

Influence of biliary anastomosis on recovery from secondary biliary cirrhosis

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Objective The influence of choledochoduodenostomy and choledochojejunostomy on the repair of hepatic lesions secondary to biliary obstruction is not well known. The aim of the present study was to compare the effects of choledochoduodenostomy and choledochojejunostomy on the recovery of these lesions in rats with biliary obstruction.

Methods Rats subjected to 4 weeks of biliary obstruction underwent choledochoduodenostomy ($n=10$) or choledochojejunostomy ($n=10$). The following variables were measured: total bilirubin, alkaline phosphatase, aminotransferases, and albumin. Hepatic mitochondrial energy metabolism was evaluated by calculating the respiratory control ratio and the oxidative phosphorylation index. Hepatic morphometry was used to estimate the mass of the hepatocytes, bile ducts, and fibrosis, as well as the hepatic stellate cell count.

Results After choledochoduodenostomy and choledochojejunostomy, there was a regression in cholestasis and a reduction in the oxidative phosphorylation index. However, the total bilirubin, alkaline phosphatase, albumin, and respiratory control ratio values improved only after choledochojejunostomy. The mass of the liver, spleen, and fibrosis was reduced after both choledochoduodenostomy and choledochojejunostomy, but the number of hepatic stellate cells increased.

After choledochojejunostomy, the hepatic mass recovered completely, and the spleen mass was significantly reduced compared with that after choledochoduodenostomy. After both choledochoduodenostomy and choledochojejunostomy, enterobiliary reflux, biliary contamination, and an exacerbation in hepatic inflammation developed.

Conclusion Choledochojejunostomy was more effective than choledochoduodenostomy, but both techniques induced enterobiliary reflux and biliary contamination, which may explain the maintenance of hepatic alterations, especially after choledochoduodenostomy. *Eur J Gastroenterol Hepatol* 24:1039–1050 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: biliary liver cirrhosis, choledochostomy, extrahepatic cholestasis, hepatic stellate cells, liver fibrosis, liver regeneration

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Introduction

In clinical practice, fibrosis and secondary biliary cirrhosis are frequently considered to be irreversible hepatic lesions [1]. However, clinical [2–4] and experimental [5–8] reports have shown that effective biliary decompression can promote the reversion of anatomopathological alterations in the liver.

Among the different methods used to treat extrahepatic cholestasis, choledochoduodenostomy (CD) and choledochojejunostomy (CJ) are frequently applied. In clinical practice, the choice between these two biliary anastomosis modalities for the treatment of an extrahepatic biliary obstruction (BO) depends more on the experience of the surgeon than on the pathogenesis of the disease being treated [9].

The use of CD in rats with secondary biliary fibrosis leads to the resolution of cholestasis, but without recovery of anatomopathological lesions and portal hypertension [10].

In similar hepatic lesions, resolution of the excretory function, regression in the liver lesions, and decreased portal pressure levels were observed after CJ [6]. In contrast, alterations in the motility of the excluded loop in conventional Roux-en-Y can lead to stasis, bacterial growth, and hepatic fibrosis [8,11,12].

Currently, it is widely believed that the therapeutic results of a BO treatment depend more on the etiology of the obstruction and the patency of the anastomosis than on the technique of biliodigestive anastomosis used [13,14]. These observations, in addition to the technical simplicity, have likely contributed to the considerable use of CD for the treatment of chronic BO, especially when using a video-laparoscopic approach.

Therefore, the aim of the present study was to comparatively evaluate the influence of CD and CJ techniques on the recovery of metabolic and anatomopathological liver lesions in rats with chronic BO.

Methods

Experimental design

Male Wistar rats with an initial weight between 232 and 317 g were divided into four groups: a BO group ($n = 6$), which included rats subjected to a bile duct ligation for 1 month; a CD group ($n = 10$) and a CJ group ($n = 10$), which included rats subjected to a bile duct ligation for 1 month and treated with either CD or CJ, respectively; and a fourth group that was subjected to a sham operation (SO; $n = 8$). The CD, CJ, and SO groups were evaluated 3 months after treatment (Fig. 1).

Surgical technique

The use of laboratory animals was in accordance with the Council for International Organizations of Medical Sciences Ethical Code for Animal Experimentation. The animals were anesthetized with pentobarbital (40–60 mg/kg by an intraperitoneal injection). The BO was performed according to a technique developed by our group involving the ligation of the biliary duct with five knots of a Prolene suture (Prolene Suture-blue monofilament 5/0, Ethicon Inc., São Paulo, Brazil) ~3 mm above the biliary–pancreatic junction. Subsequently, using the same suture, the biliary duct was circled up to ~5 mm of the lobar duct confluence, where an additional ligature was performed again with five knots [8].

Immediately before the biliary anastomosis, 2 ml blood sample was collected by a cava vein puncture, followed by

an infusion of 6 ml of a 5% glucophysiologic solution. The following procedures were then performed: a laparotomy, a liver biopsy, and an anastomosis between the anterior wall of the bile duct and either the duodenum (for CD) or the distal extremity of the jejunum (for CJ), which was sectioned 5 cm from the duodenojejunal angle. The continuity of the jejunum was established through end-to-side anastomosis 15 cm from the duct-to-jejunum anastomosis. The anastomosis was performed with a continuous suture (Vicryl 5-0, Ethicon Inc.).

At the end of the experiment, 1 month after the BO procedure for the BO group and 3 months after the anastomosis procedure for the CD, CJ, and SO groups, the animals were subjected to a laparotomy, followed by the collection of bile, splenectomy, collection of a blood sample from the vena cava, inspection of the anastomosis, and removal of the liver.

Studies performed

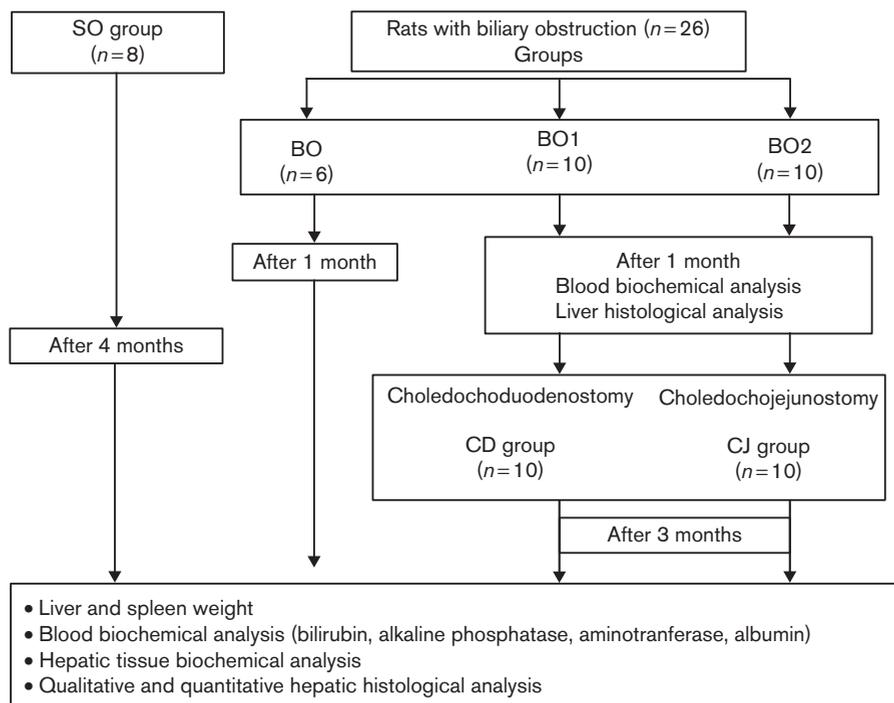
Liver and spleen wet weight

After removal, the liver and the spleen were weighed on a precision scale. The weight was in g/kg of corporeal weight.

