Title: Development and characterisation of a Porcine Liver Scaffold. ~~for Clinical Application~~

Running title: A tissue engineered liver scaffold

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Key words: porcine liver, acellular scaffold, perfusion, in vivo implantation, mesenchymal stem cells

Abstract

The growing number of patients requiring liver transplantation for chronic liver disease cannot be currently met due to a shortage in donor tissue. As such, alternative tissue engineering approaches combining the use of acellular biological scaffolds and different cell populations (hepatic or progenitor) are being explored to augment the demand for functional organs. Our goal was to produce a clinically relevant sized scaffold from a sustainable source within 24 hours, whilst preserving the extra cellular matrix (ECM) to facilitate cell repopulation at a later stage. Whole porcine livers underwent perfusion de-cellularisation via the hepatic artery and hepatic portal vein using a combination of saponin, sodium deoxycholate (SOC) and deionised water washes resulting in an acellular scaffold with an intact vasculature and preserved ECM. Molecular and immuno-histochemical analysis (collagen I and IV and laminin) showed complete removal of any DNA material, together with excellent retention of glycosaminoglycans and collagen. FTIR analysis showed both absence of nuclear material and removal of any detergent residue, which was successfully achieved after additional ethanol gradient washes. Samples of the de-cellularised scaffold were assessed for cytotoxicity by seeding with porcine adipose derived mesenchymal stem cells in vitro, these cells over a 10 day period showed attachment and proliferation. Perfusion of the vascular tree with contrast media followed by CT imaging showed an intact vascular network. In vivo implantation of whole intact non-seeded livers, into a porcine model (as auxiliary graft) showed uniform perfusion macroscopically and histologically. Using this method, it is possible to create an acellular, clinically sized, liver scaffold with intact vasculature in less than 24 hours.

Introduction

Liver disease presenting as fibrosis, cirrhosis and end stage liver failure is a worldwide health problem affecting 50 million people and is becoming an epidemic[1]. In Europe, 29 million people suffer from chronic liver disease resulting in 47,000 deaths per year [2], whilst in the US, 5 million people live with chronic liver disease. By 2020 cirrhosis will be the 12th leading cause of death [3]. Notwithstanding the financial cost, surgery related complications and the lifelong dependence on immunosuppressive drugs, the current standard of care for cirrhosis and end stage liver failure is still a liver transplantation. Worldwide, approximately 20,000 liver transplants have been carried out, between 1968 and 2009, 13,984 transplants were performed in the UK alone [2]. Europe as a whole reported more than 5,500 liver transplants per year [4]. The number of transplants performed has steadily increased due to improved survival rates (1 year survival is approximately 83% with all factors considered). However, the greatest limitation to providing this life saving procedure is a shortage in donor tissue and it is becoming increasingly clear that this demand cannot be met by tissue/organ donation alone [5].

In order to meet this demand, alternative solutions are required and innovative attempts to address this shortage have focused on cell therapy approaches such as haematopoietic and mesenchymal stem cell and human primary hepatocyte transplantations. Although the cell therapy approach has shown promising results in both pre-clinical and clinical settings, limitations to wide scale usage include access to liver tissue from which to harvest good quality cells and associated donor site morbidity [6]. In parallel, considerable work has been reported on the development of bespoke tissue engineered livers using an acellular matrix configured to resemble liver tissue and populated with cells [7]. To date, the most frequently reported matrix has been obtained using existing liver tissue made acellular using a process of de-cellularisation. Alternative matrices using 3D printing approach [8] or polymeric scaffolds have also been reported but it is currently difficult to create a liver matrix with the exact micro-architectural structure of the liver encompassing the vascular network, the biliary tree and the appropriate molecular cues with which to attract and retain cells to its surface . Biologically derived tissue matrices have a significant advantage over synthetic or 3D printed hydrogels [9], since the starting

material not only resembles the organ being “engineered” but the extra cellular matrix (ECM) has the ability to retain on its surface its molecular fingerprint.

To date different approaches have been developed for creating acellular liver matrices from different species e.g. rodents [10] [11], porcine [12-14], sheep [15] and human [16,17] using various combinations of chemicals, enzymes and physical forces. Detergents and enzymes are known to have a negative impact on the ECM of biological tissue; this damage can be mitigated by limiting the duration and exposure [18]. Additionally, efficient delivery of detergents and enzymes using existing vascular circulatory systems in larger species also helps to reduce the time and potentially provide a more uniform distribution to the deeper cellular compartment [13,15].

Several groups have studied the pig liver as a potential source for de-cellularisation and repopulation in view of the availability of pig liver and the widespread use of porcine tissues in current clinical practise. Previous studies reporting the production of acellular porcine livers using a de-cellularisation approach, have used freeze/thaw cycles followed by Triton X-100 alone, in combination with sodium dodecyl sulfate (SDS), or a combination of SDS and EDTA [19]. The time taken to produce the acellular matrix varied between 11 days and under 24 hours (this was achieved using oscillating pressure conditions [20]). All reported protocols have resulted in acellular liver tissue matrices with significant reduction in DNA content when compared to normal liver tissue. Additionally, the majority of the de-cellularised tissue was capable of supporting cells, either when engrafted or in vitro, indicating preserved ECM. Retention of the proteins within the ECM is vital for providing a platform for subsequent seeding of the organ. The ECM plays a number of roles with respect to cellular activity [21], both positive (i.e. cellular differentiation, neovascularisation, hepatocyte growth and proliferation) and negative (i.e. fibrosis and cancer progression) [22]. Consequently, the ECM can no longer be viewed as an inert or barren landscape but one which plays an active and dynamic role in the biological function of the liver as a whole. As such, the process by which liver tissue is rendered acellular is crucial and damage to the ECM should be kept to a minimum.

Different cells types have been used for re-cellularisation of de-cellularised liver matrices, including iPSC induced hepatocytes [7], human primary hepatocytes [23] and mesenchymal stromal cells. The seeding of the latter onto a liver scaffold supported hepatic differentiation and the ability to restore liver function in a severe liver failure model [11]. As such, the use of mesenchymal stromal cells as a potential cell source may be more favourable as they do not carry the potential tumorigenic risk associated with iPSC's.

