Original Article

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Reversible biliary occlusion in a small animal model: first description of a new technique

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Abstract

Background: Experimental models with reversible biliary occlusion resulted in a high mortality of the animals, up to 20–60% according to the literature. Our aim was to assess a safe and valid technique for reversible biliary occlusion with a low mortality.
Methods: We randomized 30 rats into two groups: with bile duct occlusion (BDO, n = 18) and with sham manipulation of the extrahepatic bile duct (control, n = 12). We used a removable vascular clip for temporary occlusion of the extrahepatic bile duct. The clip was removed on postoperative day (POD) 2. On POD 2, 3, and 5, we measured the hepatocellular injury and metabolic function markers in serum. Activation of mononuclear cells (HIS36) and expression of regeneration markers [cytokeratin 19, hepatic growth factor (HGF)-α, and HGF-β] were determined by immunohistochemistry.
Results: The survival rate was 96.67% (1/30); one animal died. The mortality in the BDO group was 6% (1/18) and that in the control group was 0% (0/12). BDO resulted in a sharp increase of hepatocellular injury and cholestatic parameters on POD 2 with a rapid decline till POD 3. Significantly strongest activation of Kupffer cells and expression of proliferation markers were found until POD 5 after BDO.
Conclusion: The clip technique is a safe, cheap, and valid method for reversible biliary occlusion with an extremely low mortality.

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Keywords: CK19; experimental surgery; temporary obstructive cholestasis.

Abbreviations: BDL, bile duct ligation; BDO, bile duct occlusion; BW, body weight; CK19, cytokeratin 19; HGF-α, hepatic growth factor α; HGF-β, hepatic growth factor β; HPF, high-power field; OSKST, one-sample Kolmogorov-Smirnov test; PBS, phosphate-buffered saline; POD, postoperative day.

Introduction

To study the mammalian pathophysiology of obstructive cholestasis, the most favorite model is bile duct ligation (BDL) mimicking a biliary obstruction of varying extent [1–8]. The liver parenchyma develops a histological conversion into a fibrotic or a cirrhotic pattern depending on the duration of biliary obstruction.

Primarily, the focus has been paid to histological alterations like the increase in relative size of the biliary compartment including proliferation of biliary epithelial cells (using CK19) and of interportal fibrous tissue [6, 7]. In these studies, BDL lasted up to 4 weeks [1–6, 8]. Later on, metabolic parameters such as gluconeogenesis, glycogen content, and lipid oxidation were also investigated [1–7, 9]. Several studies have shown that some of these structural and metabolic changes were reversible after relief of biliary obstruction [1–6]. BDL was performed by ligating the common bile duct with several sutures. The relief of the biliary occlusion was facilitated by Roux-en-Y choledochojejunostomy, as it is known as a standard procedure for a biliodigestive anastomosis in humans. Because of the impact of the second operation on the reduced hepatocellular regenerative capacity due to cholestasis, the mortality of the animals was mostly as high as 17–40% [2–4, 10]. To avoid mortality, we assessed a new technique for temporary bile duct occlusion (BDO) with inherently less morbidity and mortality.

Materials and methods

Animals

All surgical procedures were performed in inbred male Sprague-Dawley rats (Charles River, Germany) aged 9–10 weeks [body weight (BW) 250–280 g].

Institutional animal care and use committee statement: Rats were fed a standard laboratory diet with water and rat chow *ad libitum* until harvest. All animals were kept under constant environmental conditions with a 12-h light-dark cycle in a conventional animal facility using environmentally enriched type IV cages in groups of two to three rats. All procedures and housing of the animals were carried out according to the German Animal Welfare Legislation and approved by the local authorities (Hessisches Landesamt für Verbraucherschutz, reg.-nr. 02-025/08). Analgesic treatment was started immediately after wound closure in all animals. Buprenorphine (0.05 mg/kg BW; Temgesic®) was subcutaneously injected; the analgesic therapy was given twice a day during the first 3 days postoperatively. For postoperative monitoring, the rats were weighed daily.

Study design

The 30 animals were randomized into two groups: laparotomy with BDO (n = 18) and sham manipulation of the extrahepatic bile duct without occlusion (control, n = 12). On postoperative day (POD) 2, the clip was removed in all BDO rats. At three different time points (POD 2, 3, and 5), the animals (BDO with n = 6 per time point, control with n = 4 per time point) were sacrificed and samples (serum, liver tissue) were collected.

Surgical procedures

All groups were anaesthetized with an intramuscular injection of ketamine (10 mg/100 g BW) and xylazine (1 mg/100 g BW). The animals were shaved and placed on a small animal operating table. As the operation time for placing and removing the clip was always approximately 10 min, we avoided using a warming plate.

BDO group – laparotomy with BDO: After midline laparotomy, the common bile duct was exposed and occluded 0.5–1 cm above the pancreas using a removable vascular clip. The wounds were closed by running sutures. After 2 days (POD 2), a re-laparotomy was performed and the clip was removed. The wounds were closed.

The vascular titan clip was placed and removed using a special clip-forceps (Aesculap AG, Germany; vascular bulldog clip straight 25 mm). The used forceps ensured correct placing of the clip and simultaneously avoided overtension of the gentle clip and damage of the common bile duct or the surrounding organs. We selected a vascular clip with a low but sufficient closing force of 2.4 N with atraumatic toothing "DeBakey," as a higher closing force could lead to residual obstruction resulting in remaining dilatation of the extrahepatic bile duct.