Biochemical measures

The blood samples were collected to measure the total bilirubin (TB), the direct and indirect bilirubin fractions [15], and the amount of alanine aminotransferase (ALT), aspartate aminotransferase [16], alkaline phosphatase

Fig. 1



Research design. BO, biliary obstruction; CD, choledochoduodenostomy; CJ, choledochojejunostomy; SO, sham operation.

(ALP) [17], and albumin (ALB) [18]. All the biochemical tests were performed using Labtest Kits (Labtest Diagnostic Systems LTDA; Belo Horizonte, Minas Gerais, Brazil).

Microbiological evaluation

Bile samples were collected with surgical asepsis through a puncture of the biliary duct using a 13 × 4 hypodermic needle and placed in Eppendorf tubes containing a highly nutritious broth (brain heart infusion). After 24 h of incubation in a microbiologic stove at 35°C, the samples were seeded onto one of three selective environments: blood, mannitol, or MacConkey agar. After 24 h, a semi-automatic identification of the samples with bacterial growth was performed (autoScan-4/Dade Micro Scan Inc., Sacramento, California, USA). A standard suspension (0.5 on the McFarland scale) of the microorganisms under investigation was inoculated in each of the wells of the plate. After 15–24 h of incubation at 35°C, an automated colorimetric reading was taken to detect bacterial growth.

Histological evaluation of the liver

Liver fragments were fixed by immersion in Carnoy liquid (60% glacial acetic acid) for 3 h. The liver tissue was then transferred to a 70% alcohol solution. Hepatic parenchyma sections (5 µm thick) were stained using hematoxylin and eosin and by Masson trichrome.

To identify the hepatic stellate cells (HSC), immunohistochemistry was performed on sections using a monoclonal antidesmin antibody (IgG, Dako, Carpinteria, California, USA; dilution 1:200) following the manufacturer's specifications.

The morphometric analysis of the liver histology was performed on sections stained by Masson trichrome by a single examiner who was blinded to the slide identification codes and the respective treatments. Histological images were captured on an optical microscope Axiophot (Carl Zeiss Vision GmbH, Hallbergmoos, Germany) coupled to a digital camera (Sony Corp., Tokyo, Japan). The images were transferred to a computer through the Frame Grabber (Carl Zeiss Vision GmbH) image-capturing board. The images were captured in the RGB standard format at a 648 × 474 resolution for analysis using the graphics software KS400 V2.0 (Kontron Elektronik GmbH, München, Germany) [19]. Three distinct structures were identified for analysis: hepatocytes, which were red-stained structures; fibrosis, represented by blue-stained structures; and bile ducts, whose staining did not allow an automatic identification by the system. The areas in the three spectra that best represented the histological component under evaluation were marked.

Once the structures were identified, an automated process was initiated to obtain the areas of the selected components.

As a result, the percentage of the selected histological components present in the analyzed fragment was

calculated using the selected area and the total area of the captured frame. Through a previous pilot study, we had determined that the analysis of 30 fields at a × 256 magnification yields a representative sample of each slide. For each animal, the computer program randomly selected 30 fields per slide for morphometric analysis.

The volume fraction was obtained directly by proportional representation of the area of the histological elements of the liver (hepatocytes, fibrosis, biliary ducts, and other histological elements). The absolute fractions of the mass of these components were obtained by multiplying the volume fraction by the total mass of the liver expressed in g/kg of corporal weight [6].

The morphometric analysis of the HSC was performed in a similar manner using the same equipment described above. Using the optical framer, 30 fields were randomly selected from each slide with a × 400 magnification. The areas for HSC counting in the hepatic metabolic zone 1 were selected interactively and independently using the KS 400 software (Kontron Elektronik GmbH). The results were obtained as the sum of the number of HSC in each of the 30 fields obtained from each slide.

Mitochondrial function analysis

Isolation of the hepatic mitochondria

To study mitochondrial function, part of the right hepatic lobe was removed and placed in a physiologic solution at 4°C, fragmented with straight scissors, and conditioned in a polystyrene box containing crushed ice. The hepatic fragments were placed in a homogenized medium of 250 mmol/l sucrose, 1 mmol/l EGTA, and 10 mmol/l HEPES–KOH (pH 7.2). The liver tissues were homogenized in a Potter–Elvehjem for three cycles of 3 s each, with an interval of 1 min between the cycles. The mitochondria were isolated using differential centrifugation [20]. The resulting homogenate was centrifuged at 770g for 5 min to separate the cellular debris, the nuclei, and any intact cells. The resulting supernatant was then centrifuged at 9800g for 10 min. The resulting sediment containing the mitochondrial fraction was suspended in 10 ml of 250 mmol/l sucrose, 0.3 mmol/l EGTA, and 10 mmol/l HEPES–KOH (pH 7.2), followed by a 15-min centrifugation at 4500g. The final sediment was suspended in a solution containing 250 mmol/l sucrose and 10 mmol/l HEPES–KOH (pH 7.2). All procedures were performed at 4°C and within a 2-h period.

Determination of mitochondrial protein

Mitochondrial protein was determined using the Biuret method modified by the addition of 1% cholate [21].

Mitochondrial respiration study

The oxidative and phosphorylated mitochondrial activities were determined polarographically at 30°C using an oxygraph (Physics Institute of São Carlos – USP) equipped with a Clark electrode [22]. In these assays,

2.6 mg of mitochondrial protein was energized with 5 mmol/l succinate/4 μ mol/l rotenone in a respiration environment containing 250 mmol/l sucrose, 65 mmol/l KCl, 1 mmol/l MgCl₂, 2 mmol/l KH₂PO₄, 0.1 mmol/l EGTA, and 10 mmol/l HEPES-KOH (pH 7.4) in a final volume of 2.6 ml.

