The Impact of the Decellularized Implant on the Histopathology and Functions of the Recipient's Liver

A. Yaghoubi[1], N. Azarpira[2], Z. Vojdani[1], S, Daneshi[3], S. Karbalay-doust[3,4], T. Talaei-Khozani[1,3]*

[1]Tissue Engineering Lab, Anatomy Department, Shiraz University of Medical Sciences, Shiraz, Iran
[2]Transplantation Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[3]Histomorphometry and Stereology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[4]Anatomy Department, Shiraz University of Medical Sciences, Shiraz, Iran

ABSTRACT

Background: Decellularized livers could provide an environment for liver-specific cell migration and can be a cell or drug delivery vehicle. Synthetic glucocorticoids can be loaded on the decellularized liver to induce hepatocyte differentiation and development. Although decellularized scaffolds provide a promising approach for liver regeneration, their transplantation to the partial hepatectomized liver may have a detrimental impact on the recipients' liver function.

Objective: This study investigated the impact of the transplanted decellularized liver with/without prednisolone preloading on liver histopathology and functions.

Methods: Decellularized rat liver scaffolds were prepared by the perfusion method. After scaffold characterization, they were grafted to partially hepatectomized rats in prednisolone-free and -loaded groups. After 2 and 4 weeks, the liver and grafts were removed and evaluated by Periodic acid Schiff staining and immunohistochemistry. Serological assessments were also performed on blood sera and compared with the untreated control. The data were analyzed by ANOVA and LSD.

Results: Both grafts were invaded by hepatocytes. No histopathological symptom was detected in the recipients' liver; however, oval cells were observed within the epithelium of the bile duct and in the surrounding connective tissue. No significant variation was observed in the levels of alkaline phosphatase (ALP), but the levels of albumin and total protein were significantly reduced in both groups that received the grafts after two weeks; however, after four weeks, total protein and albumin reached the average level.

Conclusion: Decellularized liver transplantation with/without the drug is safe enough for liver recipients to be considered a promising technique in regenerative medicine.

KEYWORDS: Regenerative medicine; Liver; Prednisolone; Decellularization

INTRODUCTION

Liver architecture and extracellular matrix (ECM) changes in some chronic liver failures, such as cirrhosis [1]. Although the liver has a unique capability to regenerate itself after parenchymal injury, it loses this unique ability when the volume of the liver is too small to meet the metabolic demands [2]. In these specific clinical cases, the liver requires assistive devices to lessen complications [3]. Regenerative medicine is one of the definitive objects of modern biotechnology that enables the complete or partial production of the functional liver to treat acute or chronic liver disorders [4]. One of the important issues in tissue engineering is the long-term permanence and sequelae of the trans

*Correspondence: Tahereh Talaei-Khozani, PhD
Histomorphometry and Stereology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
ORCID: 0000-0002-8425-8871
Tel: +98-713-230-4372
E-mail: talaeit@sums.ac.ir

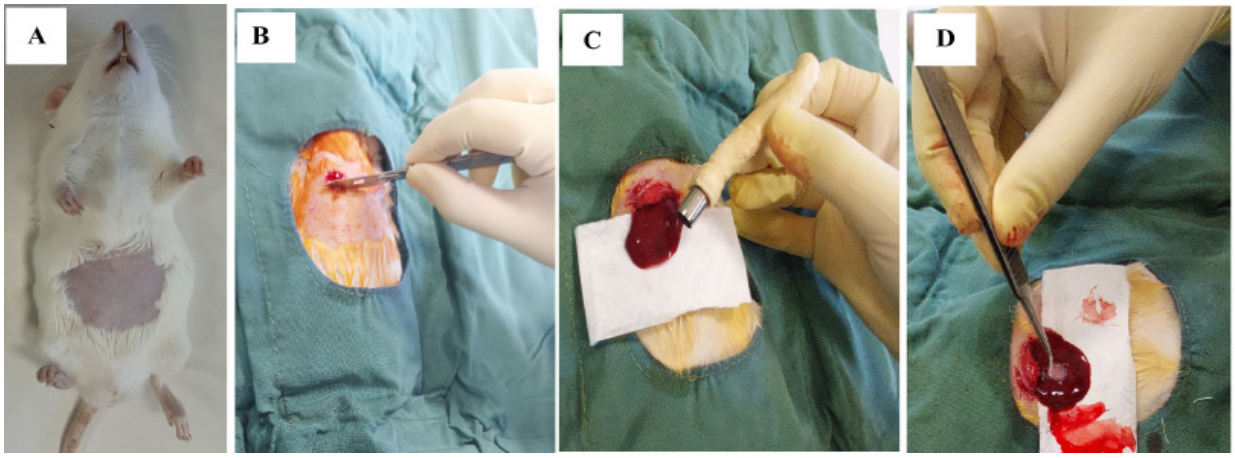

Figure 1: In the surgical procedure, the abdominal incision was performed (A), the left lobe of the liver exposed (B), a hole with a diameter of 1 cm was formed using a surgical trocar (C), and decellularized scaffold was implanted into the hole (D).