Control group – laparotomy with sham manipulation of the bile duct: These animals underwent anesthesia and laparotomy, as described, and exposure of the common bile duct without occlusion ("sham procedure"). The control animals received a second laparotomy at the time point of sacrifice.

Characterization of the animals

Food intake and BW gain were measured daily. No pair-feeding was performed. At the indicated time points of death, liver and spleen weight were measured.

Tissue preparation

At the dedicated time points and under anesthesia, a laparotomy was performed and a blood sample was collected into sterile tubes. The blood was allowed to clot. During all procedures, the samples were kept at 4 °C. Immediately after sacrifice of the animals, liver samples were placed in Shandon™ Cryomatrix™ embedding medium (Thermo Fisher Scientific, UK) and quickly cooled in liquid nitrogen for subsequent cryosectioning for immunohistochemistry. For detection of the systemic parameters, blood samples were taken from the retrobulbar venous plexus preoperatively and at days 2, 3, 4, and 5. The blood was allowed to clot. During all procedures, the samples were kept at 4 °C. All specimens were kept at −80 °C until subsequent analyses were performed. Rats do not have a gallbladder.

Laboratory measurements

Measurements in serum: The activities of alkaline phosphatase (U/L), aspartate aminotransferase (U/L), alanine aminotransferase (U/L), γ-glutamyl-transferase (U/L), and the contents of albumin (g/dL), bilirubin (mg/dL), glucose (mmol/L), cholesterol (mg/dL), triglycerides (mg/dL), and whole protein (g/dL) were measured in serum using an automated chemical analyzer (Bayer Advia 1650, Germany).

Staining and immunohistochemistry

For histological evaluation, we used a standardized sampling of three pieces of every liver. Acetone-fixed, 5-µm frozen liver sections were stained with hematoxylin and eosin in order to evaluate alterations of the liver parenchyma and bile duct hyperplasia.

Immunohistological detection of activated Kupffer cells was done by using monoclonal anti-rat-macrophage antibody [HIS36 mouse anti-rat (22231D); Pharmingen Biotechnology, Germany] diluted 1:700 in phosphate-buffered saline (PBS; pH 7.4–7.5, without Ca^{2+} and Mg^{2+}), applied to the frozen liver sections and incubated at 37 °C for 60 min. Staining procedures were performed as already published by our group [11].

Immunohistological detection of biliary neoangiogenesis [cytokeratin 19 (CK19)] was performed by using monoclonal

mouse-anti-rat ready-to-use antibody [CK19, mouse anti-rat (22231D); Pharmingen Biotechnology, Germany] with PBS (pH 7.4–7.5, without Ca^{2+} and Mg^{2+}) applied to the frozen liver sections and incubated at 37 °C for 60 min. The staining procedure was performed with a commercial set provided by Progen Biotechnik GmbH [mouse IgG2b, monoclonal antibody CK19 (Ks 19.2)]. Incubation times were set and washing procedures were performed as described in the manufacturer's manual.

The immunohistological detection of hepatic growth factors (HGF-α and -β) followed the above-described procedure with dilution of the primary antibodies at 1:50 in PBS [anti-HGF-α and anti-HGF-β (N19) sc-1356], application to the frozen liver sections, and incubation at 37 °C for 60 min. The next steps were performed with a commercial set of secondary and tertiary antibodies provided by Santa Cruz Pharmaceuticals (Goat ABC Staining System Kit; Santa Cruz Biotechnology, sc-2023). Incubation times were set and washing procedures were performed as described in the manufacturer's manual.

Negative and positive controls were made using five slides for each staining (primary antibodies) and with additional control slides from random organs.

Quantification of immunohistochemistry

Liver cryostat sections were analyzed microscopically using a morphometric analysis system (CBA 8000-Manager; Leica, Germany). We manually counted CK19-positive cells in 10 high-power fields (HPFs, 20× magnification) and calculated the ratio of CK19-positive cells per total cholangiocytes. The expression was graduated into four grades (Table 1). Kupffer cells, CK19, and HGF-α+β were evaluated according to this standardized protocol [11].

Statistics

Data are presented as mean ± standard deviation (SD). We used the parametric descriptors due to the type of data distribution and performed statistical tests in coordination with a statistician.

The data were analyzed for the type of distribution by using the one-sample Kolmogorov-Smirnov test (OSKST). Normal distribution was accepted if $p > 0.05$ (two-tailed significance, corrected for ties). For all parameters, the OSKST showed no normal distribution. Therefore, statistical evaluation for significance was performed using the Kruskal-Wallis test. Significance was accepted in all cases at $p < 0.05$ (two-tailed significance, corrected for ties). All statistical procedures were performed using SPSS® 22.0 for Windows (SPSS Science International, Chicago, IL, USA). All statistical analyses were conducted in coordination with a statistician.

Table 1: Graduation of expression of detected markers (CK19, HIS36, HGF-α+β) in immunohistochemistry according to the ratio of positively stained cells to total cells per HPF (20× magnification).

