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Original research

Absorbable bioprosthesis for the treatment of bile duct injury in an experimental model

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HIGHLIGHTS

- New absorbable bioprosthesis for the treatment of bile duct injury.
- Demonstration of reepithelization with histological and immunohistochemistry analysis.
- Permeability of the prosthesis in all the experimental models.
- No postoperative complications.
- Modeling of the bioprosthesis to allow different diameters and shapes.

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ABSTRACT

Introduction: Cholecystectomy is a common surgical procedure in which complications may occur, such as injury to the biliary tract, which are associated with high morbidity. The aim of this study was to demonstrate the efficacy of a polymer-based absorbable bioprosthesis with bone scaffold for the treatment of bile duct injury in an animal model.

Materials and methods: An absorbable bioprosthesis was used to replace the common bile duct in 15 pigs which were divided into 3 groups with different follow-ups at 1, 3 and 6 months. The animals were anesthetized at these time points and laboratory tests, Magnetic Resonance Cholangiopancreatogram [MRCP], Choledochoscopy using Spyglass and Endoscopic retrograde Cholangiopancreatogram [ERCP] were performed. After radiological evaluation was complete, the animals were euthanized and histological and immunohistochemical analyses were performed.

Results: Liver function tests at different time points demonstrated no significant changes. No mortality or postoperative complications were found in any of the experimental models. Imaging studies ([MRCP], [ERCP] and Choledochoscopy with SpyGlass™) showed absence of stenosis or obstruction in all the experimental models.

Discussion: Histological and immunohistochemical staining (CK19 and MUC5+) revealed the presence of biliary epithelium with intramural biliary glands in all the experimental models. There was no stenosis or obstruction in the bile duct.

Conclusions: The bioprosthesis served as scaffolding for tissue regeneration. There was no postoperative complication at 6 months follow-up. This bioprosthesis could be used to replace the bile duct in cancer or bile duct injury. The bioprosthesis may allow different modeling depending on the type of bile duct injury.

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1. Introduction

Background: Cholecystectomy represents one of the most common surgical procedures performed by general surgeons. Complications may arise following this surgical procedure. Iatrogenic bile duct injury, despite its low incidence, has high morbidity and harmful effects on quality of life of patients, with increase in hospital-stay and medical care costs [1]. Iatrogenic bile duct injury is defined as an injury in some part of the extrahepatic bile duct during cholecystectomy or other invasive procedures and it comprises about 95% of all benign bile duct stenosis [2,3]. Roux-en-Y hepaticojejunostomy has been the most commonly performed procedure for biliary reconstruction, especially in cases of ductal transection [4]. Biological tissues have been used for repair of bile duct injuries without satisfactory results, including synthetic materials such as latex, silastic, expanded polytetrafluoroethylene and absorbable polymers which have been utilized as biliary prostheses [5]. The absorbable bioprosthesis functions as an alternative to inserting a T-tube or biliodigestive anastomosis in patients who experienced intraoperative injury of the bile duct, or in patients who develop a postoperative stenosis. This approach provides an innovative treatment of biliary strictures that preserves the function of the papilla of Vater [1]. The importance of this study consists of proposing therapeutic strategies for the management of bile duct injuries, evaluating the usefulness of biomaterials in experimental models, and determining their effectiveness in maintaining patency of the bile duct without stenosis.

Bile duct injuries occur more often during laparoscopic cholecystectomies when compared with open surgery although there is no statistically significant difference between them [6]. Misidentification of the cystic duct and/or the common bile duct is one of the most common causes of injury. Anatomic variations are observed between 10 and 15% of patients and they are difficult to detect during preoperative studies [7,8]. There are many classifications of bile duct injuries, and the final results after repair depends on many factors [9,10]. One of the non-invasive diagnostic studies is magnetic cholangioresonance, which has nearly a 90% sensitivity for diagnosis of biliary conditions when compared with other invasive diagnostic studies such as ERCP [11]. The use of self-expanding metal stents for biliary strictures is associated with disappointing results, and its routine use is not recommended [12]. The aim of this study was to demonstrate the efficacy of an absorbable bioprosthesis with bone collagen scaffold for the treatment of bile duct injury in an animal model.

