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# TISSUE ENGINEERING LIVER: AN EMERGING THERAPY FOR HEPATIC DISEASES

**GRIGORYAN M.A.[1,2]*, SIMONYAN A.A.[1,3], SIMONYAN A.E.[1,4], KHACHATRYAN V.P.[1]**

[1] Laboratory of Tissue Engineering and Immunology, Orbeli Institute of Physiology of National Academy of Sciences of RA, Yerevan, Armenia
[2] Department of Ansesthesiology and Intensive Care, Yerevan State Medical University after M. Heratsi, Yerevan, Armenia
[3] Faculty of Zaven & Sonia Akian College of Science & Engineering, American University of Armenia, Yerevan, Armenia
[4] Laboratory of General and Molecular Genetics, Research Institute "Biology", Faculty of Biology Yerevan State University, Yerevan, Armenia

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***ABSTRACT***

*Tissue engineering holds a promise to transform transplantation medicine and drug testing. One of its most promising approaches is to grow new organs by seeding decellularized tissue with autologous cells. Here we aimed to compare the methods to reseed decellularized liver scaffold with isolated hepatocytes from rats of different ages.*

*To decellularize adult rat liver it was perfused with 1% Sodium dodecyl sulfate via portal vein. After decellularization, perfusion with Phenol Red was used to confirm the integrity of hepatic vessels. Rat hepatocytes were isolated from neonatal (1-2 day old), 6-week and 10-week old rats. Three-dimensional distribution of viable cells within the recellularized scaffold was monitored using confocal microscopy by loading cells with CellTracker Red CMTPX. Metabolic activity of engrafted hepatocytes was examined using Bradford assay for albumin quantification. Finally, histological analysis of control, decellularized and recellularized samples was performed.*

*Perfusion-based decellularization protocol successfully cleared the liver from most of the cellular content. Diminished amount of cells in 1% Sodium dodecyl sulfate perfused liver samples was confirmed by Coomassie blue staining and histology. After five days of culture, hepatocytes from neonatal rats proliferated, migrated within scaffold material. In contrast, cells from 6 and 10-week-old animals exhibited a lack of proliferation and poor adhesion. Engrafted hepatocytes from neonatal rats had significantly higher rates of albumin secretion as compared to their aged counterparts.*

*We have implemented liver decellularization and recellularization protocols using hepatocytes from rats of different ages. The data showed significant dependence of cell attachment and proliferation rates on the age of donor animal.*

***KEYWORDS:*** *liver, decellularization, primary rat hepatocytes, recellularization.*

## INTRODUCTION

The liver is an extremely powerful organ that plays an essential role in metabolism, detoxification, protein synthesis. Despite his high regenerative activity [*Michalopoulos G, 2010*], some factors (viral infection, alcohol, drugs etc.) can irreversibly damage the organ.

A large number of people over the world suffer from liver disorders leading to cirrhosis, hepatocellular carcinoma, or chronic liver failure [*Murray C et al., 2012*]. As of today, the only option for irreversible liver failure is transplantation from either a living or deceased donor. In Armenia, liver diseases are very common, including a large number of people affected by alcoholic liver disease, viral hepatitis B and C or hepatic cancers [*Andreasyan D et al., 2017*]. There are already known some new cases of liver transplantation in Armenia for these patients. However, new treatment strategies are still urgently needed.

***ADDRESS FOR CORRESPONDENCE:***
Mariam A. Grigoryan
Orbeli Institute of Physiology of National Academy of Sciences of RA
22 Orbeli Bros. Street, Yerevan 0028, Armenia
Tel.: (+374 77)70-82-45
E-mail: grigoryann.mariam@gmail.com



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With the evolution of Tissue engineering diverse therapies were developed to replace or induce regeneration of damaged organs. For the first time term Tissue engineering was used by Langer R and Vacanti J in 1993, but this field of science became popular in 1997 when Vacanti created his famous earmouse- mouse with a human ear on its back. He used cow chondrocytes to create ear shape graft on the mouse back [*Cao Y et al., 1997*]. Tissue engineering promises to transform transplantation medicine and drug testing [*Parveen S et al., 2006; Karathanasis S, 2014; Grounds M, 2018*]. This multidisciplinary field involves different scientific areas-cell biology, material science, chemistry, molecular biology, engineering and medicine. Its ultimate goal is to create fully-functional tissues and organs by combining cells, scaffolds and biologically active molecules.

