepatocytes, stellate cells, endothelial cells and various other cells can be used to repopulate the obtained scaffold. Some examples include the repopulation with mesenchymal and endothelial cell lines for vascular regeneration (Baptista et al., 2016) or the repopulation with hepatic cell lines for metabolic, viability or functional assessments (Sabetkish et al., 2015; Mazza et al., 2015). Another option deserving further thought is the recellularization with more primitive versions of said cells, such as hepatoblasts, from which both the hepatocytes and the cholangyocytes are derived. Strategies like this could potentially simplify the seeding strategies, by reducing the complexity involved with expanding multiple cell lines and mixing them in the appropriate proportions for seeding.

2 MATERIALS AND METHODS

2.1 Cell lines

The cell lines used in this project were hUVEC, hMSC, which were isolated, cultured, tagged with green fluorescent protein (GFP) and tdTomato (TOM) as preparation beforehand.

2.2 Decellularization

2.2.1 Setup assembly

Material With all the different components ready to be used for the recellularization protocol, all that is missing is the setup that allows for the procedure. First of all as vessel for the liver, various glass lab ware and lastly a custom designed acrylic vessel was used. The liquid tubing lines used were Masterflex platinum-cured silicon tubing L/S 14, 16 or 17 (Cole-Palmer Instrument Co.), depending on the PV and HA diameters. For connections, male-lock, luer 4-way stopcocks were used as well as Masterflex luer fittings (Cole-Palmer Instrument Co.), 1/16", 1/8" or 1/4", male and female variants (according to the tubing size). For the pumping action, two Masterflex L/S model 7519-06 peristaltic pumps with Masterflex L/S easy load pump head (Cole-Palmer Instrument Co.) were used. These pumps were connected with two Hugo Sachs Elektronik servo controller for perfusion and transducer amplifier modules (Harvard Apparatus) which were used to control the pressure measured at the pressure sensor in order to maintain it at a constant user-selected level. To eliminate the pulsatile action of the peristaltic pumps, two Masterflex pulse dampeners (Cole-Palmer Instrument Co.) were also used. Lastly, depending on the diameter of the target vasculature, either 24G cannulae (Terumo), 14G cannulae (Terumo) or a 1/4" straight barbed fitting (Cole-Palmer Instrument Co.) were used.
So as to prepare all of the equipment for the recellularization, all tubing lengths and fittings are cleaned with distilled water (dH_2O). Sterilization is not necessary at this stage since the liver is only sterilized after decellularization as a preparatory step for recellularization.

Assembly So as to decellularize, firstly the setup is assembled either in the laboratory or in the cold room, according to the scheme below (Figure 1), excluding the liver. The 4-way stopcocks should all be completely open, except for the one that connects to the tanks, as that one should be open to the passage of dH_2O and not of decellularization solution. Following this, the tanks are filled up with the respective liquids, being that the decellularization solution used was either 1% triton X-100 (Panreac) + 0.1% ammonium hydroxide (Panreac) in dH_2O or 1% SDS (Sigma-Aldrich) + 0.1% ammonium hydroxide in dH_2O.

2.2.2 Decellularization

To start the decellularization the tubing was primed with dH_2O and then connected into the liver, through cannulae or fittings using the PV and the HA as inlets. The liver was then lowered into the respective dH_2O filled vessel. Afterwards, the perfusion of dH_2O started in order to completely clean the vasculature of remaining blood and to start the decellularization process by submitting the cells to osmotic stress. As for this step, it is performed until there is no visible exit of blood from the outlets (infrahepatic vena cava (IVC) and suprahepatic vena cava (SVC)). The next step is to turn the valves so that the decellularization solution is perfused instead of the dH_2O. In this step, both triton X-100 and SDS were tested. The tests consisted of perfusion of just one of the detergent solutions or

perfusion of both, starting with SDS and finishing with triton X-100. This perfusion step was performed either until the liver was fully decellularized, or until no improvement was achieved from further detergent perfusion. After detergent perfusion, the liver was once again perfused with dH_2O for double the volume of that used of detergents, in order to clean the obtained DLS. If the decellularization was successful then the liver was stored in little plastic boxes submerged in water with two-three droplets of bleach while awaiting sterilization. If not, then samples were taken for deoxyribonucleic acid (DNA) assays and histology.

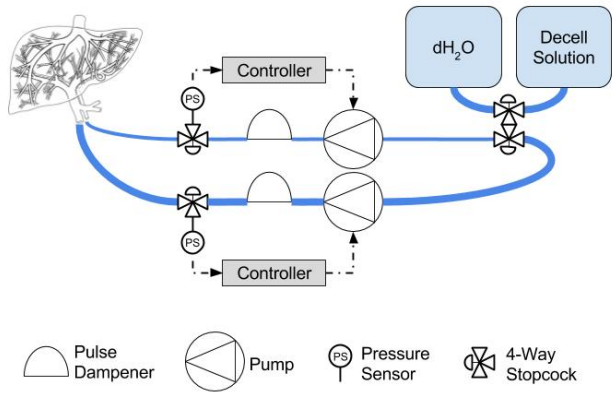

Figure 1: Scheme of the decellularization setup. In this scheme, the wider line represents the PV line and the narrower line the represents HA line. The blue boxes represent the decellularization solution and the dH_2O tanks, respectively.