The rate of oxygen consumption after the addition of 200 nmol/l ADP to the energized mitochondria with succinate (state III) and after ADP phosphorylation (state IV) was expressed in atoms of oxygen/minute/protein. The respiratory control ratio (RCR) was determined from the ratio between state III and IV. The adenosine diphosphate/oxygen (ADP/O) ratio was determined by the division of the added ADP by the oxygen consumed during the phosphorylation of all the added ADP.

Mitochondrial phosphorylation activity was calculated by multiplying the state III respiration by the ADP/O ratio.

Determination of the mitochondrial membrane potential

The mitochondrial membrane potential was determined using an SLM Aminco spectrofluorometer (SLM – Aminco, Rochester, New York, USA) with safranin, using 495 and 586 nm for the excitation and emission wavelengths, respectively. In these assays, 1 mg of mitochondrial protein was energized with 5 mmol/l succinate/4 μ mol/l rotenon in an environment of 200 mmol/l sucrose, 1 mmol/l MgCl₂, 0.03 mmol/l EGTA, and 20 mmol/l HEPES-KOH (pH 7.4) [23].

Statistical analysis

In the cases in which it was necessary to consider the dependence among multiple variables, analysis was performed using mixed-effects models. An analysis of variance was applied when the groups were independent. A significance threshold level of 5% was used. The PROC MIXED procedure of the SAS version 9 software (SAS Institute Inc., Cary, North Carolina, USA) was used for statistical analysis [24]. When a significant difference was found using ANOVA, Tukey's post-hoc test was applied [25]. The PROC GLM procedure of the SAS 9.0 software [26] was used in this analysis. In some cases, logarithmic transformations were applied to assess the residual of the models.

Results

Evaluation after the induction of cholestasis and biliary anastomosis

A total of 158 animals were subjected to BO. One hundred and seven animals (67.72%) developed signs of cholestasis (jaundice, choluria, or a visible or a palpable biliary duct dilation), 32 animals (20.25%) died, and 19 animals (12.02%) did not develop any signs of cholestasis. The mortality rate was 76.19% for the CD group and 83.05% for the CJ group. The vast majority of postsurgery deaths, 48 animals (59.2%), occurred within the first 72 h

after surgery. There were no deaths among animals of the SO group.

Upon macroscopic examination, the liver had a hard consistency and was yellow in color. The spleen was also enlarged (Fig. 2). The bilioenteric anastomoses were wide and patent, with a diameter of ~0.5 cm in all animals (Fig. 3). However, enteric contents and fur occupying the anastomoses, the biliary duct, and the CJ afferent loop were present in animals that underwent both the biliary anastomosis techniques (Fig. 4).

Biochemical analysis of the blood

When compared with the SO group, animals who had BO showed a significant increase in TB, bilirubin fractions, aminotransferase, and ALP. After CD and CJ, there was a significant reduction in the amount of TB, bilirubin fractions, aspartate aminotransferase, and ALP. A significant reduction in ALT occurred only after CJ. The ALP, TB, and indirect bilirubin values were significantly higher in the CD group than in the SO group (Table 1).

There was a significant reduction in the amount of serum ALB after BO. CD led to a significant reduction in serum ALB, whereas CJ did not alter the ALB levels (Table 1).

Microbiological analysis of bile

No bacterial growth was detected in the bile samples from the animals in the SO and the BO groups. However, of the 20 animals subjected to either CD or CJ, 16 of the animals had bacteria in their bile. Of these 16 animals, 10 were from the CD group. *Escherichia coli* were the most common bacteria isolated from these animals and were present in 10 animals (Fig. 5).

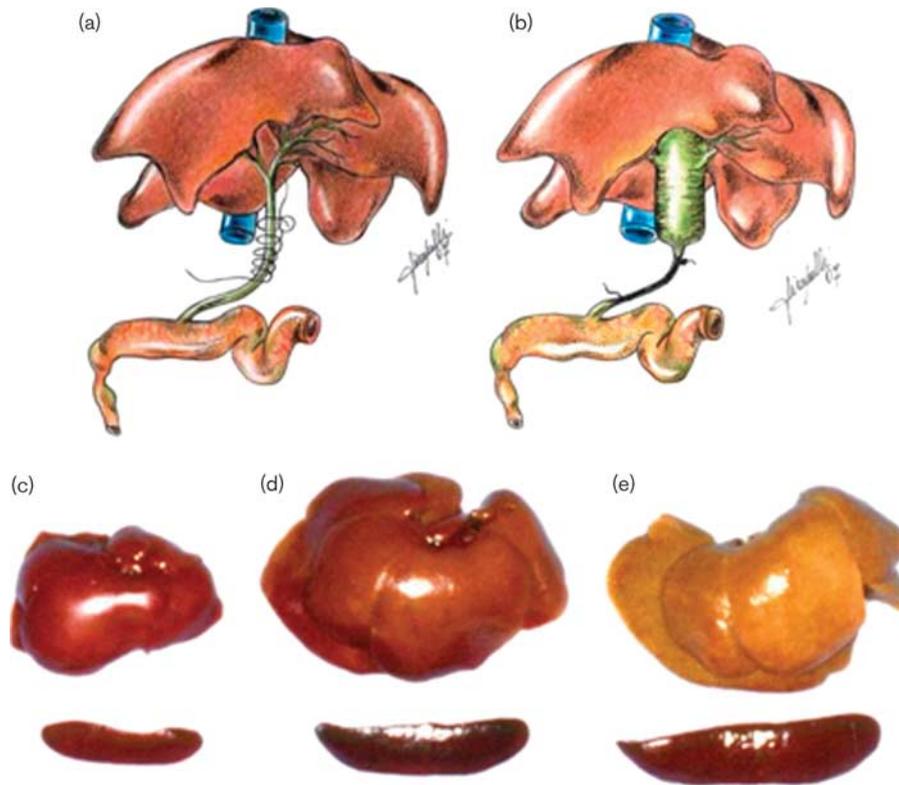
Histological analysis of the liver

Qualitative microscopic analysis

In all animals subjected to BO, an intense proliferation of the biliary ducts and fibrosis were observed. Alterations in the liver were more evident in the portal spaces. However, there was irradiation with variable intensity within the hepatic lobule, in the direction of the central vein. This process produced septation of the hepatic parenchyma with the formation of hepatocyte islands, called parenchymatous nodules. Lymphomononuclear inflammation was intermingled within the fibrosis and biliary ducts. Infiltration of neutrophils and plasmocytes was mild in zones 2 and 3 of the hepatic lobule and moderate in zone 1 of the hepatic lobule.