This study reports the development of a vascular perfusion based de-cellularisation protocol capable of producing a whole acellular porcine liver in less than 24 hours using sodium deoxyycholate (SOC) as opposed to the more commonly used sodium dodecly sulfate (SDS). The resultant scaffold was assessed for vascular integrity by engrafting the entire non seeded liver scaffold into an in vivo porcine model to determine its ability to withstand physiological perfusion pressures. Additionally, the presence of detergent residue as a potential cytotoxic residue was assessed by evaluating the de-cellularised scaffolds ability to support porcine adipose derived mesenchymal stem cells in vitro, since there are concerns that detergents remaining on the ECM matrix may be detrimental to the viability and long term differentiation potential of seeded cells. [10]

Material and methods

Regulatory guidelines: This study was carried out following Ethical permission from NPIMR Ethical Review Committee for animal experimentation and in accordance with the UK Animal (Scientific Procedures) Act 1986, which conforms to the European Convention for the protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, Council of Europe).

All chemicals and reagents were purchased from Sigma-Aldrich Ltd UK unless otherwise stated

Organ Retrieval

Female Large white/Landrace cross bred pigs (n=10) approximately 35-40kg underwent liver harvest under terminal anaesthesia. On the day of surgery each pig was pre-medicated with ketamine (5mg/kg Zoetis)/xylazine (1mg/kg) intramuscularly (Bayer UK both obtained from The National Veterinary service, Stroke on Trent UK). General anaesthesia (GA) was induced with isoflurane over oxygen and nitrous oxide. After intubation, anaesthesia was maintained with isoflurane. A mid line incision was made from the sternum to pubis and a lateral incision from the midline under the bottom left hand rib. The skin was reflected back to provide access to the bowel which was placed in a sterile bowel bag containing a moist swab for ease of access. The liver was then mobilised from the surrounding tissue until only the hepatic portal vein and hepatic artery remained as a vascular connection. The liver was freed from the diaphragm. Each animal then received 20KIU of heparin. Both the hepatic portal vein (HPV) and hepatic artery (HA) were cannulated, the thoracic aorta was clamped and the inferior and superior vena cava incised and perfusion was initiated through both the hepatic portal vein and hepatic artery simultaneously.

Scaffold production:

Whilst still in situ, the liver was de-cellularised by vascular perfusion using a Watson Marlow peristaltic perfusion pump at an RPM of 30. Soltran and heparin 20KIU (2 litres) were simultaneously perfused through HPV and HA followed by 0.25% saponin in warm saline, (2 litres) and then warm de-ionised water (2 litres). An additional soltran flush (2 litres) was repeated followed by a series of washes with

deionised water (2 litres), 0.2% SOC in warm saline (2 litres) and finally deionised water (2 litres). At this stage the liver was a pale colour and was removed from the animal and placed into an enclosed container and the de-cellularisation process continued on the bench using the same perfusion system (40rpm for the remaining protocol). The protocol was continued with a saline and heparin 5KIU flush (1litre) followed by 0.2% SOC in PBS (5 litres) and saline heparin 5KIU (1 litre). The liver was then flushed with 0.2% Tween in deionised water (15 litres) and 0.2% SOC in PBS (5 litres). This cycle was repeated three times and then followed by 0.1% SOC (2 litres). The final set of washes consisted of water only (15 litres). The entire protocol was completed within 24 hours. Following de-cellularisation the livers were either flushed with increasing concentration of ethanol (20%, 40%, 50% and 70%), with each concentration cycle lasting 30 minutes or stored in PBS in preparation for the FTIR analysis.

Sterilisation and residue assessment

To assess sterility, swabs were collected from the surface of the de-cellularised liver as well as deep to the surface through an incision and plated onto nutrient agar plates for bacterial and sabouraud dextrose for fungal presences. Plated were incubated at 37^{o}C for 48 hours. The plates were examined at 12 hours, 24 and 48 hours for any fungal or bacterial growth.

In order to detect any detergent residue within the de-cellularised tissue, FTIR analysis was undertaken in control tissue (non de-cellularised n=5) and de-cellularised tissue washed in in ethanol (n=5) or PBS (n=5). For FTIR point measurements, the measurement site was approximately 1mm x 1mm and 30 scans were co-added for each point. Scans were performed between 550 and 4000 cm^{-1} at a resolution of 4 cm^{-1} and 20 points were scanned for each sample (10 on each side of the sample). Each spectrum was subjected to an automatic baseline correction, normalised to the highest, most stable and reproduced peak and the spectra average determined (Omnic Spectra Software Nicolet iS50 Edition, ThermoFisher Scientific, UK).

Scaffold characterisation

Histology: Following de-cellularisation, up to 5 representative full thickness samples were taken from each lobe of the liver, fixed in 10% neutral buffered formal saline (NBF) and subsequently processed for routine wax embedding. Five micron paraffin sections were cut and stained with haematoxylin and eosin (H&E), Picro-sirius red combined with Miller elastin stain (PSR-ME) and a reticulin stain.

Immuno-histochemistry: The following antibodies were used to assess the basement membrane on paraffin sections: Collagen I (Abcam ab6308 mouse mAb, antigen retrieval: citrate buffer 90^{o}C for 20mins, incubation 1:200 for 1 hour), Collagen IV (Abcam ab6586 rabbit pAb, antigen retrieval: trypsin digestion at 37^{o}C for 30mins, incubation 1:100 for 2 h and Laminin (Abcam ab11575, rabbit pAb, antigen retrieval: trypsin digestion at 37^{o}C for 30mins, Incubation 1:100 for 2 hours). All washes were performed using PBS and the secondary antibody was either Impress anti-mouse or Impress anti-rabbit immunoglobulin IgG peroxidase kit, (Vector Laboratories, Peterborough, UK) for 30 mins at RT, the chromogenic substrate diaminobenzidine (Impact peroxidase substrate, Vector Laboratories, Peterborough, UK) was applied for 3 mins at RT. On completion sections were counterstained using Harris' haematoxylin.

DNA quantification and gel electrophoresis: The GenElute mammalian genomic DNA miniprep kit (Sigma-Aldrich, UK) was used for DNA extraction and quantification following the manufacturer's instructions. Total DNA was quantified in all samples by measuring the absorbance using the nano-drop spectrophotometer (NanoDrop ND1000, Thermo Scientific, Wilmington, USA), from this the absolute amount of DNA per milligram of tissue was calculated. The size, quality and purity of the extracted DNA were determined by agarose gel electrophoresis. A 1.2% agarose (Agarose Type I, low EEO; Sigma-Aldrich, Dorset, UK) gel with 1x Tris-borate-ethylenediaminetetraacetic acid (TBE - Bio Reagent, 10x, Sigma-Aldrich, UK) running buffer was run at 4 to 5 V/cm between the electrodes. Equal volumes of DNA (5µl) and 1µL of loading buffer (5x DNA loading buffer, Yorkshire Bioscience Ltd., York, UK) were loaded into each well. Visualization was achieved by staining with 1% ethidium bromide and DNA was measured via ultraviolet transillumination against a 1-kb DNA ladder (Q-Step 4 quantitative DNA ladder, Yorkshire Bioscience Ltd., York, UK).