2.3 Recellularization

After the decellularization step, the next logical step is the recellularization of the DLS. However, so as to recellularize them, a series of preparation steps must be followed in order to make possible the recellularization process. These steps are comprised of guaranteeing that the DLS, the bioreactor and the cells are all ready for this procedure.

2.3.1 Preparation

Scaffold sterilization At last, the DLS must be sterilized if not yet sterile. Several protocols for the sterilization of DLS exist. This step can be done with the perfusion of peracetic acid (PAA), ethylene oxide (EtOH), or gamma irradiation among others, being that combination methods like PAA or EtOH + gamma irradiation appears to give the best outcome(Kajbafzadeh et al., 2013). In the present case, none of these methods were followed due to time and material constraints, instead being sterilization achieved with X-ray irradiation. The DLS was placed in a 15cm tissue culture plate which was then half-filled with dH_2O. Then the lid was closed, sealed with parafilm and finally sealed in an autoclave bag. Then it was sent for X-ray sterilization using the max parameters possible with the available equipment (160kV, 6.3mA, 99.9 min). Following the sterilization, the DLS was stored at 4^oC while waiting for the recellularization.

Cell seed expansion In this project, instead of recellularizing the whole liver, the first objective was set to be the recellularization of the vascular network in a way that would prevent blood clotting, since this phenomenon would indicate that the ECM is not thoroughly covered as it is triggered by the detection of certain components of the ECM, namely collagen, by the platelets.
As such, to meet that objective, the cells chosen for the recellularization process were the hUVECs with the addition of hMSCs with more of a supporting role. Despite being considered for the objective, up to this point, smooth muscle cells (SMCs) were still not included.
An estimation based on previous work and the average porcine liver weight was made and a quantity of 100 million hUVECs and 20 million hMSCs was set as a starting point for the seedings on the recellularization attempts.
Starting from cryopreserved samples of approximately 2 million tagged cells, these were thawed and cultured until the aforementioned cell numbers. An average of 3 passages was needed to reach the required cell numbers, for both the hUVECs and the hMSCs.

2.3.2 Setup assembly

Material With all the different components ready to be used for the recellularization protocol, all that is missing is the setup that allows for the procedure.
First of all a spinner flask is used as vessel, over a Isotemp Heated Magnetic Stirrer (Fisher Scientific) with a magnet providing agitation. The liquid tubing lines used were Masterflex platinum-cured silicon tubing L/S 14, 16 or 17 (Cole-Palmer Instrument Co.), depending on the PV and HA diameters. For connections, male-lock, luer 4-way stopcocks were used as well as Masterflex luer fittings (Cole-Palmer Instrument Co.), 1/16”, 1/8” or 1/4”, male and female variants (according to the tubing size). For the pumping action, two Masterflex L/S model 7519-06 peristaltic pumps with Masterflex L/S easy load pump head (Cole-Palmer Instrument Co.) were used. These pumps were connected with two Hugo Sachs Elektronik servo controller for perfusion and transducer amplifier modules (Harvard Apparatus) which were used to control the pressure measured at the pressure sensor in order to maintain it at a constant user-selected level. To eliminate the pulsatile action of the peristaltic pumps, two Masterflex pulse dampeners (Cole-Palmer Instrument Co.) were also used. Three Masterflex female x male luer-lock smart site connections (Cole-Palmer Instrument Co.), were used in key location in order to aid on the priming process (described below), to seed the bioreactor and to collect samples for further testing and control. Additionally empty spare bottles of PBS, DMEM/F-12^{++} and hUVEC medium were used as well. Also, the spare bottles’ and the spinner

flask's caps were drilled so as to make tight entrances for the tubing. Lastly, depending on the diameter of the target vasculature, either 24G cannulae (Terumo), 14G cannulae (Terumo) or a 1/4" straight barbed fitting (Cole-Palmer Instrument Co.) were used.
So as to prepare all of the equipment for the recellularization, everything that comes in contact with the cells must be sterilized. Excluding the pressure sensor which is sterilized with a biocide, the stopcocks where EtOH is used and the filters which are already sterile (individual sterile package), everything is autoclaved.
Additionally, the sterile DLS and the bioreactor medium are also needed.
With everything ready to start the recellularization, the first step is the assembly of all the recellularization setup. For that, all of the components that do not come in contact with the cells are placed in their respective positions, either inside (pumps and magnetic stirrer) or outside (pressure controller) of the incubator. Afterwards, in the laminar flow hood, the inside of the pressure sensor is sterilized with biocide and set aside for later.