2. Materials and methods

2.1. Bioprosthesis

The polymer-based (collagen) bioprosthesis with open and interconnected pores were used (Fig. 1). It has a demineralized bone scaffold and was developed in the Biomaterials Institute Laboratory at the National Autonomous University of Mexico (UNAM) in Mexico City. The dimensions of the implants were 3-cm length, 0.32-cm outer diameter, and an inner diameter between 0.24 and 0.25 cm (Fig. 2). The prostheses were coated with ε-caprolactone (Sigma–Aldrich, 704,067) to waterproof and to prevent bile leakage. They were sterilized with hydrogen peroxide plasma. The bioprosthesis was obtained from a cuboid structure which allows different modeling according to the diameter and length of the bile duct.

2.2. Experimental model

Fifteen male Landrace pigs weighing between 30 and 40 kg each were used. The animals were divided into 3 groups with different follow-up times: 1; 3 and 6 months (5 pigs per group). The animals were anesthetized at this time points and MRCP, Choledochoscopy with Spyglass and ERCP were performed. After all the imagings were complete, the animals were euthanized and histological and immunohistochemical studied.

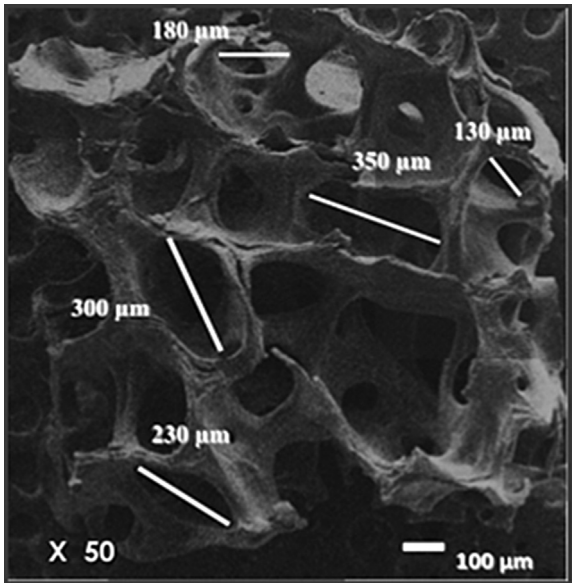

Fig. 1. Electronic microscopy: Bioprosthesis showing its porous structure.

Fig. 2. Absorbable bioprosthesis.

2.3. Ethical considerations

The protocol was approved by the Research Ethics Committees of the National Autonomous University of Mexico. The animals were housed in individual pens, and were handled and anesthetized according to the Official Mexican Norm 062-ZOO-1999 [13].

2.4. Anesthesia protocol

The animals were fasted for 12 h prior to the surgical procedure. Sedation was achieved using Azaperone (40 mg Sural, Lab. Chinoin®, México) 2 mg/kg intramuscular (i.m.) and atropine sulfate (1 mg Atropisa, Pisa livestock Aug, México) 1 mg/10 kg i.m. Subsequently, a short catheter (20-Fr) was placed in the marginal ear vein in order to have an intravenous (i.v.) line and sodium

pentobarbital anesthesia (Laboratorio Pets Pharma México) 40 mg/kg i.v. were administered. An endotracheal tube (6.5-Fr) was put in place and assisted with an automatic ventilator (Harvard app, USA). Prior to the surgical procedure, Enrofloxacin (Pisa, México) 1 g was administered i.m. as a prophylactic measure.

2.5. Surgical procedure

The animals were immobilized in a supine position and laparotomized with a supraumbilical midline incision. After transversely sectioning the common bile duct entirely with a proximal and distal anastomosis (common bile duct-bioprosthesis-common bile duct) anastomosis was performed using simple sutures with Polydioxanone 5-0 PDS suture (Ethicon, USA). The bioprostheses were adjusted in length during the surgical procedure according to the anatomical characteristics of the common bile duct of each animal (Fig. 3).

2.6. Postsurgical care

The animals were transferred to the recovery room and were administered analgesic-anti-inflammatory Meloxicam (Biochem, México) 500 mg/12 h i.m. and Enrofloxacin 1 g/24 h/7 days i.m. Close monitoring and clinical and physical examinations were performed, as well as wound healing and removal of stitches at 7 days postsurgery.