Over the last years, Tissue engineering suggests diverse therapies to accomplish or replace damaged liver-hepatocyte transplantation, bioartificial liver devices [*Mazza G et al., 2015; Yu Y et al., 2016*]. Hepatocyte transplantation has shown positive results as a treatment for liver diseases, but the clinical benefits were short-term due to low cell engraftment and survival of hepatocytes [*Fox I et al., 1998*; *Sokal E et al., 2003*; *Dhawan A, et al., 2010*; *Iansante V et al., 2018*] . In case of artificial and bioartificial liver systems, there were many limitations, including early hepatocyte death, insufficient number of cells, etc. [*McKenzie T et al., 2008*].

An effective alternative to these methods is seeding 3D scaffolds with cells [*Atala A et al., 2012*]. Several studies have shown the benefits of extracellular matrix (ECM) as a 3D scaffold [*Hurd S et al., 2015*; *Yu Y et al., 2016*]. Mechanical, physical and biological properties of the extracellular matrix affect cell functions by signal transduction [*Frantz C et al., 2010*]. Decellularization of tissues and organs is a promising tool to generate matrix materials for tissue engineering and regenerative medicine. The aim of decellularization is to remove cells and cell materials from tissue using detergents, salts, enzymes, physical means and preserve the structure of the extracellular matrix. The potential applications of decellularized matrix in tissue engineering have been demonstrated for a number of tissues, including bladder [*Yoo J et al., 1998*], artery [*Dahl S et al., 2003*], esophagus [*Nieponice A et al., 2006*], skin [*Schechner J et al., 2003*] and trachea [*Macchiarini P et al., 2008*]. The technique can also be used to create extracellular hepatic matrix with vessels that can be used to seed liver scaffolds with cells [*Ye J et al., 2015*].

Recellularized liver matrix supports specific biological hepatic functions including albumin and urea secretion, and provides mechanical properties typical for a native liver [*Bobrova M et al., 2015*]. Successful implementation of methods in decellularization and further recellularization of rat livers were shown by several authors [*Uygun B et al., 2010*; *Bobrova M et al., 2015*; *Lee S et al., 2015*].

As a cell source for recellularization primary human [*Uygun B et al., 2010; Soto-Gutierrez A et al., 2011*; *Yagi H et al., 2013*] and porcine hepatocytes [*Yagi H et al., 2013*], immortalized hepatocytes [*Totsugawa T et al., 2007*], human hepatic cell lines, stem cells [*Shafritz D et al., 2006*; *Basma H et al., 2009*] and induced pluripotent stem cells [*Du C et al., 2014*] have been used. The main limitation of primary hepatocyte culture is the deterioration of functional stability when cultured for long time [*Walldorf J et al., 2004*]. This limitation can be overcome by the use of stem cell-derived hepatocytes.

The aim of this study was to compare the main methods of recellularization involved in liver tissue engineering. By developing local expertise in this new field of science, we hope to raise awareness and speed up adoption of tissue engineering protocols for the benefit of the Armenian public.

### *Materials and methods*

***Animal surgery:*** Sprague-Dawley rats of different ages were acquired from the vivarium of L.A.Orbeli institute of physiology NAS RA. Animals were weighed and heparinized (150*U* for 1*kg*). Heparin (5000 *U/mL*, Belmedpreparaty, Republic of Belarus) was diluted in normal saline at 1:10000 ratio and injected intraperitoneally. After, chloroform anesthesia was performed, which was verified by a lack of reaction to a tail pinch. Animal abdomen was then open, followed by liver excision.

