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Three-dimensional Collagen Matrix Scaffold Implantation as a Liver Regeneration Strategy

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TITLE:

Three-dimensional Collagen Matrix Scaffold Implantation as a Liver Regeneration Strategy

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SUMMARY:

Liver diseases are induced by many causes that promote fibrosis or cirrhosis. Transplantation is the only option for recovering health. However, given the scarcity of transplantable organs, alternatives must be explored. Our research proposes the implantation of collagen scaffolds in liver tissue from an animal model.

ABSTRACT:

Liver diseases are the leading cause of death worldwide. Excessive alcohol consumption, a high-fat diet, and hepatitis C virus infection promote fibrosis, cirrhosis, and/or hepatocellular carcinoma. Liver transplantation is the clinically recommended procedure to improve and extend the life span of patients in advanced disease stages. However, only 10% of transplants are successful, with organ availability, presurgical and postsurgical procedures, and elevated costs directly correlated with that result. Extracellular matrix (ECM) scaffolds have emerged as

an alternative for tissue restoration. Biocompatibility and graft acceptance are the main beneficial characteristics of those biomaterials. Although the capacity to restore the size and correct function of the liver has been evaluated in liver hepatectomy models, the use of scaffolds or some kind of support to replace the volume of the extirpated liver mass has not been assessed.

Partial hepatectomy was performed in a rat liver with the xenoimplantation of a collagen matrix scaffold (CMS) from a bovine condyle. Left liver lobe tissue was removed (approximately 40%), and an equal proportion of CMS was surgically implanted. Liver function tests were evaluated before and after the surgical procedure. After days 3, 14, and 21, the animals were euthanized, and macroscopic and histologic evaluations were performed. On days 3 and 14, adipose tissue was observed surrounding the CMS, with no clinical evidence of rejection or infection, as was vessel neoformation and CMS reabsorption at day 21. There was histologic evidence of an insignificant inflammation process and migration of adjacent cells to the CMS, observed with the hematoxylin and eosin (H&E) and Masson's trichrome staining. The CMS was shown to perform well in liver tissue and could be a useful alternative for studying tissue regeneration and repair in chronic liver diseases.

INTRODUCTION:

The liver is one of the most important organs involved in maintaining homeostasis and protein production[1]. Unfortunately, liver disease is the leading cause of death worldwide. In advanced stages of liver damage, which include cirrhosis and hepatocellular carcinoma, liver transplantation is the clinically recommended procedure. However, due to the scarcity of donors and the low rate of successful transplants, new techniques in tissue engineering (TE) and regenerative medicine (RM) have been developed[2,3].

TE involves the use of stem cells, scaffolds, and growth factors[4] to promote the restoration of inflamed, fibrotic, and edematous organs and tissues[1,5,6]. The biomaterials used in scaffolds mimic the native ECM, providing the physical, chemical, and biological cues for guided cellular remodeling[7]. Collagen is one of the most abundant proteins obtained from the dermis, tendon, intestine, and pericardium[8,9]. Furthermore, collagen can be obtained as a biopolymer to produce two- and three-dimensional scaffolds through bioprinting or electrospinning[10,11]. This group is the first to report the use of collagen from a bone source for the regeneration of liver tissue. Another study reports the use of scaffolds synthesized from bovine collagen, which was obtained from skin, with homogeneous and closely situated pores, without any communication between them[12].

Decellularization preserves the native ECM, allowing the subsequent incorporation of cells with stem cell potential[13,14]. However, this procedure is still in the experimental phase in the liver, heart, kidney, small intestine, and urinary bladder from mice, rats, rabbits, pigs, sheep, cattle, and horses[3,14]. Currently, the resected liver mass volume is not replaced in any of the animal hepatectomy models. However, the use of additional support or network (biomaterials) that enables cell proliferation and angiogenesis could be essential for the prompt restoration of liver parenchymal functions. Thus, scaffolds could be employed as alternative approaches to

regenerate or repair tissue in chronic liver diseases, in turn, eliminating limitations due to donation and the clinical complications of liver transplantation.

PROTOCOL:

The present research was approved by the ethics committee of the School of Medicine (DI/115/2015) at the Universidad Nacional Autónoma de México (UNAM) and the ethics committee of the Hospital General de Mexico (CI/314/15). The institution fulfills all technical specifications for the production, care, and use of laboratory animals and is legally certified by national law (NOM-062-ZOO-1999). Male Wistar rats weighing 150–250 g (6–8 weeks old) were obtained from the Laboratory Animal Facility of the School of Medicine, UNAM, for this study.

1. Obtaining collagen matrix scaffolds from bovine femur

1.1. Obtain the condyle from bovine femur from a slaughterhouse certified by health and agricultural authorities of Mexico.

1.1.1. Carefully dissect the condyle fat, muscle, and cartilage with a surgical instrument. Cut the condyle fragments into 3 cm x3 cm fragments using a saw cutter and clean the fat and blood with a towel. Wash the condyle fragments with water.

1.1.2. Boil (92 °C) the fragments with 1 L of an anionic detergent (10 g/L) for 30 min. Wash the condyle fragments twice to remove any remnants of the anionic detergent.

1.1.3. Dry the condyle fragments for 3 h using filter paper (0.5 mm).

1.2. Prepare a triangular (1 cm x1 cm x1 cm) CMS of thickness 0.5 cm from the fragments mentioned in step 1.1 (**Figure 1A**).

1.2.1. Demineralize the fragments in 100 mL of 0.5 M HCl for 10 min with constant agitation. Remove the HCl.

NOTE: Neutralize the HCl with sodium hydroxide (10 M).

1.2.2. Rinse the fragments three times with 100 mL of distilled water, 15 min each time, with constant agitation. Dry the CMS with filter paper (0.5 mm) for 1 h.

1.2.3. Use a stereo-microscope[15] to analyze the structural properties of the CMS (size of pores, pore formation, and porous interconnection) (**Figure 1B**).

1.2.4. Use a scanning electron microscope[16] to analyze the rough surface of the CMS trabeculae (**Figure 1C**).

1.2.5. Use the dissection instrument for blending and stretching to evaluate the mechanical changes (plasticity and flexibility) of the CMS (**Figure 1D**).

1.2.6. Pack the CMS into the sterilization pouch and sterilize it with hydrogen peroxide plasma for 38 min. Store the sterile CMS in the original pack in a dry area at 20–25 °C until use.

2. Preparation of the surgical area and handling and preparation of the animal model

2.1. Sanitize the surgical area, worktable, microsurgery microscope, and seat with 2% chlorhexidine solution. Sterilize all surgical instruments, surgical sponge, swabs, and disposable surgical drape through heat sterilization (121 °C/30 min/100 kPa)

2.2. Assign the rats (n=5) into three groups of five rats per group: 1. sham, 2. hepatectomy, and 3. hepatectomy plus CMS, and follow all the groups on days 3, 14, and 21 days.

2.2.1. Administer ketamine (35 mg/kg) and xylazine (2.5 mg/kg) intramuscularly in the hind limb.

NOTE: The sedative period typically lasts for 30–40 min.

2.2.2. Shave the abdominal skin (5 cm x 2 cm) using surgical soap and a double-edged blade, and disinfect the skin using topical 10%povidone-iodine solution in two rounds.

2.2.3. Place the animal on a warm plate in the decubitus dorsal position, with the neck hyperextended to maintain a permeable airway **(Figure 2)**.

2.2.4. Evaluate the depth of the anesthesia through the respiratory pattern and loss of the withdrawal reflex in the limbs.

2.2.5. Place a disposable surgical drape around the shaved skin and make an incision (2.5 cm) on the albous line with a scalpel, using the xiphoid process as a reference point. Avoid the abdominal wall blood vessel to prevent bleeding.

2.2.6. Put the abdominal retractor in place and observe the abdominal cavity. Using the dissection forceps, extract the left liver lobe and place it on the metal plate **(Figure 3A)**.

NOTE: In the sham group, only extract the left liver lobe and then return the liver to the abdominal cavity. Suture the abdominal wall and skin with a 3-0 nylon suture.

2.2.7. In the experimental groups with and without CMS, use a scalpel blade and sterile scalpel blade (#15) to perform hepatectomy (approximately 40%) with two cuts. Use a triangular metallic template (1 cm x 1 cm x 1 cm) to perform the hepatectomy **(Figure 3B)**.

2.2.8. To prevent bleeding of the liver, maintain surgical compression with a swab on the edge

of the liver for 5 min.

2.2.9. Hydrate the CMS in sterile saline solution for 20 min before the surgical procedure. Implant the CMS in the hepatectomy site with four stitches sutures between the liver tissue and the CMS to prevent displacement of the biomaterial. Use 7-0 non-absorbable polypropylene sutures **(Figure 3C)**.

NOTE: Do not remove the sutures in a second surgery; sutures can be used as a reference to identify the site of CMS implantation.

2.2.10. Return the liver to the abdominal cavity and suture the abdominal wall and skin with a 3-0 nylon suture. Clean the surgical incision with a surgical iodine-soaked sponge in two rounds. Observe and monitor the vital signs of the animals.

3. Postoperative care

3.1. Administer meglumine flunixin (2.5 mg/kg) intramuscularly in the hind limb.

3.2. For anesthesia recovery, place the animals in individual polycarbonate boxes with laboratory animal bedding in a noise-free area with temperature control (23 °C).

3.3. Observe the recovery of the animals and monitor their water and food consumption for 2 h.

4. Evaluation of liver function in serum

4.1. Collect blood samples (500 µL) from the lateral tail veins of the sedated animals before the surgical procedure (baseline values) and at evaluation days 3, 14, and 21.

4.2. Centrifuge the blood samples at 850 × *g*/10 min at room temperature (23 °C); separate the serum and store it at -80°C until use.

4.3. Perform a panel of liver function tests: serum albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TB), and direct bilirubin (DB) (**Table 1**).

5. Euthanasia and tissue management

5.1. Sedate the animals for each evaluation (days 3, 14, and 21) by following the protocol described above.

5.2. Perform an incision (5–6 cm) on the **albous line** with a scalpel to observe the organs of the abdominal cavity and take photographs of the hepatectomy area of the sham and the experimental groups, with and without the CMS.

5.3. Take liver samples (2 cm x 2 cm; 0.40–0.45 g) from all the study group animals and place them in a 4% formaldehyde solution for 24 h for subsequent histological evaluation.