2.7. Statistical tests

For statistical analysis, the SPSS 21 Statistics software program and the IBM test were used. Comparisons between pre- and postoperative values were analyzed by the Wilcoxon and Student *t* tests. A $P < 0.05$ was considered significant.

2.8. Assessment of hepatic function

Blood samples were taken from the jugular vein 24 h prior to the surgical procedure (Time 0), and at 1, 3, and 6 months to determine the serum enzyme levels of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (AP), Total bilirubins (TB), Direct bilirubins (DR), and Indirect bilirubins (IB). Each sample was placed in an Eppendorf tube and centrifuged at 5000 *g* for 10 min. The serum was obtained using a Pasteur pipette, transferred into new Eppendorf tubes, and processed routinely employing a Universal Kit (Bayer, Leverkusen, Germany).

2.9. MRCP

The pigs were anesthetized with the anesthetic protocol by adding Isoflurane (Baxter, USA) at 2%. Three and 7 T equipments (Philips, Achieva model) were utilized, performing sequences in T1 and T2 with transverse, coronal, and sagittal abdominal regions for the bile duct reconstruction. Seven Tesla equipments were employed solely for the bile duct portion after its resection.

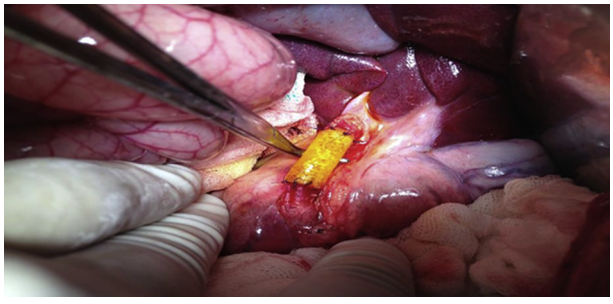

Fig. 3. Bioprosthesis in common bile duct.

2.10. ERCP

The pigs were anesthetized with the anesthetic protocol as described in the surgical procedure previously. Using a X-Ray C-Arm (Phillips, BV Libra) and under fluoroscopy guidance, the endoscope was introduced through the oral cavity to the ampulla of Vater. To confirm the anatomical site, Ultravist® 300 (Bayer, México) contrast media was utilized.

2.11. Choledochoscopy with SpyGlass™

For the Choledochoscopy with SpyGlass™ study, the pigs were anesthetized with the anesthesia protocol as previously described. Employing endoscopic guidance, cannulation of the ampulla of Vater was done. The SpyGlass™ Direct Visualization System (Boston Scientific Corp., Natick, MA, USA) was advanced into the biliary tract of the anesthetized pigs and visualization of the common bile duct and the biliary tract was achieved.

2.12. Histopathological study

Bile duct regeneration was assessed in the fixed, paraffin-embedded bile duct samples. We assessed the following variables: presence of biliary duct epithelium at the site of prosthesis insertion; presence of intramural biliary glands, inflammatory changes in addition to fibrosis. Hematoxylin-Eosin (H&E) stain was performed.

2.13. Immunohistochemistry of MUC5 Ac and cytokeratin 19

The surgical specimens of the common bile duct were collected according to the National Institutes of Health (NIH) guidelines with approval by the Institutional Review Board (IRB) of the Roswell Park Cancer Institute (RPCI) and the University of North Carolina School of Medicine at Chapel Hill. The common bile duct was resected together with the bioprosthesis. Tissue microarray was performed as follows: formalin-fixed, paraffin-embedded tissue microarray sections were incubated at 70 °C for 20 min, deparaffinized in Xylene, and rehydrated by means of graded alcohols into a phosphate-buffered saline (PBS) solution. Endogenous peroxidase was blocked using 3% H_2O_2 in 83% methanol for 10 min at room temperature. For antigen retrieval, slides were placed in 0.01 sodium citrate and 0.01 M citric acid, pH 6.0 for 20 min in a microwave oven put on a high setting followed by 15 min of cool-down incubation in citrate solution [14].

