A three-dimensional scaffold from decellularized human umbilical artery for bile duct reconstruction

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Konstantine Chakhunashvili*, Merab Kiladze**, David G. Chakhunashvili*, Lia Karalashvili*, Zurab Kakabadze*

**Tbilisi State Medical University, Georgia*
***Javakhishvili Tbilisi State University, Georgia*

A three-dimensional scaffold from decellularized human umbilical artery for bile duct reconstruction

AIM: *The main purpose of the study was to create in vitro bile duct equivalent out of decellularized human umbilical cord artery and use it to reconstruct common bile duct obstruction with preservation of sphincter of Oddi.*
MATERIAL AND METHODS: *SDS and Triton X-100 were used for decellularization of the artery. Allogeneic isolated cholangiocytes were seeded onto the inner surface of the decellularized artery. Experimental study was held and 12 domestic pigs of both sexes, weighing 25-30 kg were used. They were divided in equivalent two groups. Common bile duct obstruction model was created in all animals. Animals of the first group (n=6) received no further treatment and were under observation. Animals of the second group (n=6) underwent relaparotomy after two days of initial intervention, lesion site (2 cm) was incised and defect was reconstructed with the bile duct equivalent with the size of 2-2,5 cm. Maximum observation period was 84 days.*
RESULTS: Laboratory, morphologic and radiologic investigations showed good integration with the host organism.
DISCUSSION: *Bile duct reconstruction is still a major of HPB surgery. This fact prompted this study to assess the efficacy of the novel method for bile duct reconstruction the experimental study by using appropriate laboratory, morphologic and radiologic investigations.*
CONCLUSION: *Preliminary results obtained with the described method allows us to say that bile duct equivalent created by us with decellularized human umbilical artery and cholangiocytes can be successfully used for bile duct reconstruction with inclusion of the sphincter of oddi.*

KEY WORDS: Bile Duct Obstruction, Bile Duct Reconstruction, Decellularization, Human Umbilical Artery

Introduction

Treatment of bile duct obstruction caused by congenital or acquired diseases, such as biliary atresia, bile duct cancers and etc., still poses a major challenge since complication after highly utilized biliary-enteric anastomoses occur at high rate, even though, there are a lot of new modifications utilized to improve the outcome [1-3]. There are several types of surgical interventions utilized to treat this condition, such as endoscopic stenting, biliary-digestive (choledocoduodeno, choledocojejuno anastomoses, hepato-enteric and hepato-gastric anastomoses) and biliobiliary anastomoses. However, the choice of surgical treatment depends on the level of the biliary stricture [5-8]. The method of choice in total and subtotal strictures is Roux-en-Y choledocojejunostomy or hepato-enteric anastomoses. Although, those methods, even when executed by the most skilled surgeons, are proven to be complicated postoperatively in more than 50%, causing recurrent cholangitis, biliary cirrhosis, portal hypertension and

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Correspondence to: Chakhunashvili Konstantine, Tbilisi State Medical University, Georgia (e-mail: vitacorpge@yahoo.com)

increase risk of hepatocellular carcinoma [8-10]. These are mainly attributed to the exclusion of the sphincter of Oddi that frequently occurs following the biliary-digestive anastomoses. A perfect method for common bile duct reconstruction would be to restore its anatomical structure and preserving function of sphinchter of Oddi.

Similar complications are observed in Kasai operation, when hepato-enteric anastomoses are formed in patient with biliary atresia. Multi-center studies show that 10-year survival rate after Kasai operation with native liver (without liver transplantation) is very low - 25%-60% - and significant increase in GGT levels is a predictor of a poor outcome [12-15].

It is believed that majority of those complication can be avoided by including sphincter of Oddi in the reconstructed bile drainage system, therefore, multiple synthetic tubes and autologous conduits (vascular grafts, skin grafts and etc.) had been used to achieve a successful long-term drainage, although, none of the above methods were proven to be efficient [16]. However, when small intestinal submucosa was tested in animals to patch bile duct it showed promising results in vivo [17].