6. Histological analysis

6.1. Preserve the liver tissues in 4% formaldehyde and dehydrate the tissue using a series of alcohol concentrations (60%, 70%, 80%, 90%, 100%); place them in xylene (1h) and embedded them in paraffin[16].

6.2. Cut the paraffin blocks with a microtome into 4 µm-thick sections for the preparation of eight slides.

6.3. Perform H&E and Masson's trichrome staining[16].

6.4. Observe the stained sections under a light microscope to select the representative areas of the liver, with and without CMS. Obtain photomicrographs at 4x, 10x, and 40x magnification and process the images using appropriate software[17] (see the **Table of Materials**).

REPRESENTATIVE RESULTS:

Bone demineralization affects the mechanical properties of CMS without altering the original shape or interconnection of its pores**.** CMS can have any shape, and therefore, can be adjusted to the size and shape of the selected organ or tissue[18]. In the present protocol, we used a triangular CMS (**Figure 1A–D**). A rat model was used to evaluate the regenerative capacity of the CMS xenoimplant in the liver. Although the liver is a friable and soft organ, the surgical procedure performed in this protocol ensured that the CMS remained in place (**Figure 2** and **Figure 3A–C**). The partial hepatectomy of 40% of the left lobe enabled a portion of the organ to be assessed, keeping the rest of the liver intact, so that the changes in the implant and the native parenchyma could be compared. Additionally, we obtained blood samples to evaluate liver function before and after CMS implantation.

There were no differences in baseline concentrations of the biochemical parameters between the sham group and the experimental group with and without CMS implantation at 3 and 14 days; they remained within the reference values (**ALB**: 0.43–2.41 g/dL; **ALP**: 134–357.3 U/L; **ALT**: 41–83.1 UI/L; **AST**: 61.4–276.2 UI/L; **TB:** 0.01–0.43 mg/dL; **DB**: 0.1 mg/dL)[19]. In addition, there were no differences in the albumin, ALP, ALT, AST, TB, and DB levels between the sham group and the experimental groups at 21 days, indicating that the CMS does not disrupt liver function (**Table 1**).

During euthanasia, exploratory laparotomy revealed the typical color and correct size and shape of the liver. No inflammation or infection was observed at the implantation site in any of the cases. On day 3, there were no changes in the organ in relation to the abdominal cavity (**Figure 4A**). In a portion that contained liver tissue and CMS, blood was observed to have infiltrated the CMS, with incipient adherent omental fat (**Figure 4B**). On day 14, the amount of

fat increased; there was blood vessel neoformation and the integration of the CMS into recipient liver tissue but no changes in the small intestine or organs of the abdominal cavity (**Figure 5A**). Omental fat was higher in the anterior zone (**Figure 5B**) compared with the visceral zone (**Figure 5C**) in the CMS implantation site. On evaluation day 21, CMS incorporation into the liver was more evident (**Figure 6A**). The increase in omental fat could be used as an indicator of the progress of regeneration, as it is a source of mesenchymal stem cells[20]. Moreover, at 21 days, the liver presented dense areas that corresponded to degradation and absorption, changing the size and original shape (**Figure 6B**).

To analyze the microstructure of the liver with the CMS implant, we performed histological analysis of a representative fragment of tissue from the implantation site, which was compared with tissue from the right liver lobe (control). Biopsies performed on days 3, 14, and 21 were processed and subjected to H&E and Masson's trichrome staining. The normal structure of the liver parenchyma was observed in the sham group at day 21 (**Figure 7A** and **Figure 7E**). On day 3, the histological analysis showed that the presence of the CMS in the liver did not promote a foreign body reaction, and the conspicuous presence of lax connective tissue was observed (**Figure 7B** and **Figure 7F**). On days 14 and 21, the lax connective tissue was more abundant, with the CMS trabeculae surrounded by hepatocytes, suggesting that hepatocytes had migrated to the CMS (**Figure 7C** and **Figure 7G**; **Figure 7D** and **Figure 7H**). The results also showed areas of hepatocytes that were irrigated by a vessel (**Figure 8A**). A close inspection revealed that the hepatocytes that had adhered to the CMS trabeculae were sometimes surrounded by lax connective tissue (**Figure 8B**–**D**). Two double-blinded independent pathologists performed the histopathological analysis. Immunohistochemistry and polymerase chain reaction-based assays are underway to evaluate the different proteins and genes related to the hepatic regeneration process.

Thus, the macroscopic and microscopic observations and the biochemical values support our proposition that the CMS is an ideal biomaterial for supporting and promoting liver regeneration. Nevertheless, CMS implantation must be evaluated for more than 21 days to determine its absorption time and complete liver tissue restoration. In addition, studies that evaluate the different proteins and genes related to hepatic regeneration in the presence of the CMS should be conducted.

FIGURE AND TABLE LEGENDS:

Figure 1: Bone specimen and the CMS. (**A**) A triangular sample of the bone specimen was utilized to prepare the CMS. (**B**) The CMS, as seen under a stereo-microscope. (**C**) A detailed view of the pores and interconnection or trabecular connection of the CMS, as observed by electron microscopy. (**D**) CMS manipulation with an instrument was carried out to verify the modification of mechanical properties. Abbreviation: CMS = collagen matrix scaffold.

Figure 2: Preparing the animal for surgery. The animal is in the decubitus dorsal position, showing the shaved abdominal area, prepared for hepatectomy and CMS implantation. Abbreviation: CMS = collagen matrix scaffold.

Figure 3: Hepatectomy of the left lobe of the liver. (**A**) The left lobe was extracted and placed on the metallic plate to perform the hepatectomy. (**B**) Hepatectomy of 40% of the left lobe was carried out in a triangular shape, like the CMS. (**C**)The area of the hepatectomy is replaced by the CMS. Abbreviation: CMS = collagen matrix scaffold.

Figure 4: Day 3 of evaluation. (**A**) Macroscopic observation of the implantation site on evaluation day 3. The liver implanted with (filled star) the CMS (dotted line) did not change in color or size. The small intestine (empty star) and the rest of the abdominal structures showed no alterations. (**B**) Sample of the liver (filled star) and the CMS (white arrow) covered with omental fat (red arrow). Abbreviation: CMS = collagen matrix scaffold.

Figure 5: Day 14 of evaluation. (**A**) Macroscopic observation of the implantation site on evaluation day 14. The liver tissue (filled star) with the CMS did not change in color or size. The small intestine (empty star) and the rest of the structures and organs of the abdominal cavity showed no alterations. Sample of the liver with the implanted CMS: (**B**) anterior view; (**C**) posterior/visceral view. Omental fat (red arrow) covering the area. The CMS was integrated into the liver tissue. The omental fat (red arrow) mainly covers the area in the anterior view. The dotted line indicates the site of CMS implantation; sutures (blue thread). Abbreviation: CMS = collagen matrix scaffold.

Figure 6: Day 21 of evaluation. (**A**) Macroscopic observation of the implantation site at evaluation day 21. The recipient liver (filled star) implanted with the CMS (dotted line) did not change color or size. The small intestine (empty star) and the rest of the abdominal structures showed no alterations. (**B**) Sample of the liver with the implanted CMS (dotted line) showing changes in size and shape. Abbreviation: CMS = collagen matrix scaffold.

Figure 7: Histology of the liver and the liver with CMS. (**A**) Normal liver (SHAM) showing the characteristic tissue architecture. (**B**) Day 3 of CMS implantation showing lax connective tissue in the trabeculae of the CMS (dashed ovals). (**C**) Transition zone between the native liver (right side) and the CMS (left side), with the trabeculae of the CMS (arrow). (**D**) Native liver in contact with the CMS (dotted lines) at day 21. (**E**) Normal liver with Masson's trichrome stain. (**F**) The CMS implanted in the liver; invasion of lax connective tissue at day 3 (dashed oval). (**G**) On day 14, native liver with the CMS trabeculae, showing an area of hepatocytes (filled star) outside the native liver, indicating that the native hepatocytes had migrated through the CMS. (**H**) On day 21, the native hepatocytes have migrated toward the CMS (arrow). **A** and **E:** Scale bars = 200 µm: 4x, **B–D** and **F–H**: Scale bars = 100 µm: 10x. **A–D:** H&E staining; **E–H:** Masson's trichrome staining. Abbreviations: CMS = collagen matrix scaffold; H&E = hematoxylin and eosin.

Figure 8: Histology of the liver and CMS. (**A**) Pool of hepatocytes (white arrow) present in the trabeculae of the CMS (black arrow). The hepatocytes are irrigated by a vessel (filled star). (**B**) Hepatocytes (white arrow) have migrated and adhered to the trabeculae of the CMS (black arrow). (**C**) A group of hepatocytes (dashed circle) are observed between two trabeculae (black arrows) of the CMS. (**D**) Hepatocytes have adhered to trabeculae (black arrows) of CMS in the

presence of lax connective tissue (dashed oval). **A** and **C**: Scale bars = 100 µm: 10x. **B** and **D:** Scale bars = 20 µm: 40x. Abbreviation: CMS = collagen matrix scaffold.

Table 1: Liver function. Liver function tests were performed for the sham and experimental groups with and without CMS at 3, 14, and 21 days. Abbreviations: CMS = collagen matrix scaffold; ALB = Albumin; ALP = Alkaline phosphatase; ALT= Alanine aminotransferase; AST = Aspartate aminotransferase; TB = Total bilirubin; DB = Direct bilirubin.

DISCUSSION:

Organ transplantation is the mainstay of treatment in patients with liver fibrosis or cirrhosis. A few patients benefit from this procedure, making it necessary to provide therapeutic alternatives for patients on the waiting list. Tissue engineering is a promising strategy that employs scaffolds and cells with regenerative potential[2,4,13]. The removal of a portion of the liver is a critical step in this procedure because of the profuse bleeding of this vascularized organ. Therefore, hemostasis of the surgical bed must be performed to prevent this complication. Furthermore, the binding of the liver tissue to the CMS, which is essential for ensuring tissue-biomaterial interaction, is facilitated through the use of sutures. However, it must be done carefully to avoid tearing the liver and causing later bleeding.

Although the biomaterial proposed herein is of bovine origin (xenogenic), there are no data of biomaterial reaction in rats, making selective hepatectomy possible. In contrast, the 2/3 hepatectomy model has been shown to cause the death of the animals because of the removal of a large amount of liver tissue[14]. Moreover, we developed a stainless steel metallic template because we had difficulty standardizing the size in the hepatectomy and the CMS. Sterilization of the CMS was challenging because the existing techniques modified the structure of collagen and its biochemical properties. Hence, sterilization by heat, gamma radiation, and ethylene oxide was explored as sterilization methods before determining that the plasma of hydrogen peroxide was the optimal technique[13].