The slides were incubated at room temperature for 1 h with primary antibodies coupled with conjugated peroxidase (Gene Tex, TX, USA) as follows: MUC 5AC (1:100 BIOCARE, USA), and CK19 (1:100 BIOSB, USA), followed by 30 min incubation at room temperature with the BIOSB (Mouse/Rabbit polydetector HRP/DAB Detection System) kit. Sections were incubated for 5 min with 3, 30 Diamino-benzidine (DAB Peroxidase Substrate BIOSB) and counterstained with hematoxylin (Dako, CA, USA). APERIO ImageScope software was used to capture and store the images.

3. Results

In order to evaluate the regenerative process of the bioprostheses implanted in the pigs, clinical laboratory tests, imaging ([MRCP] Magnetic Resonance Cholangiopancreatogram, [ERCP],

Choledochoscopy using SpyGlass™, histopathological (H&E) and immunohistochemical studies (cytokeratin 19, MU5 Ac) were performed. No animals died before they were sacrificed.

3.1. Hepatic function

In this study, we used 5 different liver function indicators to assess the patency of the bioprostheses without any obstruction. This serum enzyme assay is regularly used in clinical practice to determine liver damage or biliary tract obstruction. The results are shown in Table 1. The patterns of change in the serum activity for the 1, 3 and 6-month follow-up groups showed no significant elevations during the study.

3.2. MRCP, ERCP and Choledochoscopy with Spyglass

Magnetic Resonance Cholangiopancreatogram studies performed in all the animals showed no absence of stenosis or obstruction. (Fig. 4) Seven Tesla Resonance of the surgical piece demonstrated presence of epithelium surrounding the bioprosthesis in the 6- month follow-up model. In the 1-month follow-up group, complete reepithelization took place and the prosthesis served as a scaffold to allow epithelial formation surrounding the prosthesis (Fig. 5). Evaluation with ERCP and Choledoscopy demonstrated the bioprosthesis showed no evidence of stenosis in any of the animals (Fig. 6). The presence of a trabecular pattern at the site of the neo-bile duct formation was observed using SpyGlass™ (Fig. 7) in the 1-month model, and it was possible to visualize the biliary tract, demonstrating the absence of stenosis or biliary leak in all the experimental models.

3.3. Histopathological report

Histological stained section showed normal biliary epithelium, fibrous wall, and a variable number of intramural biliary glands, some with pyloric metaplasia in the 1-month model. The bioprostheses with trabecular structures were also visualized. These histological changes were found in the groups of 1 and 3 months. At 6 months, histological analysis demonstrated a large number of intramural biliary glands in the lamina propia of the neo-bile duct with increased mucus production and a fibrous wall of variable thickness. The biliary epithelium exhibited normal characteristics. Other histological section demonstrated that the prosthesis was still detectable, but with less bone scaffolding (Fig. 8). Bile ducts of animals without injury and prosthesis were examined and served as a control for histological and immunohistochemical analysis.

3.4. Immunohistochemical study

The neo-bile duct stained positive for CK 19 and MUC 5 Ac in all the experimental models. At 6 months, the neo-bile duct exhibited biliary cells organized identical to those of the native bile duct. This portion tested positive for CK 19 (Fig. 9), resembling those observed in the controls. Also, the bile duct portion tested positive for MUC 5 Ac (Fig. 10), demonstrating intramural biliary glands and reepithelization of the neoduct with cells corresponding to bile duct epithelium.

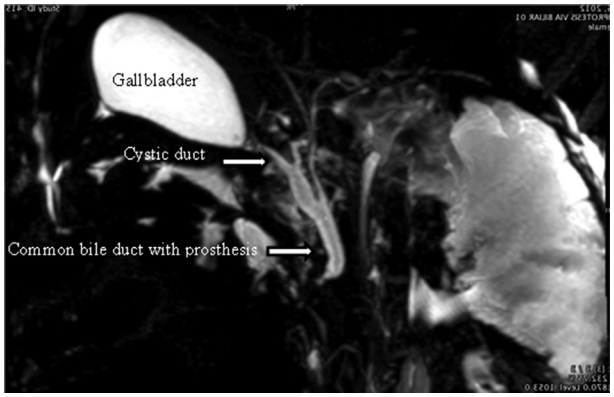

Fig. 4. MRCP in 6-month model demonstrating adequate permeability.