planted engineered tissue in the host [5]. The decellularization method is a valuable process to prepare natural scaffolds because it can preserve the function of primitive microvascular systems [6], the extracellular matrix (ECM) compositions, and architecture, growth factors, and mechanical characteristics, as well as the elimination of immunogenic cellular materials [7]. Promising results were obtained by transplantation of decellularized liver scaffolds in partially hepatectomized rats. The scaffolds were invaded by liver-specific cells, blood vessels, and immune cells. It has been shown that the invading immune cells to the graft destroy the extracellular matrix of the scaffold by inducing fibrogenesis [8]. This may interfere with the reorganization of the migrated hepatocytes. To reduce immunoreaction, some researchers suggested loading glucocorticoids such as prednisolone in fabricated tissues [9]. In one study, prednisolone and mesenchymal stem cell (MSC) loading improved the volume of the regenerated liver in the decellularized liver implant and modulated the immune cell infiltration [10].

Corticosteroids have a critical role in hepatocyte maturation and liver functions. It has been reported that synthetic glucocorticoid such as dexamethasone upregulates the expression of HNF4 and C/EBPα, which are essential for liver development and hepatocyte differentiation even in the absence of hepatogenic growth factors such as hepatocyte growth factor (HGF) and epidermal growth factor (EGF). In combination with the hepatogenic growth factors, Dexamethasone also induces bile duct formation through the upregulation of HNF6-β [11]. Glucocorticoids, such as prednisolone, are anti-inflammatory remedies and can suppress the immune system [12]. Although prednisolone has been reported to play a significant role in treating liver disease, some research also showed the liver repair declaration after a prednisolone prescription in a rat model [13]. Therefore, we preloaded the decellularized liver scaffold to improve the hepatogenic property along with limiting the immune cell migration. Partial liver transplantation is a choice to overcome the shortage of the donors' liver [14]. As the size of the human liver is large and fabricating the engineered liver in such dimensions is too hard, the same strategy may be suggested for the implantation of the engineered liver. A decellularized liver with or without cells or preloading of hepatogenic agents can be transplanted, and in vivo recellularization happens as a result [8]. As a promising technique, partially engineered liver implantation should be safe for the rest of the recipients' liver. Therefore, in the current study, we investigated the safety of the decellularized scaffold with or without the drug after implantation on the histopathology and functions of the recipients' liver.

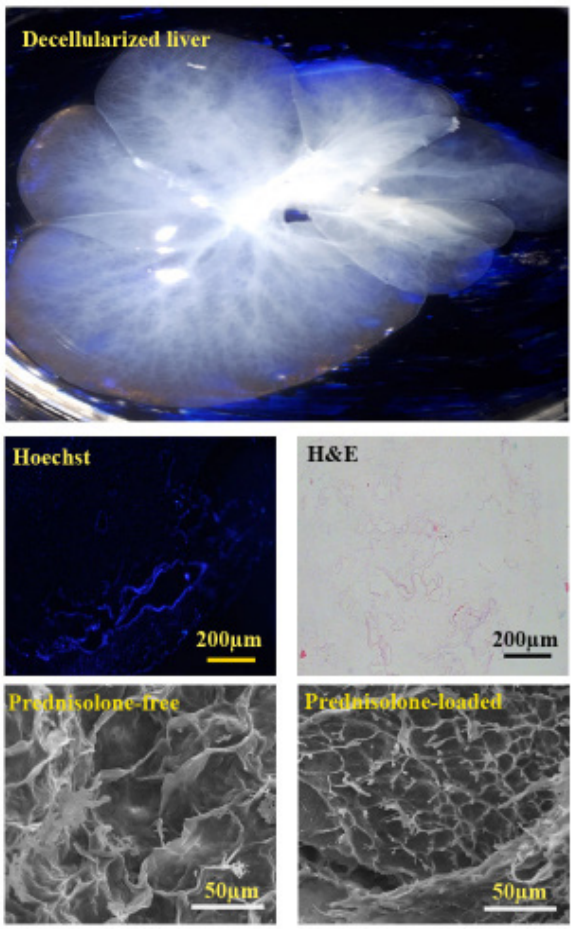

Figure 2: The whole liver decellularized that prepared by perfusion with SLES. The vascular structures were preserved and are visible. Hoechst and H&E staining indicated cell depletion of the liver bioscaffolds. Scanning electron microscopy images showed preservation of the ultrastructure of the liver extracellular matrix after perfusion procedure.