It is essential to determine the maximum size of the CMS that could be implanted in the liver and evaluate the biological response at a systemic level. Further, it is necessary to optimize and investigate the efficacy of this technique in a larger animal species. Furthermore, it is important to investigate the effects of the CMS implantation in the liver for a longer period (>30 days), which will allow the assessment of the extent of the biosorption of the CMS and the regeneration of the hepatic tissue. Further, the effects of the CMS implantation must be examined in animal models with liver damage. This implantation method has opened the door to strategies exploring the restoration of the shape and volume of resected tissue, thereby reducing anesthesia, surgical duration, and recovery time[4,13].

The CMS was obtained from a natural source and preserved its physical and chemical properties compared to synthetic biomaterials made using complex methodologies, such as bioprinting or electrospinning, which are not biocompatible or bioabsorbable[2,3]. The primary component of this CMS is collagen type I, which is the main protein of the ECM that enables cell adhesion and proliferation[21]. Additionally, the trabeculae of the CMS pores enable cell

migration and the continuous flow of growth factors, blood, and other mediators of the regeneration process. According to the tissue to be repaired, this research group has experience designing the CMS in different shapes, preserving its 3D structure. For example, cylindrical CMS were implanted in dog urethra and bile duct in pigs and yielded promising results in tissue regeneration[22,23].

The implantation of this CMS could be an alternative treatment to stimulate tissue regeneration and restore the fibrogenesis–fibrolysis balance in liver cirrhosis due to different etiologies (e.g., virus, alcohol, metabolic factors). Proliferation, migration, and inflammation assays must be performed to identify the molecular and cellular mechanisms triggered by the CMS. In conclusion, this paper describes a reproducible procedure for hepatectomy as well as the examination of the regeneration process through the xenoimplantation of biomaterials.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