Analysis of extra-cellular matrix components

Glycosaminoglycan (GAG) quantification: To quantify the GAG content for both control and de-cellularised liver samples, the Blyscan GAG assay kit (Biocolor, Carrickfergus, Northern Ireland) was used. In brief, 50mg of finely minced wet tissue was placed in a micro-centrifuge tube and incubated with 1mL of papain digestion buffer at 65°C for 18 h. Aliquots of each sample were mixed with 1,9-dimethyl-methylene blue (DMMB) dye and reagents from the GAG assay kit. 200µL of each sample was added in triplicate into a 96-well plate. The absorbance was measured using a plate reader (Versamax, Molecular Devices LLC, USA) at 656nm and the absolute GAG content was calculated per milligram of tissue.

Collagen extraction. Collagen was extracted from the control and de-cellularised samples according to published protocol [24] Briefly, samples were digested using a solution of guanidine hydrochloride buffer (4M) (Sigma-Aldrich, Poole, Dorset., UK) with 12µL of protease inhibitors (Sigma-Aldrich, Poole, Dorset, UK) left for 18 h at 4°C with agitation. Samples were then centrifuged for 10 min at 12,000g to separate tissue debris. Supernatant was transferred to a micro-centrifuge tube and nuclear material was digested by using RNAse enzyme (Sigma-Aldrich, Dorset, UK; activity of 140EU/mL) for 2 min at RT, followed by DNAse I (Sigma-Aldrich, Dorset, UK; activity of 100 EU/mL). Samples were agitated without vortexing and incubated at 37°C for 1 h, and then dialyzed against distilled water overnight at 4°C. After dialysis the supernatant was transferred to an amicon ultra filter 2 (Ultra-4 3kDa Ultracel-PL memb, Merk Millipore, Bilerica, MA, USA) to remove the hydrolysed DNA and salt, and then centrifuged for 30 min at 4,000g. For protein isolation, the centrifuged dialysate was added to 50µL of Tris base (pH 9.5) with ice-cold phenol-chloroform-isoamyl alcohol (PCIA, Sigma- Aldrich, Dorset, UK) and allowed to stand for 24h at 4°C, followed by centrifugation for 10 min at 12,000g. The upper layer (containing DNA/RNA) and the lower organic phase (containing solvents) were individually retrieved by pipetting. The interface containing proteins was carefully removed and transferred to another micro-centrifuge tube. Protein was precipitated using ice-cold ethanol and centrifuged for 10

min at 15,000g at 4°C. The resultant pellet was allowed to dry and solubilized with 100 µL of ultrapure acidic water (pH=3 with acetic acid).

Collagen quantification: Collagen was quantified using Sircol collagen assay kit (Biocolor, Carrickfergus, Northern Ireland). Briefly, 90 µL of each sample was subjected to sirius red dye binding, followed by washing with ice-cold acid-salt reagent to remove unbound dye. Samples were centrifuged, and dye bound to collagen was released from the pellet with alkali reagent (1 N NaOH). Extracts were placed in a 96-well plate in triplicates and spectrophotometric readings were taken at 555nm on a microplate reader (Versamax, Molecular Devices LLC, USA). Absolute values were attained with a standard curve composed of type I bovine skin collagen solution (0.5mg/mL) in the range of 5-100µg per 0.1mL. Total collagen was normalized per milligram of tissue.

In vitro cytocompatibility assessment using adipose derived mesenchymal stem cells

Cells isolation and culture: Adipose tissue was collected from Large White/Landrace crossbred pigs. Under anaesthesia and using aseptic techniques, (the abdomen was cleaned using 10% Iodinated iodine followed by sterile saline (Baxter 0.9% NaCl)) a 3cm-transverse cutaneous incision was made. Approximately 5g of adipose tissue was harvested from beneath the dermis, cut into small pieces and prepared for digestion. Collagenase 0.1% in PBS was added to the tissue and allowed to digest for up to 2 hours at 37^{o}C with agitation (50rpm). The stromal-vascular fraction (SFV) was separated from the remaining fibrous material and the floating adipocytes separated by centrifugation at 300 g for 10min. The sedimented SVF-cells were filtered through a 100µm pore filter followed by an incubation step in an erythrocyte lysing buffer (160mM NH4Cl) for 10 min. For initial cell culture and expansion of the AD-MSC, low-glucose DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100Ug/mL streptomycin was used. Cultures were washed with phosphate-buffered saline (PBS) 24 h after plating to remove unattached cells and then fed with fresh medium. Cultures were maintained at 37^{o}C with 5% CO2 and medium was changed 3 times per week. Cells were grown to confluence after the initial plating (P=0), typically within 7 days. Once confluent 80%, the adherent

cells were released with 0.5% trypsin–EDTA and then either plated at $25x10^3$ per cm^2 or used for experimental analysis. All cells used for analysis were early passage (passages 2 to 4).

In vitro cyto-compatibility assessment

Prior to the *in vitro* assay, liver scaffolds were prepared as described above. Briefly, de-cellularised samples were cut into 1x1x0.2cm samples and individual pieces placed into a 24-well plate and incubated in growth medium (DMEM with 10% FCS and 1% AA) overnight at 37°C including 5% CO_2. AD-MSC were released from the culture flasks using a non-enzymatic solution, counted and seeded onto each de-cellularised liver sample at 2.5 x 10^5 cells per cm^2 in triplicate. Cells were left undisturbed to adhere for 2 h and then fresh growth medium was added into each well. The medium was changed every other day. After 1, 2, 4, 7 and 10 days of culture, the liver scaffolds were collected, gently washed with PBS, fixed in NBF 10% for 24h and processed for histological analysis.

In Vivo assessment

Vascular integrity by contrast perfusion and CT Imaging: In order to assess the integrity of the vasculature following de-cellularisation, a single de-cellularised liver was perfused with contrast media (ex vivo) (Omnipaque GE HealthCare, Uk) initially through the hepatic artery and then through the hepatic portal vein and imaged (GE Healthcare Innova 4100) as an assessment for leakages and or blockages.

In- vivo implantation: Two intact de-cellularised livers were implanted into two separate pigs under terminal anaesthesia (i.e. non –recovery) following left sided nephrectomy to evaluate 1) whether the de-cellularised liver would be re-perfused under physiological conditions 2) the technical aspects of vascular reconstruction and 3) uniformity and pattern of the perfusion. This procedure was undertaken without clamping or removal of the hosts native liver.