Grade	Description
0	No positively stained cells are visible
1	1% > $x \leq$ 25% positively stained cells
2	25% > $x \leq$ 50% positively stained cells
3	50% > $x \leq$ 75% positively stained cells
4	75% > $x \leq$ 100% positively stained cells

Results

Safe and easy administration of the vascular clip for temporary biliary occlusion

In all BDO animals, the clip was easy and safe to place at the extrahepatic bile duct above the pancreas. As we placed the clip under the control of a microscope and by using the clip-forceps, we avoided any impairment of the branches of the hepatic artery, portal vein, and pancreas. Furthermore, the microscope and forceps assured the placement of the clip below the bile duct branches of the lower liver lobes (right lobes and caudate lobes), preventing a non-intended incomplete BDO (Figure 1A–D). In all BDO animals, we found a macroscopically visible dilatation of the extrahepatic bile duct above the clip (Figure 1A–D) until POD 2. We have not seen any dislocation of the clip or damage of the neighboring liver lobes. On POD 2, the clip was easy to remove using the clip-forceps without any additional surgical manipulation (e.g. adhesiolysis, thermic or mechanic coagulation) besides the re-laparotomy. Interestingly, shortly after (≤5 min) the retrieval of the clip, the massive dilatation of the main bile duct was reduced to a slightly dilated common bile duct. No persistent dilatation of extrahepatic bile duct has been noted on POD 3 and POD 5, respectively. In all BDO animals, we found a slightly green staining of the liver indicating intrahepatic cholestasis after 48 h of BDO (POD 2). Until 72 h (POD 5) after the removal of the clip, the tissue staining disappeared in all BDO animals. Few drops of clear ascites were found only in two BDO animals (2/18): in n = 1 on POD 3 and n = 1 on POD 5.

The control animals showed neither ascites nor a modified coloring of the liver.

Situs pictures

Survival and recovery of the animals

The survival rate of all animals was 96.67%; only one animal (1/30, 3.33%) died. Death occurred in a BDO animal on POD 3 (24 h after clip removal). The mortality rate in the BDO group was 6% (1/18) and that in the control group was 0% (0/12). Autopsy could exclude surgical complications like bleeding, ischemia, organ damages, or peritonitis.

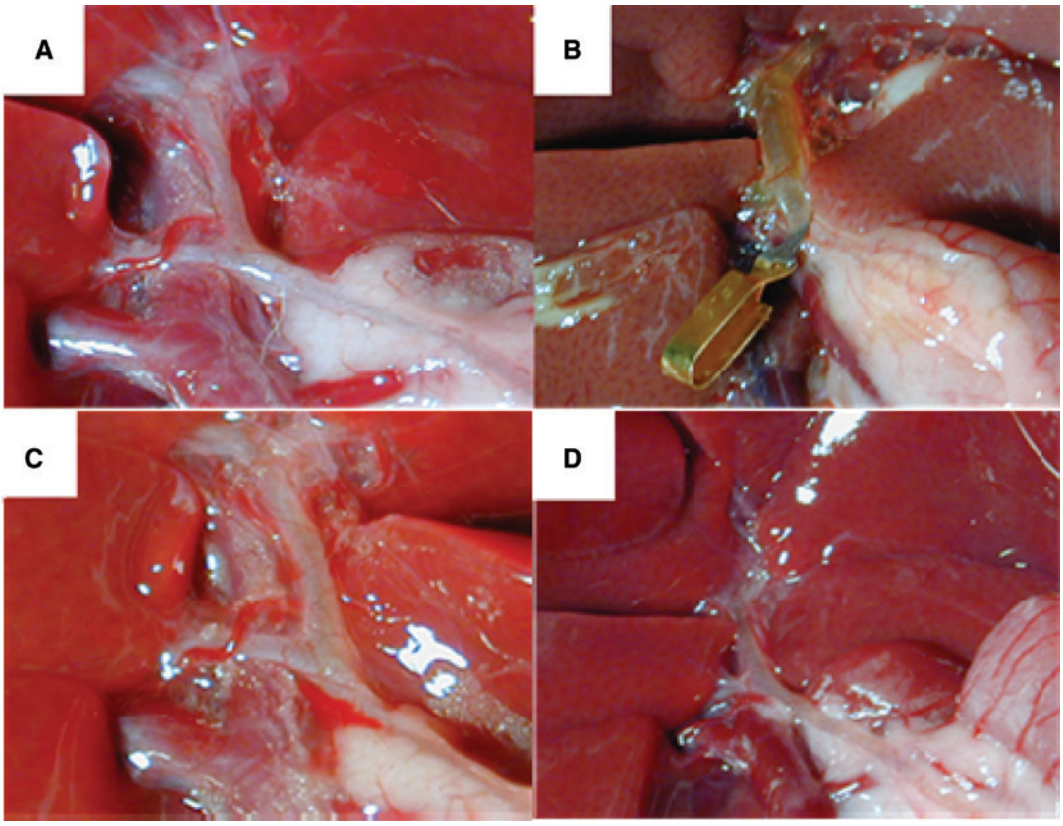

Figure 1: Pictures with macroscopically visible dilatation of the extrahepatic bile duct during temporary BDO with the vascular clip (B) and after restored bile flow (C and D).
(A) Normal situs of the liver hilus of a rat (control, POD 2). (B) Liver hilus with extrahepatic bile duct after 2 days of BDO. The titan clip occluded the common bile duct below the branches of the lower liver lobes (right lobes and caudate lobes) (BDO, POD 2). (C) Liver hilus with extrahepatic bile duct 2 min after the removal of the titan clip (BDO, POD 2). (D) Liver hilus with extrahepatic bile duct 3 days after the removal of the titan clip (BDO, POD 5).