After biliodigestive anastomosis, there was restoration of the hepatic architecture, with a reduction in ductal proliferation and parenchymatous nodules. The reduction in fibrosis was more discrete, with the persistence of the septum in the portal spaces (Fig. 6). Lymphomononuclear inflammation was observed after anastomosis. Neutrophil and plasmocyte infiltration was mild in zones

Fig. 2



Schematic representation of the biliary obstruction technique through ligation, followed by circling the biliary duct (a), with fibrosis and a ductal dilation zone (b). In the lower panel (from left to right), the rat liver (top) and spleen (bottom) subjected to the sham surgery can be seen (c) 2 weeks after biliary obstruction, where there is a pronounced hepatosplenomegaly (d), and 4 weeks after biliary obstruction (e). A discrete retraction of the liver is observed after 4 weeks, whereas the spleen is enlarged. These data suggest the progression of hepatic lesions (e).

2 and 3 of the hepatic lobule and moderate in intensity in zone 1 of the hepatic lobule.

The number of HSC was not altered in the BO group compared with the SO group. After biliary anastomosis, the number of HSC increased. This increase in the number of HSC was more noticeable in the animals that underwent CD (Fig. 6).

Analysis of the estimated liver mass and quantitative microscopic analysis of histological components

When compared with animals in the SO group, animals in the BO group showed a significant increase in the estimated hepatic mass. After CD and CJ, the estimated mass of the liver was significantly reduced compared with the BO group. The CD and CJ groups had estimated mass values that were equivalent to the SO group. However, the hepatocyte mass was not altered in the BO group or after biliodigestive anastomoses (Table 2).

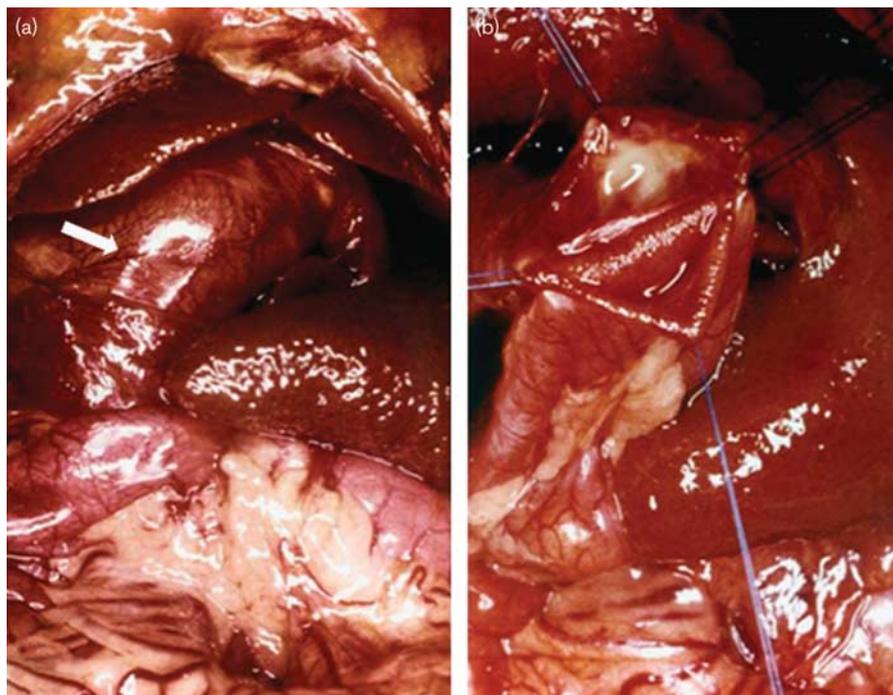
The BO group showed a significant increase in the relative fibrotic mass and an increase in the mass of the bile ducts compared with the SO group. After

biliodigestive anastomoses, there was a significant reduction in the estimated mass of fibrosis compared with the BO group. However, after CD or CJ, there was no reduction in the mass of the bile ducts. The fibrotic mass and bile duct mass of the CD and CJ groups remained significantly higher compared with the values of the SO group (Table 2).

In terms of the relative mass of other histological elements, there was a significant increase in the BO group compared with the SO group. After biliodigestive anastomoses, there was a significant reduction in the other histological elements compared with the BO group. However, there were no differences in the other histological elements in the CD and CJ groups compared with the SO group (Table 2).

Compared with the SO group, the increase in the number of HSC observed in the BO group was not significantly different. A significant increase in the number of HSC was observed in the CD group compared with the BO group. After CD and CJ, the number of HSC remained significantly higher than that of the SO group (Table 2).

Fig. 3



Dilated hepatic duct in a rat after 4 weeks of biliary obstruction (arrow) and liver with a granular surface (a). (b) Duodenum and the biliary duct with interior mucus can be observed as open and overlapped for the performance of the anastomosis.

Analysis of the estimated mass of the spleen

After BO, the relative mass of the spleen was significantly increased compared with that of the SO group. After biliodigestive anastomosis, a significant reduction in the estimated mass of the spleen was observed compared with the BO group. However, CJ induced a more significant reduction in the splenomegaly (Table 2).

Biochemical analysis of liver tissue

Mitochondrial function

After BO, oxygen consumption during state 3 mitochondrial respiration was reduced. However, the difference was not significant compared with the SO group. After CD or CJ, there was a significant increase in oxygen consumption during state 3 compared with the BO group. In terms of oxygen consumption during state 4 mitochondrial respiration, there was a significant increase in the BO group compared with the SO group, which after CD and CJ reverted to the same level as the SO group. Oxygen consumption after CJ was significantly lower than that after CD. However, oxygen consumption remained significantly higher in both the CD and the CJ groups compared with the SO group (Table 3).

The RCR and the ADP/O ratio were significantly reduced in the BO group compared with the SO group. After either CD or CJ, there was a significant increase in the ADP/O ratio. A significant increase in the RCR was found