Procedure 1: The de-cellularised liver was primed with 5000IU of heparin in 1 litre of RT saline (Baxter) through the HP and HPV. A left sided nephrectomy was performed in the host animal and

hepatic artery of the de-cellularised liver connected to the recipient left renal artery, the de-cellularised inferior vena cava to the host left renal vein and the de-cellularised hepatic portal vein to the host hepatic portal vein. The de-cellularised superior vena cava was tied closed. The liver was allowed to perfuse under constant monitoring for approx. 90 minutes

Procedure *2:* The de-cellularised liver was primed using 2 x 5000IU of heparin in 500 ml of RT saline. Implantation of the de-cellularised liver was via direct anastomosis as described as above. The de-cellularised inferior vena cava was sutured to the renal vein, the de-cellularised hepatic portal vein to the host portal vein and the de-cellularised hepatic artery to the host renal artery. The liver was allowed to perfuse under constant monitoring for approx. 70 minutes

For each animal, vital signs including blood pressure, heart rate and oxygen saturation were monitored. Following termination, the implanted was liver removed, samples taken from each lobe and prepared for histological evaluation as previously described.

Results

Scaffold characterisation:

Following de-cellularisation, the entire liver tissue mass turned a pale golden colour; the gallbladder became white, (indicating sufficient perfusion through the HPV and HPA), as well as all the blood vessels (HPV and HPA) associated with the liver. Histological assessment of H&E stained sections showed an intact hepatic lobular structure together with all the main features of the portal triad (bile ducts hepatic portal vein and artery) could be easily identified. All structures showed complete absence of any nuclear material or cellular structures. The absence of cellular and nuclear material was also confirmed by DNA molecular analysis, which showed a 70% reduction in DNA content of the de-cellularised tissue compared to native liver tissue (control 634.4ng/mg ±14.6 ng/mg vs De-cell 3.36ng/mg ± 7.66ng/mg) and no visible DNA bands were observed by gel electrophoresis (Figure 1).

Following de-cellularisation, microbiology confirmed sterility of the tissue for both bacterial and fungal contamination over a period of 10 days (data not shown).

FTIR spectra for the three groups, control (i.e non de-cellelurised), de-cellularised (washed in PBS), and de-cellularised (Ethanol wash) are shown in Figure 2. Regions of the spectra associated with proteins, lipids and/or polysaccharides and nucleic acids were identified and key peaks numbered 1-10. No differences were observed between groups for peak 1 (3100-3600 cm^{-1}) relating to proteins, water and alcohols, peak 4 (1600-1700cm^{-1}) identifying amide I and peak 5 (1500-1600 cm^{-1}) associated with amide II bands of protein in the three groups suggesting preserved ECM integrity. However, peak 2 (2800-3000 cm^{-1}, -CH stretching) and peak 6 (1300-1500 cm^{-1}, -CH bending) associated with lipids and or polysaccharides showed significantly stronger transmittance in the de-cellularised-PBS wash tissue than in both the control and de-cellularised-ethanol samples showing the same transmittance. This difference may be attributed to residual deoxycholine retained from the de-cellularisation process within the de-cellularised PBS washed samples. Strong peaks observed at 2800-3000 cm^{-1} and 1700-1725 cm^{-1} are characteristic of deoxycholate and lipids. The intensity of peak 3 (1700 cm^{-1}, associated with Carbonyl and lipid groups) was reduced in both de-cellularised samples but substantially more in the

de-cellularised PBS wash; an ethanol wash did not restore the levels to that seen in control tissue. This suggests potential ECM alteration by the de-cellularisation process as characterised by increased carbonyl groups in the liver ECM. Crucially however, peaks (800-1200 cm^{-1}) associated with deoxyribose stretching and symmetric and asymmetric PO_2^- groups was stronger in the control sample (peak 9, 1026 cm^{-1} and peak 10, 1078 cm^{-1}) than in both the de-cellularised samples where it had shifted to weaker peak at 1065 cm^{-1}, with the de-cellularised-PBS-Ethanol sample being lower of the two. It appears that, a final wash with ethanol removed residual detergent and lipids whilst also removing nucleic materials without any detrimental impact on the ECM as demonstrated by the restoration of the FTIR spectral profile after ethanol wash to closely match that of the control (non de-cellularised).

As the functionality of the ECM of the de-cellularised liver will be vital for cell repopulation, when implanted at a later stage, detailed analysis was undertaken. PSR-ME stained sections clearly showed intact boundaries of the hepatic lobes (i.e. interlobular septa); whilst at a higher magnification intact elastin within each blood vessel wall could also be seen. When viewed under polarised light, the collagen structure showed excellent birefringence with the collagen appearing as bright orange/red indicating potential for functionality (Figure 3 A-C). Reticulin fibres were also well preserved, illustrating preservation of the fine fibrillary structure of the stroma within the connective tissue, this collectively demonstrated retention of key ECM components (Figure 3D). Detailed IHC staining of the sections showed the appropriate location and presence of laminin (outlining the basement membrane of the blood vessels Figure E-F), collagen 1 (liver capsule and portal stroma Figure G-H) and collagen IV (basal lamina of blood vessels and bile duct, Figure 3I-J) within the stroma of the de-cellularised tissue. Both the molecular collagen (control: 141.4µg/mg±18 µg/mg vs de-cellularised: 104µg/mg± 22.44µg/mg; 26% decrease $p < 0.05$) and GAG data (control:0.12 µgsGAG/mg±0.03 *vs* de-cellularised: 0.08 µgsGAG/mg ±0.007, $p<0.05$ representing a 28% decrease in the de-cellularised tissue) were in accordance with the morphology data (Figure 3 K-L).

***In vitro* analysis:**

Samples of sterile de-cellularised liver matrix were used to confirm cyto-compatibility of the tissue using porcine AD-MSC. AD-MSC were seeded onto the parenchymal (Figure 4 A-E) and serosal (Figure F-J) surface individually over a 10 day period. Cells readily attached to both surfaces and showed good morphology with little cell death accompanied by cellular migration into the tissue. Twenty four hours post–seeding, cells were easily identifiable on both surfaces, by day 2, both surfaces showed cells with elongated morphology. By day 4, cells seeded onto the cut parenchymal surface had multiplied and were seen to migrate into the tissue whilst those on the serosal surface were beginning to a form a multi-layered feature consisting of elongated viable cells. By day 10 both surfaces showed cells in various stages of differentiated morphology based on appearance.

Liver Perfusion analysis: in order to determine whether an intact vasculature had been retained following exposure to the detergents as part of the de-cellularisation process, a single liver was perfused with contrast media and CT imaged initially through the hepatic portal vein (Figure 5 A-B) and then the hepatic artery (Figure 5C-D). In both cases the venous and arterial vasculature was uniformly perfused to the peripheral edges of the liver including all the smaller capillary vessels. No leakage was seen indicating that the blood vessels were intact throughout the liver.