***Liver decellularization:*** A modified protocol from Uygun B. and co-authors [*Uygun B et al., 2010*] was used for whole liver decellularization. In brief, the portal vein was cannulated using 20 *G* angiocath, followed by perfusion using pre-warmed ($37^{O}C$) normal saline at 10 *ml/min* flow rate. The blanching of the liver indicated successful cannulation. Afterwared, a cut was made at inferior vena cava to allow efflux. Light pressure with a sterile swab on the inferior vena cava was appleid as a further test for successful cannulation; all labes of the



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liver quickly began to swell. After confirmation of cannulation the liver was resected by cutting ligaments and surroundding tissues. The excised liver was transferred to a normal saline-filled Petri dish and was perfused for an additional 30 *minutes*. To start decellularization, cannula port was switched to 1% Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, USA) in double-distilled water. The decellularization lasted on average 72 hours.

***Neonatal rat hepatocytes isolation*** [*Vandergriff A et al., 2015*]: Livers from 7-8 neonatal pups were rinsed in cold calcium- and magnesium-free Hanks' buffered salt solution, minced into ~1-$mm^3$ pieces, and incubated overnight at 4°C in the same media supplemented with 0.1 *mg/ml* trypsin (Mediatech, USA). The next day tissue chunks were washed with calcium and magnesium-free Hank's balanced salt solution (CMF-HBSS) (HiMedia Laboratories, India) and treated with 0.4 *mg/ml* soybean trypsin inhibitor (Invitrogen, USA). The tissue was collected into Leibovitz's medium (L15 medium, Invitrogen, USA) containing 0.8 *mg/ml* collagenase type II (Worthington Biochemical Corp., USA) and shaken for 20 *min* at 37°*C*. Then the cells were gently triturated, passed through a cell strainer to remove any undigested pieces, and centrifuged for 3 *min* at 17.5 *g*. Finally, cell pellet was gently resuspended in Dulbecco's modified essential medium (DMEM) (Invitrogen, USA) supplemented with 10% FBS (Sigma-Aldrich, USA).

***6 and 10 week rat hepatocytes isolation:*** Primary rat hepatocytes from 6 and 10 week old rats were isolated by the perfusion protocol using collagenase type II [*Shen L et al., 2012*]. Percoll density gradient centrifugation was used to separate hepatocytes from erythrocytes (Fig. 1). The cannulation, perfusion with normal saline and excision performed as described above (see Materials and Methods: Decellularization). After 30 *min* of normal saline perfusion, the liver was perfused with 0.075% collagenase type II (Worthington Biochemical Corp., USA), solution at 10 *ml/min* flow during for 15 *minutes*. Under sterile conditions,with a cell scraper the cells were dispersed into DMEM within a sterile Petri dish and filtered the cell dispersion through a 100 $\mu m$ pore size cell strainer into a 50 *ml* Conical tube in order to remove connective tissues and undigested tissue fragments. Later the cells were suspended in 40 *ml* DMEM and centrifuged at 50 *g* for 3 *min* at 4°C. The supernatant was aspirated, and cells were gently resuspended in 40 *ml* cold DMEM to wash the cells. Centrifugation was then repeated. The supernatant was aspirated, and cells were gently resuspended with 25 *ml* DMEM. 25 *ml* 90% Percoll solution (GE Healthcare, USA) in Phosphate-buffered saline (PBS) (MP Biomedicals, USA) was added into the tube, gently mixed and centrifuged at 200*g* for 10 *min* at 4°C (Centrifuge, MPW-360, Mechanika precyzyjna BCM, Poland). The erythrocytes were aspirated from the top of the gradient because the viable hepatocytes remain at the bottom of the Percoll gradient. The cell pellet was suspended in 30 *ml* warm DMEM, then centrifugation was repeated and the cell pellet was resuspended in 20 *ml* warm DMEM.