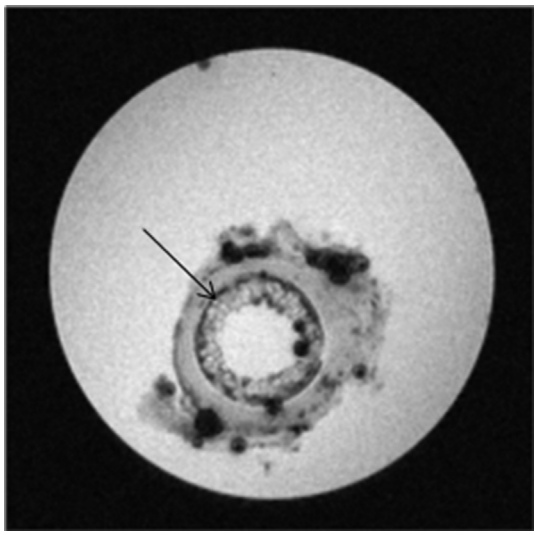

Fig. 5. Common bile duct 7 T Resonance with prosthesis (arrow) in a 6-month model, demonstrating re-epithelization and formation of the neo-bile duct surrounding the bioprosthesis.

4. Discussion

The prostheses currently used for bile duct injury repair have not shown any convincing results, and long-term models have not been studied. In some cases, follow-up has been studied up to 6 months [5]. This model is just a specifically transection type of

Table 1
Liver function tests at 1, 3 and 6 months follow-up. No significant elevations were found after the procedure or during the follow-up period. No cholestatic or obstructive pattern was found. *Alanine aminotransferase* (ALT), *Aspartate aminotransferase* (AST), *Total Bilirubins* (TB), *Direct Bilirubins* (DB) and *Alkaline Phosphatase* (AP).

	Control	1 month $n = 15$		3 months $n = 12$		6 months $n = 9$	
		Post	*P*	Post	*P*	Post	*P*
ALT	23.3 ± 1.5 (22–25)	22 ± 4.3 (17–25)	0.72	23 ± 2 (23–26)	0.80	21.3 ± 1.5 (20–23)	0.42
AST	26 ± 7.2 (20–34)	20 ± 3.4 (16–22)	0.41	18.3 ± 6.6 (14–26)	0.42	21.6 ± 1.15 (21–23)	0.74
TB	0.16 ± 0.16 (0.05–0.35)	0.08 ± 0.09 (0.02–0.19)	0.20	0.05 ± 0.04 (0.02–0.10)	0.60	0.20 ± 0.16 (0.03–0.35)	0.66
DB	0.11 ± 0.08 (0.04–0.20)	0.09 ± 0.06 (0.02–0.15)	0.25	0.12 ± 0.08 (0.03–0.19)	1	0.14 ± 0.05 (0.10–0.20)	0.31
AP	185 ± 36 (151–224)	203 ± 40 (165–246)	0.68	114.6 ± 33 (78–142)	0.96	145 ± 12 (132–156)	0.66

Fig. 6. ERCP with no evidence of stenosis in a 6 months model.

injury repaired by interposition of a graft. Further research is needed to include other types of injury.

This work proposes the use of a new, natural bioprosthesis with bone scaffold. It is absorbable, porous and impermeable, with interconnections that allow epithelial regeneration through formation of blood vessels and cells, demonstrated by conventional microscopy and immunohistochemical techniques. The function of the prosthesis was evaluated using different methods, including laboratory, MRCP and ERCP, showing proper bioprosthesis functions [11], with satisfactory results.

On postoperative follow-up of this biological substitutions, there was no mortality, stenosis, bile duct leakage or collection in the experimental animals studied. Choledochoscopy with SpyGlass™, demonstrated the absence of obstruction or stenosis in all the models studied. There was no migration of the bioprosthesis from its insertion site and no necrosis of the bile duct was observed.

At macroscopic evaluation, reepithelization was seen. There was a trabecular pattern in the neoduct, which permitted appropriate vascularization required to maintain a functional epithelium.

Histopathology with H& E staining showed the presence of intramural biliary glands. On Immunohistochemistry (IHC), all experimental models stained positive for cytokeratin 19, MUC5Ac, keratins 7 and 19 for the detection of biliary epithelium [15,16], demonstrating the presence of biliary epithelium in the absorbable bioprosthesis.