Assembly and priming After that, the setup is assembled and primed in sterile conditions, according to the scheme below (Figure 2), by following the following protocol. Working in sterile conditions entails not only working in the laminar flow hood but also using a sterile laboratory coat, sterile gloves, and a face mask.

1. Connect all the parts, excluding the pressure sensors;
2. Turn the stopcocks 1 and 2 so that the stopcock 2 is diagonal (fully closed) and the stopcock 1 is open towards the tubing A;
3. Prime the tubing A through the smart connector, with biorreactor medium;
4. Connect either the cannula or the fitting on the DLS to the tubing A carefully to avoid the entrance of bubbles;
5. Turn the stopcock 1, so as to open the smart connector towards stopcock 2. By doing so, the DLS and tubing A are already primed closed off, so that the DLS cannot be disturbed.
6. Insert the liver in the vessel;
7. Disconnect the stopcocks 1 and 2 and close the top cap of the spinner flask, through where the liver entered;
8. Turn the stopcock 2 so that the pressure sensor spot is opened towards the tubing B;
9. Guarantee that the stopcock 3 is open from the tubing B towards either the tubing D or H;
10. From the sensor position, prime the line until the pulse dampener, then fill the pulse dampener until the required level (or slightly upwards). Finally prime the tubing until the stopcock 3;
11. Close of the tubing length C by turning stopcock 3 so that the tubing H is connected to the tubing D;
12. Reconnect the stopcocks 1 and 2;
13. Turn the stopcock 2 so that there is an open line between both stopcocks connecting the smart site connector to the place where the pressure sensor connects;
14. Guarantee that the system is not tangled. If it is tangled, it is possible to disconnect the stopcocks 5 and 6 to disentangle the lines. The fittings in the stopcock 4 can also be disconnected temporarily to remove tangles ;
15. After guaranteeing everything is connected and closed besides the pressure sensor, finally connect it in its place, prime it through the smart site connector and close it so as to avoid leaks and contamination;
16. Double-check everything is closed and either open the connected medium bottle to fill it up or exchange it for a filled one if the bottle is empty;
17. Move the setup into its place inside of the CO_2 incubator (which should be at 37°C and 5% CO_2) and attach the tubing to its respective pumps;
18. Turn the stopcocks 4, 5 and 6 so that there is an open path all the way from the medium bottle, through the pump, and up until the spinner flask (tubing lengths D, H, F and G);
19. Program the pump so that it pumps from the medium bottle to the spinner flask and fill the vessel up to the desired level;
20. Reverse the pumping direction and turn the stopcocks 1, 2, and 3 so as to open all of the main line, the smart site connector branch in the stopcock 1 and the pressure sensor branches in the stopcock 2 (only the branch that connects to the medium bottle in the stopcock 3 should be closed). At this stage, there should be an uninterrupted pathway that connects the tubing lengths G, F, H, C, B and A, completing the bioreactor recirculation loop;
21. Lastly, input the desired parameters and start the pump. Wait for a minimum of 4 hours (overnight would be better) before starting the seeding.

2.3.3 Seeding

After waiting at the very least four hours, the DLS can finally be seeded.
The first step of the seeding is to calculate how much and how often can the seedings take place. For that, the amount of time it takes for the entire volume inside the system to go 5 times through the liver was calculated with equation 1, being that value used as time between seedings.

$$t_{seeding} = \frac{5 \times V_{medium}}{F_{pump}} \quad (1)$$

$t_{seeding}$ is the time between seedings, in min;

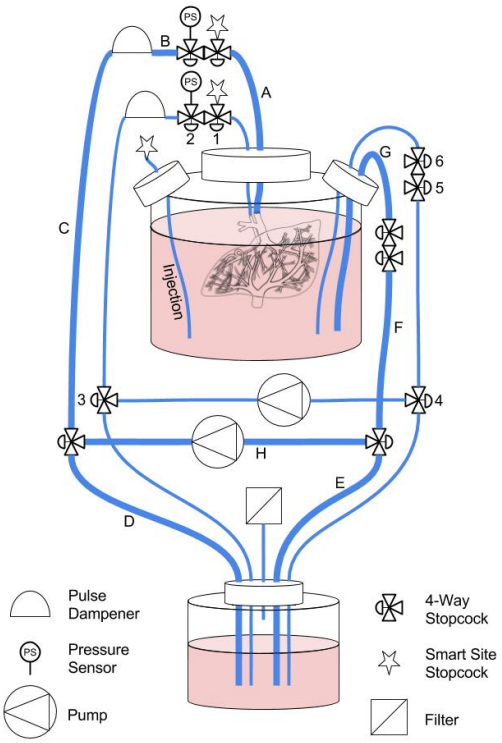

Figure 2: Recellularization setup. In the left figure, a scheme of the recellularization setup can be seen. In this scheme, in the PV line (wider) the tubing lengths are labeled and on the HA line (narrower) the 4-way stopcocks are labeled. In the right figure the actual setup is displayed, assembled inside of the CO_2 incubator.