Current advances in the rapidly developing tissue bioengineering are of great importance to us and medical community, in general. A lot of tissues and organs have been created in vitro, tested in animal models and some translated into clinical practice, such as: urethral grafts, skin grafts, blood vessels, heart, lungs, trachea, ligaments, GI tract grafts and etc. [18-31]. Although, there still is a long way before tissue and organ bioengineering can impact daily medical routine and tackle the issue of organ shortage. We can apply principles of this field to substitute existing and researched bile duct grafts, which were not implemented in clinical practice (enteric conduits, vascular conduits, synthetic conduits and etc) [16]. Our hypothesis was to create bile duct equivalent in vitro by using decellularized human umbilical artery, seeding cholagiocytes onto it and cultivating it. The bile duct equivalent would later be used to reconstruct bile duct stricture, without bypassing sphincter of Oddi, in animal models to test the efficacy and assess the integration of the tissue with the host tissue, which will be presented in this paper.

Material and Methods

Decellularization

We used human placenta to acquire three-dimensional scaffold from umbilical arteries. All placentas were retrieved with an informed consent from healthy mothers with gestation age between 38-42nd weeks. Umbilical cord with size of 15-20 cm was removed from placenta and was transported to the laboratory within a sterile box. Upon delivery, umbilical cord was placed on the procedural table and all the vessels were cannulated. The vessels were rinsed with 1% heparin saline solution 400 ml. After rinsing the umbilical cord was frozen at – 80° C for 24 hours. After that umbilical cord was thawed at room temperature and was connected to the pump perfusion system (Heidolph, Germany). Decellularization process was ongoing for 72 hours. In the first 24 hours we used 0.01% SDS deionized water solution, in the second 24 hours – 0.1% SDS deionized water solution, in the last 24 hours – 1% SDS deionized water solution. To remove residual SDS the artery was rinsed with deionized water for 15 minutes. The following step was to rinse the artery with 1% triton X-100 (sigma) deionized water solution. The last step was to perfuse the artery with PBS for 4 hours.

Assessment of the Purity

To assess the purity of decellularization decellularized human umbilical artery underwent the DNA assay (Sigma), which was done by instruction provided by the seller, and was also stained with Hematoxylin and Eosin and Masson trichrome.

Cell Isolation and Seeding

To isolate and seed porcine cholangiocytes, extrahepatic bile ducts and gall-bladder was harvested at the Tbilisi central slaughterhouse. Harvested organs were put into sterile cold box and transported to the laboratory to perform the following procedure. Resected common bile duct was cannulated and other ducts were ligated to prevent the leak. The whole extrahepatic tree including gall bladder were rinsed with HBSS (Sigma) until transparent fluid was flowing. Afterwards, collagenase IV 0.25% HBSS (Sigma) solution was infused and gall bladder was incubated at 37° C for 30 minutes. After incubation the fluid was collected in several falcon tubes (50 ml) and falcon gradient solution was added. Tubes were centrifuged at 1200 RPM for 10 minutes. After removing supernatant, cells were placed onto lumen of decellularized umbilical arteries which were placed in a culture plate. After seeding plates were placed into a CO^2 incubation at 37° C. After 15 minutes DMEM/f12 (Sigma), FBS (Sigma), Penicilin/streptomycin, vitamin solution, epidermal growth factor, dexamethazon and foscolin was added to the cultivation plate and cultivation continued for 12 hours in CO_2 incubator 37° C (Romanov et al).

Assessment of the Cell Seeding and Cultivation Efficacy

To assess the above said, samples of bile duct equivalent were subjected to freeze-drying for preparation of

scanning electron microscopy and fixation in 10% formalin to later stain samples for Hematoxylin and Eosin, Masson Trichrome, CK-19 (Biocare) and Ki-67 (IHC-tek).

Bile duct obstruction model and reconstruction

The study was conducted on 12 domestic pigs of both sexes with the weight of 25-30 kg. According to protocol approved by Tbilisi State Medical University Ethics Committee (approval by meeting record 12, 15.09.2015) animals of the both groups got intervention to create bile duct obstruction model. Animals of the first (control) group (n=6) did not undergo any further procedures and did not receive any treatment, thus, the first group was a control. Animals of the second (experimental) group (n=6) after two days of model creation underwent relaparotomy, part of common bile duct was resected and reconstruction was done by bile duct equivalent engineered by us with the size of 2-2.5 cm. Maximum observation period was 84 days.
General anesthesia was induced by intramuscular injection of ketamine 15 mg/kg and atropine 1 mg was used intramuscularly. Upon relaparotomy, in the second group the organs were visualized and ligated common bile duct was isolated. 2-2,5 cm of the ligated common bile duct was resected and the defect was reconstructed with the bile duct equivalent (size 4 cm in length, 4 mm in diameter) created by us. Before applying end-to-end anastomoses, polyvinyl drainage tube was place inside the lumen of the bile duct equivalent and then into proximal and distal ends of the resected common bile duct. End-to-end anastomoses were performed with intermittent sutures with 7/0 ETHICON atraumatic needle under a surgical microscope (Zeiss, Germany).
One animal from each group was euthanized on 7th, 21st, 42nd, 56th, 70th, 84th days. Blood was harvested from all the animals on baseline, 2nd, 4th, 7th, 14th, 28th, 56th and 84th days to asses liver function tests (Total Serum Bilirubin, Albumin, AST, ALT). Post-mortem examination and revision of the abdominal cavity was done in all animals and radiological studies of biliary tree were done; Entire common bile duct was also resected for the further histological studies.