Mucins are glycosylated proteins that are normally expressed in the ductal and glandular epithelium of epithelial tissues [17]. The use of MUC 5 Ac (serum mucin) has been studied as a marker for bile duct cancer and as a diagnostic method and, in some cases, as a prognostic evaluation [18,19]. In this study, it was also possible to

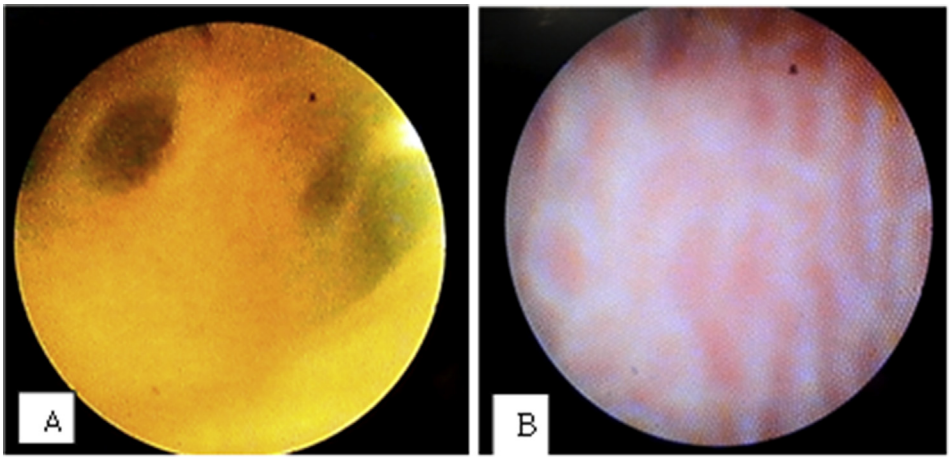

Fig. 7. A) Visualization of the biliary tract using SpyGlass™ in a 1-month model. B) Trabecular pattern of the neo-bile duct visualized with SpyGlass™ in a 1-month model.

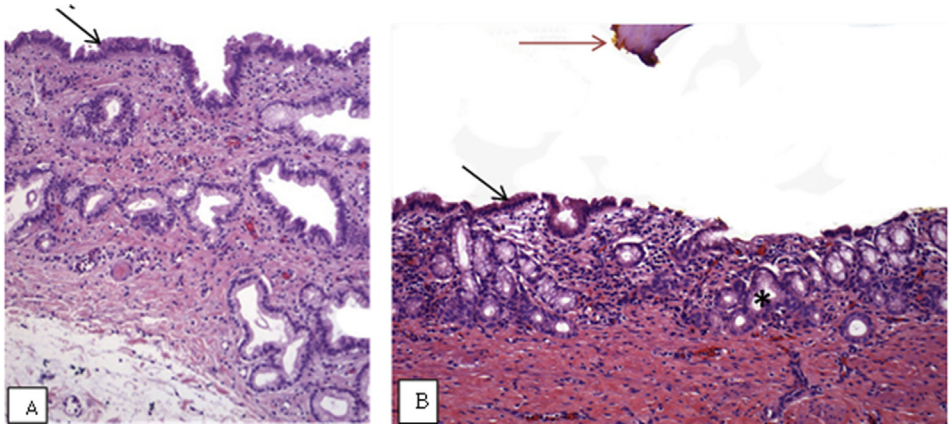

Fig. 8. A) Control: Bile duct columnar epithelium (arrow) and intramural bile glands (*). B) Prosthesis on 6-months model: bile duct columnar epithelium (black arrow) and biliary intramural glands hyperplasia (*). Bioprosthesis remnant (red arrow).