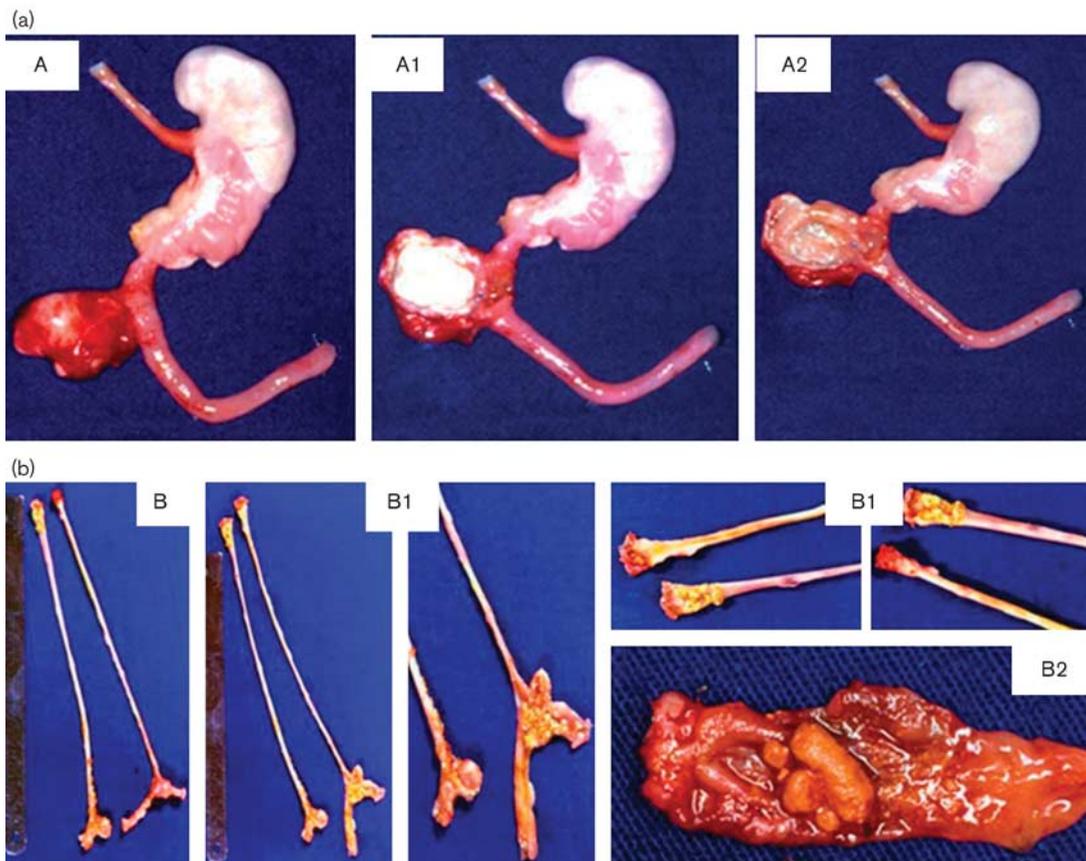
only in the CJ group. A recovery in the RCR and ADP/O ratio was not observed in the CD group compared with the SO group (Table 3). Furthermore, the mitochondrial membrane potential was not altered in the presence of BO or after biliodigestive anastomoses (Table 3).

The oxidative phosphorylation index was significantly reduced in the BO group compared with the SO group. In the CD and CJ groups, the level of oxidative phosphorylation reached a level similar to that of the SO group (Table 3).

Discussion

Quantitative and qualitative anatomopathological lesions, excretory function, and energy metabolism of the liver have been widely studied in models of experimental chronic extrahepatic cholestasis [7,27–29]. However, few reports have evaluated the effects of biliodigestive anastomosis on the regression of the hepatic lesions in these models of chronic extrahepatic BO [6,7,10,30]. Studies comparing these conditions in the long term, evaluation of CD and CJ performance, and the possible effects of these treatments on the liver and the biliary ducts are rare [8]. The use of CD because of the anatomical, functional, and technical advantages (especially in the video-laparoscopy era) and the possibility of a hepatic transplant for the treatment of secondary biliary cirrhosis with portal hypertension justify this study.

Fig. 4



A, A¹ and A² – The necropsy product of an animal subjected to CD with en-bloc extraction of the esophagus, stomach, duodenum, and biliary duct (a). The biliary duct had an organized mass containing hair and food, similar to a calculus with a consistency of clay (A¹), although the anastomosis was wide and patent (A²). B, B¹ and B² – The necropsy product of an animal subjected to CJ with an excluded loop of 15 cm. There is a bulging of the CJ and the jejunum-jejunal anastomotic confluence (b). Detail of the jejunum-jejunal confluence with food stasis (B¹), and the presence of a CJ with an organized mass containing hair and food, similar to a calculus of clay-like consistency (B²). CD, choledochoduodenostomy; CJ, choledochojejunostomy.

Table 1 Biliary obstruction effect (BO, BO1, and BO2) and the effect of choledochoduodenostomy and choledochojejunostomy on canalicular and cellular enzymes and albumin, expressed in terms of mean±SD

	SO (n=8)	BO (n=6)	BO1 (n=10)	CD (n=10)	BO2 (n=10)	CJ (n=10)	
TB	0.28±0.09	8.45±1.01	9.35±1.40	0.40±0.10	9.58±2.34	0.32±0.09	*1
DB	0.09±0.05	6.32±0.62	6.07±1.08	0.08±0.05	6.63±1.62	0.08±0.02	*2
IB	0.023±0.21	2.05±0.48	3.28±1.22	0.33±0.07	2.95±1.13	0.24±0.07	*1
ALT	95.25±34.67	234.83±103.87	209.70±55.27	195.60±259.96	188.90±74.01	101.70±31.87	*3
AST	194.13±70.77	493.58±131.12	473.00±110.32	326.60±291.94	455.20±215.81	194.40±50.49	*2
ALP	80.88±21.07	369.83±86.31	450.70±66.47	154.10±51.81	415.60±110.92	84.80±23.37	*4
ALB	2.41±0.13	1.87±0.27	2.29±0.37	1.90±0.50	2.29±0.27	2.28±0.13	*5

ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BO, biliary obstruction; CD, choledochoduodenostomy; CJ, choledochojejunostomy; DB, direct bilirubin; IB, indirect bilirubin; TB, total bilirubin.

*1 – Multiple significant comparisons: SO versus BO, BO1, and BO2; BO1 versus CD; BO2 versus CJ; and SO versus CD.

*2 – Multiple significant comparisons: SO versus BO, BO1, and BO2; BO1 versus CD; and BO2 versus CJ.

*3 – Multiple significant comparisons: SO versus BO, BO1, and BO2 and BO2 versus CJ.

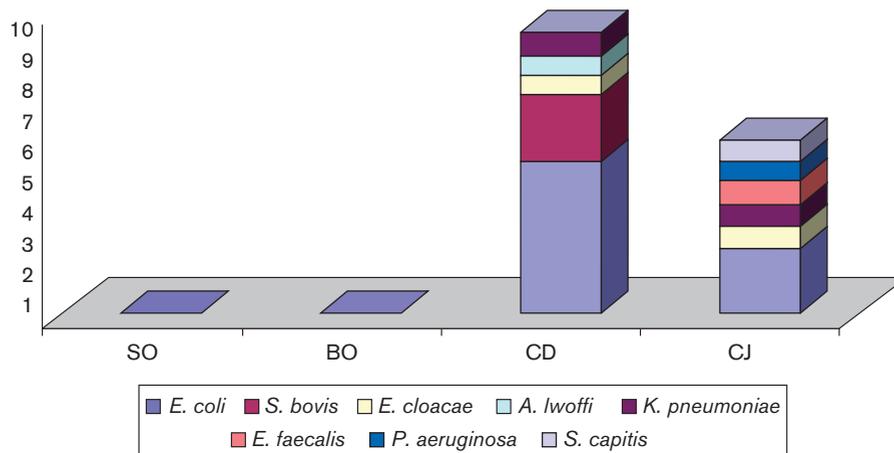
*4 – Multiple significant comparisons: SO versus BO, BO1, and BO2; BO2 versus CJ; CD versus CJ; and SO versus CD.