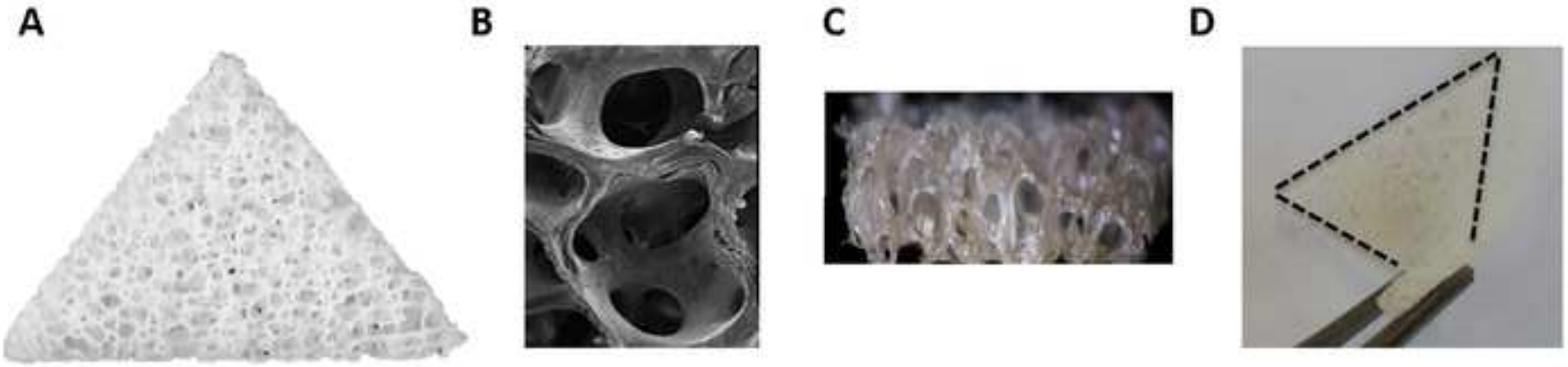

Figure 2

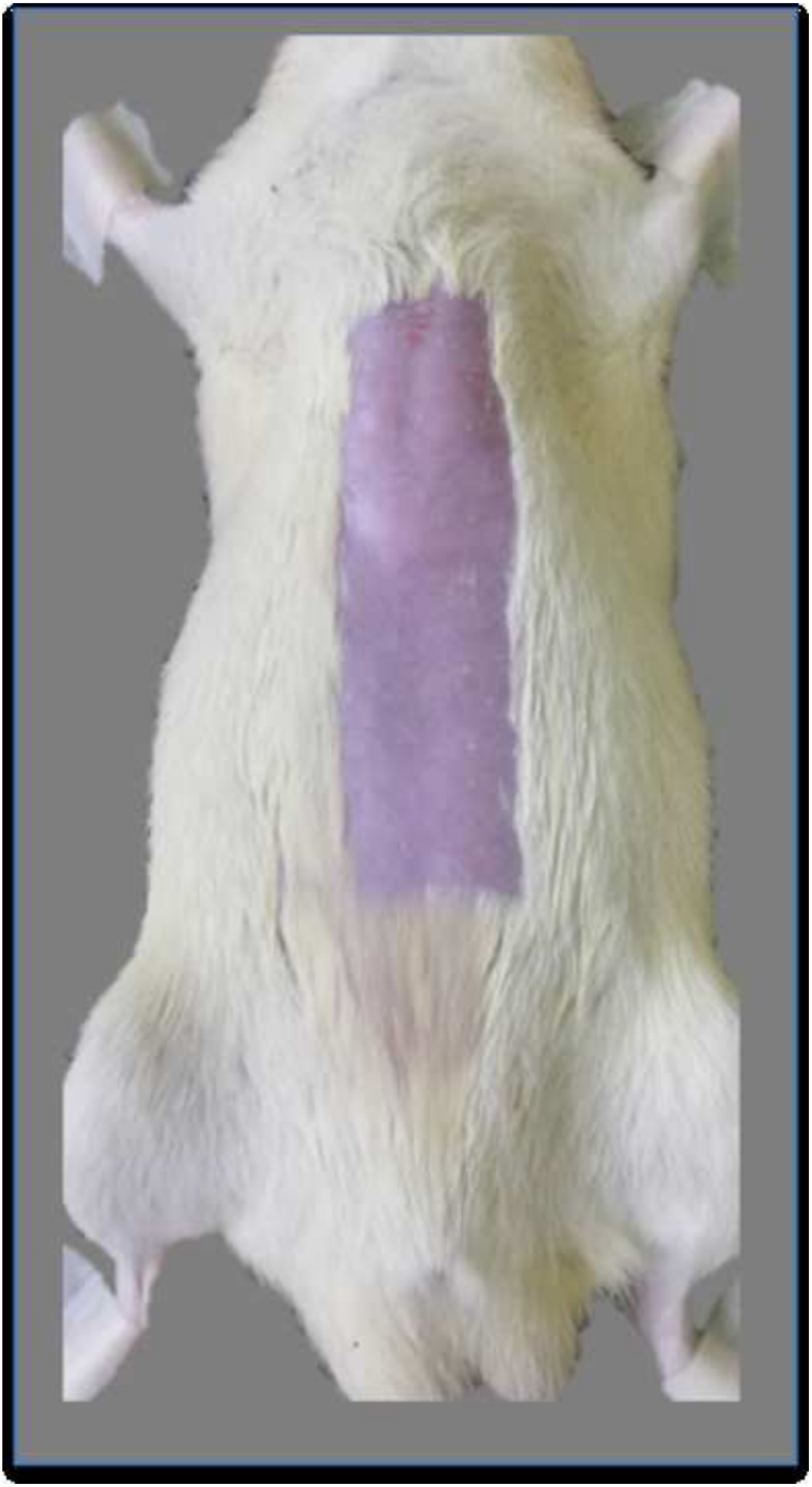

Figure 3

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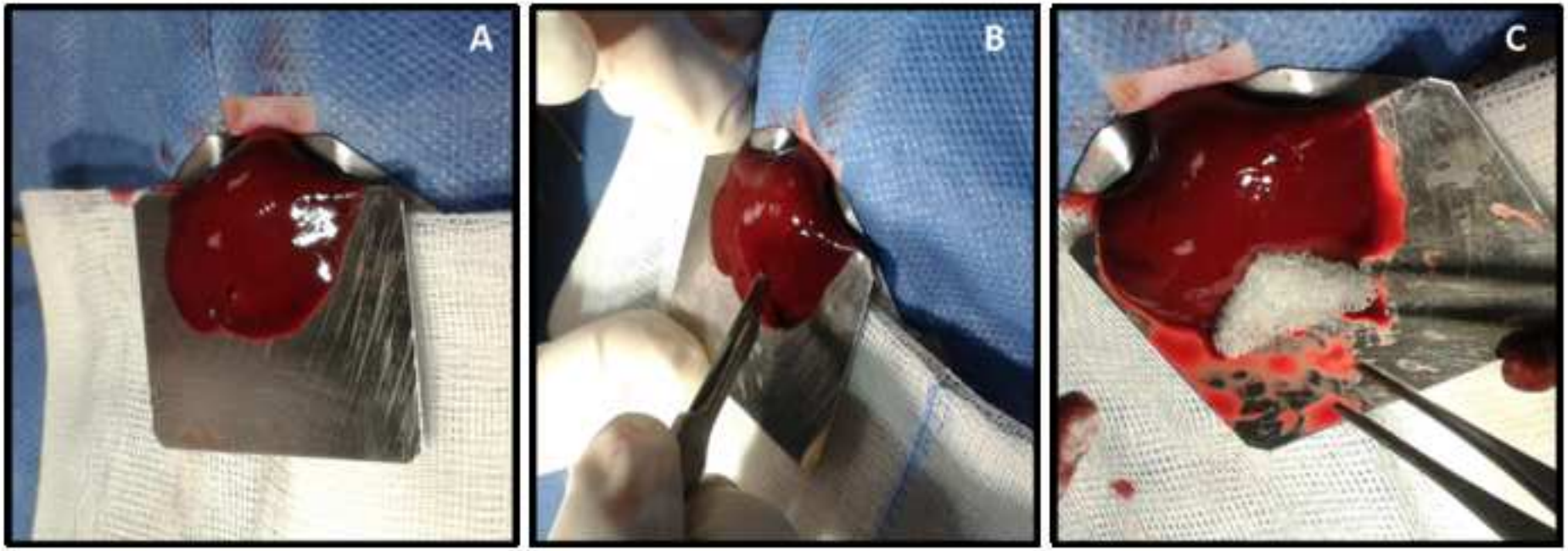