***In Vivo* Analysis:**

Following priming de-cellularised liver scaffolds were implanted into two animals in non-recovery procedures (Figure 6A-B). Perfusion of hosts blood into the liver scaffold occurred slowly but over time resulted in the entire liver scaffold taking on the characteristic colour and consistency of a normal liver. However, darker patches of potentially pooled or coagulating blood became apparent macroscopically. The first animal was monitored for 90 minutes but was terminated due to circulatory failure, accompanied by a loss of blood pressure. For the second animal, the liver scaffold was primed with a lower volume of saline (500ml versus 1 litre) This animal was monitored for 70 minutes and terminated due to marked elevation in heart rate (>240bpm). Although each animal was given Hartmann fluid throughout the procedure no additional blood was administered, the increased heart rate was probably due to large volume of blood being shunted into the implanted liver scaffold.

Following termination of each animal, the liver scaffold was removed and macroscopic inspection showed complete perfusion of the liver and some change in the colour of the gall bladder (Figure 6C-D). Each lobe was truncated and showed perfusion deep into the liver tissue but accompanied by some dark patches. Analysis of H&E stained sections showed engorged blood vessels but also occasional extravasation of red blood cells into the tissue matrix (Figure 6 E-H). Histologically the tissue was not uniformly perfused with some areas showing accumulation of blood and other areas with little to no presence of red blood cells. Numerous nucleated white cells could be seen within the red blood cell population (Figure 6G)

Discussion

This study was undertaken to develop a biologically derived liver scaffold applicable for human clinical application. Porcine livers were chosen as a starting material due to availability, clinical size, ethical acceptability and to avoid any competition with human liver tissue destined for transplantation. Our approach demonstrated the production of a whole porcine acellular 3D scaffold suitable for future clinical translation in approximately 18 hours which is less than the previously reported 24 hours used for human livers [25]. Our scaffold had a preserved vascular circulatory system, was capable of supporting adipose derived mesenchymal stem cell growth and migration, was capable of withstanding normal physiological perfusion pressures when implanted *in vivo* and was perfused uniformly by the host's blood. The overall protocol is very effective with respect to time and cost and has the potential to be automated and translated to eventual clinical usage. An advantage in using porcine livers to create liver scaffolds for future human clinical implantation, is the readily available supply of healthy tissue from food chain animals. This source of material limits and conflict with the requirement for human livers which can be retrieved for transplantation. However, future studies would need to address any safety concerns around zoonosis; specifically, viral infections and issues surrounding potential alpha - GAL epitope.

Both the histological and molecular data demonstrated removal of all nuclear material leaving behind an intact ECM composed of collagen, elastin and GAG's. Crucially to confirm that the entire liver had been de-cellularised, representative histological samples were taken from each liver lobe (approx. 5 per lobe) and analysed individually. Unlike previously published studies [26] our scaffold did not become translucent as seen in other tissues from different species (e.g. rodents) but remained a pale gold or beige colour. This pale gold or beige appearance has been previously reported in both porcine and human de-cellularised livers and described as a possible consequence of lipofuscin accumulation which occurs with increasing age. Verstegen et al., (2017) noted the presence of brown deposits on unstained and H&E stained histological sections from human liver de-cellularised tissue; they confirmed the presence of lipofuscin using Sudan Black B stain [17]. The pig livers used in this study were all from animals less than 50kg in weight (approximately 12 weeks old) therefore it is debatable whether the

beige colour of the scaffold at the end of the process is due to lipofuscin accumulation and is unlikely to be age related. Additionally, no brown deposits were seen on H&E stained sections either prior or post de-cellularisation.. Our previous study on porcine oesophagus scaffolds, also showed a pale colouration of the scaffold tissue following complete de-cellularisation which was confirmed both molecularly and histologically. [27]

Porcine livers were harvested using a similar approach to that employed for harvesting human livers for transplantation. The liver's inherent vascular circulatory system (HPV and HA) was used to deliver de-cellularising reagents into the tissue under peristaltic flow; the latter chosen in order to mimics normal liver physiology. Similar to previous studies using this route, the intricate vascular network remained intact, as did the internal elastic lamellae on small blood vessels. This is crucial as it may help to prevent blood clotting when re-perfused. The absence of endothelium and exposure of collagen fibres from the basement membrane to circulating blood was thought to activate the clotting cascade and cause vascular thrombi noted in previously implanted de-cellularised tissues [28]. Additionally, blood and cell culture media both exhibit different mechanical flow properties (non-Newtonian versus Newtonian) and as such perfusion *ex vivo* may not replicate or mimic the *in vivo* scenario.

Soltran is an organ preservation solution and has been used clinically for many years. It was heparinised and used to flush the liver of red blood cells and any thrombi in preparation for cell lysis; this was accelerated by using saponin which acts by causing the lipid bilayer to become more permeable to macromolecules. The use of SOC is a departure from the use of SDS or Triton x-100 as the main detergent. SDS as an ionic detergent causes proteins to denature, deoxycholic acid is naturally produced as bile acid by the body and the sodium salt of deoxycholic acid is known to solubilise cellular and membrane components by acting specifically on lipids. As a mild detergent it has previously been used to de-cellularise various tissues with varying outcomes depending upon tissue thickness [26]. Significantly, it has been used to de-cellularise 3mm-thick slices of porcine liver but at a much higher concentration of 4% [29] compared to the 0.2% initially used followed by 0.1% used in this study. The

sequential use of saponin followed by SOC was designed to increase the overall impact on cell breakdown whilst keeping its impact on the extra cellular matrix to a minimum.

The predominant component of our de-cellularisation protocol was deionised water and 0.2% Tween 20, the latter acting as a non-ionic nontoxic surfactant and was essential in removing the cell breakdown products from the scaffold. Previous use of SDS at concentrations of 0.01%, 0.1% and 1% when combined with Triton x-100 have all resulted in the production of acellular liver scaffold. However, residual SDS on the ECM has proven to be toxic to cells when scaffolds have been repopulated and ideally SDS should be avoided during the latter stages of any de-cellularisation protocol [30]. Therefore, the final stage of our protocol was completed washes using water. This was followed by an ethanol rinse to assess whether this would remove any residual detergent bound to lipid breakdown products.