Liver Function Tests

Blood collected from animals were assessed for the levels of total serum bilirubin (Human), plasma albumin (Human), AST (Human) and ALT (Human). All of which was done by spectroscopic method; all the assays were conducted by reagents Human Diagnostic Worldwide and official protocols provided by the producer were pursued.

Radiology

Urografin (Bayer) was injected through the common bile duct to visualize intra and extrahepatic biliary tree and assess for the obstruction and dilation of the above said pathways.

Morphology

Upon revision liver and all the organs were assessed macroscopically and only after that histological samples were harvested (resection of the common bile duct). The samples were fixed in 10% formalin solution and underwent staining with Hematoxylin and Eosin, Masson Trichrome, CK-19 (Biocare) and Ki-67(IHC-tek).

Immunohistochemical Staining

Immunohistochemical staining with CK-19 was performed according to the producer's manual. After applying CK-19 (1:100 dilution, Biocare) the samples were incubated with a tertiary polymer at the room temperature for 15 minutes, after which it was incubated with DAB solution (Biocare), afterwards it was counterstained with hematoxylin and rinsed with deionized water. In the end, Tacha's bluing solution (Biocare) was applied for 1 minute and the samples then were rinsed with deionized water.
Immunohistochemical staining with Ki-67 was also performed to the producer's manual. Sections were rinsed in PBS-tween 20 for 2 minutes. After applying Ki-67 (1:100 dilution, IHC-tek) the samples were incubated for 1 hour at room temperature, after which they were also rinsed in PBS tween 20 for 6 minutes. The samples were placed in peroxidase blocking solution for 10 minutes at room temperature. Biotinylated secondary antibody (IHC-tek) was applied and incubated for 30 minutes at the room temperature. The samples were rinsed with PBS-tween 20 for 6 minutes. The samples were then put into ABC-peroxidase solution (IHC-tek) for 30 minutes at room temperature. The sample were rinsed with PBS-tween 20 for 6 minutes.
Counterstaining was done with Mayer's Hematoxylin Solution (IHC-Tek). The samples were rinsed in running water for 5 minutes and then were dehydrated in Ehtanol (1 minute in 95% ethanol, 100% ethanol 3 times for 2 minutes). The samples were cleared with xylene for 10 minutes and in the end the coverslip was applied.

Scanning Electron Microscopy

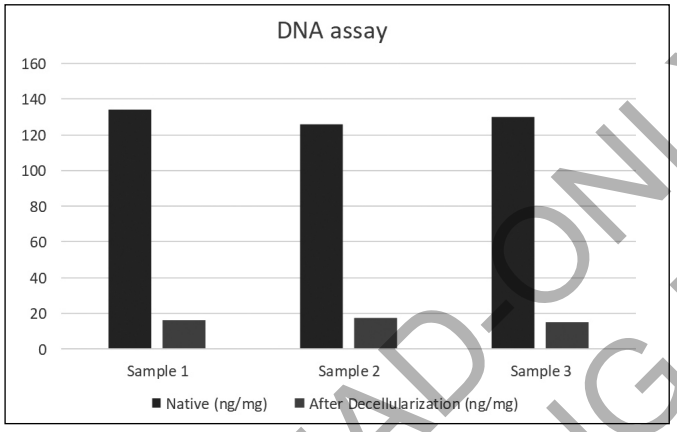
The samples, which prior to subjection to scanning electron microscopy were fixated in 3% glutaraldehyde solu-

tion and freeze-dried (Biobase, China), underwent procedure with Oxford University JEOL JSM-5510 apparatus, which works on a low-level vacuum and can give resolution of 3.5 nm and maximum power is 30 kV.