V_{medium} is the volume of medium in the bioreactor (vessel + tubing), in mL;

F_{pump} is the sum of the flow rate in both lines (HA + PV), in mL/min;

As a reference value from previous work developed in the laboratory, a maximum of 5 million cell/min was admitted for seeding, since the entry of too many cells at once in the DLS can cause clogging. Taking this into consideration, the number of cells per seeding was calculated with equation 2

$$N_{seeding} = \frac{5 \times F_{cells}^{Max}}{F_{pump}} \quad (2)$$

$N_{seeding}$ is the maximum number of seedable cells per seeding;

F_{cells}^{Max} is the maximum number of cells seeded, in cell/min;

F_{pump} is the sum of the flow rate in both lines (HA + PV) in mL/min;

With these data, the DLS can finally be seeded through the smart connector that leads directly into the vessel.
With two different cell samples (equal types, but distinct tags) the seedings for the first condition are performed until the sample is all used up, and then the pumps are turned off to let the cells adhere better without a shear stress source. Four hours later the pumps are re-started and the next seeding cycle can start. As the conditions may have varied greatly due to the colonization of the DLS by the first cells seeded, it is advisable that the seeding parameters are recalculated.

The different cell tags were used since the two seeding cycles were performed with different set-points in the pressure control, to assess the pressure that would obtain an optimal penetration to cell death ratio of the seeded cells inside the DLS.

2.3.4 Control Assays

In terms of bioreactor monitoring, apart from the control over the pressure and flow which is part of the control loop, and the control over temperature, pH, and dissolved gases that exists due to the placement of the bioreactor in a CO_2 incubator, additional mechanisms are used to monitor the recellularization process.

Firstly, due to the presence of phenol red indicator in the medium, one can program the medium changes depending on the color of the bioreactor medium. Due to the acidifying effect of the cellular metabolism, as the consumption of nutrients in the medium advances, the medium gets increasingly acid and that is visible as the medium starts shifting from a pink-reddish to an orange and finally to a yellow hue.

Secondly, since the objective entails the culture of anchorage-dependent animal cells and medium is by itself clear, increase of turbidity of the medium is an indicator of possible contamination, that if detected early, can be treated in the case of microbial origin. Fungal contaminations in this stage are extremely difficult to remove.

Additionally glucose and lactate concentrations are used as indicators of nutrient consumption and cell metabolism.

For this, collected samples were centrifuged at 3000 rpm for 5 minutes and then the supernatants transferred to falcon tubes. These were stored at -20°C until the end of the respective experiment so that all samples could be processed simultaneously.

The glucose concentration was assessed with blood glucose test strips and the lactate concentration was determined with the Pierce LDH Cytotoxicity Assay Kit (ThermoFisher Scientific).

The protocol for determining the glucose consists of placing a droplet of the relevant sample on a clean surface and then measuring the glucose concentration with the glucometer.

The protocol used for the LDH measurements was the one described in its user manual without any alteration.

2.4 Quality testing

2.4.1 Microscopy Staining

So as to assess the extent of both the decellularization and the recellularization, DLSs and recellularized livers were fixated by perfusing for 15 to 20 minutes and then immersing them in formaldehyde overnight. Afterwards they were sent for tissue processing and hematoxylin and eosin (H&E) staining.

3 RESULTS AND DISCUSSION

3.1 Decellularization

3.1.1 Liver preparation

To prepare the DLS for recellularization, two steps may have to be followed depending on the protocol followed.
Firstly, considering the objective, a partial hepatectomy may have to be done to the DLS. The existence of the partial hepatectomy in the protocol is dictated by the final objective of the project. As in this project, the final objective is to transplant only the right lobe of the liver, the procedure must be integrated into the protocol.
On another note, the partial hepatectomy does not need to be performed post-decellularization. It can also be performed pre-decellularization, which leads to some advantages and disadvantages, when we compare both approaches. On one hand, if the partial hepatectomy is performed first then the liver submitted for decellularization is smaller which helps save time and reagents in the process. On the other hand if the decellularization is performed first then all of the vasculature is visible and as such the partial hepatectomy is easier. Additionally, as the decellularization is done with an intact liver there is no risk diffusional issues that would result in poorly decellularized patches. This issues are related with the possible vascular damage the liver may suffer during decellularization (which is minimized with the post-decellularization approach) and with possible strangulation resulting from the sutures. This resistance to decellularization can easily be seen in figure 3 where it is clear how the tightness of the suture can greatly limit the reach of the detergents perfusion in the sutured area.
Additionally, a mixed approach was tried, in which the main vascular branch connecting the right lobe and the right central lobe was ligated without performing partial hepatectomy. The objective of this approach was reaping the advantages of performing a partial hepatectomy while leaving the vast majority of the liver untouched to prevent the disadvantages associated with suturing. The result reached with this approach is evidenced in Figure 4. In this picture, apart from the effect of the ligation in the decellularization pattern, the effect of immersion