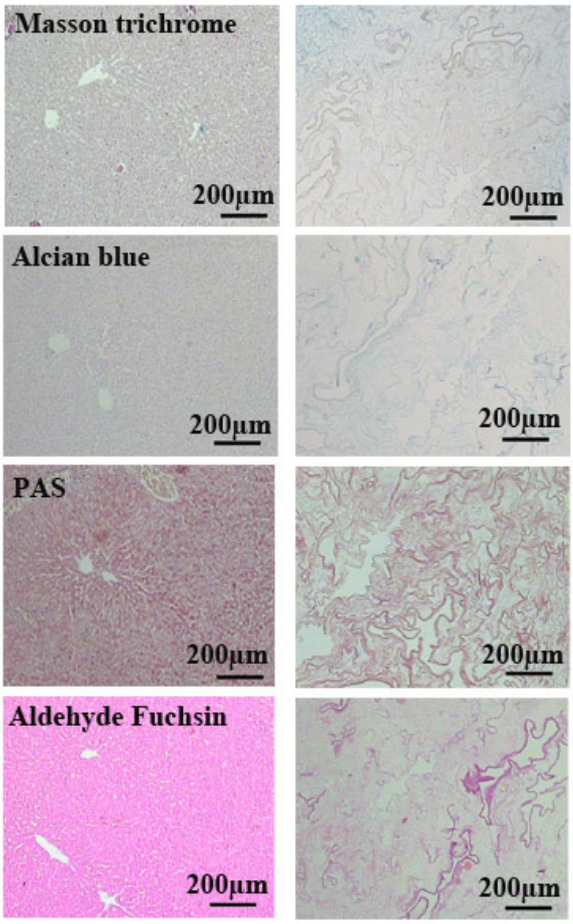

Figure 3: The retention of important liver extracellular matrix compounds was evaluated by histochemical tests. Masson Trichrome, Alcian blue, PAS, and Aldehyde-fuchsin detected preservation of collagen fibers, acidic glycosaminoglycans, neutral carbohydrate, and elastic fibers respectively in the decellularized liver compared to the intact one.

MATERIALS AND METHODS

Preparation of Acellular Liver Scaffold

A perfusion technique was performed to decellularize the rat liver. To do this, the male Sprague Dawley rats weighing 200±20 g were purchased from the Comparative and Experimental Medicine Center at Shiraz University of Medical Sciences. The perfusion was performed under anesthesia with Ketamine 10% (100 mg/kg, Alfasan, Netherlands) and Xylazine 2% (10 mg/kg, Alfasan, Netherlands). Initial rinsing of the liver with 400 mL of deionized water was performed by cannulation of the portal vein with a tube made of polymer plastic with a diameter of 2 mm. After the liver was flushed out of the blood, the decellularization process was initiated by 400 mL sodium lauryl ether sulfate 1% (Kimia Sanaat Ataman Co, Tehran, Iran). After clearing the liver, the final wash was performed with 400 mL of deionized water, and then the decellularized scaffold was sterilized by perfusing 0.1% peracetic acid. All steps were performed at a speed of 3/5 mL/min peristaltic pump (WT600-1F, Longer pump Co., USA). Eventually, the liver was removed after separating the connections from the surrounding tissues.

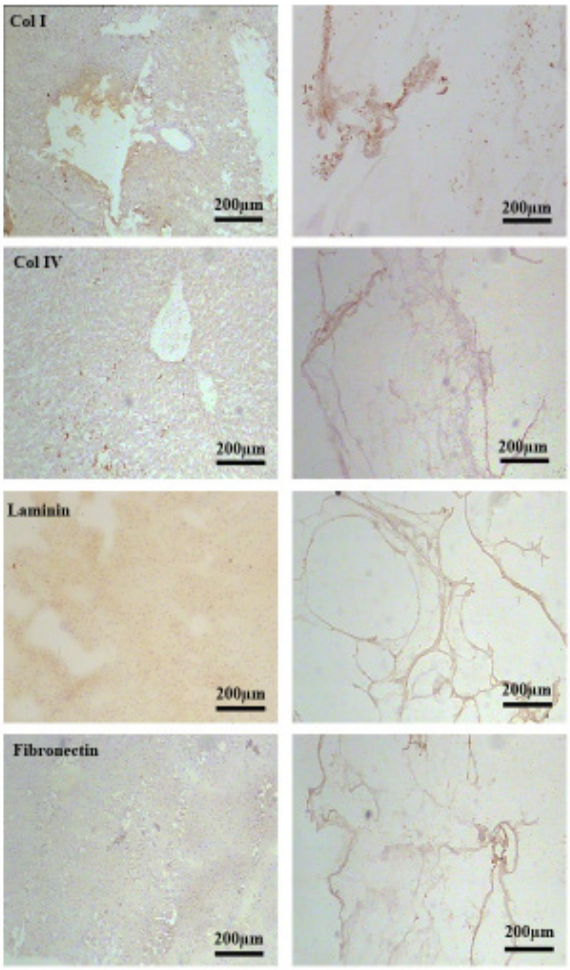

Figure 4: Immunohistochemistry demonstrated the retention of collagen I, IV, laminin, and fibronectin in the acellularized liver in comparison to intact tissue.