Results

Purity And Integrity After Decellularization

Histological staining for Hematoxylin and Eosin, Masson trichrome of the decellularized umbilical artery showed us the acellular matrix with porous intact collagen structure. Scanning electron microscopy demonstrated that ultrastructure and filament alignment and structure were intact, but no cells were to be visualized. DNA assay showed the content of genetic material after decellularization was less than 16 ng/mg (Graphic 1, Figs. 1, 2, 3).



Graphic 1: DNA assay of native human umbilical artery and decellularized human umbilical artery.

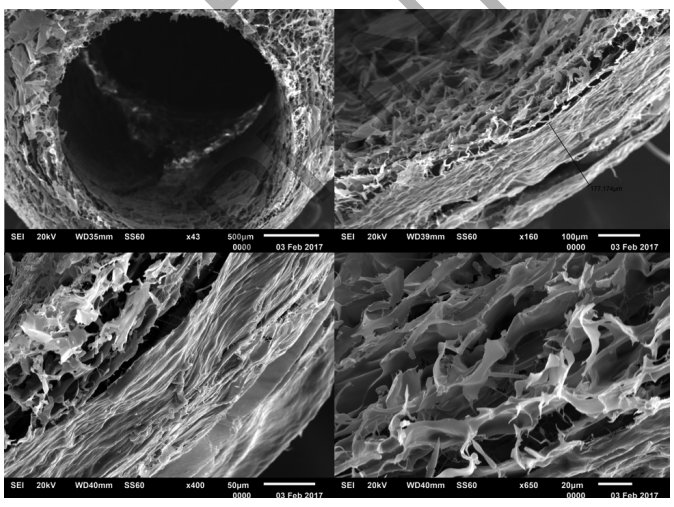


Fig. 1: Scanning electron microscopy of native human umbilical artery.

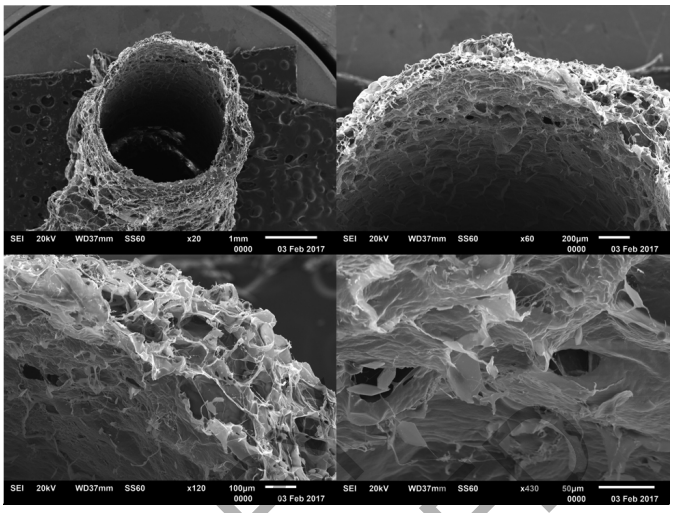


Fig. 2: Scanning electron microscopy of decellularized human umbilical artery.

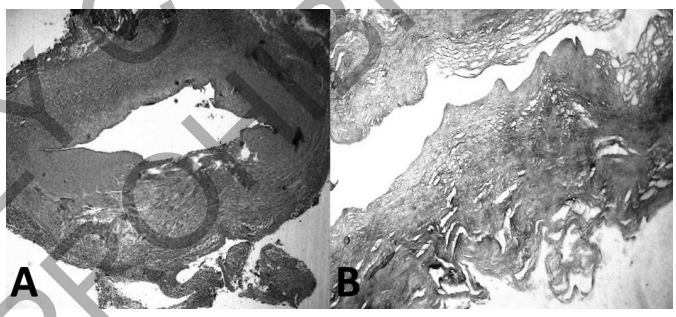
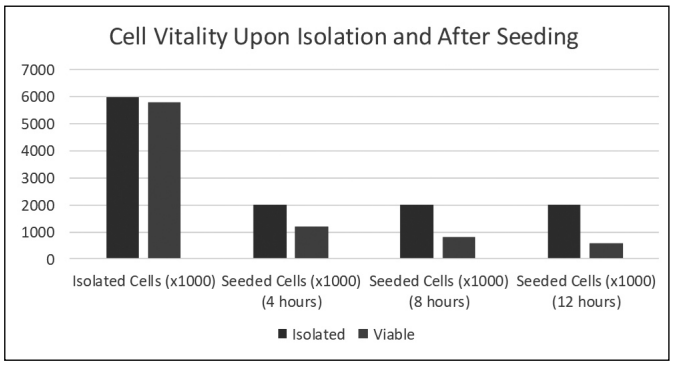


Fig. 3: A. Native human umbilical artery (H&E) x 200. B. Decellularized human umbilical artery (H&E) x300.