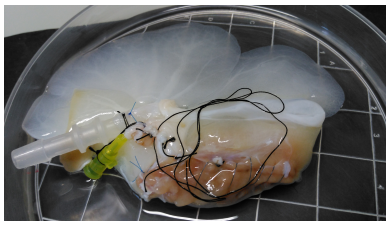

Figure 3: Decellularized liver scaffold which was partially hepatectomized prior to decellularization. For reference, the DLS is placed in a 15cm tissue culture plate.

is also observable in the edges of the non-decellularized liver lobes. This image makes it clear why a perfusion-based approach is needed in thick solid organs such as the liver and why one reliant only on diffusion would not cut it. Additionally, as this particular liver was poorly extracted, having the HA being cut too short for canullation, the decellularization was performed using the PV as the only inlet in the procedure. As the result was satisfactory it suggests that for decellularization there is no need for HA canullation and perfusion, which is advantageous as it allows simplification of both the procedure and design/construction of the automatic machine.
With that said, more experiments would need to be conducted to validate these results, and the structural integrity of these scaffolds would need to be tested to assess the viability of the methods.

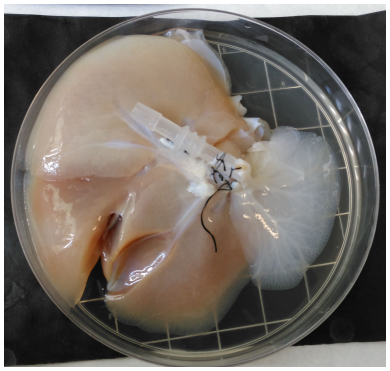

Figure 4: Decellularized liver scaffold which was ligated prior to decellularization. For reference, the DLS is placed in a 15cm tissue culture plate.

3.1.2 Decellularization

As a non-ionic detergent triton X-100 is preferred since it has been shown to be less damaging than SDS. When both were used sequentially, triton X-100 was used after so that it could help clean up the remaining SDS, which dissolves very poorly in water and as such is difficult to remove. To counteract the denaturing effect of the detergents in the ECM the ammonium hydroxide was added since it has been shown to help stabilize said structure.
In total, 21 decellularizations were performed, 10 with livers extracted from 2Kg piglets and 11 from 5Kg piglets.

The exact protocols varied in time, volumes perfused, pressures used and detergent compositions and concentrations.
From those 21, 5 yielded good decellularization results (Figure 5). These were characterized as good as they were transparent after the procedure. As these DLSs were later used for recellularization experiments[1], no H&E stains or other immunohistochemistry assays of well decellularized scaffolds were performed to verify the decellularization.
Even though the details on the protocol followed varied and not enough replicas were performed in any set of conditions to allow for conclusive results some general ideas can still be captured from the experiments performed:

1. As the first fully decellularized liver was decellularized with just seven liters of 1% triton X-100 and 0.1% ammonium hydroxide solution, it is definitely possible to decellularize without using more damaging detergents like SDS;
2. The results were better on a overall analysis when several conditions started being better controlled and stabilized. For instance, the usage of support structures (Figure 6) that prevent the movement of the liver during decellularization (such as that caused by flotation) aided in getting more reliable results;
3. Good bleeding is a fundamental step. Blood clots/cells can severely hinder the decellularization process;
4. If the liver has badly decellularized patches, gentle massage can improve the results by promoting redistribution and eliminating the preferential pathways that may have been formed;
5. The last experiment suggests that the best option in terms of liver preparation is the ligation of the blood vessels between the right and right central lobes as this process appeared to garner good results with the most simple protocol.

3.2 Recellularization

3.2.1 Cell seed expansion

It is worthy of being noted that in the biorreactor stage, the 100M mark for the hUVECs was never reached, since cells started to expand slower overall. This can probably be due to some degree of cell senescence as the samples were already in high passages (passage 10-11) when seeded for expansion. In the last two recellularization experiments it was found out that there was also some hMSC contamination in some of the hUVEC samples, which can also contribute for the disparity between the objective and the results as these cells are bigger and as such don't reach densities as high as the hUVECs do.