Therefore, it seems that an anesthesia-related problem led to the death of the animal. All surviving animals showed an uncomplicated recovery during the observation period. No significant differences between the groups were found regarding BW, food intake, and liver and spleen weights (data not shown).

Serum parameters

BDO resulted in a significantly strong increase in all systemic parameters compared to control on POD 2 (Table 2). With restored bile flow, nearly all parameters declined to normal values on POD 3 (Table 3). Only the activities of aspartate aminotransferase and alkaline phosphatase remained significantly elevated until POD 5 (Table 2). Glucose was significantly depressed after BDO until POD 3 and returned to normal levels until POD 5 (Table 3). Cholesterol was increased on POD 2 after BDO, followed by a rapid decline to normal values (Table 3). Whole protein content and triglycerides were not significantly altered after BDO or in control.

Immunohistochemistry

We found a three-fold increase of the expression of HGF-α and HGF-β after BDO till POD 5 in comparison to control (Table 4). HGF-α was predominantly expressed in the area between the central veins and the portal fields, whereas HGF-β was expressed in the periportal areas and near the central veins (Figures 2A–D and 3A–D).

Following BDO, CK19 showed a constant and nearly two-fold increase till POD 5 (Table 4). A strong expression was localized around the portal fields and a weaker expression toward the central veins (Figure 4A–D).

Activation of Kupffer cells (HIS36) showed a significant increase on POD 3 after BDO, followed by a stepwise decrease till POD 5 in comparison to control. The strongest increase in the numbers of resident macrophages was

Table 2: Hepatocellular injury and cholestasis parameters.

	Control (n = 12)	BDO (n = 18)	p-Value (BDO vs. control)
Preoperative			
Aspartate aminotransferase (U/L)	72 ± 15.4	64 ± 40.2	[a]
Alanine aminotransferase (U/L)	35 ± 3.7	35 ± 14.6	[a]
Alkaline phosphatase (U/L)	278 ± 70.5	238 ± 10.4	[a]
Bilirubin (mg/dL)	0.1 ± 0.1	0.15 ± 0.05	[a]
γ-Glutamyl-transferase (U/L)	3 ± 0.4	3 ± 0.6	[a]
POD 2			
Aspartate aminotransferase (U/L)	48 ± 28.1	544 ± 262	0.001
Alanine aminotransferase (U/L)	41 ± 5	478 ± 235	0.001
Alkaline phosphatase (U/L)	227 ± 34	579 ± 244	0.002
Bilirubin (mg/dL)	0.05 ± 0.04	0.75 ± 0.1	0.001
γ-Glutamyl-transferase (U/L)	3 ± 0.6	9 ± 3	0.002
POD 3			
Aspartate aminotransferase (U/L)	90 ± 2	154 ± 84	0.003
Alanine aminotransferase (U/L)	51 ± 5	91 ± 21	0.056
Alkaline phosphatase (U/L)	191 ± 21	298 ± 44	[a]
Bilirubin (mg/dL)	n.d.	0.3 ± 0.2	n.d.
γ-Glutamyl-transferase (U/L)	2.5 ± 0.5	2.9 ± 1.1	[a]
POD 4			
Aspartate aminotransferase (U/L)	55 ± 1	57 ± 3	[a]
Alanine aminotransferase (U/L)	39 ± 0	41 ± 8	[a]
Alkaline phosphatase (U/L)	n.d.	260 ± 4	n.d.
Bilirubin (mg/dL)	0.1 ± 0.01	0.2 ± 0.01	[a]
γ-Glutamyl-transferase (U/L)	2.5 ± 0.5	2.8 ± 1.3	[a]
POD 5			
Aspartate aminotransferase (U/L)	34 ± 5	55 ± 8	0.004
Alanine aminotransferase (U/L)	29 ± 4	33 ± 5	[a]
Alkaline phosphatase (U/L)	204 ± 2	282 ± 67	[a]
Bilirubin (mg/dL)	0.1 ± 0.01	0.2 ± 0.01	[a]
γ-Glutamyl-transferase (U/L)	2.5 ± 0.5	3.3 ± 0.5	[a]

[a] $p > 0.05$ vs. control; n.d., not determined. The rats studied had either BDO for 2 days or a sham laparotomy (control). On POD 2, the bile flow was restored by removal of the clip devise. Data are given as mean ± SD.

found at the portal fields, whereas a weaker activation was found toward the central veins (Figure 5A–D).

Discussion

Within the last decades, numerous studies explored the impact of temporary biliary occlusion on liver function and liver histology [1–3, 5, 6]. Only few authors reported about their survival data; the mortality of the animals was mostly as high as 17–40% [2–4, 10]. Most of them described the highest mortality within 1–5 days after the biliary drainage operation [3, 5]. The authors identified the combination of additional surgical trauma, the pro-inflammatory response, and the increased hepatocellular metabolic and proliferative demand due to the second operation with a reduced hepatic regenerative capacity due to the biliary occlusion to be the critical factors for the high mortality in this experimental setting [2–5].

To avoid these unfavorable results, we searched for an alternative technique for temporary BDO with inherently less morbidity and mortality. We used a titan vascular clip to occlude the common bile duct (Figure 1A–D). The clip was easy to place and to remove without any additional surgical trauma within the second operation. In our study, only 1 of the 30 animals died (survival: 96.67% with 29/30; mortality: BDO 6% with 1/18 vs. control 0% with 0/12). Autopsy could exclude surgical complications. The application of the clip devise was always done under direct control of a microscope, assuring the ascertained arterial and portal-venous blood supply. Therefore, the new technique for temporary BDO using a vascular clip is a safe, fast, and inexpensive method without significant mortality. Similar observations concerning safety and usage of a

Table 3: Liver metabolic parameters.