Graphic 2: Isolated cholangiocyte count and their vitality after 4 hours, 8 hours and 12 hours of seeding.

Cell Seeding And Cultivation Efficacy

Histological (Hematoxylin and Eosin, Masson Trichrome) studies showed that cells were distributed in an equal fashion in whole extracellular matrix of decellularized umbilical artery. Immunohistochemical staining showed good expression and survivability of cholangiocytes after seeding and cultivation. Scanning electron

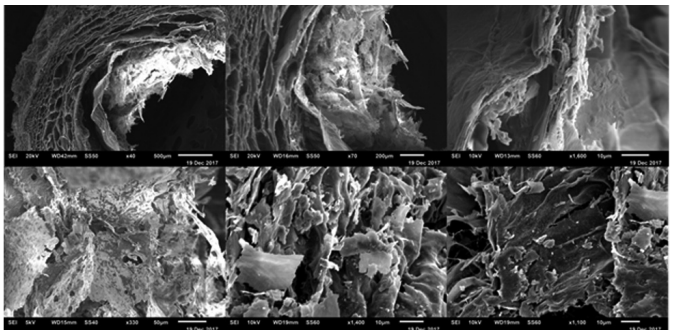


Fig. 4: Scanning electron microscopy of seeded cells (in vitro) onto inner surface of decellularized umbilical artery.

microscopy showed that cells were randomly and equally distributed in the upper (seen) layers of the decellularized umbilical artery. However, there were no intercellular connections formed as of after 12 hours of cultivation (Graphic 2, Fig. 4).

In Vivo Results

Animals of each group did not develop any complications during surgical intervention or in early post-operative period. We did not observe infection, spontaneous opening or herniation in the incision site. In animals of the both group jaundice was developed on 2nd day after operation, at fist scleral icterus and yellowish discoloration of visible mucous membranes were noticeable. Acholic fecal masses were also demonstrated after day 2. In the first group (control), out of 6 animals, 1 animal died on 50th day after operation and second on 62nd day after operation. Necropsy showed that the main reason was the fulminant liver failure. Rest of the animals were under observation till the predisposed dates of euthanasia. In second group, reconstruction was well tolerated in all the animals both in early and late post-operative period (Table I).

Table I - *Observation period and euthanasia dates for the first and second group. Cause of death for animal #4 and 5 was fulminant hepatic failure, developed after progression of biliary cirrhosis.*

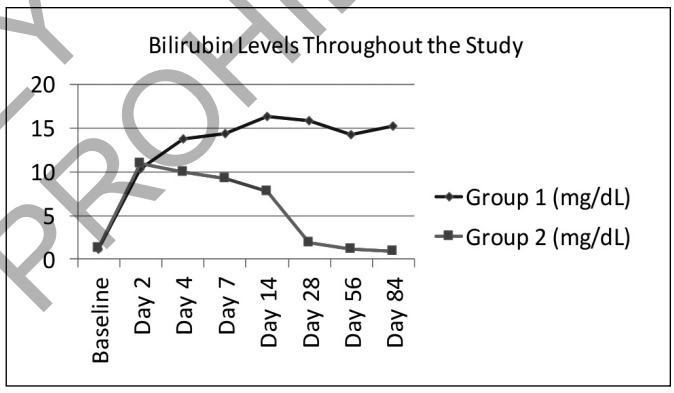
Group I		Group II	
Animal #	**Day of Euthanasia/Death**	**Animal #**	**Day of Euthanasia/Death**
1	Day 7	2.1	Day 7
2	Day 21	2.2	Day 21
3	Day 42	2.3	Day 42
4	Day 50 (complication)	2.4	Day 56
5	Day 62 (complication)	2.5	Day 70
6	Day 84	2.6	Day 84