[1]The fifth one, was not recellularized as the PV was damaged during isolation. It was instead used for partial hepatectomy practice

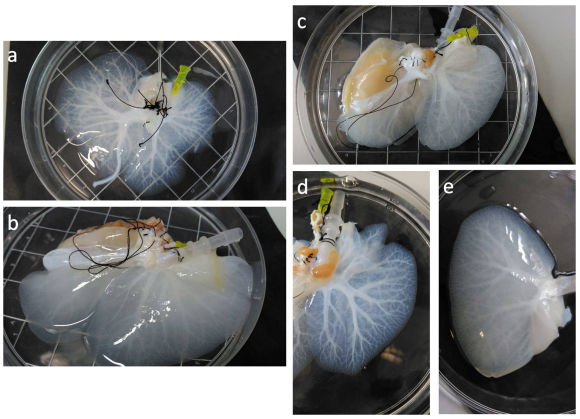

Figure 5: Livers which were considered well decellularized. (a) First well decellularized liver and the only one extracted from a 2Kg piglet. It was used for the first recellularization experiment having been the only full liver recellularized. (b) Liver which was partly hepatectomized prior to decellularization having the left and central left lobes removed. After decellularization the central right lobe was also removed, removing the badly decellularized part where it was sutured (Same liver as in Figure 3). (c) and (d) represent livers which were also partly hepatectomized prior to and post decellularization, the difference being that, these livers were not sutured for decellularization. (e) represents a liver that was only decellularized through the PV, with the circulation between the right lobe and the rest of the liver stopped by ligation of the main blood vessel (See Figure 4). For reference, all DLSs are placed in a 15cm tissue culture plate.

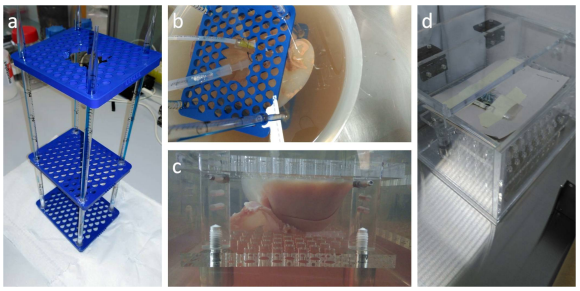

Figure 6: Liver support structures. (a) First structure made with the aim of supporting and immobilizing the liver during the decelularization procedure was made with four 5mL pipettes and three tops from 1mL micropipette tip boxes.

3.2.2 Recellularization

As the project is focused in vascular regeneration, the usage of hepatocytes and other important liver cells was also still unconsidered, being hUVECs and hMSCs the only seeded cell types.
In total, 4 recellularizations were performed. The first one, was done with a DLS obtained from a liver extracted from a 2Kg piglet while the others were done with DLSs obtained from livers extracted from 5Kg piglets. These DLSs correspond to those portrayed in Figure 5 (a), (b), (c) and (d).
For the first recellularization, the DLS portrayed in Figure 5 (a) was used. This was the only recellularization performed on a whole liver, as well as the only performed

on a DLS from a 2Kg piglet, being this the reason for the utilization of the whole liver. Additionally, it was also the only DLS that went through a 7-days protocol as it was used to practice and identify possible problems with said protocol.

Seeding was done in two days. On the first, GFP-labeled cells were seeded at high pressure, and on the second TOM-labeled ones were seeded at low pressure.

Due to mismanagement of the cell lines in the last days before seeding, high cell mortality was observed and as such, only 37.0 million hUVECs and 24.2 million hMSCs were seeded on the first day, while only 73.8 million hUVECs and 28.0 million hMSCs were seeded on the second day. The pressures programmed for the seedings were of 50mmHg and 25mmHg in the HA and PV, respectively, for the first seeding and 25mmHg and 10mmHg in the HA and PV, respectively, for the second seeding.

Between seedings, the bioreactor was set at a maintenance pressure of 4mmHg in both inlets, in order to promote the adhesion of the cells to the DLS by decreasing the shear stress to which they were submitted to. What effectively happened was that, at the end of the first day of seeding the HA canulla fell off and so the protocol was followed only for portal recellularization and the pump associated to the HA line was stopped.

At the end of the seedings, the system was left in the maintenance pressure of 4mmHg and manually increased over time at an approximate rate of 2mmHg per day until a pressure of 10mmHg was reached. This gradual increase is performed to avoid sudden drastic increases in shear stress, which can be harmful to the cells, DLS or may flush the cells out from the scaffold.

At day 5, the PV canulla also fell of leaving the liver suspended. Due to that, the experiment was temporarily stopped, and the liver recanullated. Afterwards the portal pressure was set at 10mmHg, as it was the value before the recanullation and the arterial pressure was initially set at 50mmHg, which proved unsuccessful as the pumping action varied wildly leading to the entry of some air into the scaffold. At this point the flow rate seemed to be unrelated to the inlet pressure[2], and due to that, a direct flow rate control was used to obtain a flow rate between 3 and 4 times smaller than that observed in the portal setup.

Medium samples were collected daily for LDH measurements, which were used as means of tracking cellular stress.

Microscopy observation of the H&E stains (Figure 8 (a), (b) and (c)) revealed that they were mostly empty with some populated areas with high cell density. The cells were in their majority MSCs, with only residual amounts of hUVECs. High cell mortality was observed, which suggests that the pressures used were too high.

From this first experiment, it was observable that pressures like the ones used are damaging to the cells, especially the hUVECs, which indicates that lower pressures should be used.