	Control (n=12)	BDO (n=18)	p-Value (BDO vs. control)
Preoperative			
Proteins (g/dL)	5.8±0.3	5.8±0.3	[a]
Albumin (g/dL)	4.1±0.01	3.9±0.2	[a]
Glucose (mmol/L)	7.3±5.4	7.7±5.7	[a]
Triglycerides (mg/dL)	121.4±9.3	120±5.1	[a]
Cholesterol (mg/dL)	79.2±19.8	79.8±27.8	[a]
POD 2			
Proteins (g/dL)	5.6±0.4	5.3±0.3	[a]
Albumin (g/dL)	2.7±0.4	2.5±0.4	[a]
Glucose (mmol/L)	10.9±5.2	9.1±2.6	0.05
Triglycerides (mg/dL)	117±9.3	113±9.7	[a]
Cholesterol (mg/dL)	75.2±18.2	148.5±68.9	0.001
POD 3			
Proteins (g/dL)	5.1±0.3	4.5±0.2	[a]
Albumin (g/dL)	2.5±0.3	2.0±0.1	[a]
Glucose (mmol/L)	18.4±0.6	8.7±1.2	0.006
Triglycerides (mg/dL)	119±6.5	124±9.7	[a]
Cholesterol (mg/dL)	85.0±32	86.6±17	[a]
POD 4			
Proteins (g/dL)	5.3±0.4	5.6±0.1	[a]
Albumin (g/dL)	2.8±0.5	2.3±0.01	[a]
Glucose (mmol/L)	10.8±0.1	12.6±0.8	0.035
Triglycerides (mg/dL)	119.9±10.1	117±7.2	[a]
Cholesterol (mg/dL)	71.0±12.73	84.0±7	[a]
POD 5			
Proteins (g/dL)	5.0±0.15	4.9±0.39	[a]
Albumin (g/dL)	2.2±0.1	1.9±0.15	[a]
Glucose (mmol/L)	22.3±0.1	17±3.5	[a]
Triglycerides (mg/dL)	121±7.3	120±5.3	[a]
Cholesterol (mg/dL)	67.0±19.8	78.4±8.3	[a]

[a]$p > 0.05$ vs. control. The rats studied had either BDO for 2 days or a sham laparotomy (control). On POD 2, the bile flow was restored by removal of the clip devise. Data are given as mean±SD.

Table 4: Results of immunohistochemistry.

	Control (n=12)	p-Value (BDO vs. control)	BDO (n=18)	p-Value
POD 2				(BDO: POD 2 vs. POD 3)
HIS36	1.0±0.07	0.0001	2.28±0.39	0.001
CK19	1.05±0.25	0.156	1.44±0.58	0.015
HGF-α	1.44±1.22	0.257	1.6±0.59	0.005
HGF-β	0.83±0.39	0.002	1.61±0.44	0.001
POD 3				(BDO: POD 3 vs. POD 5)
HIS36	0.9±0.07	0.0001	3.17±0.69	0.001
CK19	1.0±0.2	0.0001	2.14±0.37	0.004
HGF-α	1.38±1.19	0.003	2.5±0.81	0.047
HGF-β	0.8±0.3	0.0001	2.56±0.54	0.003
POD 5				(BDO: POD 5 vs. POD 2)
HIS36	1.1±0.09	0.0001	2.16±0.7	0.669
CK19	1.1±0.25	0.0001	2.53±0.38	0.001
HGF-α	1.40±1.2	0.0001	3.06±0.15	0.0001
HGF-β	0.75±0.45	0.0001	3.33±0.53	0.0001

The rats studied had either BDO for 2 days or sham laparotomy (control). On POD 2, the bile flow was restored by removal of the clip devise. Data are given as mean±SD.