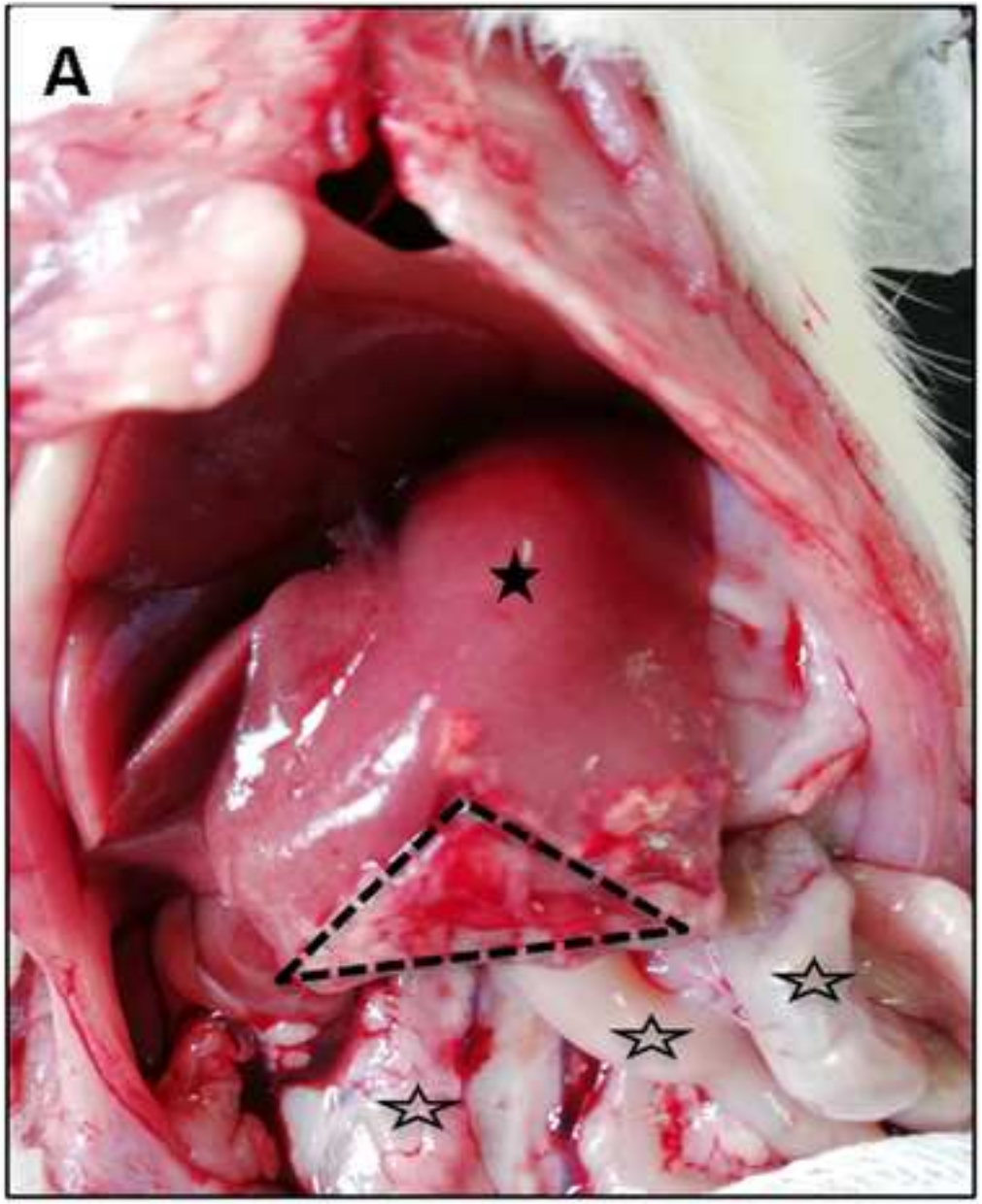


Figure 4



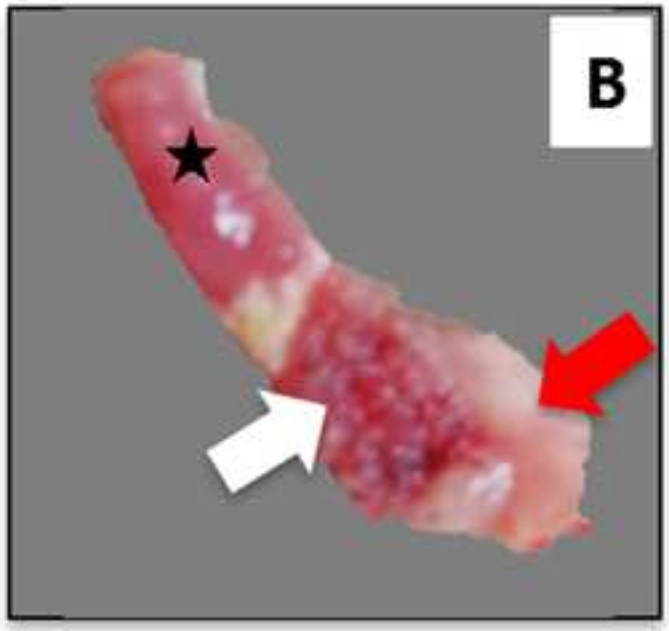


Figure 5

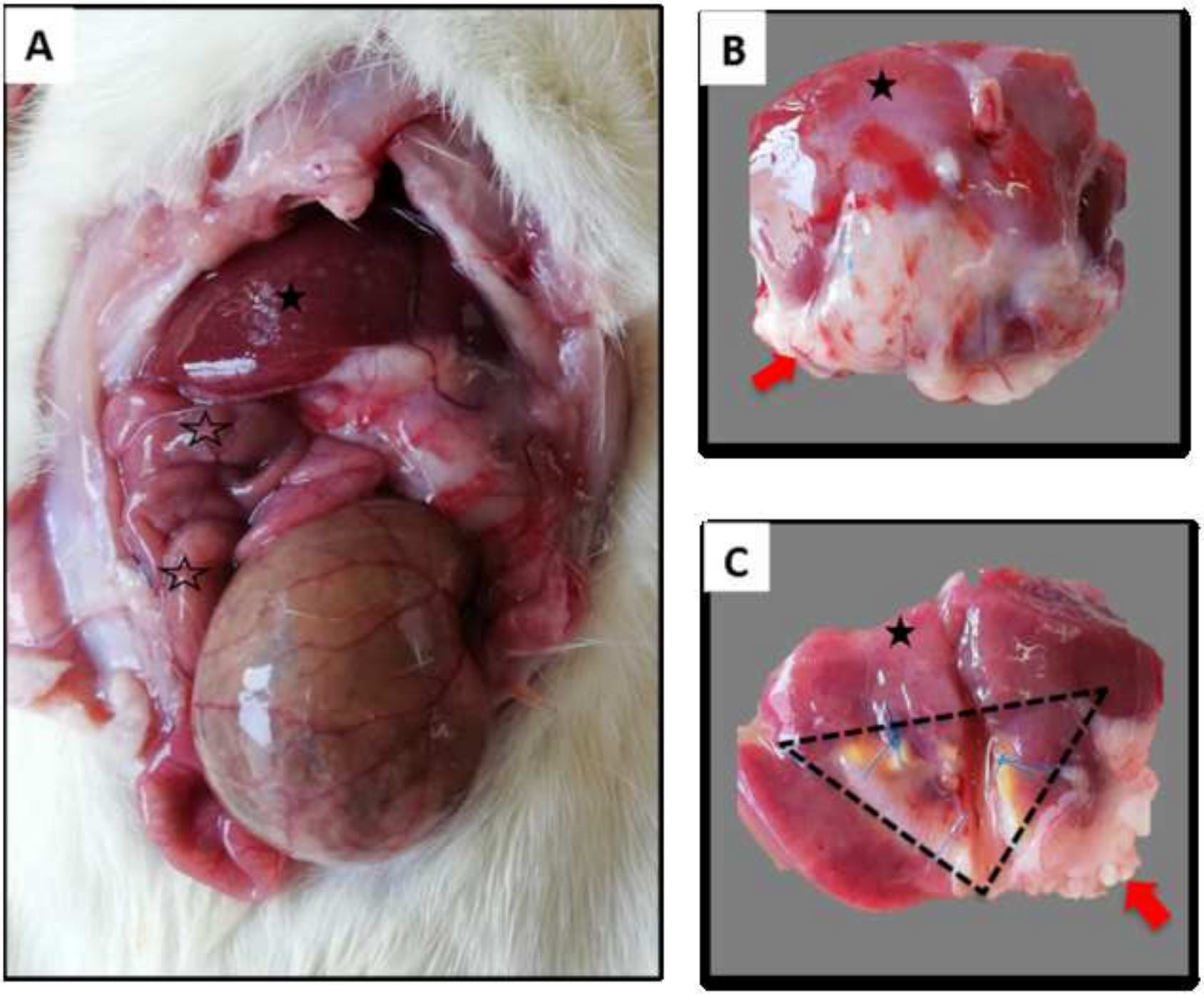
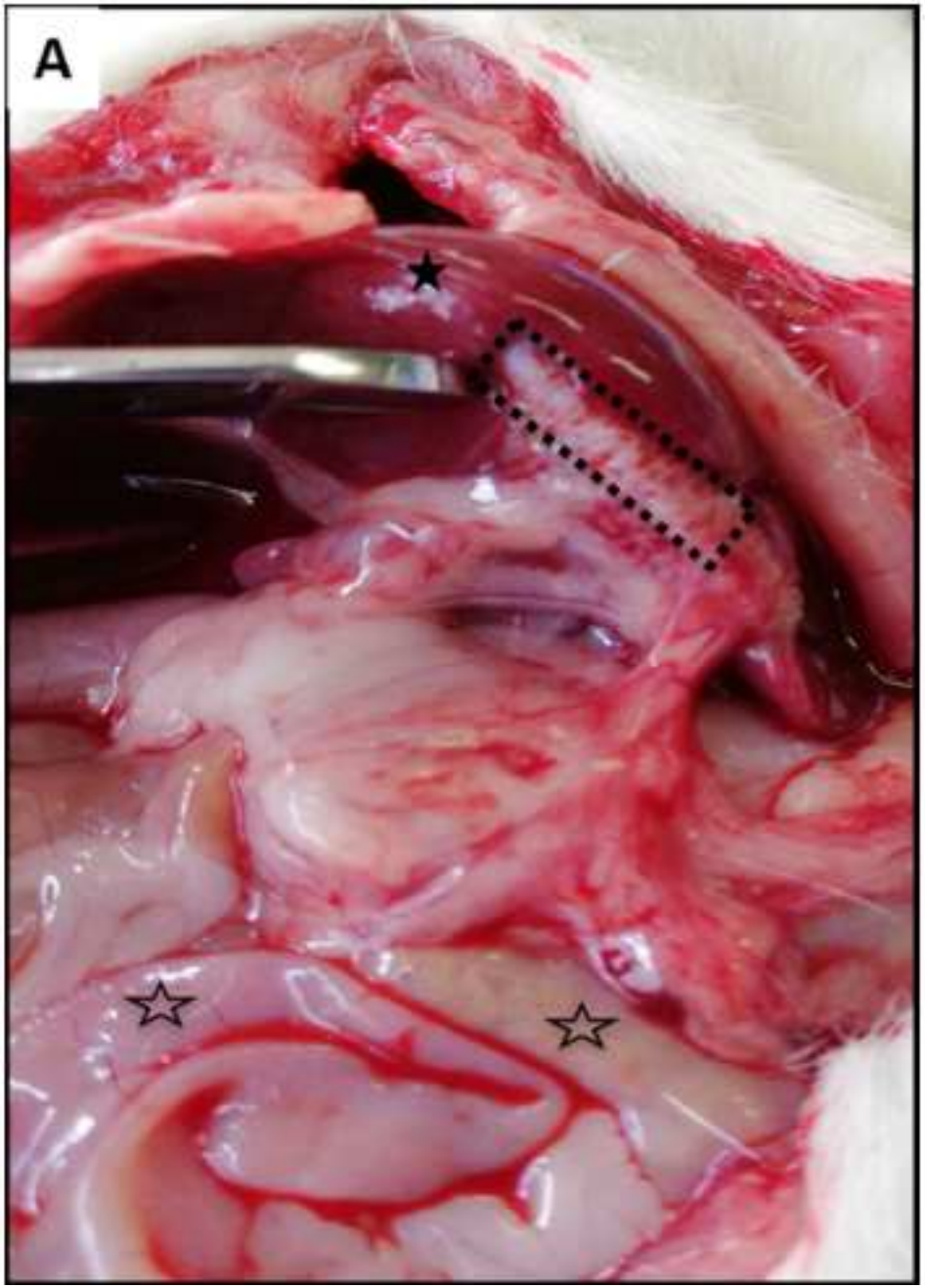