For the second, third and fourth recellularizations, the DLS portrayed in Figure 5 (c) (b) and (d) were used, respectively. As mentioned before, the protocol followed for these recellularizations was a 24h protocol, instead of the 7-day one used for the first DLS.

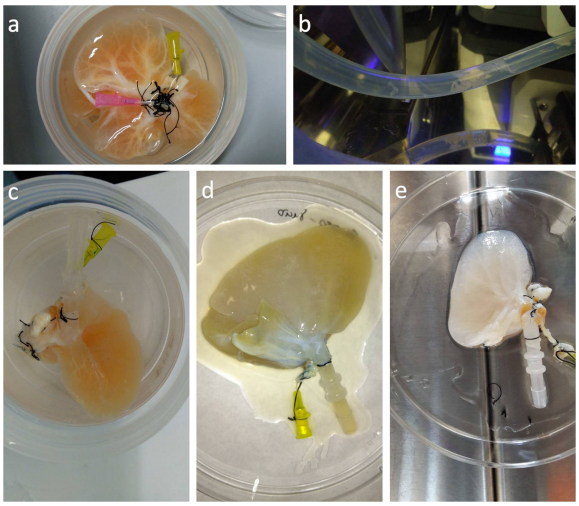

Figure 7: Recellularization results. (a) First recellularized DLS. (b) Floating structures found in bioreactors 2 and 3. (c) Second recellularized DLS. (d) Third recellularized DLS. (e) Fourth recellularized DLS.

For the second recellularization, the planned conditions were 16mmHg and 30mmHg, for the PV and HA respectively in the first seeding and 8mmHg and 15mmHg, for the PV and HA respectively in the second seeding. For maintenance, a constant pressure of 4mmHg was selected. When starting the first seeding, it was noticed that so as to maintain the pressures planned the correspondent flow rates were very high and possibly damaging to the cells in the case of the PV(256ml/min at 8mL/min) and very low in the case of the HA (0-0.04 ml/min at 50mmHg). Due to this, the actual pressures used were adjusted to 4mmHg and 50mmHg, for the PV and HA respectively in the first seeding and 2mmHg and 25mmHg, for the PV and HA respectively in the second seeding and for maintenance pressure. Even so, a flow rate of 180mL/min was obtained with the 4mmHg in the PV.

Seeding was done in two days. On the first, GFP-labeled cells were seeded at high pressure, and on the second TOM-labeled ones were seeded at low pressure. 50.0 million hUVECs and 36.4 million hMSCs were seeded on the first day, while only 39.8 million hUVECs and 23.3 million hMSCs were seeded on the second day.

Contamination of the hUVEC tissue culture plates by hMSCs was observed, which can partially justify the low cell

[2]Over a 42 hour interval, the pressure varied over 30mmHg (from 14mmHg to 52,7mmHg) in a constant flow setup (2mL/min), being that when the pumps were stopped the pressure did not decrease to 0. Additionally, after recanullation, the initial pressure measured was of -15.7mmHg for a 3.1 mL/min flow rate.

numbers seeded, as these cells are considerably larger than hUVECs and as such, occupy more space leading to lower cell numbers when confluence is reached.

After the second seeding, and stopping the pumps for 4 hours to promote attachment, the bioreactor was left to run at maintenance conditions for 24h before retrieving and fixating the liver for staining. Just before retrieving the liver, at the 24 hour mark, a medium sample was collected for LDH analysis.

Additionally to the scaffold, some floating structures which appeared to be either cell aggregates or DLS fragments (Figure 7 b).

When observed under the microscope after H&E staining (Figure 8 (d), (e) and (f)), the DLS was mostly empty yet dotted with some high density populated areas, as was observed in the first bioreactor. Lower cell mortality was observed when compared with the first bioreactor, but still the cell mortality was high enough to suggest that in the third experiment the pressures should be decreased even further. It is worth noting that all the differences in results seen between the first and second recellularizations can be explained by the fact that the protocols differed in length of time being the second one a shorter experiment which would inevitably lead to less expansion after adhesion in the scaffold.

For the third recellularization, the planned conditions were 8mmHg and 50mmHg, for the PV and HA respectively in the first seeding and 4mmHg and 25mmHg, for the PV and HA respectively in the second seeding. For maintenance, a constant pressure of 2mmHg in the PV and 10mmHg in the HA was selected.

In the first seeding, 76.3 million hUVECs and 26.3 million hMSCs were seeded. The second seeding was not done since when it was to be performed the bioreactor was noted to be contaminated with bacteria. Instead, the liver was fixated with formaldehyde skipping the remainder of the experiment entirely.

When observed under the microscope, the DLS was filled with bacteria which appeared to be from the *bacillus* genus. Apart from that, minor amounts of hUVECs and hMSC could also be seen, even though most of them appeared to be dead (Figure 8 (g), (h) and (i)).