Characterization of Liver Extracellular Matrix

Hematoxylin & Eosin and Hoechst (10 ng/mL in H2O) staining were accomplished on 5µm paraffin-embedded sections to evaluate the cell removal from the liver scaffold. Extracellular matrix contents, including collagen, elastic fibers, acidic glycosaminoglycans, and neutral carbohydrates, were assessed by staining the sections with Masson's trichrome, Aldehyde fuchsin, Alcian blue, and Periodic acid Schiff (PAS), respectively. In addition, the preservation of collagen I, IV, laminin, and fibronectin was evaluated by immunohistochemistry after preparing frozen sections from decellularized and intact livers at 8µm thickness. To do this, 3% H2O2 in methanol was used for 20 min

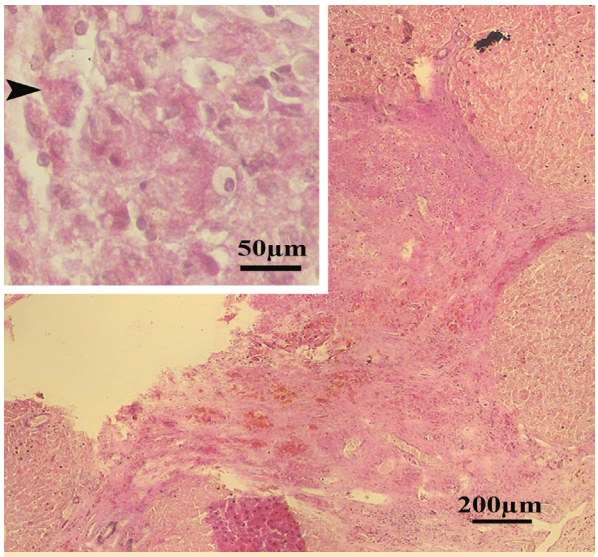

Figure 5: PAS staining showed that all grafts occupied with hepatocytes contain different degrees of glycogen deposition and different stages of hepatocyte differentiation.

to inactivate endogenous peroxidase activity. Then the sections were incubated in 300µL PBS containing 10% goat serum and 5% bovine serum albumin (BSA) to block non-specific binding sites. The sections were incubated with biotinylated anti-collagen type I (1:250), anti-collagen type IV (1:500), anti-fibronectin (1:250), and anti-laminin (1:100) antibodies overnight at 4°C (All from Abcam PLC, Cambridge, MA, USA). The samples were treated with 200–400 µL streptavidin-HRP (1:10000; Abcam, USA; ab7403) for 20–30 min for color development, and after that, the sections were incubated in 3, 3'-Diaminobenzidine tetrahydrochloride (DAB, Sigma Aldrich, D5905). Finally, hematoxylin was used for counterstaining. Then, the prepared slides were observed by light (Olympus BX61, Tokyo, Japan) and microscopes equipped with a digital camera (Olympus DP73).

Also, scanning electron microscopy was performed to assess the retaining architecture of the decellularized liver scaffolds. To do this, lyophilized scaffolds were coated with gold using Q150R- ES sputter coater (Quorum Technologies, UK) and observed by a VEGA3 microscope (TESCAN, Czech Republic).

Transplantation of Decellularized Scaffolds

To transplant the liver grafts, 20 mature male

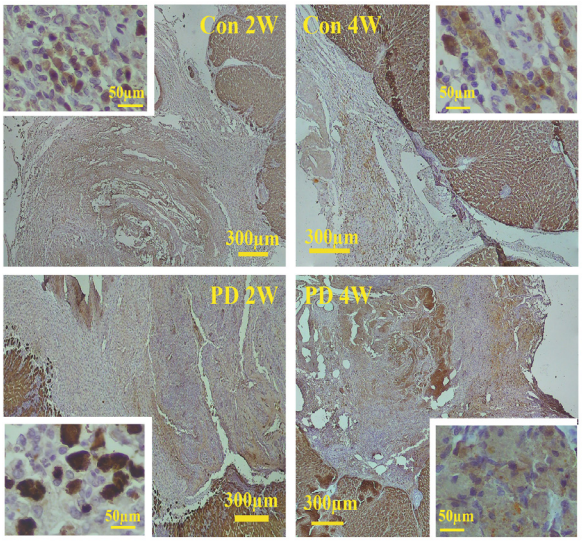

Figure 6: HSA marker also indicated the presence of both mature and immature hepatocytes in all bioscaffolds after implantation. DC: decellularized, PD: prednisolone, Con: control.

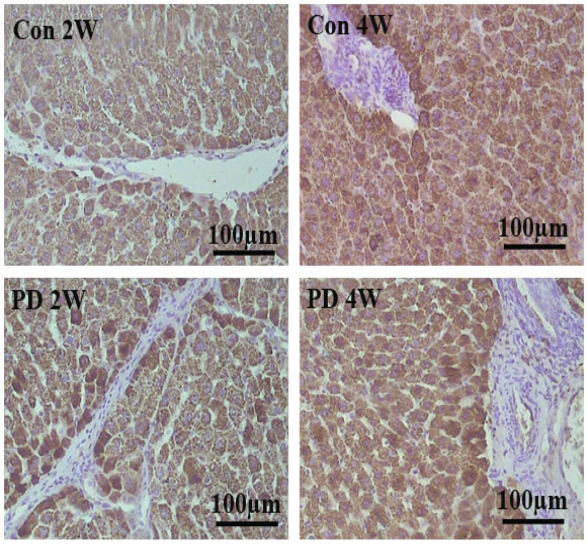

Figure 7: HSA staining showed that the hepatocytes in the recipient's liver were stained normally in both groups treated with prednisolone-free and loaded scaffolds.