For gaining cell count the counting chamber (hemocytometer) was used. First it was cleaned with 70% ethanol and lens paper. The coverslip is then gently placed atop the counting chamber. A small sample of cell suspension was taken using a pipette and the pipette is placed near the edge of the chamber, allowing the cell suspension to enter the counting chamber by capillary action. Cell viability was

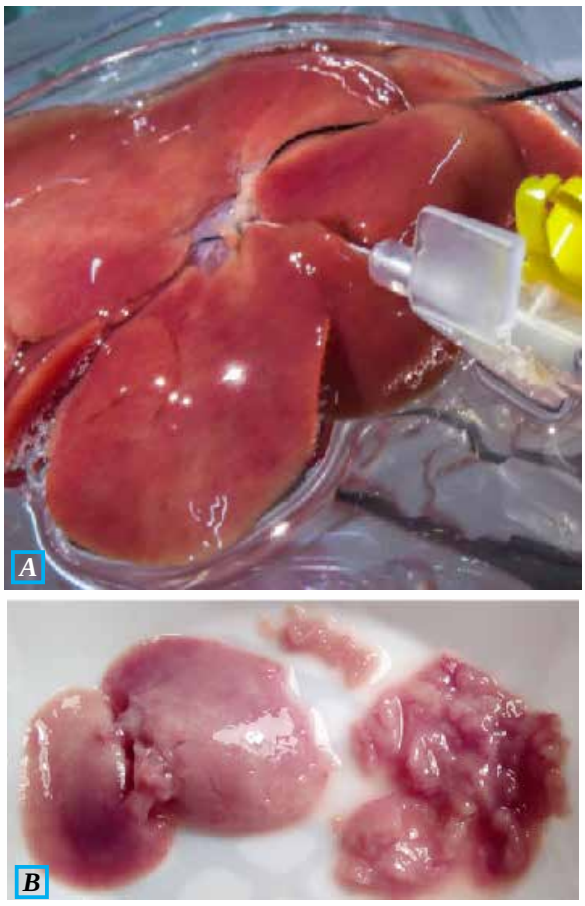


***FIGURE 1.*** *Primary rat hepatocytes isolation*
***(A)*** *The liver was cannulated from portal vein,*
***(B)*** *perfused with collagenase solution and minced*



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determined, using trypan blue exclusion test. Trypan blue is an azo dye that which is used as a vital stain to selectively color dead tissues or cells. Trypan blue dye 0.4 % liquid (Sigma-Aldrich, USA) added to the cell suspension in a 1:1 ratio. Live cells or tissues with intact cell membranes are not colored and dead cells appearing blue due to the permeability of their damaged membranes to trypan blue.

***Recellularization:*** Decellularized scaffolds were cut into ~1$cm^3$ pieces and sterilized under UV (Tool sterilizer, China) for 10 *minutes*. Each piece was then placed in a 24-well plate. 0.5 *ml* cell suspension of freshly isolated hepatocytes were then injected into each piece of the decellularized matrix with 20 *G* syringe, followed the addition of 0.5 *ml* of cell culture media (DMEM + 10% Fetal bovine serum (FBS) (Sigma-Aldrich, USA) + penicillin/streptomycin (Gibco, USA)). Recellularized livers were cultivated for 5 days in cell culture incubator (CO2 incubator, ICO105med, Memmert GmbH + Co KG, Germany).

***Cell and tissue characterization:*** CellTracker Red CMTPX (Invitrogen, USA) fluorescent dye was used the monitor the distribution of viable cells after recellularization using confocal fluorescence microscopy (Inverted microscope, DMi8, Leica microsystems, Germany). Culture media was removed, 10 *µl* pre-warmed dye solution (10 *µM*) was added and recellularized scaffolds were incubated 30 *minutes* a $CO_2$ incubator. Than dye was removed, scaffolds were washed 2 times with Phosphate-buffered saline and added 2 *ml* DMEM (without Phenol Red). We used 480-550 *nm* excitation and 560-650 *nm* emission wavelengths.