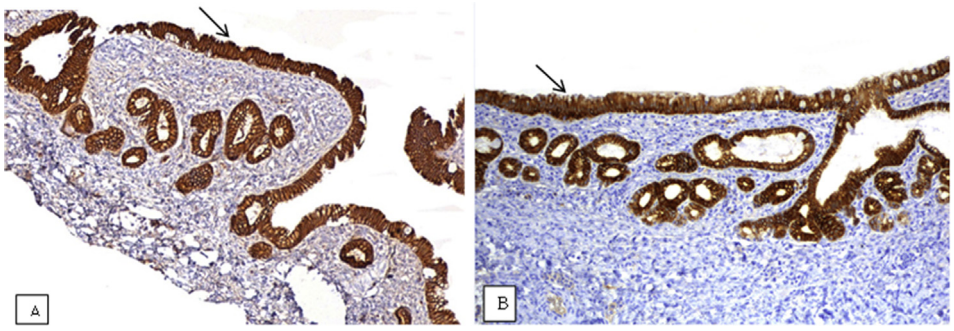

Fig. 9. A) Control: Cytokeratin 19 specific for biliary epithelium (arrow) and intramural biliary glands (*). B) Prosthesis in 6-month model: Cytokeratin 19 demonstrating the presence of biliary epithelium (arrow) and the presence of biliary glands (*).

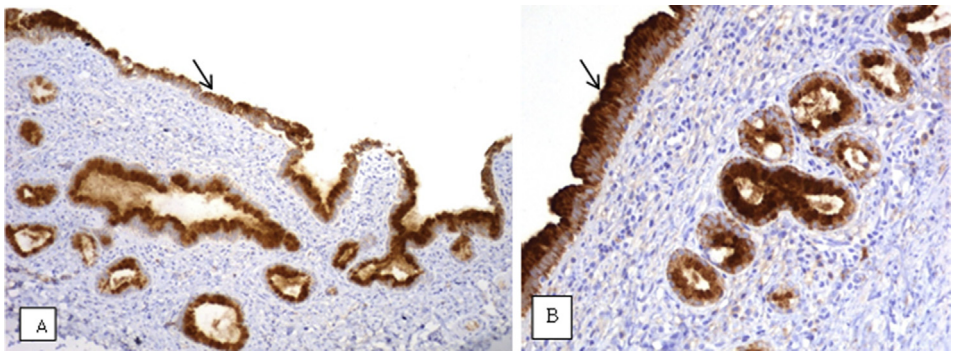

Fig. 10. A) Control: MUC 5 Ac demonstrating biliary epithelium (arrow) and intramural biliary glands (*). B) Prosthesis in 6-month model: MUC 5 Ac demonstrating the presence of biliary epithelium (arrow) and the presence of biliary glands (*).

detect, with the use of MUC 5 Ac, the presence of biliary tissue in the neo-bile duct formation.

No mortality or complications on follow-up were detected.

5. Conclusions

The bioprosthesis served as a scaffolding for tissue regeneration without any postoperatively complications at 6 months follow-up. It could be used to replace the bile duct in cancer or in other pathological entities requiring replacement of the affected biliary segment or in cases of bile duct injury. Close monitoring of the experimental models ruled out any potential early postoperative complications.

Employing absorbable bioprosthesis as an accessible and inexpensive therapeutic option. The prosthesis has yet to be tested in inflamed or infected fields, as in the case with bile duct injury resulting from cholecystectomy. Importantly, due to the process of obtaining and modeling the prosthesis from the cube structure, it would allow a Y-shaped graft to connect more than 1 duct different types of bile duct injury or cancer.

Ethical approval

Approved by the Research Ethics Committees of the National Autonomous University of Mexico (026-2012).

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Author contribution

Montalvo-Javé Eduardo E.- Study design, data analysis, writing.

Mendoza Barrera Germán E.- Study design, data collections, data analysis, writing.

Valderrama Treviño Alan I.- Study design, data collections, writing.

Piña Barba María C.-Data analysis.

Montalvo-Arenas César- Data analysis.

Rojas Mendoza Fernando – Data analysis.

León Mancilla Benjamin- Data collections, data analysis, writing.

García Pineda Manuel A. – Data collections, data analysis.

Jaime Limón Álvaro – Data collection.

Albores Saavedra Jorge – Data analysis.

Tapia-Jurado Jesús – Study design.

Conflict of interest

The authors declare no conflict of interest.

Guarantor

Eduardo Esteban Montalvo Javé.

Germán Eduardo Mendoza Barrera.

Disclosure

The following additional information is required for submission. Please note that failure to respond to these questions/statements will mean your submission will be returned. If you have nothing to declare in any of these categories then this should be stated.

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