Sprague-Dawley rats weighing 230±10 g were divided randomly into two groups: those receiving prednisolone-free and those treated with prednisolone-loaded decellularized implants. Each group was partitioned into two subgroups for 2 or 4 weeks [8, 10, 15]. At first, the animals were anesthetized by intramuscular injection of 10 mg/kg xylazine (Alfasan, Netherlands) and 100 mg/kg ketamine (Alfasan, Netherlands). Then, a transverse incision was made on the abdomen under sterilized conditions. The left lateral lobe of the liver was exposed, and a hole with a diameter of 1 cm was formed using a surgical trocar. Finally, sterile hepatic scaffolds were sutured to this area with prolene 8-0 and after that, the abdominal wall and skin were closed by vicryl and nylon (3-0) sutures (Fig 1).

Blood Collection

At 2 and 4 weeks after implantation, blood samples were taken from the left ventricle with a 5 mL syringe under the deep anesthesia that killed the animal. Samples were stored in serum gel separator tubes and centrifuged at 3000 rpm for 30 minutes. Blood serum was then collected in a microtube and conserved at -20 °C until analysis. Chemical parameters, including alkaline phosphatase (ALP), hepatic factors albumin, total protein, and bilirubin were measured by the commercial kits (all from Biorex diagnostics kits, Fars, Iran) according to the manufacturer's instructions. The same assessments were done on the blood samples from the untreated control group.

Evaluation of Grafts after Transplantation

The grafts were harvested after 2 and 4 weeks. The presence of hepatocytes in the grafts was assessed by Periodic acid Schiff staining, anti-Hepatocyte specific antigen (HAS), and c-kit antibodies after paraffin-embedded tissue processing and provision of 5 μm sections.

The immunohistochemistry was performed by heating the samples in citrate and Tris-EDTA buffers to retrieve the antigen. Blocking endogenous enzymes and non-specific binding sites was done by incubating the samples in 10% H2O2 in distilled water and PBS containing goat serum, respectively. Pre-diluted hepatic specific antigen (HAS), c-kit, and glial fibrillary acidic protein (GFAP) antibodies (Dako, Denmark) were used to label hepatocytes, oval cells, and hepatic stellate cells, respectively, followed by incubating in polymer-HRP for 30 min, and then, the antibody was visualized by incubation in diaminobenzidine as chromogen.

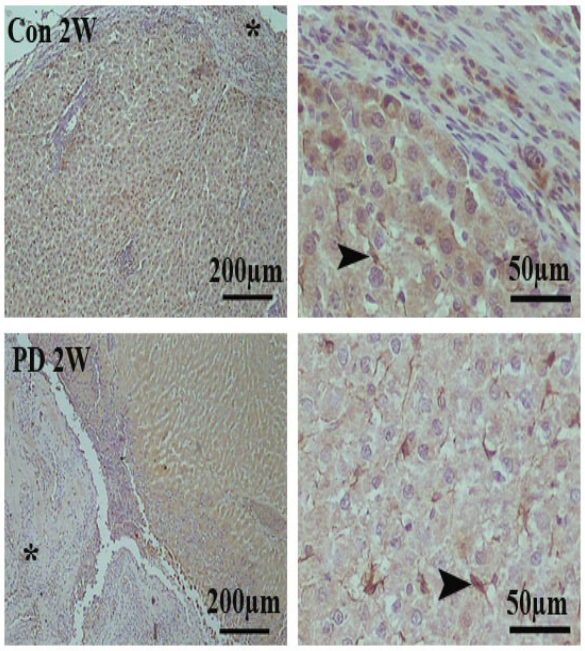

Figure 8: Staining with GAFP antibody showed hepatic stellate cells (arrow). The hepatic stellate cells were normal in both groups treated with prednisolone-free and loaded scaffolds. The right micrographs show the higher magnification. The implanted scaffold was labeled with *. PD: prednisolone, Con: control.

Ethical Consideration

The animal handling and treatment were performed according to the guidelines approved by the Shiraz University of Medical Sciences ethics committee (IR.SUMS.REC.1398.1210) in the present experiment.

Statistical Analysis

The data were presented as mean values ± standard deviation (SD), and statistical analyses were applied using one-way ANOVA analysis and the Tukey test. Graph Pad Prism 9 was used for analyses and depicting the graphs. A p-value less than 0.05 was considered a significant difference.

RESULTS

Liver Scaffold Assessment before Transplantation

Assessment of liver scaffolds by H&E and Hoechst staining showed that the cells and their nuclei were effectively removed from the tissue. Scanning electron microscopy images indicated that both the extracellular matrix's ultrastructural and the tissue's vascular architecture were preserved after the decellularization procedure (Fig 2). Comparison of scaffolds stained by Masson's trichrome, Aldehyde fuchsin, Alcian blue, and PAS with intact tissues proved that collagen, elastic fibers, acidic glycosaminoglycans, and neutral carbohydrates were reasonably preserved during the decellularization process (Fig 3). Furthermore, immunohistochemistry confirmed that extracellular matrix proteins, such as collagen type I and IV, laminin, and fibronectin, were retained properly compared to the intact liver (Fig 4).