Figure 6



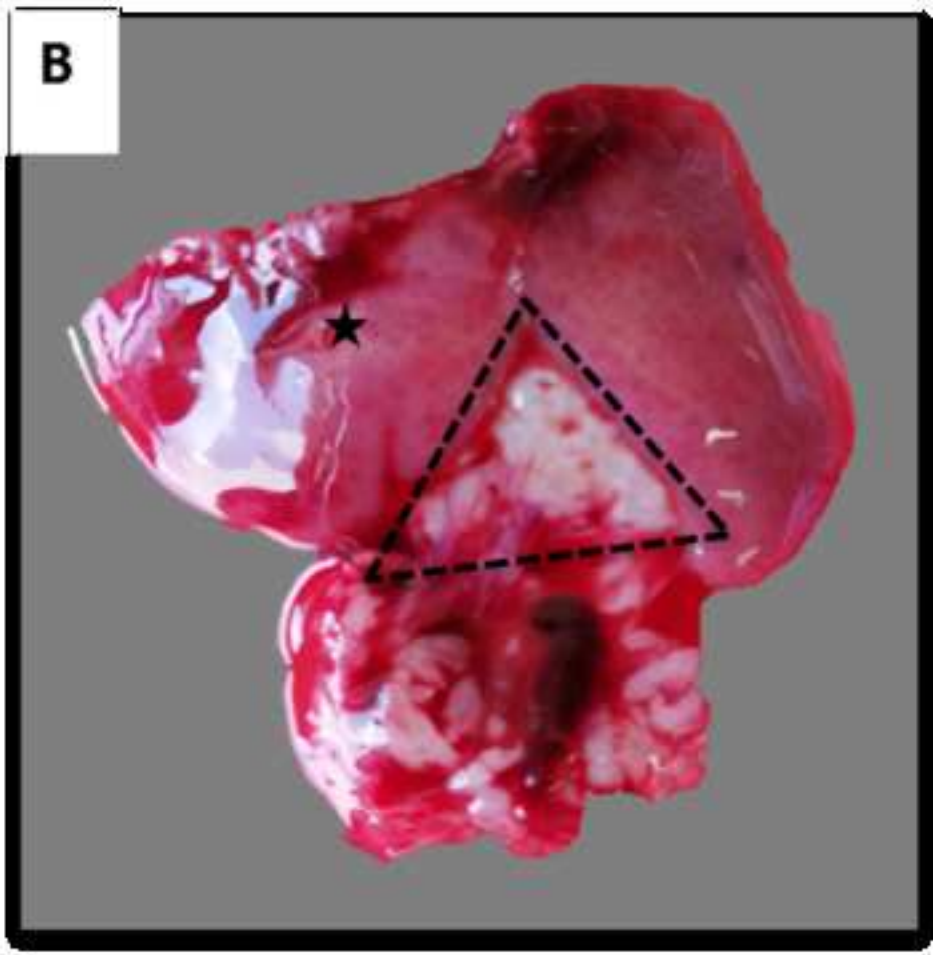


Figure 7

Click here to access/download;Figure;Figure 7.tif

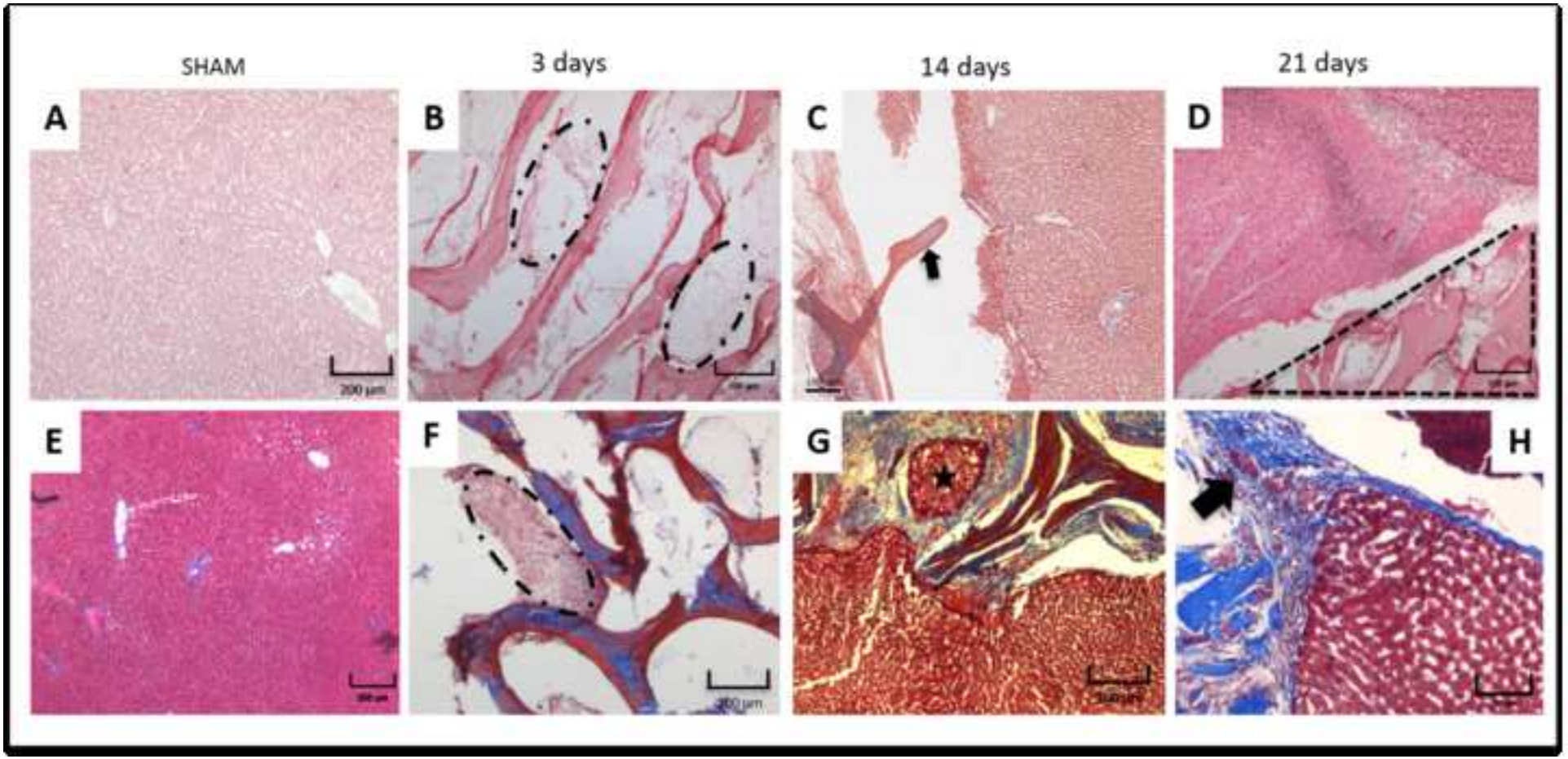


Figure 8

Click here to access/download;Figure;Figure 8.tif

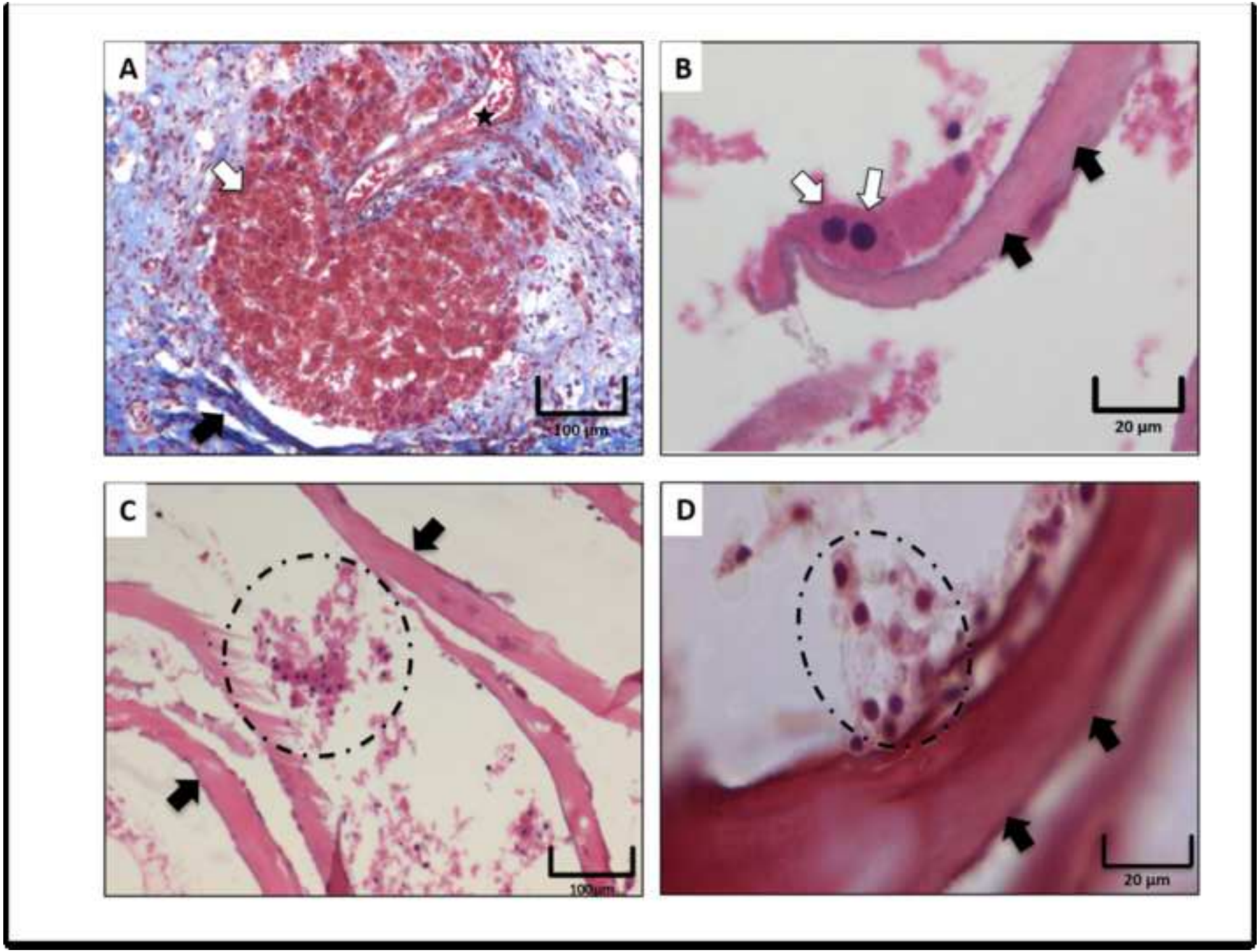


Table 1. Values of liver function

	Days	SHAM	Hepatectomy	Implantation CMS
ALB (g/dL)	3	1.4±0	1.25±0.07	1.4±0
	14	1.3±0.5	1.85±0.07	1.8±0.14
	21	1.3±05	1.6±0.1	1.7± 0
ALT (IU/L)	3	73±1.4	3.5±0.7	54.6±6.4
	14	84±0	59.5±4.9	57±4.2
	21	57±0	73.5±14.8	73.5±14.8
AST (IU/L)	3	106±1.4	80±6.6	90±1.4
	14	127±5.5	94±21.2	83.5±17.6
	21	123.7±27.3	92.5±24.7	101±31.1
TB (mg/dL)	3	0.33±0.05	0.25±0.07	0.3±0
	14	0.5±0	0.2±0	0.15±0
	21	0.3±0	0.4±0	0.4±0.14
DB (mg/dL)	3	0.1±0	0.05±0.07	0.1±0
	14	0.1±0	0.1±0	0.1±0
	21	0.1±0	0.1±0	0.1±0

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Anionic detergent	Alconox	Z273228	
Biopsy cassettes	Leica	3802453	
Camera DMX	Nikon	DXM1200F	
Centrifuge	Eppendorf	5424	
Chlorhexidine gluconate 4%	BD	372412	
Cover glasses 25 mm x 40 mm	Corning	2980-224	
Eosin	Sigma-Aldrich	200-M	CAS 17372-87-1
Ethyl alcohol, pure	Sigma-Aldrich	459836	CAS 64-17-5
Flunixine meglumide	MSD	Q-0273-035	
Glass slides 75 mm x 25 mm	Corning	101081022	
Hematoxylin	Merck	H9627	CAS 571-28-2
Hydrochloric acid 37%	Merck	339253	CAS 7647-01-0
Ketamine	Pisa agropecuaria	Q-7833-028	
Light microscopy	Nikon	Microphoto-FXA	
Microtainer yellow cape	Beckton Dickinson	365967	
Microtome	Leica	RM2125	
Model animal: Wistar rats	Autónoma de México		
Nylon 3-0 (Dermalon)	Covidien	1750-41	
Polypropylene 7-0	Atramat	SE867/2-60	
Povidone-iodine10% cutaneous solution	Diafra SA de CV	1.37E+86	
Scaning electronic microscopy	Zeiss	DSM-950	
Sodium hydroxide, pellets	J. T. Baker	3722-01	CAS 1310-73-2
Software ACT-1	Nikon	Ver 2.70	
Stereoscopy macroscopy	Leica	EZ4Stereo 8X-35X	
Sterrad 100S	Johnson and Johnson	99970	
Surgipath paraplast	Leica	39601006	
Synringe of 1 mL with needle (27G x 13 mm)	SensiMedical	LAN-078-077	
Tissue Processor (Histokinette)	Leica	TP1020	
Tissue-Tek TEC 5 (Tissue embedder)	Sakura Finetek USA	5229	

Trichrome stain kit	Sigma-Aldrich	HT15	
Unicell DxC600 Analyzer	Beckman Coulter	BC 200-10	
Xylazine	Pisa agropecuaria	Q-7833-099	
Xylene	Sigma-Aldrich	534056	CAS 1330-20-7

Authors: We are grateful for you comments that undoubtedly improve the quality of the manuscript.

Editorial comments:

Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Answer: The manuscript was revised by a native English speaker.

2. Please provide an email address for each author.
Answer: We added email address of all the authors. Also, it is very important to mention that in the first version we forgot included a co-author Jorge García-Loya: galj@unam.mx, which participated in the manufacture and design of Collagen Matrix Scaffold. All the co-authors are agreeing to include this co-author in the manuscript.

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Gabriela Gutiérrez-Reyes: gabgurey@yahoo.com.mx

3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).
Answer: We changed the style of the references in accordance with the editorial instructions

4. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
Answer: We included the ethics statement in line 73-77

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Alconox®, Nikon, etc.
Answer: We removed all the trademark symbols (™), registered symbols (®), and company names from in the entire manuscript.

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.
Answer: We correctly adjusted numbering in the protocol section.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be

described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Answer: Thank you very much. We have rewritten all the sentences of the protocol section in the imperative tense.

8. Please add more details to your protocol steps. Please ensure you answer the “how” question for each step, i.e., how is the step performed?

Answer: We added more details to protocol steps, as indicated.

9. How do you obtain the bovine femur?

Answer: Bovine bone is obtained from a healthy animal, with no zoonotic diseases (i.e. tuberculosis, bovine spongiform encephalopathy). The femoral condyle is obtained from the meat trail, certified by the Mexican Health and Agriculture authorities.

The meat trail only provides either the proximal (blue circle) or distal femoral condyle (red circle).

Figure of the bovine femur

Figure of bovine femur. Proximal (blue circle) and distal femoral condyle (red circle).

10. 1: What do you visually look for when checking under the CMS under stereo-microscope or EM? How do you assess the plasticity and flexibility and why?

Answer: We evaluated the porosity of the CMS utilizing a stereoscopic microscope: the size, shape, and arrangement of the pores.

The characterization of the rough surface of the trabeculae of the pore and their interconnectivity were evaluated by electronic microscopy.

The plasticity and flexibility are evaluated through manipulation of the CMS with a dissection forceps as shown in figure 1D.

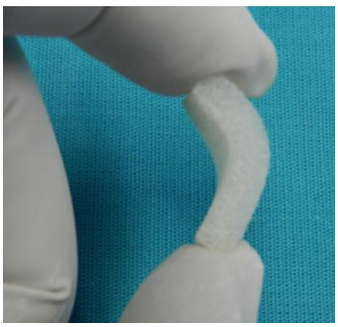


Figure showing manipulation of the CMS.

11. 2: How do you sterilize all the instruments? How do you check the depth of anesthesia? How do you shave? How many rounds of ethanol -iodine scrub is performed? How do you maintain sterility?

Answer: The CMS are placed in special bags for hydrogen peroxide plasma sterilization. Line 104-105

The depth of anesthesia was evaluated with the respiratory pattern and sensitivity to pain in the limbs. Line 124-125

We shaved the abdominal skin using surgical soap and a double-edged blade. We also disinfected the skin with two rounds of topical 10% povidone-iodine solution. Line 120-121. We didn't use ethanol-iodine scrub to disinfect the skin.

The surgical area and all material were sterilized with chlorhexidine solution 2% and through heat sterilization, respectively. Line 110-113

All procedures were carried out in the operating room and the personnel followed the safety and hygiene protocols, reducing the risk of contamination.

12. Please expand the hepatectomy procedure to include all action associated with it. How long can you store the CMS and how?

Answer: We included more details about the hepatectomy procedure in the protocol section.

As to CMS storage, after sterilization, the CMS were kept in their original sterilization pouch until use.

13. 5: What is the timeline for performing blood draw? How do you perform the blood draw?