FTIR analysis is not routinely used for assessing the effect of de-cellularisation on the ECM of biological tissue with only few studies reporting its use. However, FTIR can assess the composition of the extracellular matrix [31] and can produce standardised repeatable results. To date, it has been used to assess the ECM finger print in fibrocartilage bio-scaffold for bone-tendon interface [32] and in the evaluation of de-cellularised human tracheal samples [33]. It has not to our knowledge been used to determine the presence of any residual detergent or enzymes remaining in biological tissue following de-cellularisation. An additional advantage in using FTIR is its potential to be used as a quality control method for scaffolds destined for clinical application with respect to retained detergent or enzyme residue. Our data clearly demonstrated that additional treatment of the de-cellularised liver using ethanol-gradient washes helps to eliminate both detergent residue and sterilise the scaffold. It is possible that during the de-cellularisation process a point is reached where the ratio of detergent to lipid break down product favours the lipids, and as such the detergent becomes bound up in the lipid forming a complex. This would then potentially remain behind as a residue and may cause cellular toxicity, as shown by previous *in vitro* studies where the seeded cells did not attach to the matrix [30]. Additional wash steps using water alone (data not shown) were not sufficient to remove any remaining residue and therefore the use of an organic solvent such as ethanol which is gentle on the ECM was used to capture

the lipid–detergent complex and remove it from the tissue. Overall the FTIR data suggests that the final washes with ethanol both removed any detergent residue, leaving behind a sterile matrix capable of supporting AD-MSC in vitro.

Alcohols have been reported to be effective at removing lipids, but this is accompanied by tissue dehydration, which leads to cell lysis and elimination of cellular materials [34,35]. However, alcohols have also been reported to precipitate some proteins [34-36], with the potential to alter ECM ultrastructure, particularly structural proteins such as collagens, elastin and growth factors. As such, the benefits of ethanol use observed in this study may not be applicable to other tissue types and should be used conservatively. However, it is worth noting that any residual chemical reagents and detergents retained in the ECM after de-cellularization can be cytotoxic to colonising cells and potentially lead to graft failure in vivo. It has been suggested that the use of alcohols be limited to the elimination of phospholipids from tissue, since the preservation of phospholipids in the tissue could cause calcification [34,35].

Retention of a potentially functional ECM is important for future re-cellularisation and implantation, and similar to previous studies our scaffold showed preservation of collagen (I and IV), laminin and reticulin. Preservation of the basement membrane is important for re-cellularisation since it controls cellular growth and differentiation during embryogenesis whilst directing cellular migration and epithelialisation during tissue repair [37]. The major components of the basement membrane include collagen IV, laminin, perlecan and nidogen, which have been retained in our de-cellularised scaffold. Additionally, the internal elastic lamella of the basement membrane in smaller blood vessels was also preserved. This adds a level of confidence in using the vasculature as a route of delivery for re-cellularisation at later date.

Although analysis of ECM in our scaffold demonstrated retention of the major ECM components, no attempt was made to determine whether key cytokines or growth factors normally associated with the ECM had been retained following the de-cellularisation [38]. Along with the molecular integrity of the

scaffold the structural/mechanical integrity must also be assessed, since both are known to influence cell behaviour (specifically hepatocytes) at least in *in vitro* cell culture [39]. An unstable or damaged liver ECM is unlikely to retain cellular phenotype and may deter cellular dedifferentiation. The presence of reticulin outlining the lobule boundaries indicates that a mostly complete ECM framework has been retained along with all the porous infra structure. This suggests that a significant degree of mechanical integrity has been retained; however, this can only be verified following large scale seeding and in vivo implantation.

In order to assess whether the de-cellularised scaffold was capable of supporting cells (any cells), it was seeded with porcine derived AD-MSC's as a simple cytotoxicity study over a short period of time in vitro. Had the cells all died within the first few days then the de-cellularisation process would have been considered unsuitable and would have required modification. Detergent residue left from the de-cellularisation process can impact on cellular attachment, the FITR analysis was undertaken partially to address this issue of detergent residue. Since functionality of the cells was not relevant and nor were the cells likely to differentiate into liver specific cells, no further analysis was undertaken. We specifically did not set out to differentiate the AD-MSC's into liver specific or hepatic lineages in this study.

Previous studies have used numerous cell types to seed liver matrices, including iPSC, which showed significant expression of hepatic markers [40] following implantation and mesenchymal stromal cells. MSC's hold promise on various levels, when seeded onto a liver matrix they have been shown to differentiate into hepatocytes and when transplanted restore liver function in a lethal liver failure model [11]. Our scaffolds were seeded for 10 days with AD-MSC's and although they did not express any markers of differentiation (HNF –alpha or CK 19 – data not shown), probably due to the short seeding period, the cells showed changes in cellular morphology and migration indicative of a degree of cellular–matrix cross talk. The use of primary porcine hepatocytes has also been reported as a potential cell source [41]; however, concern surrounding immune rejection and the possibility of xeno zoonosis

have yet to be fully addressed. The advantage in using a xenogenic cell source is that there is no limitation to donor tissue availability for re-cellularisation unlike the current clinical situation.

Characterisation of our scaffold clearly demonstrates that it has the potential to support cells, whilst the intact vascular system points to a favourable route for the delivery of cells for re-cellularisation. However, this observation is based on a limited number of animals (n=2) and was used primarily to demonstrate that it is 1) surgically possible to suture the de-cellularised blood vessels to normal tissue and 2) whether the entire liver is capable of being uniformly perfused. Follow up studies are required with a larger cohort of animals using seeded (hepatic progenitors) in a recovery model. A lack of an integral vascular supply and network to any tissue engineered organ runs a significant risk of failure due to the inability to keep the cell supplied with sufficient nutrients and oxygen. Our ex vivo perfusion study using contrast media clearly showed that both the arterial and venous vascular system retained an intact basement membrane allowing for complete perfusion with no evidence of extravasation into the surrounding tissue. On implantation in vivo, the entire liver was perfused with blood demonstrating that it might be possible to deliver cells and nutrients into the thickest portion of the liver and maintain oxygen exchange within the tissue. Crucially, if the structural integrity of the ECM can be maintained following de-cellularisation, molecular cues on the ECM may promote matrix-cell- cross talk resulting in a more optimised re-cellularisation process [42]. In a previous porcine small bowel decellularized scaffold, we demonstrated that by connecting the unseeded scaffold onto the renal artery and vein it was possible to see host cells in the villi (located in the mucosa) of the small bowel tissue, following 24 hour implantation.[43]. This was possible because the scaffold had retained its overall structural integrity.