Intact Liver and Graft Assessments after Transplantation

Immunohistochemical and PAS staining revealed that the hepatocytes were formed or migrated in post-transplant grafts. Most of the cells that occupied the graft were PAS and HSA-positive, indicating that hepatocytes occupied a wide volume of each graft compared with other cells. The staining intensity of the PAS-positive cells was varied, indicating the different degrees of glycogen deposition and stages of hepatocyte differentiation. The intensity of the reaction to HSA was also different, confirming the presence of both mature and immature hepatocytes in both scaffolds (Fig 5 and 6). HSA antibody, along with normal histology of the intact part of the liver, indicated the drug-free and drug-loaded decellularized implants had no impact on the integrity of the rest of the liver (Fig 7).

No pathological changes or immune cell infiltration were found in the recipient's liver. Staining with GFAP antibody also showed that the hepatic stellate cells had normal appearance and distribution (Fig 8). Although the structure of the portal space remained normal, some c-kit-positive cells were detected around the bile ducts, which indicates the development of oval cells. Some epithelial cells covering the bile ducts also reacted with a c-kit antibody. The oval cells were present in the recipient's liver of animals treated with drug-free and loaded decellularized implants (Fig

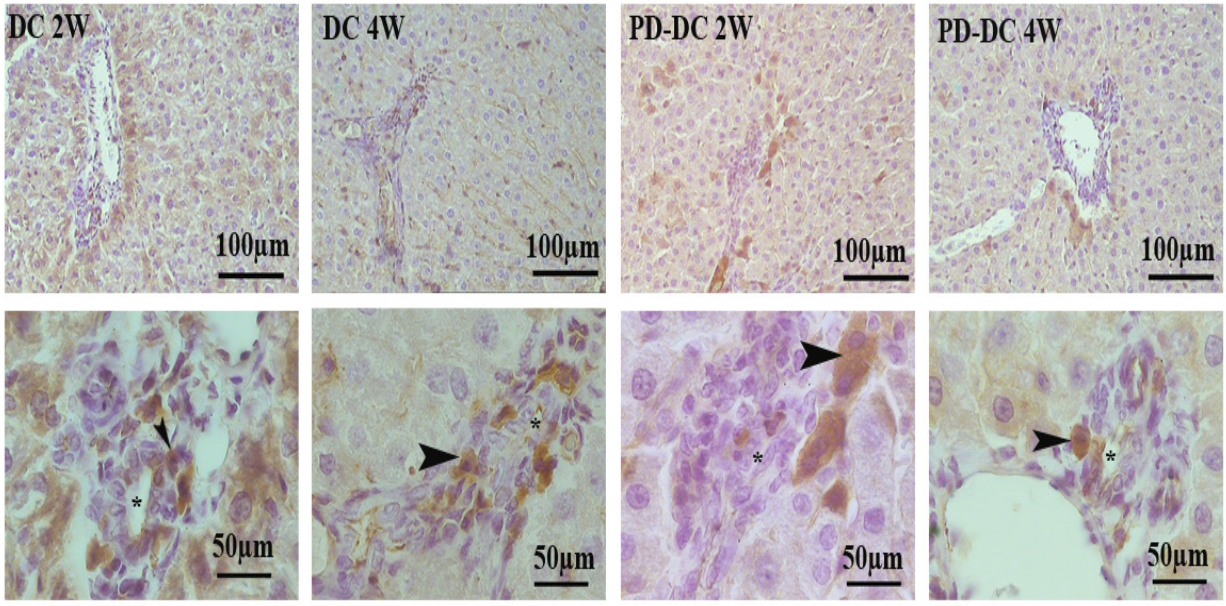

Figure 9: C-kit maker confirmed the presence of oval cells around the bile ducts in the recipient's liver of animals treated with both decellularized implants. Arrowhead showed oval cells and * shows the lumen of the bile duct. Some epithelial cells that cover the bile ducts are also c-kit positive.

9). The normal reaction to both PAS staining indicated that the glycogen deposits did not influence by both decellularized implantations (Fig 10).

Assessment of Serum Biochemical Parameters

To evaluate the possible impact of the decellularized scaffold on the function of the intact liver, ALP activity along with the liver productions, including albumin, total protein, and bilirubin (total and direct), were measured two and four weeks after transplantation of the drug-free and drug-loaded scaffolds. The results indicated that the level of ALP activity in the sera of the implant-treated animals was statistically the same as in the control group. The drug loading also had no impact on the ALP activity. However, the amount of albumin and total protein in all the partial hepatectomy groups significantly declined compared to the control group after 2 weeks ($P<0.0001$ for all). At the 4th week, the total protein and albumin amounts returned to a normal level, and the bilirubin level was higher in the groups that both received grafts (Fig 11).

DISCUSSION

According to our experiment, it was found that albumin and total protein levels decreased postoperatively. However, after 4 weeks, these two levels were statistically similar to the control group. Robert and White noted that the albumin concentration dropped sharply 24 hours after partial hepatectomy in the rat [16]. Chandler and Snider reported that serum albumin levels and seromucoid fraction increased 14 days after partial hepatectomy [17]. Decreased total protein concentrations 12 to 36 hours after partial hepatectomy was also observed in the Sekas and Cook study. They attributed this decline to reducing globulin levels after surgery [18]. Besides, the serum bilirubin level increase in our study is similar to the Sekas and Cook observations [18].