To assess the metabolic activity of engrafted hepatocytes, the Bradford assay for albumin quantification was used (https://bio-protocol.org/bio101/e45). For this purpose 100 *mg* Coomassie Brilliant Blue G 250 (Sigma-Aldrich, USA) was dissolved in 50 *ml* 95% ethanol, added 100 *ml* 85 % phosphoric acid, after dye solvation 1000 *ml* distilled water was added. We used 96-well plate for spectrophotometry. 100 *µl* DMEM + 5% Fetal bovine serum (culture media of recellularized scaffold) was used as control and 100 *µl* media were added in wells from each recellularized scaffold (neonatal, 6 week and 10 week old) in triplicate. The medias were collected at 2nd and 3rd days of cultivation. 10 *µl* of dye was added in each well. After 2 *minutes* we measure the absorbance with spectrophotometer (Microplate Photometer, HiPo MPP-96, Biosan, Latvia) at 568 *nm* wavelength.

Histological analyses with hematoxylin and eosin (H&E) were performed for control, decellularized and recellularized liver samples. The latter were fixed with 10% formalin, processed for staining with hematoxylin and eosin by Histogen lab personnel.

***Statistical analysis:*** The statistical analysis was conducted with the software STATGRAPHICS Centurion 16.2 (StatPoint Technologies, Inc. USA; Warrenton, VA). Mean ± Standard Error (SE) was calculated from the data of each group. Student t-test was used for the comparison of data between different groups. Difference of **p<0.01 were considered statistically significant.

## Results

***Results of decellularization*:** Upon visual examination, perfusion with 1% Sodium dodecyl sulfate successfully cleared the liver from the cells (Fig. 2A-C). We next checked the integrity of hepatic vasculature of decellularized sample using Phenol red dye (Taylor technologies, USA). The dye path outlined the liver vascular network, gradually moving from larger vessels to smaller capillaries (Fig. 2D). The scaffold was then examined

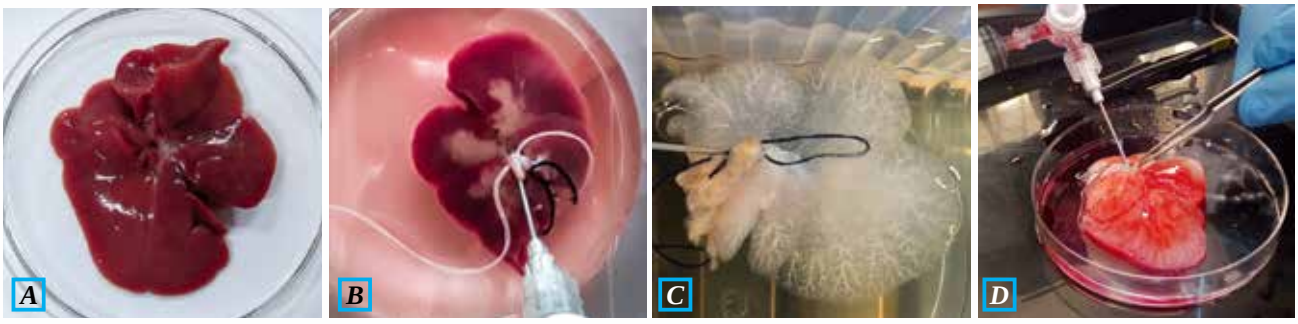


***Figure 2.*** *Decellularization process*
*Intact rat liver (A) after cannulation and perfusion with detergent became pale (B) and finally it became transparent and we could see vessels in it (C). For verification of vascular integrity we injected decellularized matrix with red dye*

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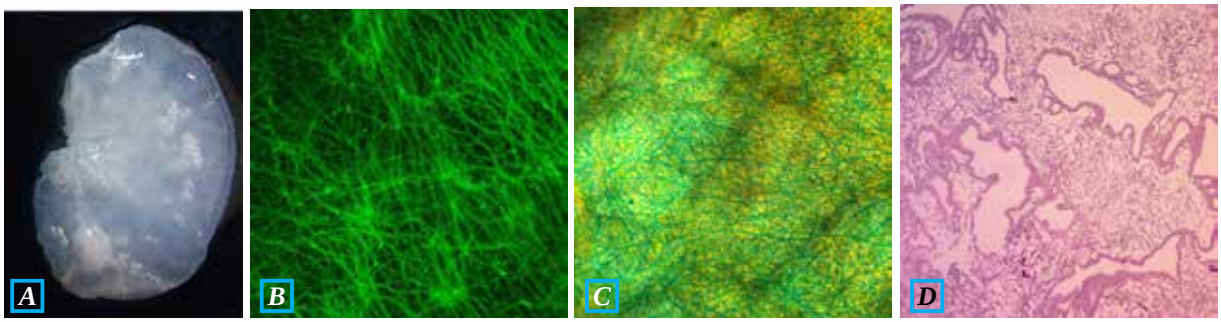