Answer: The blood sample was taken from the lateral tail vein (500 uL) in the sedated animals, prior to the surgical procedure (baseline values) and at days 3, 14, and 21 of evaluation of the model. Line 163-165

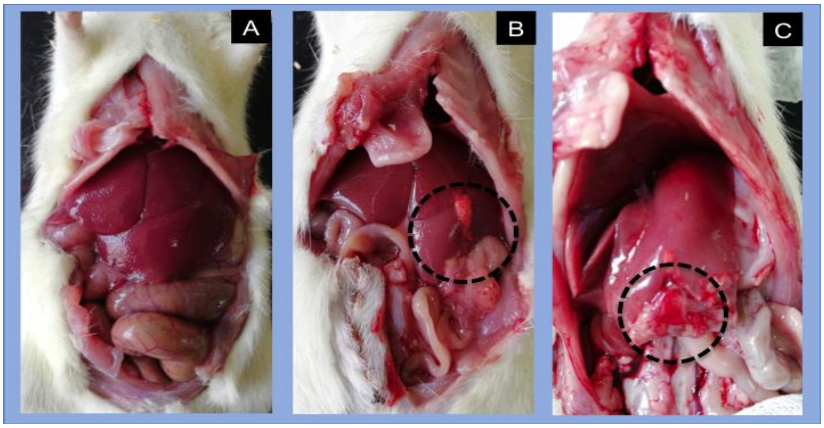
With a 1 mL syringe we drew 500 uL of blood, then removed the needle (27G) and transferred the blood into microtainer tubes, after which the tubes were centrifuged at 850g / 10min, the serum was separated, and then stored at -80ºC, until use.

14. What is the difference between sham operated and experimental group? Do you also perform this procedure in animals having liver damage?

Answer: In the sham group only the left liver lobe was extracted and then returned to the abdominal cavity, suturing the abdominal wall and skin with a 3-0 nylon suture. Line 132-133

In the experimental groups, with and without CMS, we performed hepatectomy (approximately 40%), with two cuts in the liver in a triangular shape, using a scalpel and sterile scalpel blade. For CMS implantation at the hepatectomy site, we made four stitches between the liver tissue and the CMS, to prevent biomaterial displacement. We then returned the liver to the abdominal cavity and sutured the abdominal wall and skin.

Regarding liver damage in animals, we developed a fibrosis model to evaluate this strategy to improve liver regeneration after liver damage.



Animal groups after 3 days. A. Sham, B. Hepatectomy without CMS, C. Hepatectomy with CMS

15. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).
Answer: We changed rpm to centrifugal force. Line 164

16. Please ensure all figures are referenced in order.
Answer: We complied with your indications.

17. Please include a single line space between each step of the protocol.
Answer: We complied with your indications.

18. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Answer: We complied with your indications.

19. Please ensure the results are described in the context of the presented technique. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.
Answer: We complied with your indications.

20. Please obtain explicit copyright permission to reuse any figures/tables from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure/table has been modified from [citation]."
Answer: We complied with your indications.

21. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique

d) The significance with respect to existing methods
e) Any future applications of the technique
Answer: We complied with your indications.

a) Critical steps within the protocol
The critical step is the removal of a portion of liver because the bleeding is very profuse, because this organ is highly vascularized. Therefore, hemostasis of the surgical bed is very important to avoid this complication. Furthermore, the binding of liver tissue with CMS is important to ensure tissue-biomaterial interaction; this procedure is facilitated with the use of sutures. However, it must be done carefully to avoid tearing the liver and later bleeding.

b) Any modifications and troubleshooting of the technique
Although the biomaterial proposed herein was of bovine origin (xenogeneic), no rejection data were observed to enable the reproduction of selective hepatectomy, in this technique the tissue fragment was minor compare with hepatectomy of 2/3 that is remove a large amount of tissue, that can promote the death of the animal. (14) Because we had technical problems with the standardization of the size in the hepatectomy and the CMS, we developed a stainless-steel metallic template that allowed us to homogenize the size and shape of all the hepatectomy as well as CMS obtained.
Sterilization of the CMS turned out to be a challenge because the existing techniques modified the structure of collagen and their biochemical properties, for which it was necessary to try sterilization by heat, gamma radiation, and ethylene oxide, determining that the plasma of hydrogen peroxide was the optimal technique. (13)

c) Any limitations of the technique
The size of the implants CMS is a limitation to evaluate a greater biological response; therefore, in the future it will be necessary to evaluate this technique in a larger animal species. Furthermore, it is important to evaluate the implantation of CMS in liver a longer time (> 30 days) which allows to determine the total bioabsorption of the CMS and to determine as much as the *novo* hepatic tissue was regenerated. On the other hands, it is necessary evaluate the implantation of CMS in animal model with liver damage.

d) The significance with respect to existing methods
This method opens the door of possibility to restore the shape and volume of the resected tissue, reducing anesthesia, surgical duration, and recovery time compared with existing methods (13, 14).
The CMS was obtained of a source natural that preserve its physical and chemical properties, compared to synthetic biomaterial, that are made with complex methodology, such as bioprinting or electrospinning, establish their properties, which are not biocompatible or bioabsorbable. (2,3)

e) Any future applications of the technique
In the future, in the liver cirrhosis from different etiologies (virus, alcohol, metabolic factors, etc) the implantation of CMS could be an alternative of treatment for stimulate tissue regeneration and restore the fibrogenesis-fibrolysis balance.
In perspective, the studies should be including proliferation, migration, and inflammation assays to determine the molecular and cellular mechanisms triggered by the presence of CMS.

In conclusion, we believe that this procedure of hepatectomy was reproducible and exist the possibility to evaluate liver regeneration process through the xenoimplantation of biomaterials.

22. Please upload all tables separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.
Answer: We changed the format of the table of the manuscript and the details were included.

Reviewers' comments:

Authors: We would like to thank you for your insightful review of our manuscript and appreciate the time that you took to review it. Below there are all the comments to your concerns.

Reviewer #1:
Manuscript Summary:

This manuscript presents a method to obtain a collagen scaffold and transplant it onto a hepatectomized rat liver. It is unclear why the authors want to publish this as a method; collagen has been used as a scaffold for liver regeneration previously and there are many publications on this.
Answer: As the reviewer mentioned, the collagen has been used as a scaffold in multiple research protocols. However, until now, there have been no reports on the use of collagen from a bone source for the regeneration of liver tissue.
A methodological search in the pubmed database support our claim, using the following key words: collagen, scaffolds, liver regeneration and bovine collagen.
We found that there are no similar studies with the proposed biomaterial we propose for the regeneration of liver tissue.
The most closely related study reports on the use of scaffolds synthesized from bovine collagen, which was obtained from skin. The authors report that the biomaterial that their obtained presents homogenous and close pores, no communication between them. That scaffold was incubated in vitro with human endothelial cells to evaluate the biocompatibility and proliferation. (Chan E, et al. Three dimensional collagen scaffolds promotes intrinsic vascular for tissue engineering applications. *PLoS One.* 11:e0149799 (2016))
Additionally, our systematic revision reveals that the proposed surgical procedure (triangular hepatectomy) has not been published in any article. Also, the use of the biomaterial that replaces the section of liver tissue damage has not been published.
Additional references can be revised:
-(Takimoto, Y., et al. De novo liver tissue formation in rats using a novel collagen-polypropylene scaffold. *Cell Transplantation*. 12: 413-421. (2003))
-(Dingle, A. et al. (Qiang, L., et al. Extrahepatic bile duct regeneration in pig using scaffolds loaded with human collagen-binding bFGF. *Biomaterials*. 33: 4298-4308. (2012))
- (Perez, R., et al. Biomaterials and culture technology for regenerative therapy of liver tissue. *Adv Healthc Mater*. 6: 1-23 (2016))
-(Mazza, G., et al. Liver tissue engineering: from implantable tissue to whole organ engineering. *Hepatol Commun. 2*:131-141. (2017))

-(Characterization of isolated liver sinusoidal endothelial cells for liver bioengineering. *Angiogenesis.* (2018))
-(Sherlock, S., et al. Regenerative hepathology: In the quest for a modern Prometheus? *Dig Liv Dis*. 52: 1106-1114 (2020))
-(Ali, M., et al. Biomaterial-based cell delivery strategies to promote liver regeneration. *Biomater Res*. 25:5 (2021))

The authors should include a discussion of the particulars of this protocol to make it clear why it will be of interest to other scientists in the field and what is efficacious about their method compared to existing ones.
Answer:
<u>Surgical Procedure:</u> Usually the hepatectomy is performed in 2/3 in tissue in order to evaluate biological and molecular regeneration process. However, we evaluate the xenoimplantation of CMS in the liver, which is a friable tissue. This methodology could be developed in other animal species, e.g. rabbits and pigs.
<u>Collagen Matrix Scaffolds:</u> this natural biomaterial was obtained from a bovine condyle; and our evidence strongly suggests that it does not cause rejection in the liver tissue of rat. The primary component of this CMS is collagen type I, it is well known that is the main protein of the extracellular matrix that enables cell adhesion and proliferation.
(Badylak, SF. The extracellular matrix as a biological material. *Biomaterials.* 28: 3587-3593 (2007))
Additionally, the trabeculae of CMS pores enable cell migration and the continuous flow of growth factors, blood, and other mediators of the regeneration process.
Our research group has experience with designing the CMS in differents shapes, preserving its 3D structure, according to the tissue to be repaired. For example, cylindrical CMS implantedin dog urethra and bile duct in pigs both with promising results in tissue regeneration.
Montalvo-Jave, et al. Absorbable bioprosthesis for the treatment of bile duct in an experimental model. *Int J Surg*. 20: 163-169. 2015
Acevedo, GC. Xenoimplante de colágena en uretra de perro. Specialty in Urology Thesis. 2011. UNAM
Another advantage of CMS is that it is not dependent on a decellularized process and/or engineering process for obtention (bioprinting or electrospinning).
Nevertheless, the evaluation of different CMS shapes and thicknesses is necessary, to know the degradation process of scaffolds in liver tissue.

There are also a few details lacking in the sections, which are described below. Overall, this manuscript should restructure in the introduction and discussion to make it clear why this should be published as a methods manuscript
Answer: Thank you very much, we complied with your indications.

Major Concerns:

- The authors need to demonstrate that their method is either new or an improvement on an existing method in some way. They should state clearly how it is an improvement or what is efficacious about it compared to previously used methodology.
Answer: Thank you very much, we have complied with your indications.

- In section 1c of the protocol, there are no details given on the methodology for scaffold

characterization or sterilization. Can you provide references for the procedures of determining the structure and biological properties of the scaffold, and its sterilization? If this is to be included as part of the methods then details should be provided or it should be removed.

Answer: Thank you very much for your comment. It is important to mention that the scaffold characterization and sterilization procedure was previously reported by our group.

Briefly, in the physical and chemical characterization, amide groups that correspond to collagen type I were identified by infrared spectroscopy (FITR), whereas the size, shape and interconnection of the pores and the rough area in the trabeculae were determined by Scanning Electronic Microscopy (SEM).

We also included the most relevant information in the protocol section.