Previous studies of implanted de-cellularised liver scaffolds in pre-clinical models have highlighted a number of hurdles, including thrombosis and haemorrhage, which have prevented human clinical translation. A potential cause for the formation of blood clots in the re-perfused liver is the interaction between blood and the blood vessel wall. In some cases blood vessels within the de-cellularised livers lacked an intact endothelium and unless particular attention has been paid to the reagents used during

de-cellularisation, the internal elastic lamina may also be damaged. The consequence is that blood flowing through the vessels may be in direct contact with the collagen fibres and may activate the clotting cascade. Clinically, damage to the endothelium exposing the collagen within the basement membrane can result in intimal hyperplasia initiated by thrombus formation [44].

Conclusion

It is possible to create a clinically relevant sized acellular liver scaffold which has the potential following additional investigation to address the clinical human liver transplantation shortage. A tissue engineering approach to creating a liver scaffold with both macro and micro architectural anatomy incorporating an intact vascular and biliary network is possible. The use of porcine livers as the starting material represents an appropriately sized tissue which can be readily obtained. Additionally, the use of porcine livers will not compete with any human livers destined for transplantation. An integrated vascular network provides the optimum route for re-cellularisation using any number of different cell types and supplies appropriate route for nutrient delivery. Our method is a low cost approach and coupled with FTIR analysis for quality control, presents a potential pathway for creating tissue engineered liver for future human application.

Acknowledgements

The authors would like to acknowledge the Paul Foundation for its generous financial support. Additional thanks go to the husbandry and theatre staff at NPIMR and Theoni Demcollari & Hemal Shah for technical assistance

Author Disclosure Statement

All authors state that they have nothing to declare and no conflict of interest.

References

1. Meziere A, M Blachier, S Thomas, M Verny, S Herbaud, O Bouillanne, O Henry, JP David, A Le Thuaut, F Canoui-Poitrine and E Paillaud. (2013). Neuropsychiatric symptoms in elderly inpatients: a multicenter cross-sectional study. Dement Geriatr Cogn Dis Extra 3:123-30.
2. Blachier M, H Leleu, M Peck-Radosavljevic, DC Valla and F Roudot-Thoraval. (2013). The burden of liver disease in Europe: a review of available epidemiological data. J Hepatol 58:593-608.
3. Murray CJ and AD Lopez. (1997). Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. Lancet 349:1498-504.
4. Rana A, A Gruessner, VG Agopian, Z Khalpey, IB Riaz, B Kaplan, KJ Halazun, RW Busuttil and RW Gruessner. (2015). Survival benefit of solid-organ transplant in the United States. JAMA Surg 150:252-9.
5. Arulraj R and J Neuberger. (2011). Liver transplantation: filling the gap between supply and demand. Clin Med (Lond) 11:194-8.
6. Iansante V, A Chandrashekran and A Dhawan. (2018). Cell-based liver therapies: past, present and future. Philos Trans R Soc Lond B Biol Sci 373.
7. Park KM, KH Hussein, SH Hong, C Ahn, SR Yang, SM Park, OK Kweon, BM Kim and HM Woo. (2016). Decellularized Liver Extracellular Matrix as Promising Tools for Transplantable Bioengineered Liver Promotes Hepatic Lineage Commitments of Induced Pluripotent Stem Cells. Tissue Eng Part A 22:449-60.
8. Lee H, S Chae, JY Kim, W Han, J Kim, Y Choi and DW Cho. (2019). Cell-printed 3D liver-on-a-chip possessing a liver microenvironment and biliary system. Biofabrication 11:025001.
9. Hiller T, J Berg, L Elomaa, V Rohrs, I Ullah, K Schaar, AC Dietrich, MA Al-Zeer, A Kurtz, AC Hocke, S Hippenstiel, H Fechner, M Weinhart and J Kurreck. (2018). Generation of a 3D Liver Model Comprising Human Extracellular Matrix in an Alginate/Gelatin-Based Bioink by Extrusion Bioprinting for Infection and Transduction Studies. Int J Mol Sci 19.
10. Geerts S, S Ozer, M Jaramillo, ML Yarmush and BE Uygun. (2016). Nondestructive Methods for Monitoring Cell Removal During Rat Liver Decellularization. Tissue Eng Part C Methods 22:671-8.
11. Zhu LQ, WT Jiang, C Pan, YH Liu and Y Thian. (2014). Liver injury possibly related to drug interaction after liver transplant: a case report. J Clin Pharm Ther 39:439-41.
12. Lang R, MM Stern, L Smith, Y Liu, S Bharadwaj, G Liu, PM Baptista, CR Bergman, S Soker, JJ Yoo, A Atala and Y Zhang. (2011). Three-dimensional culture of hepatocytes on porcine liver tissue-derived extracellular matrix. Biomaterials 32:7042-52.
13. Yagi H, K Fukumitsu, K Fukuda, M Kitago, M Shinoda, H Obara, O Itano, S Kawachi, M Tanabe, GM Coudriet, JD Piganelli, TW Gilbert, A Soto-Gutierrez and Y Kitagawa. (2013). Human-scale whole-organ bioengineering for liver transplantation: a regenerative medicine approach. Cell Transplant 22:231-42.
14. Struecker B, KH Hillebrandt, R Voitl, A Butter, RB Schmuck, A Reutzel-Selke, D Geisel, K Joehrens, PA Pickerodt, N Raschzok, G Puhl, P Neuhaus, J Pratschke and IM Sauer. (2015). Porcine liver decellularization under oscillating pressure conditions: a technical refinement to improve the homogeneity of the decellularization process. Tissue Eng Part C Methods 21:303-13.
15. Kajbafzadeh AM, N Javan-Farazmand, M Monajemzadeh and A Baghayee. (2013). Determining the optimal decellularization and sterilization protocol for preparing a tissue scaffold of a human-sized liver tissue. Tissue Eng Part C Methods 19:642-51.
16. Mazza G, K Rombouts, A Rennie Hall, L Urbani, T Vinh Luong, W Al-Akkad, L Longato, D Brown, P Maghsoudlou, AP Dhillon, B Fuller, B Davidson, K Moore, D Dhar, P De Coppi, M Malago and M Pinzani. (2015). Decellularized human liver as a natural 3D-scaffold for liver bioengineering and transplantation. Sci Rep 5:13079.
17. Verstegen MMA, J Willemse, S van den Hoek, GJ Kremers, TM Luider, NA van Huizen, F Willemssen, HJ Metselaar, IJ JNM, LJW van der Laan and J de Jonge. (2017). Decellularization of Whole Human Liver Grafts Using Controlled Perfusion for Transplantable Organ Bioscaffolds. Stem Cells Dev 26:1304-1315.