For the fourth recellularization, the planned conditions were 8mmHg and 50mmHg, for the PV and HA respectively in the first seeding and 4mmHg and 25mmHg, for the PV and HA respectively in the second seeding. For maintenance, a constant pressure of 2mmHg in the PV and 8mmHg in the HA was selected.

In the first seeding, 70.5 million hUVECs and 20.4 million hMSCs were seeded. After the last seeding the pumps were stopped as per protocol for 4 hours. When they were going to be turned on, it was noticed that even though the pumps were stopped, the pressures registered in the sensors, were of 5.2mmHg and 14.8mmHg in the PV and HA respectively, instead of the expected 0mmHg. since these are higher than the maintenance conditions, to ensure that the cells would not be without perfusion overnight, fixed flows of 4mL/min and 1mL/min were set for the PV and HA respectively. In the next morning, due to the fixed flow conditions, the pressures observed were of 15mmHg and 100mmHg. So as to try to lower these pressures, the medium flow was changed so that it passed for a few minutes by the lower bottle, which is connected to the atmosphere by a filter. Even though this did lower the pressures, these rapidly rose when the original pumping path was restored, stabilizing at 10mmHg and 95mmHg. Using the same line of thought as before, the second seeding was also performed using fixed flow rates and not fixed pressures.

The flow rates used were 8mL/min and 2mL/min for the PV and HA respectively, and 43.6 million hUVECs and 17.2 million hMSCs were seeded.

At seeding the pressures registered were 13.8mmHg and 78.5mmHg, at the last seeding before stopping the pumps the pressures were 14.5mmHg and 82.6mmHg.

After 24 hours, medium was recovered for LDH testing and the scaffold was fixated in formaldehyde and sent for H&E staining.

Like what happened in the second bioreactor experiment, some floating structures were found and these too were sent for staining.

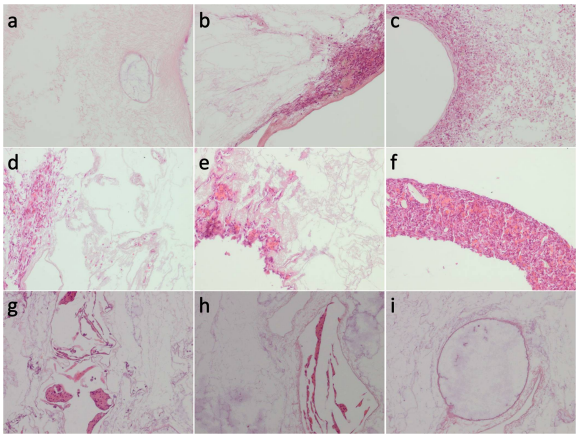

Figure 8: H&E stains of the recellularized scaffolds. (a) Staining of the hilum of the first recellularized DLS . (b) Staining of the left lobe of the first recellularized DLS. (c) Staining of the central right lobe of the first recellularized DLS. (d) Staining of the hilum of the second recellularized DLS. (e) Staining of the left lobe of the first recellularized DLS. (f) Staining of the fragments observed on the second experiment. (g) Staining of the right lobe of the third recellularized DLS. (h) Staining of the right lobe of the third recellularized DLS. (i) Staining of the right lobe of the third recellularized DLS.

One further detail worth mentioning that could improve the results is the method of calculation used for the seedings. As of these experiments, the weight of the livers was not taken into account, which is an easy alteration that could lead to more reliable results than those obtained.

4 CONCLUSIONS

In conclusion, even though the data collected is not nearly enough to make any statements, there are several leads that can be taken from the experiments performed.
In terms of the decellularization of porcine livers, a 1% triton X-100 and 0.1% ammonium hydroxide solution seems enough to decellularize a liver extracted from a 2Kg piglet, which suggests that the use of strong detergent solutions is not needed. Also, the stabilization of the liver seems to be important for the process as well.
Even though 21 livers were decellularized, only 5 had good results. Nevertheless, the degree of decellularization improved over time, as the process control and stabilization improved.
So as to further improve the decellularization protocol as well as amplify the reproducibility of the results, another project stemmed from this one, in which the construction of an automatic decellularization machine was the aim. Regarding that project, no conclusions are made as it is not advanced enough to do so.
Regarding the recellularization of the DLSs, no major conclusions can be made but the results obtained suggest that the pressures used were to high and could cause cell damage. Since there were some issues pertaining to how the flow rates developed and how different they were from liver to liver even in similar conditions, further
One of the major shortcomings in this project was the lack of replicates, which is something that will necessarily be dealt with as the project continues and more results are collected.
Summing up, even though the results obtained were lacking in number and no conclusions can be extracted from them, they give us an ample room to adjust the protocols used, and to better understand the processes of decellularization and recellularization. It is also important to mention that as part of a larger project, the main objective of this thesis was building enough knowledge that could be used as a stepping stone for the continuation of the project.

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