*5 – Multiple significant comparisons: SO versus BO; BO versus BO1 and BO2; BO1 versus CD; CD versus CJ; and SO versus CD.

In the present study, chronic BO was induced through ligation and circling of the extrahepatic biliary duct for 4 weeks [8]. Animals subjected to BO in this study lost

their hepatic architecture because of duct proliferation and hepatocyte island formation induced by fibrous septa that established bridges between the portal spaces and

Fig. 5



Incidence of bacterial contamination of the biliary ducts and frequency of bacterial species found in animals in the SO, BO, CD, and CJ groups. BO, biliary obstruction; CD, choledochoduodenostomy; CJ, choledochojejunostomy; SO, sham operation.

fragmented the hepatic parenchyma. This histological pattern is referred to as secondary biliary cirrhosis [6,28,30–33]. From functional and metabolic viewpoints, there were signs of cell damage, which translated into hypoalbuminemia, decoupling of the electron transportation chain in accordance to the reduction of the mitochondrial RCR, and a reduction in the oxidative phosphorylation index.

In the present study, the postsurgery mortality was high (CD: 76.19% and CJ: 83.05%). The animals that died after a bile shunt were subjected to autopsies, which did not indicate any mechanical or infectious causes, dehiscence of the anastomoses, or abdominal bleeding that would justify death. On the basis of this information and on the data of other studies, we deduce that the high mortality rate found in the present study might have been related to the different degrees of the hepatocellular lesion resulting from the BO [34,35], metabolic, anesthetic, and hemodynamic effects [6,36] and the hepatic injury of ischemia and reperfusion [37,38].

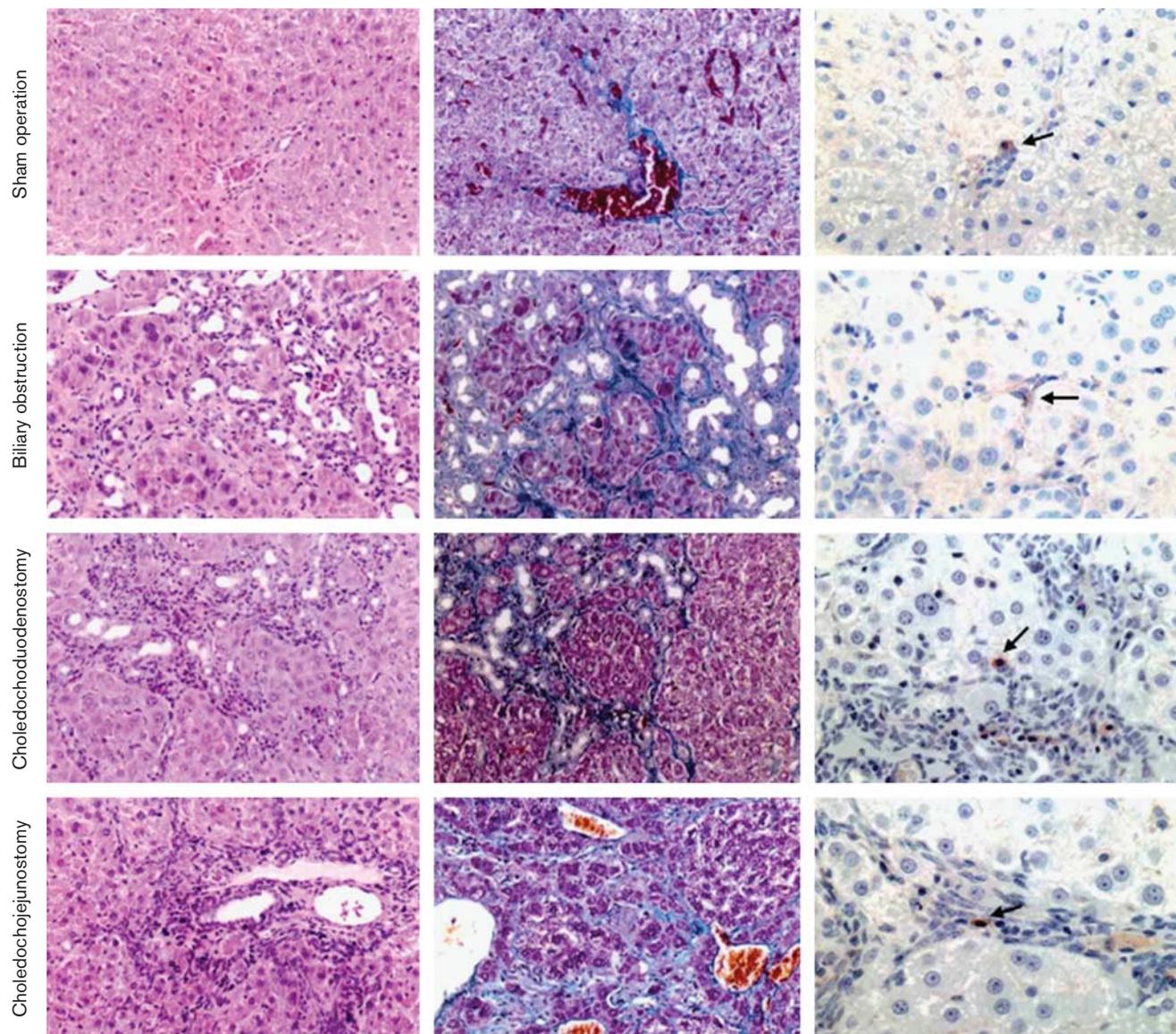
In the present study, food that refluxed from the intestine to the biliary duct was observed in the biliary channels and in the anastomoses. The food was organized as friable calculus in both types of biliary anastomoses that were used in this study. The excluded loop of the Roux-en-Y, despite having 15 cm of extension (proportionally corresponding to 40 cm in humans), was not enough to avoid the reflux and the stasis. These findings may be the cause of the transitory episodes of cholestasis and cholangitis described previously in humans [39] and experimental models [10,11], which contradicts the common belief that a wide and pervious anastomosis is enough to ensure the resolution of BO without additional problems [6,40,41].

The findings presented here reinforce the hypothesis that the biliary duct acts as a passive structure in the biliodigestive anastomosis [42]. After biliary anastomosis, there is a reduction in the biliary duct pressure resulting from the functional elimination of the sphincter mechanism. The propulsive power of the duodenal wall adds to the biliary duct pressure, leading to duodeno-biliary reflux. However, the sectioned jejunal loop for the Roux-en-Y ceases to receive the duodenal pacemaker and the continuity of phase III of contraction [43]. A new pacemaker could develop in the middle of the excluded loop, but at a low frequency of contraction and possibly in a retrograde direction [44].