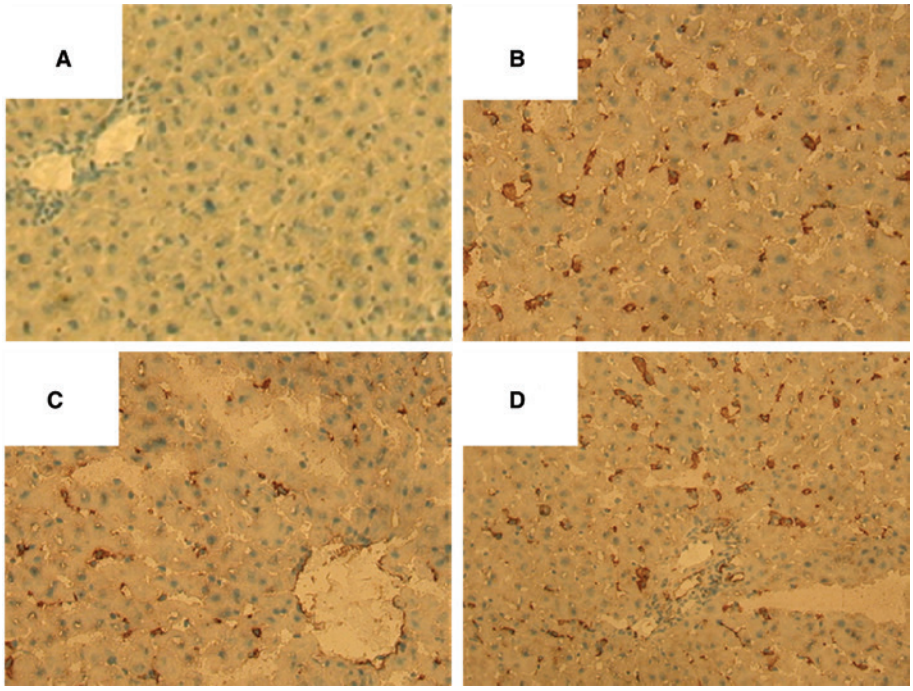

Figure 2: Immunohistochemical assessment of the liver after BDO for 2 days or control.
Staining for HGF-α (brown: HGF-α; blue: nuclei, 200× magnification). (A) Control, POD 2: control animal without BDO. (B) BDO, POD 2: after 2 days of BDO. (C) BDO, POD 3: 24 h after relief of BDO. (D) BDO, POD 5: 72 h after relief of BDO.

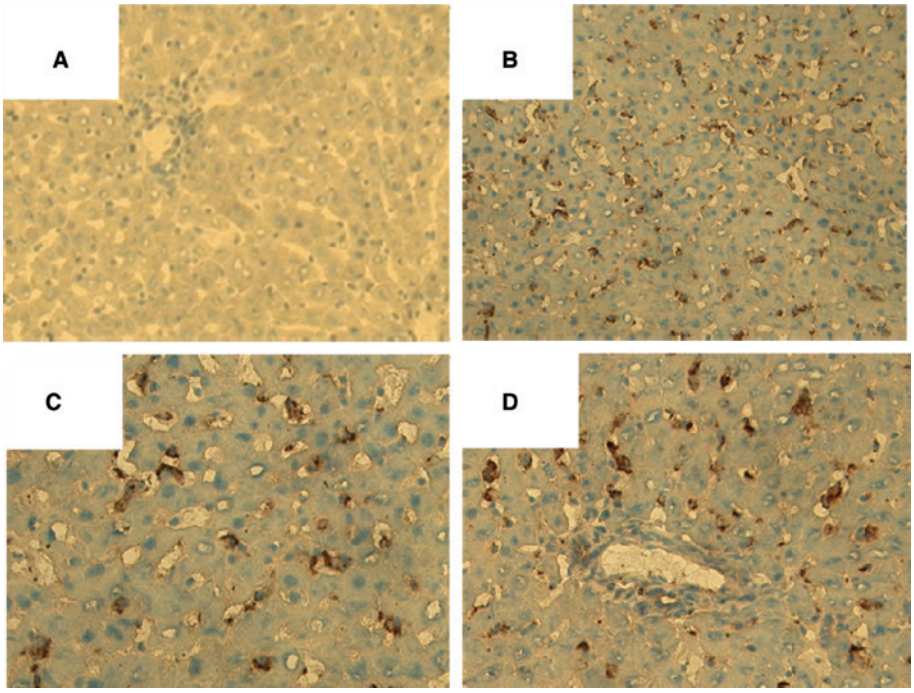

Figure 3: Immunohistochemical assessment of the liver after BDO for 2 days or control.
Staining for HGF-β (brown: HGF-β; blue: nuclei, 200× magnification). (A) Control, POD 2: control animal without BDO. (B) BDO, POD 2: after 2 days of BDO. (C) BDO, POD 3: 24 h after relief of BDO. (D) BDO, POD 5: 72 h after relief of BDO.

vascular clip device were published by Jorge with experimental intermittent hepatic pedicle clamping and temporary choledochal clamping (~10 min) in rats [7, 12, 13].

The second endpoint in our study was the suitability of the clip to induce comparable systemic and histological alterations, as already described with the "ligation