***FIGURE 3.*** *Staining of decellularized liver scaffolds*
*(A) Macroscopic view of decellularized liver lobe. (B) ×10, Staining with CellTracker™ Red CMPTX to show the absence of cells (C) ×10, Coomassie Brilliant Blue staining of collagen fibers. (D) ×10, H&E Staining also shows the scaffold has no cells*

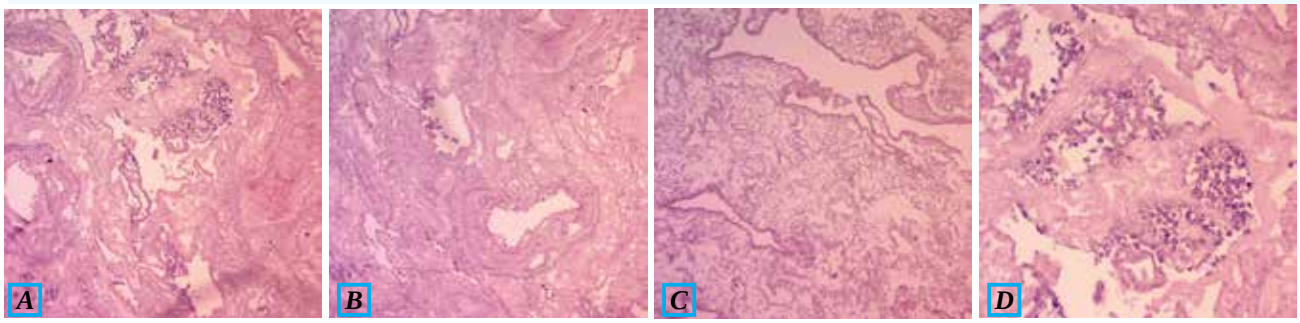


***FIGURE 4.*** *Recellularized scaffolds*
*(A) ×10, H&E, Scaffold recellularized with hepatocytes from neonatal rats. (B) ×10, H&E, Scaffold recellularized with hepatocytes from 6week old rat. (C) ×10, H&E, Scaffold recellularized with hepatocytes from 10week old rat. (D) ×10, H&E, Staining shows a minimal amount of cells in the case of 6 week and 10-week-old rats, but in case of neonatal, we have a lump of cells (blue dots)*

for the absence of cells by fluorescence imaging, Coomassie blue staining and hematoxylin and eosin [*Crapo P et al., 2011*] (Fig. 3B-D). The later confirmed the minimal presence of cells, with images displaying mainly the extracellular matrix.

***Results of recellularization:*** A partial recellularization of the liver scaffold was achieved with islands of re-seeded hepatocytes clearly visible using both histological and fluorescent staining (Fig. 4 A-C, Fig. 5). To verify the functional performance of reseeded hepatocytes albumin quantification was performed. It revealed that albumin concentration was higher in the recellularized scaffolds compared to the media. With additional time in culture, hepatocytes from 6 and 10 week old animals started to lose albumin producing capacity, while neonatal rat cells have increased production of albumin (Fig. 6). A maximum number of cells were found after liver scaffold recellularization with neonatal rat hepatocytes (Fig. 4A).

### *DISCUSSION*

Decellularization and recellularization of liver protocols were successfully implemented using available reagents and equipment. The data revealed a strong dependence between hepatocyte donor age and cell attachment and proliferation rates. Specifically, during five-day culture, neonatal rat hepatocytes were proliferating and migrating within scaffold material. In contrast, cells from 6 and 10 week old animals were not dividing even though few cells adhered were adhered to the scaffold material.