Free circulation of the enteric content to the biliary duct may induce transitory episodes of mechanical obstruction. These mechanisms can justify the persistence of an increase in ALP and ALT in the animals subjected to CD, as well as the presence of food in the biliary duct in almost all of the treated animals. These mechanisms may also explain the transitory episodes of cholangitis that are present in humans without signs of mechanical obstruction [12,39,45,46].

In the present study, animals with a BO had a discrete mononuclear inflammatory infiltrate in the portal spaces and no bacterial contamination of the bile. In addition to the contamination of the bile with multiple gram-negative bacteria (normally found in biliary infections), a mixed inflammatory infiltrate of variable intensity was found in the animals treated with biliary anastomosis. These findings reinforce the possibility of ascending contamination of the biliary duct as a cause of cholangitis after biliary anastomosis [47–50]. These alterations can explain the increase in ALP, hypoalbuminemia, and ALT, in addition to explaining the increased number of stellate

Fig. 6



Histological sections of the livers from animals in the SO, BO, CD, and CJ groups. Tissue sections were stained (from the left to the right) with H&E, Masson trichrome, and antidesmin immunohistochemistry ($\times 200$). Arrows point to stellate hepatic cells staining positive for desmin. A more significant increase in stellate cells was found in the CD group compared with the SO, BO, and CJ groups. BO, biliary obstruction; CD, choledochoduodenostomy; CJ, choledochojejunostomy; SO, sham operation.

cells (especially in animals in which CD was performed and reflux may have been more intense).

Both anastomotic techniques provided the re-establishment of biliary flow and a regression in the hepatic-splenomegaly and the fibrotic mass. However, rats exposed to 3 or 4 weeks of BO and treated with CD or CJ showed a regression in the fibrosis, but not a correction of the portal hypertension [6,10,30]. The reason for the delay in the repair of the portal pressure, despite fibrosis regression, is not clear. This delay in recovery has been attributed to the proliferation of HSC

that seems to be more strongly correlated with portal hypertension than with fibrosis itself [30].

In the present study, even after the resolution of BO, there was a significant increase in the number of HSC after CD. However, there was no change in the number of HSC after CJ. The lack of normalization of the number of HSC and portal hypertension suggests that the adverse effects of the biliary anastomoses, such as enterobiliary reflux and bacterial contamination of the bile ducts, not only aggravate the inflammatory process (especially in zone 1 of the hepatic lobules), but maintain the

Table 2 Effect of biliary obstruction and choledochoduodenostomy and choledochojejunostomy on the relative mass of the liver and spleen (g/kg), liver histological components (g/kg), and the number of hepatic stellate cells (mean±SD)

	SO (n=8)	BO (n=6)	CD (n=10)	CJ (n=10)	
Hepatic mass	33.72±3.37	60.71±6.38	35.13±6.75	35.78±3.35	*1
Splenic mass	1.74±0.13	6.81±1.39	4.23±1.53	3.08±0.57	*2
Hepatocytes	22.71±3.19	23.19±6.22	21.07±5.49	22.74±2.42	NS
Fibrosis	0.07±0.05	6.64±2.19	1.29±0.56	0.84±0.31	*3
Biliary ducts	0.02±0.02	6.24±1.77	0.65±0.43	0.41±0.33	*4
Other components	10.61±1.96	23.37±3.15	12.12±2.42	11.90±2.42	*1
Stellate cells	24.13±12.86	38.67±13.06	71.50±32.68	56.00±21.97	*5

BO, biliary obstruction; CD, choledochoduodenostomy; CJ, choledochojejunostomy; SO, sham operation.

*1 – Multiple significant comparisons: SO versus BO and BO versus CD and CJ.

*2 – Multiple significant comparisons: SO versus BO; BO versus CD and CJ; CD versus CJ; and SO versus CD and CJ.

*3 – Multiple significant comparisons: SO versus BO; BO versus CD and CJ; and SO versus CD and CJ.

*4 – Multiple significant comparisons: SO versus BO and SO versus CD and CJ.

*5 – Multiple significant comparisons: BO versus CD; and SO versus CD and CJ.

Table 3 Biliary obstruction effects and the effect of choledochoduodenostomy and choledochojejunostomy on liver oxidative phosphorylation (mean±SD)

	SO (n=8)	BO (n=6)	CD (n=10)	CJ (n=10)	
State 3	89.26±18.07	70.61±8.03	107.97±21.07	107.89±23.23	*1
State 4	22.77±5.53	44.43±5.33	47.75±12.21	35.40±9.14	*2
ADP/O	1.54±0.11	0.78±0.09	1.24±0.40	1.35±0.26	*3
RCR	4.10±1.21	1.58±0.20	2.23±0.63	3.14±0.67	*4
Membrane potential	149.50±2.50	150±0	149.6±0.84	148.60±0.96	NS
Oxidative phosphorylation index	136.89±28.36	55.42±10.51	133.71±45.35	144.24±38.39	*5

ADP/O, adenosine diphosphate/oxygen; BO, biliary obstruction; CD, choledochoduodenostomy; CJ, choledochojejunostomy; RCR, respiratory control ratio; SO, sham operation.

*1 – Multiple significant comparisons: BO versus CD and CJ.

*2 – Multiple significant comparisons: SO versus BO; CD versus CJ; and SO versus CD and CJ.

*3 – Multiple significant comparisons: SO versus BO; BO versus CD and CJ; and SO versus CD.

*4 – Multiple significant comparisons: SO versus BO; BO versus CJ; CD versus CJ; and SO versus CD.

*5 – Multiple significant comparisons: SO versus BO; and BO versus CD and CJ.

stimulation of HSC proliferation [30]. Moreover, these findings reinforce the possibility that HSC themselves alter the sinusoidal space through the contraction of their dendritic spines and therefore maintain portal hypertension [51–53]. In the present study, despite the regression of fibrosis, splenomegaly remained unaltered after CD. However, the splenic mass regressed, but without complete recovery after CJ. These data reinforce the association between HSC mass and portal hypertension [30].

The majority of the cholestatic and metabolic alterations, in addition to the anatomopathological lesions in the liver, reversed after efficient biliary anastomosis. However, bilioenteric reflux leads to intraductal and intracanalicular obstruction with cholestasis and a transitory increase in the pressure within the biliary duct, with no changes in ductal proliferation. The presence of enteric contents in the anastomoses and biliary ducts of all the animals (especially in those treated with CD) reinforces the notion that the communication between the biliary duct and the intestine, even in the presence of a wide and pervious anastomosis, does not ensure normal biliary flow.

The results of the present work reinforce the concept of reversibility of cholestatic and metabolic alterations after

the relief from cholestasis. These alterations can be reversed even in advanced lesions with fibrosis and in secondary biliary cirrhosis. These observations are in agreement with the concept developed from clinical [4] and experimental [5–7,34] observations. These data indicate that, whenever possible, effective biliary desobstruction should precede hepatic transplant in patients with a treatable secondary biliary cirrhosis and portal hypertension through biliodigestive anastomosis.