A bilirubin increase may happen due to post-hepatectomy bile flow reduction [19]. The other reason for bilirubin elevation may be postoperative bile leakage from the transaction margin. In some human cases, it happens 3 to 21 days after liver resection [20, 21]. As our data shows, as time progresses, the serum albumin, total protein, and bilirubin level im

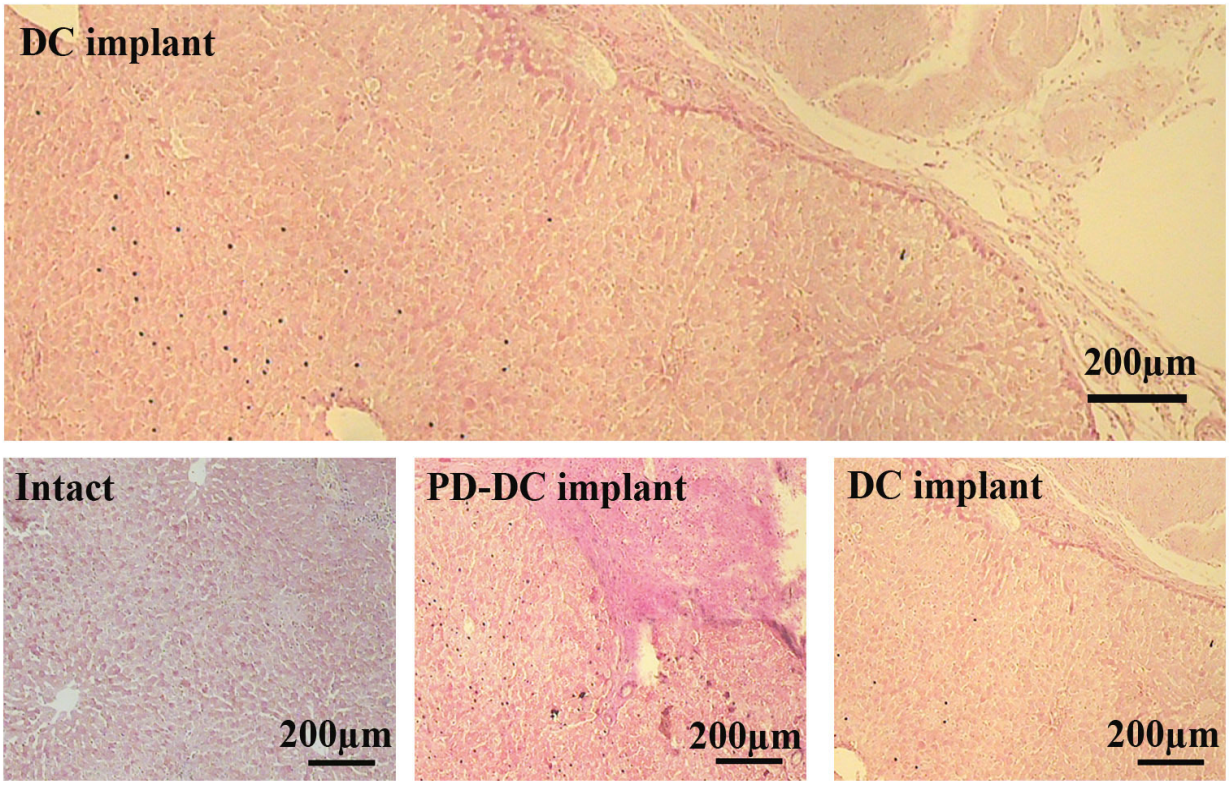

Figure 10: PAS staining showed the glycogen deposition was not modified by implantation of both drug-free and loaded decellularized implants compared to intact liver.

prove, and biliary leakage may be responsible for the delay in bilirubin elevation up to 4 weeks.

Since the histopathology of the recipient's liver was expected, the reduction in total protein or albumin and the increase in bilirubin may be related to the surgical operation rather than the presence of a decellularized implant. Albumin and total protein loss were detected in rats who underwent abdominal surgery [22]. Laparoscopy has been reported to lead to a decrease in polyribosome liver cell fraction [23], which can be a cause for reducing the liver's total protein level and protein synthesis [24]. Besides, albumin synthesis also decreases during laparoscopy surgery [25]. A decrease in albumin has been reported in patients who have undergone liver resection with or without liver cirrhosis 3 days after surgery [26]. Also, Hyperbilirubinemia is a common complication after abdominal surgery [27].

The results of our study indicate that after partial hepatectomy, there is no change in the production of the ALP in the animals that received implants. It has been recorded that ALP levels reach normal levels 72 h post-operation in patients who underwent laparoscopic cholecystectomy [28].