Our observations point to the crucial importance of cell potency in the recellularization. Particularly, we see that already 6-week-old and older rat hepatocytes exhibit insufficient expansion ca-

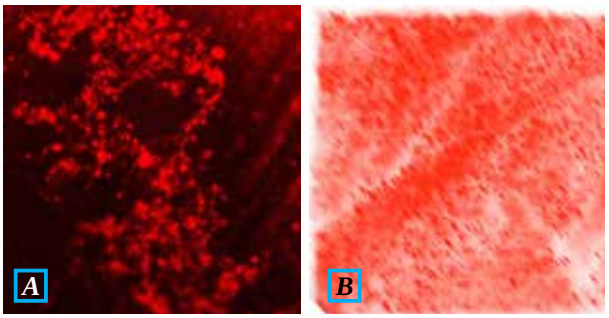


***FIGURE 5.*** *Fluorescent staining of recellularized liver (A) ×10, CellTracker Red CMTPX. (B) ×10, CellTracker Red CMTPX, 3D distribution of cells*



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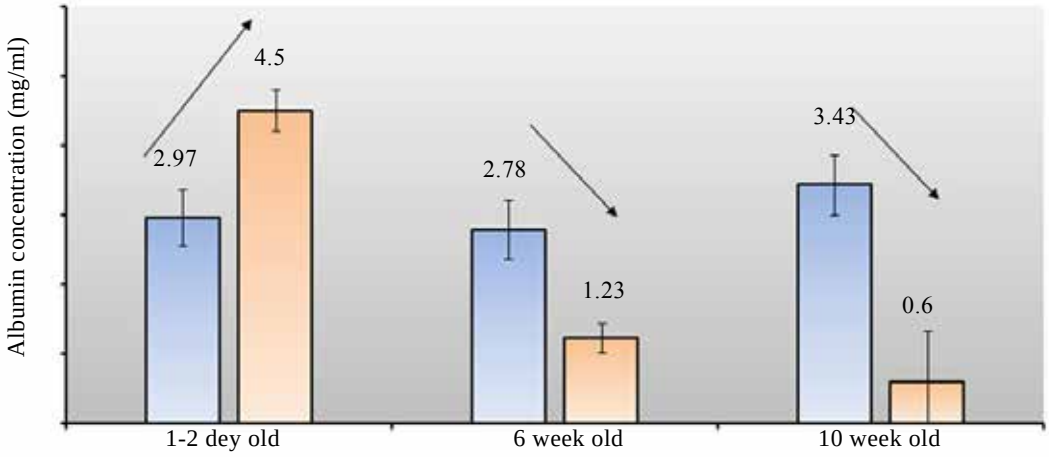


***Figure 6.*** *Bradford Assay for protein quantification. Medias were collected at 2nd (▭) and 3rd (▭) days of cultivatiom*

pacity in the scaffold, notwithstanding the factual high regeneration rate of liver tissue [*Andersen K et al., 2013*]. Thus, while considering the sources of cells for liver recellularization one may exclude the use of hepatocytes from the elderly patient. Alternatively, the extracted cells from such patients might need to be re-differentiated toward their pluripotent state. Yet another option can be the isolation of liver resident stem cells, which, while present in small quantities, are activated under conditions where the regenerative capacity of mature hepatocytes is impaired [*Best J et al., 2015*].

In contrast, decellularization was successfully implemented regardless of the age of the donor tissue. Thus, we may suggest that the donor age is not a significant factor for the cell extraction from the surrounding extracellular matrix. The success of decellularization in rats opens a floor for the local implementation of the same protocol for liver lobes extracted from larger animals or donated human tissue from deceased patients. Future studies can also include the examination of factors that are likely to affect cell attachment such different species (i.e., rat vs cow), organ difference (i.e., liver vs heart scaffold), cell cultivation times before seeding and recellularization method. The establishment of the most efficient methodology will take us closer to creating local expertise to liver tissue engineering – first in animals and later,

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