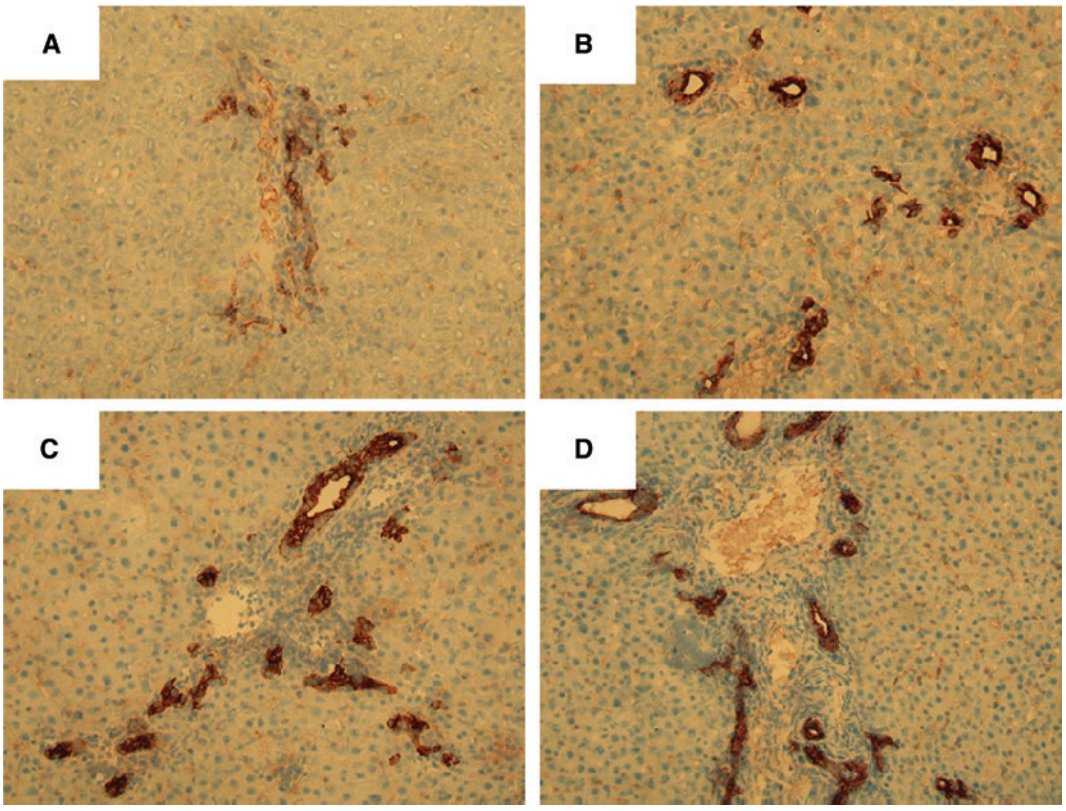

Figure 4: Immunohistochemical assessment of the liver after BDO for 2 days or control.
Staining for CK19 (red: CK19; blue: nuclei, 200× magnification). (A) Control, POD 2: control animal without BDO. (B) BDO, POD 2: after 2 days of BDO. (C) BDO, POD 3: 24 h after relief of BDO. (D) BDO, POD 5: 72 h after relief of BDO.

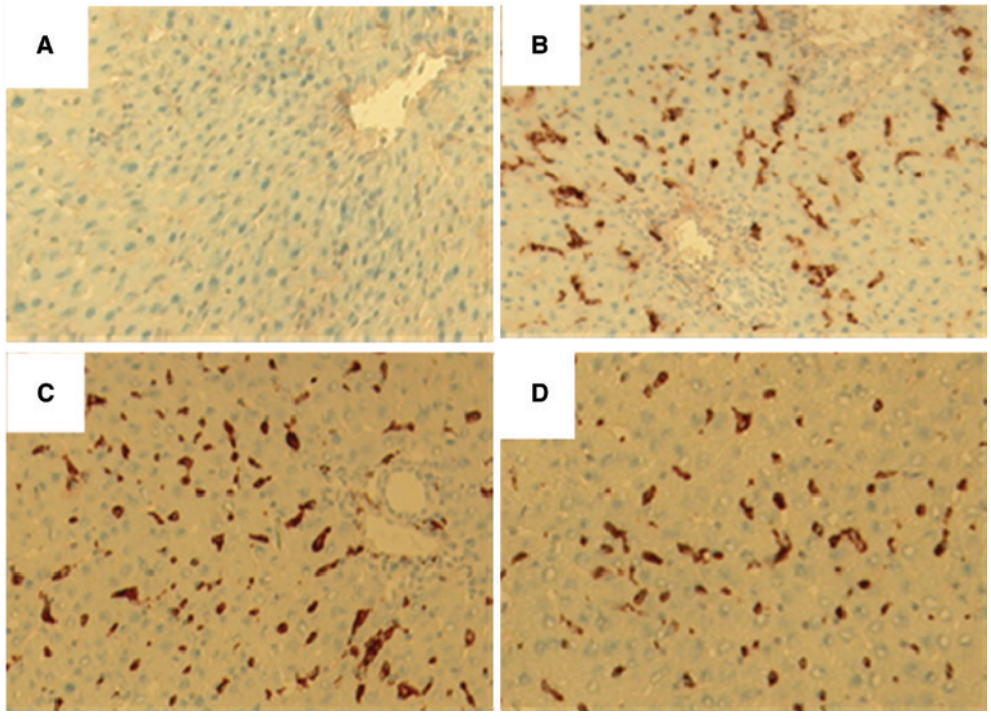

Figure 5: Histologic assessment of the liver after BDO for 2 days or control.
Staining for HIS36 (brown: HIS36; blue: nuclei, 200× magnification). (A) Control, POD 2: control animal without BDO. (B) BDO, POD 2: after 2 days of BDO. (C) BDO, POD 3: 24 h after relief of BDO. (D) BDO, POD 5: 72 h after relief of BDO.

technique of BDL" [7, 8, 12–15]. BDO led to a strong increase in systemic cholestatic parameters, while after removal of the clip we determined a rapid decline of these parameters [7, 8, 12–15]. Interestingly, in our setting, the common bile duct showed no remaining dilatation after the removal of the vascular clip. In contrast, Jorge described in his setting (his clip was removed after 10 min) a residual dilatation of the main bile duct for up to 28 days [12, 13]. As Jorge described no further details of the used vascular clip, it led to the suspicion that the closing force is a crucial factor for visible and remaining dilatation of the main bile duct. Therefore, we selected a vascular clip with a low but sufficient closing force of 2.4 N with atraumatic toothing "DeBakey." A higher closing force could lead to residual obstruction resulting in remaining dilatation of the extrahepatic bile duct. We found no dislocation of the vascular clip or signs of mechanical damage of the neighboring organs (e.g. liver lobes, pancreas, branches of hepatic artery, and portal vein) by the clip.