Our data showed the transplantation of the prednisolone-loaded decellularized scaffolds had no impact on the serum level of liver markers compared to the scaffolds without prednisolone. Prednisolone is one of the most common glucocorticoids for treating liver disease and cancer, which prevents the progression of hepatocellular carcinoma by inducing apoptosis [29, 30]. Although low doses of synthetic corticosteroids are secure for the liver, a case report in 2011 showed that long-term intravenous administration and high doses of corticosteroids, such as methylprednisolone, induced intensive liver harm and raised liver enzymes and proteins [31]. It seems that the local administration of prednisolone is safe.

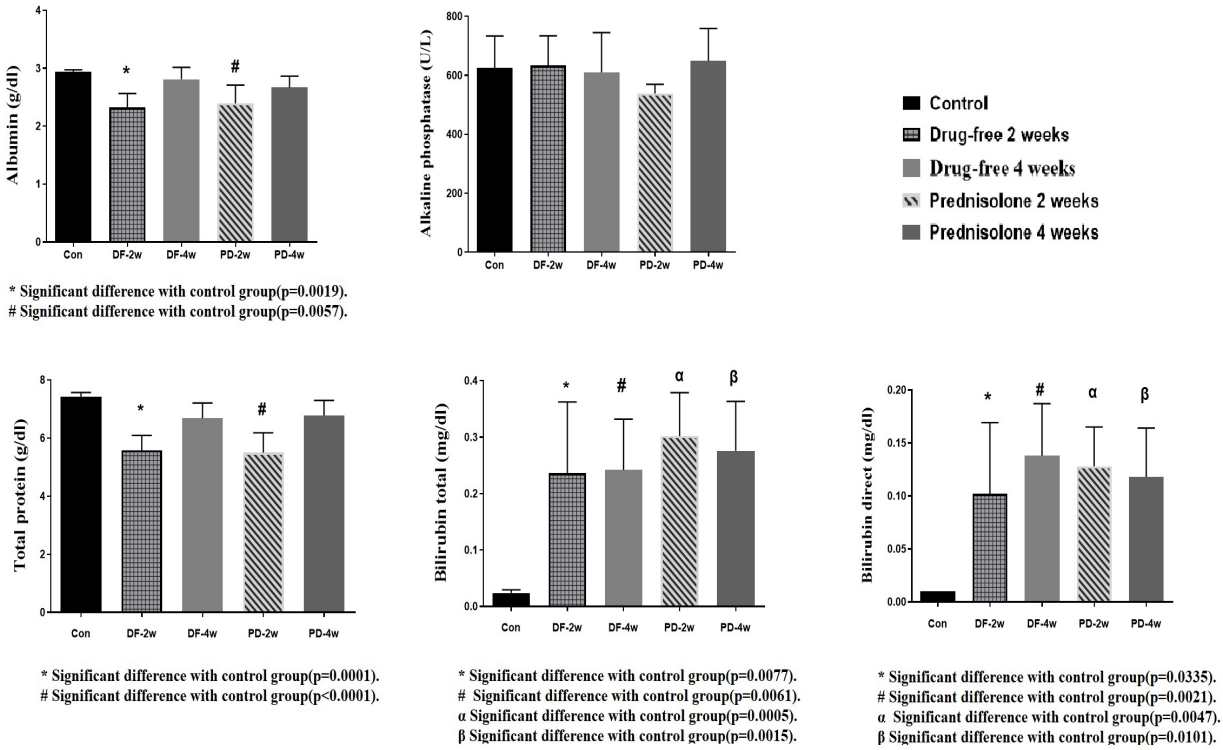

Figure 11: The serological assessments for liver function two and four weeks after scaffold transplantation.

Previously, prednisolone was loaded in scaffolds such as 3D printed poly (vinyl alcohol) [32], Hyaluronic Acid/Collagen [9], polylactic acid [33], and chitosan [34] for various aims. Mohammadi et al. (2018) implanted the drug-loaded scaffold in the rat Osteoarthritis model to reduce inflammation [9]. Prednisolone-loaded decellularized liver implanted into the liver reduced immune cell infiltration [10]. Prednisolone-loaded chitosan is also implanted into the artery to prevent restenosis while releasing a drug that inhibits cell proliferation [33]. The loading of prednisolone on decellularized scaffolds in the current study had no significant reverse effect on liver regeneration and hepatic function, so it can be used to reduce immune cell migration.

In a study, glycogen deposit was assessed by PAS staining, and glycogen accumulation in hepatocytes was found 72 h after partial hepatectomy [35]. However, in the other study, a decrease in glycogen deposition was detected in the animals executed portal vein ligation and partial hepatectomy after 1, 2, and 4 weeks [36]. Our data showed that the glycogen deposition was not changed after partial hepatectomy and scaffold transplantation, that may be due to the extent of the liver tissue removal, the time of sampling, or the compensatory effect of recellularized implant contained migratory or differentiated functional hepatocytes.

In conclusion, this study demonstrated that both drug-loaded and drug-free liver bioscaffolds were invaded by hepatocytes at different stages of differentiation two weeks after transplantation without being influenced by prednisolone. In addition, the results showed that loading prednisolone on grafts did not affect the production of ALP and proteins. Implantation of the decellularized liver with or without prednisolone is safe for the recipient's liver, while it can provide a promising scaffold for hepatocyte migration and liver reconstitution.

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CONFLICTS OF INTEREST: None to be declare.

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