Liver Function Test Results

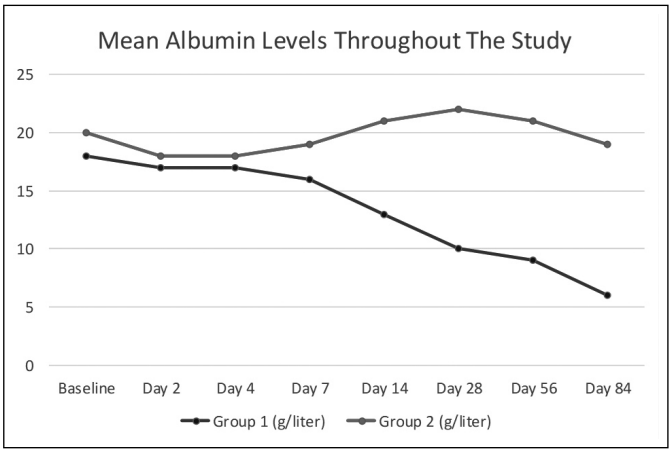
In both group after modeling common bile duct obstruction rapid rise in total bilirubin level was seen by the day 2 (>10mg/dl) (Table II, Graphic 3). Later on there was a further rise of total bilirubin level, AST and ALT

Table II. *Mean total serum bilirubin levels throughout the observation period in the first and second group.*

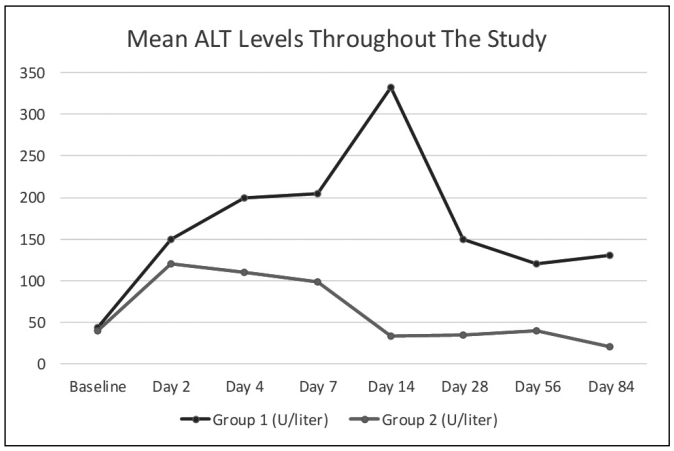
Day	Group 1 (mg/dL)	Group 2 (mg/dL)
Baseline	1,08	1,26
Day 2	10,3	10,9
Day 4	13,8	10
Day 7	14,4	9,26
Day 14	16,3	7,73
Day 28	15,8	1,91
Day 56	14,2	1,12
Day 84	15,2	0,9



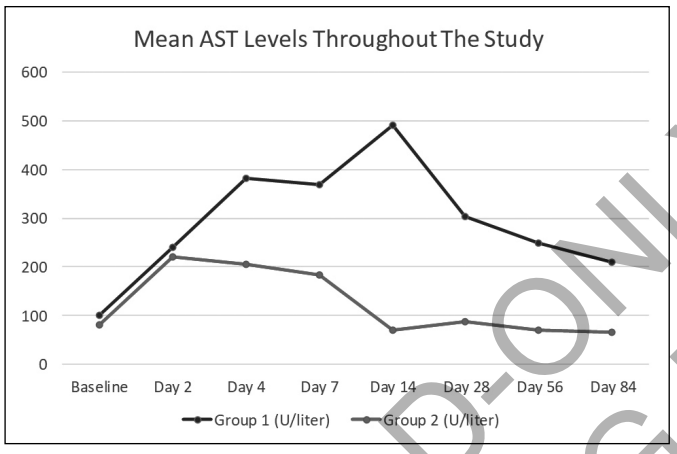
Graphic 3: Mean total serum bilirubin levels throughout the observation period in the first and second group.



Graphic 4: Mean serum ALT levels throughout the observation period in the first and second group.



Graphic 5: Mean serum AST levels throughout the observation period in the first and second group.



Graphic 6: Mean serum Albumin levels throughout the observation period in the first and second group.

and decline of albumin levels in the first group compared to baseline values. However, in the second group we saw a gradual decline of total bilirubin levels, which were normalized by the day 28th; AST and ALT also declined and normalized by the day 14 and serum albumin level staid unchanged (Graphics 4, 5, 6).