The impressive and fast expression of proliferation markers underlines the fast response and the high activity level of the biliary epithelial cells even in our short observation time. However, our data are in line with former publications of short-term cholestasis using the "ligation technique" [6, 7, 16, 17]. Therefore, BDO using the clip induced a comparable regenerative response of the liver parenchyma, as described with the "ligation technique." Furthermore, the fast and notable activation of Kupffer cells correspond to the results of Gregory and Tracy using the "ligation technique" [16–18].

Hence, our "clip technique" led to comparable results like the "ligation technique" and resulted in a significantly lower mortality of the animals. Therefore, our clip technique was a safe and valid method for induction of reversible occlusive cholestasis in small animals.

Conclusion

In summary, the vascular clip is a safe, minimally invasive, inexpensive, and valid technique for the induction of obstructive cholestasis without the risk of a high mortality of the animals as described with the "ligation technique."

Further investigations regarding the appropriateness of the clip device for long-term obstructive cholestasis (≥2 weeks) and comparable restoration of the bile flow are needed and currently under way.

Author Statement

Research funding: Authors state no funding involved. Conflicts of interest: The authors have declared that no competing interests exist. Informed consent: Informed consent is not applicable. Ethical approval: The research related to the animal use complies with all the relevant national regulations and institutional policies. All procedures and housing of the animals were carried out according to the German Animal Welfare Legislation and approved by the local authorities (Hessisches Landesamt für Verbraucherschutz, reg.-nr. 02-025/08).

Author Contributions

Beate Richter: conceptualization; data curation; formal analysis; investigation; methodology; project administration; software; validation; writing – original draft; writing – review and editing. Semik Khodaverdi: investigation. Wolf Otto Bechstein: financial support. Carsten Gutt: financial support. Lukas Krähenbühl: financial support. Thomas Schmandra: supervision; writing – review and editing.

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Supplementary Material: The article (https://doi.org/10.1515/iss-2018-0021) offers reviewer assessments as supplementary material.

Reviewer Assessment

Beate Richter*, Semik Khodaverdi, Wolf Otto Bechstein, Carsten N. Gutt, Lukas Krähenbühl and Thomas C. Schmandra

Reversible biliary occlusion in a small animal model: first description of a new technique

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Reviewers' Comments to Original Submission

Reviewer 1: anonymous

Jun 24, 2018

Reviewer Recommendation Term:	Accept
Overall Reviewer Manuscript Rating:	90

Custom Review Questions	**Response**
Is the subject area appropriate for you?	5 - High/Yes
Does the title clearly reflect the paper's content?	5 - High/Yes
Does the abstract clearly reflect the paper's content?	5 - High/Yes
Do the keywords clearly reflect the paper's content?	4
Does the introduction present the problem clearly?	4
Are the results/conclusions justified?	5 - High/Yes
How comprehensive and up-to-date is the subject matter presented?	4
How adequate is the data presentation?	5 - High/Yes
Are units and terminology used correctly?	5 - High/Yes
Is the number of cases adequate?	4
Are the experimental methods/clinical studies adequate?	4
Is the length appropriate in relation to the content?	4
Does the reader get new insights from the article?	5 - High/Yes
Please rate the practical significance.	4
Please rate the accuracy of methods.	4
Please rate the statistical evaluation and quality control.	4
Please rate the appropriateness of the figures and tables.	4
Please rate the appropriateness of the references.	4
Please evaluate the writing style and use of language.	3
Please judge the overall scientific quality of the manuscript.	5 - High/Yes
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:

This is a really nice study with a significant result. It shows clearly that the described clip technique is a safe and easy method for reversibible biliary occlusion with a very low mortality. The clip is easy to remove in a second small surgery. In other studies, the animals needed a biliodigestive anastomosis to reverse the biliary occlusion with higher mortality perhaps also because of the biger surgical trauma. The laboratory measurements show a fast recovery after removing the clip. The paper is well structured, giving a good overview about the published literature. The methods and results are clearly described by the author.

Reviewer 2: anonymous

Jul 05, 2018

Reviewer Recommendation Term:	Accept
Overall Reviewer Manuscript Rating:	76

Custom Review Questions	**Response**
Is the subject area appropriate for you?	4
Does the title clearly reflect the paper's content?	5 - High/Yes
Does the abstract clearly reflect the paper's content?	5 - High/Yes
Do the keywords clearly reflect the paper's content?	4
Does the introduction present the problem clearly?	4
Are the results/conclusions justified?	4
How comprehensive and up-to-date is the subject matter presented?	5 - High/Yes
How adequate is the data presentation?	5 - High/Yes
Are units and terminology used correctly?	5 - High/Yes
Is the number of cases adequate?	4
Are the experimental methods/clinical studies adequate?	5 - High/Yes
Is the length appropriate in relation to the content?	5 - High/Yes
Does the reader get new insights from the article?	4
Please rate the practical significance.	3
Please rate the accuracy of methods.	4
Please rate the statistical evaluation and quality control.	5 - High/Yes
Please rate the appropriateness of the figures and tables.	4
Please rate the appropriateness of the references.	4
Please evaluate the writing style and use of language.	5 - High/Yes
Please judge the overall scientific quality of the manuscript.	4
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:

The author presents in this experimental paper with accurate methods a modified technique of reversible biliary occlusion resulted in a reduces mortality compared to former studies. As described in the conclusions further investigations with long-term-outcomes are needed to prove superiority of these methods.