18. Gilbert TW, TL Sellaro and SF Badylak. (2006). Decellularization of tissues and organs. Biomaterials 27:3675-83.
19. Badylak SF, D Taylor and K Uygun. (2011). Whole-organ tissue engineering: decellularization and recellularization of three-dimensional matrix scaffolds. Annu Rev Biomed Eng 13:27-53.
20. Struecker B, A Butter, K Hillebrandt, D Polenz, A Reutzel-Selke, P Tang, S Lippert, A Leder, S Rohn, D Geisel, T Denecke, K Aliyev, K Johrens, N Raschzok, P Neuhaus, J Pratschke and IM Sauer. (2017). Improved rat liver decellularization by arterial perfusion under oscillating pressure conditions. J Tissue Eng Regen Med 11:531-541.
21. Akhmanova M, E Osidak, S Domogatsky, S Rodin and A Domogatskaya. (2015). Physical, Spatial, and Molecular Aspects of Extracellular Matrix of In Vivo Niches and Artificial Scaffolds Relevant to Stem Cells Research. Stem Cells Int 2015:167025.
22. Wang Y, CT Nicolas, HS Chen, JJ Ross, SB De Lorenzo and SL Nyberg. (2017). Recent Advances in Decellularization and Recellularization for Tissue-Engineered Liver Grafts. Cells Tissues Organs 203:203-214.
23. Navarro-Tableros V, MB Herrera Sanchez, F Figliolini, R Romagnoli, C Tetta and G Camussi. (2015). Recellularization of rat liver scaffolds by human liver stem cells. Tissue Eng Part A 21:1929-39.
24. Greco KV, L Francis, M Somasundaram, G Greco, NR English, JA Roether, AR Boccaccini, P Sibbons and T Ansari. (2015). Characterisation of porcine dermis scaffolds decellularised using a novel non-enzymatic method for biomedical applications. J Biomater Appl 30:239-53.
25. Buhler NE, K Schulze-Osthoff, A Konigsrainer and M Schenk. (2015). Controlled processing of a full-sized porcine liver to a decellularized matrix in 24 h. J Biosci Bioeng 119:609-13.
26. Crapo PM, TW Gilbert and SF Badylak. (2011). An overview of tissue and whole organ decellularization processes. Biomaterials 32:3233-43.
27. Wang F, Y Maeda, V Zachar, T Ansari and J Emmersen. (2018). Regeneration of the oesophageal muscle layer from oesophagus acellular matrix scaffold using adipose-derived stem cells. Biochem Biophys Res Commun 503:271-277.
28. Caralt M, E Velasco, A Lanas and PM Baptista. (2014). Liver bioengineering: from the stage of liver decellularized matrix to the multiple cellular actors and bioreactor special effects. Organogenesis 10:250-9.
29. Coronado RE, M Somaraki-Cormier, S Natesan, RJ Christy, JL Ong and GA Halff. (2017). Decellularization and Solubilization of Porcine Liver for Use as a Substrate for Porcine Hepatocyte Culture: Method Optimization and Comparison. Cell Transplant 26:1840-1854.
30. Rieder E, MT Kasimir, G Silberhumer, G Seebacher, E Wolner, P Simon and G Weigel. (2004). Decellularization protocols of porcine heart valves differ importantly in efficiency of cell removal and susceptibility of the matrix to recellularization with human vascular cells. J Thorac Cardiovasc Surg 127:399-405.
31. Khanarian NT, MK Boushell, JP Spalazzi, N Pleshko, AL Boskey and HH Lu. (2014). FTIR-I compositional mapping of the cartilage-to-bone interface as a function of tissue region and age. J Bone Miner Res 29:2643-52.
32. Zhou Y, C Chen, Z Guo, S Xie, J Hu and H Lu. (2018). SR-FTIR as a tool for quantitative mapping of the content and distribution of extracellular matrix in decellularized book-shape bioscaffolds. BMC Musculoskelet Disord 19:220.
33. Tint D, CT Stabler, A Hanifi, F Yousefi, G Linkov, K Hy, AMS Soliman and N Pleshko. (2018). Spectroscopic Analysis of Human Tracheal Tissue during Decellularization. Otolaryngol Head Neck Surg:194599818806271.
34. Levy RJ, N Vyavahare, M Ogle, P Ashworth, R Bianco and FJ Schoen. (2003). Inhibition of cusp and aortic wall calcification in ethanol- and aluminum-treated bioprosthetic heart valves in sheep: background, mechanisms, and synergism. J Heart Valve Dis 12:209-16; discussion 216.
35. Clark JN, MF Ogle, P Ashworth, RW Bianco and RJ Levy. (2005). Prevention of calcification of bioprosthetic heart valve cusp and aortic wall with ethanol and aluminum chloride. Ann Thorac Surg 79:897-904.

36. Prasertsung I, S Kanokpanont, T Bunaprasert, V Thanakit and S Damrongsakkul. (2008). Development of acellular dermis from porcine skin using periodic pressurized technique. J Biomed Mater Res B Appl Biomater 85:210-9.
37. Mak KM and R Mei. (2017). Basement Membrane Type IV Collagen and Laminin: An Overview of Their Biology and Value as Fibrosis Biomarkers of Liver Disease. Anat Rec (Hoboken) 300:1371-1390.
38. Bedossa P, D Dargere and V Paradis. (2003). Sampling variability of liver fibrosis in chronic hepatitis C. Hepatology 38:1449-57.
39. Ji R, N Zhang, N You, Q Li, W Liu, N Jiang, J Liu, H Zhang, D Wang, K Tao and K Dou. (2012). The differentiation of MSCs into functional hepatocyte-like cells in a liver biomatrix scaffold and their transplantation into liver-fibrotic mice. Biomaterials 33:8995-9008.
40. Chen HL, HL Chen, RH Yuan, SH Wu, YH Chen, CS Chien, SP Chou, R Wang, V Ling and MH Chang. (2012). Hepatocyte transplantation in bile salt export pump-deficient mice: selective growth advantage of donor hepatocytes under bile acid stress. J Cell Mol Med 16:2679-89.
41. Yang YG and M Sykes. (2007). Xenotransplantation: current status and a perspective on the future. Nat Rev Immunol 7:519-31.
42. Gilbert TW. (2012). Strategies for tissue and organ decellularization. J Cell Biochem 113:2217-22.
43. Nowocin AK, A Southgate, SM Gabe and T Ansari. (2016). Biocompatibility and potential of decellularized porcine small intestine to support cellular attachment and growth. J Tissue Eng Regen Med 10:E23-33.
44. Kipshidze N, G Dangas, M Tsapenko, J Moses, MB Leon, M Kutryk and P Serruys. (2004). Role of the endothelium in modulating neointimal formation: vasculoprotective approaches to attenuate restenosis after percutaneous coronary interventions. J Am Coll Cardiol 44:733-9.