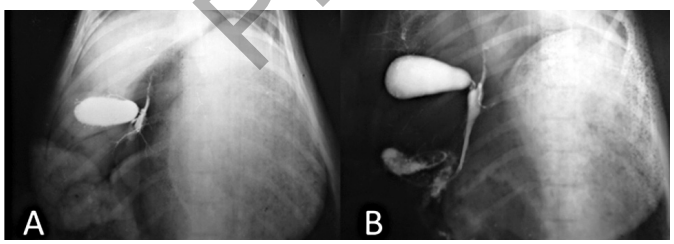


Fig. 5: A. Model of bile duct obstruction (postoperative day 7), the x-ray shows no bile drainage to the duodenum. B. Common bile duct is reconstructed with bile duct equivalent (postoperative day 7), there is no sign of stricture or bile flow obstruction.

Radiology Results

X-ray images showed an obvious and successful obstruction of bile drainage in the post-operative period (Fig. 5). In the second group, however, the bile duct drainage was good, thus, no dilation was observed at any period (Fig. 5).

Histology Results

Hematoxylin and Eosin and Masson trichrome staining demonstrated a well developing epithelial layer without fibrosis or transmural inflammation (Figs. 6, 7).
Immunohistochemical assays showed that epithelial layer in internal lumen of the transplanted bile duct equivalent was well differentiated cholangiocytes by the 48th day, which was proven by CK-19 staining; whilst, Ki-67 staining demonstrated a good proliferative activity in the transplanted bile duct equivalent.

Discussion

Bile duct obstruction, due to various diseases and caus-

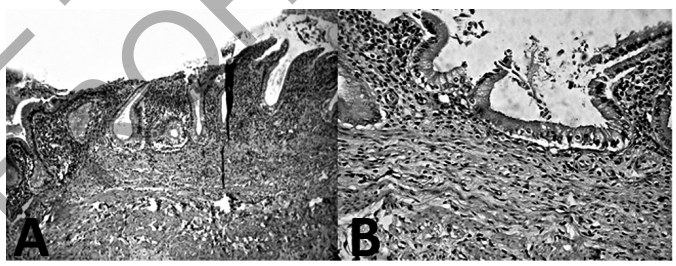


Fig. 6: A. Bile duct equivalent 48 days after reconstruction (H&E) X200. B. Bile duct equivalent 48 days after reconstruction (H&E) X400.

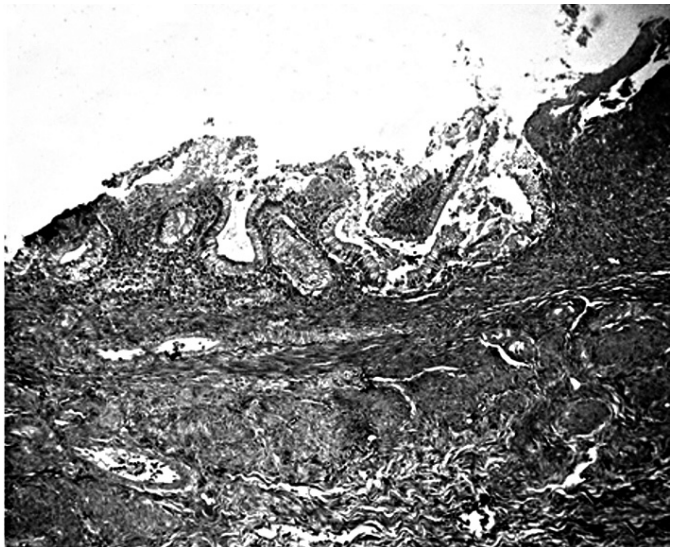


Fig. 7: Bile duct equivalent 48 days after reconstruction (Masson Trichrome) X200.

es, still is a major challenge in surgery and it affects millions of people worldwide [1-3]. Many researches and studies have been done to introduce a new method, which would allow us to reconstruct extrahepatic biliary tract without further complications; The vast literature review that was done by us, clearly demonstrates that many methods that had been offered to successfully reconstruct obstruction in the extrahepatic biliary tract were not established as a gold standard and variations of The Roux-en-Y hepatijojejunostomy stay as a gold standard [33-38]. The variations of Roux-en-Y hepatijojejunostomy has its own very frequent complications, such as recurrent cholangitis. Those complications may lead to chronic liver failure, that by itself requires a liver transplant, which is a huge problem since there is a huge shortage of donor organs worldwide [8, 39-42].