***(León-Mancilla B. Physico-chemical characterization of collagen scaffolds for tissue engineering. J App Res Tech.*14: 77-85 (2016))**

To preserve the biological and biochemical properties of collagen, we utilized the plasma gas sterilization method.

We included the information in the protocol section, and added the respective reference.

- In Figure 8, how were the cells observed identified as hepatocytes? Could they be immune cells that have infiltrated into the CMS? Immunostaining should be performed to confirm the identity of these cells

Answer: The histopathology analysis was performed by two double-blinded independent pathologists. We currently carry out immunohistochemistry and PCR assays to evaluate the different proteins and genes related to the hepatic regeneration process.

Minor Concerns:

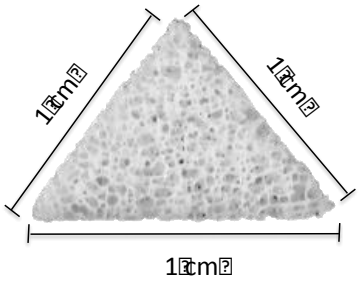
- Animal preparation section could include more detail. For example, was there support for animals during surgery? (e.g., heating pad), what approximate size area is shaved on the abdomen? Are the rats **male** or female?

Answer: We added the following details to in the protocol section:

- **Place the animal on a warm plate in the supine position, with the neck hyperextended, to maintain a permeable airway. Line 122-123**
- **Shave the abdominal skin (5 cm x 2 cm), using surgical soap a double-edged blade. To disinfect the skin, we used two rounds of topical 10% povidone-iodine solution, twice rounds. Line 120-121**
- **Male Wistar rats, weighing 150-250 g, were obtained from the Laboratory Animal Facility of the School of Medicine, UNAM. Line 114-115**

- How is the shape and size of the triangular piece of the liver cut out determined? Is there a standardized way of cutting the same shape and size in every animal?

Answer: To standardize the size and shape standardization in the hepatectomy, a metallic template was used, which was sterilized previous each procedure. The next image shows the shape and size of the CMS.



Form and size of CMS

Metallic template use for hepatectomy

- At what location are the sutures placed in the CMS to attach it to the liver? Do the sutures need to be removed in a second surgery as they are non-resorbable?

Answer: Sutures were placed between the CMS and the liver tissue. Those non-absorbable sutures were also the reference site of the CMS (Figure 5 C). The sutures were not removed in a second surgery.

- Explain all acronyms the first time they are used (e.g., ALB, ALP, etc)
Answer: We defined all acronyms in the first time they were used in the manuscript.
Serum Albumin (ALB), Alkaline phosphatase (ALP), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Total bilirubin (TB), and Direct bilirubin (DB). Line 161-163

- Line 158 of results correct the word "friable"
Answer: Friable refers to tissue that tears, sloughs, and bleeds more easily when touched (https://www.merriam-webster.com/medical)
We add an example of the use of the term.
Khanamir, R.A., et al. Histophatological and serological analysis of aborted ewes and neonatal death with Toxoplasma gondii in Duhok City, Kurdistan-Iraq. *Arch Razi Inst*. 75: 241-248 (2020)

- In figure 6A, it looks like there may be some connective tissue deposited and causing adhesion between the liver and the underlying tissues - is this a problem for the regenerative process or proper liver function?

Answer: As the reviewer stated, some connective tissue is deposited during the regeneration process. When, we compared the regeneration in the hepatectomy alone Vs hepatectomy plus CMS we observed more deposition of connective tissue in underlying tissues in the second condition.
It is important to mention that connective tissue that includes the adipose tissue plays a role, because it function as a source of the Mesenchymal Stem Cells that are important in the regeneration process.
(Tsuchiya, A., et al. Mesenchymal stem cells therapies for liver cirrhosis: MSC as "conducting cells" for improvement of liver fibrosis and regeneration. *Inflamm Regen*. 39:18. (2019))

Future studies that evaluate the role or impact of connective and adipose tissue in regenerative process are needed.

- Figure 7 labels are blurry and the images are of low quality
Answer: We changed the quality and labels in all the Figures of the manuscript.

- Figure legends use wrong word - "evolution" should be evaluation?
Answer: We wrote evaluation instead of evolution in the figure legends.

- What concentration of Meglumineflunixin is administered for postop?
Answer: The concentration of meglumine flunixin was 2.5 mg/kg.
We added that information in Line 152.

Reviewer #2:
Authors: We are grateful for you comments that undoubtedly improve the quality of the manuscript.

The authors demonstrated the protocols of the experiment for liver regeneration in rats with 40% partial hepatectomy by implanting CMS demineralized from a bovine condyle. Even though the concepts of this study are interesting, the protocols presented in this version are quite difficult to follow.
Answer: Thanks you for your valuable time and observations.

The authors should consider the following:
In lines 79-80, how to cut into 1x1x1 fragments that are 0.4-0.5 cm thickness? This instruction is very confusing.
Answer: We clarified the shape and size of the CMS fragments. Line 90-91

In line 90, please add more information about animals like gender, age, and the number of animals. In addition, please add the animal information, such as gender, age, and company, in the table of materials.
Answer: We used male Wistar rats, weighting 150-200 gr (6-8 weeks old), for each group, evaluating five male Wistar rats. The animals were provided by the Laboratory Animal Facility of the School of Medicine, UNAM. We added the information in the manuscript in line 114-117

In lines 99-110, please add the experimental procedure of preparing the sham group. For example, after partial hepatectomy how to do instead of implant the CMS in the sham group.
Answer: We added the experimental procedure for preparing the sham group. Line 132-133

In line 115, please add the amount of Meglumineflunixin.
Answer: The concentration of meglumine flunixin was 2.5 mg/kg.
We added that information in Line 152.

In lines 119-127, please add more information such as equipment (already listed in the materials) or add some comments/descriptions in the table of materials.
Answer: We added more details and more information in the table of materials.

In the table1 and protocol #5, please match the terms, such as TB vs. BT, BD vs. BD.

Answer: We matched the terms in the protocol and in the table.

In line 140, why the authors washed with water after dehydrated liver tissue in the embedding step? It does not make sense.
Answer: Thanks you, we corrected that mistake.

In line 146, please check 'MC'.
Answer: We corrected that mistake, changing MC to CMS.

Some of the materials in the table does not mention in the protocols. Please make it specific instruction. For example, in line 148, the 'Nikon software' might be ACT-1.
Answer: We corrected the error in the table.

The authors must correct the result section. It seems that there are errors and missing texts (in lines 172-190, Figures 1-8)
Answer: We corrected the errors and missing texts in the manuscript and matched them with the figure numbers.

How many rats were used in the experiment for serum biochemical test and histological analysis? Please add the number of rats used in the experiment. Especially, the number of used animals and statistical analysis should be provided to address therapeutic effects of the CMS in rats after partial hepatectomy.
Answer: We used male Wistar rats, weighing150-200 g (6-8 weeks old), for each group and evaluated five male Wistar rats. We added the information in the manuscript in Line 114-117
In this preliminary study we did not perform a statistical analysis because its main focus was the evaluation of the feasibility and biocompatibility of the CMS xenoimplant in the liver. Currently, we are performing immunohistochemistry and PCR assays to evaluate the different proteins and genes related to the hepatic regeneration process, and consider that said data will be enough for a statistical analysis.

The authors added the reference data in table 1. However, the values of the serum enzymes (ALB, ALP, ALT, AST, BT, and BD) are not clear whether the blood was collected from the damaged rats after partial hepatectomy or healthy rats. The authors should add the proper group as control instead of adding reference data.
Answer: the Sham group was used as the control. In the new, revised version of the manuscript we added the serum values of the hepatectomy group without CMS. We used as reference values the data published by León, A. in 2011, also reference values, including in representative results section of manuscript. (León, A. Hematological and biochemical in Sprague Dawley laboratory rats breed in CEPALAB, CenpSPRD.*REDVET.* 12: 1-10 (2011)).
The data of the table with the present values correspond to damaged rats after partial hepatectomy.
In the new version, the modified table is the follow:

Table 1. Values of liver function

	Days	SHAM	Hepatectomy	Implantation CMS
ALB (g/dL)	3	1.4±0	1.25±0.07	1,.4±0
	14	1.3±0.5	1.85±0.07	1.8±0.14
	21	1.3±05	1.6±0.1	1.7±0
ALT (UI/L)	3	73±1.4	3.,5±0.7	54.6±6.4
	14	84±0	59.5±4.9	57±4.2
	21	57±0	73.5±14.8	73.5±14.8
AST (UI/L)	3	106±1.4	80±6.6	90±1.4
	14	127±5.5	94±21.2	83.5±17.6
	21	123.7±27.3	92.5±24.7	101±31.1
TB (mg/dL)	3	0.33±0.05	0.25±0.07	0.3±0
	14	0.5±0	0.2±0	0.15±0
	21	0.3±0	0.4±0	0.4±0.14
DB (mg/dL)	3	0.1±0	0.05±0.07	0.1±0
	14	0.1±0	0.1±0	0.1±0
	21	0.1±0	0.1±0	0.1±0

The authors should check figure 7. Is it really the sham group?
Answer: Yes, Figure 7A and 7E correspond to sham group. We correct errors and missing texts in the manuscript and matched them with the figure numbers.

The authors should increase the resolution of images.
Answer: We changed the quality of all the Figures of the manuscript.

Reviewer #3:

Authors: We are grateful for you comments that undoubtedly improve the quality of the manuscript.

Manuscript Summary:
The manuscript represents the xeno-implantation of bovine condyle derived scaffolds into rats. The procedures are well organized and written that are easy to understand for the readers. There are a couple of questions to be answered before the acceptance.

Minor Concerns:
1. Why the authors chose "right lobe" of the rat liver which is located most behind the abdomen and IVC passes through. The picture looks like that the authors handle the right component of the middle lobe. Please make sure if it is really right lobe.
Answer:
- We chose the left lobe because it is the most superficial lobe in the abdominal cavity.
- We corrected that mistake. According to an image obtained in a publication by Martins and Neuhaus, hepatectomy was performed in the left lateral lobe (LLL). We changed the error throughout the document Martins, P., Neuhaus, P. Surgical anatomy of the liver, hepatic vasculature and bile ducts in the rat. *Liv Int.* (2007)

2. In figure 8, the hepatic tissue in the scaffold has been shown which is important in this manuscript. Please provide a multiple pictures with different sections to show the hepatic tissue infiltration more clearly.

Answer: Thank you very much. Below, we have provided more pictures to show the hepatic tissue infiltration.