Current advances in tissue engineering and stem cell research has demonstrated that it can be applied in various field of medicine, especially in reconstructive medicine. In regards to reconstruction of luminal structures with tissue engineered organs, the most of the research has been done in urethral reconstruction; There has been a lot of papers publishing that acellular matrices of various sources has been used (both seeded cells and without, cultivated and not cultivated) for reconstruction of urethra in humans and animal models [43-45].

We hypothesized that decellularized human umbilical artery could be a good a three-dimensional scaffold for creation of bile duct equivalent, that would give us a possibility to use is for restoration anatomical integrity of hepaticocholedochus.

Data acquired from experimental group, in which reconstruction was done by our bile duct equivalent demonstrated promising results. In all animals of the second group, there was a relative rapid drop in total bilirubin levels, which was normalized by the post-operative day 28. This gives irrefutable laboratory evidence that bile duct drainage was successfully achieved. Normalization of AST, ALT levels and no changes in albumin levels demonstrates that there was no liver function impairment of any pathogenesis.

Radiological evidence collected from the second group also demonstrated a fine drainage of extrahepatic biliary tree; no stricture was shown in any part of the bile duct, whilst there was a major dilation observed in the control group.

Histochemical and immunohistochemical assays demonstrated that the integration of bile duct equivalent developed by us was smooth and without any impending complications. The major success that can be seen from the Hematoxylin and Eosin and Masson trichrome staining is the full reepithelization of the internal lumen, which is a major factor of integration to the host organism. Immunohistochemical staining for CK-19 clarified that the epithelial cells demonstrated in the Hematoxylin and Eosin and Masson trichrome stained samples were indeed cholangiocyte population, whilst Ki-67 demonstrated a good proliferative activity; both cholangiocyte population detection and their good proliferative activity without evidence of inflammation can be interpreted as a good remodeling of the graft. Although, the above mentioned facts lets us speculate that in the future there would have been no fibrosis or graft rejection, we think that further studies should be held to evaluate long term adaptation of the bile duct equivalent with the organism and account for long term complications related to the graft.

Conclusion

Decellularized human umbilical artery is a good three-dimensional scaffold for creating bile duct equivalent. Reconstruction of hepaticocholedochus with total or subtotal stricture using bile duct equivalent allows us to restore its anatomical integrity, while including sphincter of Oddi in bile drainage. This might help us prevent complications such as recurrent cholangitis, biliary cirrhosis, portal hypertension and hepatocellular carcinoma, which frequently appear in biliary-digestive anastomoses. These results are encouraging, however, there is necessity for big series of investigations and longer follow-up period to make the final statement about the efficacy of this method of bile duct reconstruction.

Acknowledgement

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Riassunto

Scopo principale di questo studio è la creazione sperimentale di dotto biliare in vitro a partenza da una arteria ombelicale umana decellularizzata al fine della ricostruzione di un epato-coledoco danneggiato, conservando lo sfintere di Oddi.

Sono stati utilizzati SDS e Triton X-100 per la decellularizzazione dell'arteria ombelicale, e con inseminazione dei colangioliti allogenici isolati sulla superficie interna dell'arteria decellularizzata.

Si è trattato di uno studio sperimentale utilizzando 12 suini domestici di entrambi i sessi, del peso di 25-30 kg, divisi in due gruppi equivalenti, creando in tutti gli animali un modello di ostruzione dell'epatocoledoco.

Un primo gruppo di 6 animali è rimasto sotto osservazione senza ulteriori interventi. I sei animali del secondo gruppo è stato rioperato dopo due giorni dall'intervento iniziale, il sito della lesione (2 cm) è stato inciso e il difetto è stato ricostruito con il dotto biliare equivalente alla dimensione di 2-2,5 cm. Il periodo di osservazione massimo è stato di 84 giorni.

Le indagini di laboratorio, morfologiche e radiologiche hanno mostrato una buona integrazione del neo-dotto con l'organismo ospite.
La ricostruzione del dotto biliare è ancora un importante intervento chirurgico in ambito epato-biliare, e per questo motivo è stato affrontato questo studio sperimentale per valutare l'efficacia del nuovo metodo di ricostruzione del dotto biliare, utilizzando indagini di laboratorio, morfologiche e radiologiche appropriate.
I risultati preliminari ottenuti con il metodo descritto ci consentono di affermare che il neo-dotto biliare creato con l'utilizzo dell'arteria ombelicale umana decellularizzata e colangioliti allogenici può essere utilizzato con successo per la ricostruzione del dotto biliare conservando lo sfintere di Oddi.

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