The novel contribution of this work validates the advantages of CJ and supports the need for clinical studies and the acquisition of more information on the selection of the type of biliary anastomosis used for treatment. Although the enterobiliary reflux is present in the two types of anastomosis, the quantity of food and the organization of the refluxed material in the biliary duct after CD were larger. In addition, the incidence of biliary contamination was higher after CD. The data suggest the probability of easy access of the enteric contents derived from the duodenum into the biliary duct that allows transitory closure, which increases the intrabiliary pressure. This phenomenon could be sufficient to maintain the TB and ALP at a higher level. The perpetuation of cholestasis delays the repair of histological lesions and the

metabolic recovery of the mitochondria. The presence of biliary duct contamination and the resulting inflammation may contribute to the increased number of HSC and consequently to a less significant regression of the splenomegaly, especially after CD.

Conclusion

Biliary interventions should minimize the intensity of the effects resulting from enterobiliary reflux and biliary duct stasis. The effects of biliary interventions justify the evaluation of new technical and therapeutic techniques for benign diseases related to extrahepatic cholestasis, as suggested previously. Moreover, even with concerns regarding the generalization of these experimental results to humans, caution should be exercised in the frequent use of CD. The choice of a biliary intervention should be made carefully, especially in the video-laparoscopic era, for cases of chronic BO with a good prognosis.

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Conflicts of interest

There are no conflicts of interest.

References

- Friedman SL. The cellular basis of hepatic fibrosis: mechanisms and treatment strategies. *N Engl J Med* 1993; **328**:1828–1835.
- Blumgart LH. Biliary tract obstruction: new approaches to old problems. *Am J Surg* 1978; **135**:19–31.
- Yeong ML, Nicholson GI, Lee SP. Regression of biliary cirrhosis following choledochal cyst drainage. *Gastroenterology* 1982; **82**:332–335.
- Hammel P, Couvelard A, O'Toole D, Ratouis A, Sauvanet A, Fléjou JF, *et al.* Regression of liver fibrosis after biliary drainage in patients with chronic pancreatitis and stenosis of the common bile duct. *N Engl J Med* 2001; **344**:418–423.
- ADBEl-Aziz G, Lebeau G, Rescan PY, Clément B, Rissel M, Deugnier Y, *et al.* Reversibility of hepatic fibrosis in experimentally induced cholestasis in the rat. *Am J Pathol* 1990; **137**:1333–1342.
- Zimmermann H, Reichen J, Zimmermann A, Sägeser H, Thenisch B, Höflin F. Reversibility of secondary biliary fibrosis by biliodigestive anastomosis in the rat. *Gastroenterology* 1992; **103**:579–589.
- Aronson DC, Chamuleau RAFM, Frederiks WM, Gooszen HG, Heijmans HS, James J. Reversibility of cholestatic changes following experimental common bile duct obstruction: fact or fantasy? *J Hepatol* 1993; **18**:85–95.
- Dutra RA, Santos JS, Araújo WM, Simões MV, de Paixão-Becker AN, Neder L. Evaluation of hepatobiliary excretion and enterobiliary reflux in rats with biliary obstruction submitted to bilioduodenal or biliojejunal anastomosis. *Dig Dis Sci* 2008; **53**:1138–1145.
- Uchiyama K, Onishi H, Tani M, Kinoshita H, Kawai M, Ueno M, *et al.* Long-term prognosis after treatment of patients with choledocholithiasis. *Ann Surg* 2003; **238**:97–102.
- Franco D, Gigou M, Szekely A, Bismuth H. Portal hypertension after bile duct obstruction. *Arch Surg* 1979; **114**:1064–1067.
- Klaus A, Weiss H, Kreczy A, Eigentler A, Neher C, Margreiter R, *et al.* A new biliodigestive anastomosis technique to prevent reflux and stasis. *Am J Surg* 2001; **182**:52–57.
- Hashimoto N, Kotoura Y, Ohyanagi H. Hepatobiliary scintigraphy after biliary reconstruction Roux Y and RY-dj. *Hepatogastroenterology* 2005; **52**:200–202.
- Vogt DP, Hermann RE. Choledochoduodenostomy, choledochojejunostomy or sphincteroplasty for biliary and pancreatic disease. *Ann Surg* 1981; **193**:161–168.
- Nagai T, Yamakawa T. Experimental model with bilioenteric anastomosis in rats. Technique and significance. *Hepatogastroenterology* 1992; **39**:309–313.
- Sims FH, Horn C. Some observations on Powell's method for the determination of serum bilirubin. *Am J Clin Pathol* 1958; **29**:412–417.
- Henry JR, Chiamori N, Golub OJ, Berkman S. Revised spectrophotometric methods for the determination of glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, and lactic acid dehydrogenase. *Am J Clin Pathol* 1960; **34**:381–398.
- Roy AV. Rapid method for determining alkaline phosphatase activity in serum with thymolphthalein monophosphatase. *Clin Chem* 1970; **16**:431–436.
- Doumas BT, Watson WA, Biggs HG. Albumin standards and the measurement of serum albumin with bromocresol green. *Clin Chim Acta* 1971; **31**:87–96.
- Fazan VPS, Salgado HC, Barreira AA. Descriptive and quantitative light and electron microscopy study of the aortic depressor nerve in normotensive rats. *Hypertension* 1997; **30**:693–698.
- Pedersen PL, Greenawalt JW, Reynafarje B, Hullihen J, Decker GL, Soper JW, *et al.* Preparation and characterization of mitochondria and submitochondrial particles of rat liver and liver-derived tissues. *Methods Cell Biol* 1978; **20**:411–481.
- Kaplan RS, Pedersen PL. Characterization of phosphate efflux pathways in rat liver mitochondrial. *Biochem J* 1983; **212**:279–288.
- Chance B, Williams GR. Respiratory enzymes in oxidative phosphorylation. *J Biol Chem* 1956; **217**:409–427.
- Vercsei AE, Bernardes CF, Hoffmann ME, Gadelha FR, Docampo R. Digitonin permeabilization does not affect mitochondrial function and allows the determination of the mitochondrial membrane potential of Trypanosoma cruzi in situ. *J Biol Chem* 1991; **266**:14431–14434.
- Littell RC, Milliken GA, Sroup WW, Wolfinger RD. *SAS System for Mixed Models*. Cary: SAS Institute; 1996.
- Montgomery DC. *Design and analysis of experiments*. 5th ed. New York: John Wiley & Sons; 2000.
- SAS Institute. *SAS/STAT® user's guide. Version 9*. Cary: SAS Institute; 2002.
- Gall JAM, Bhathal OS. A quantitative analysis of the liver following ligation of the common bile duct. *Liver* 1990; **10**:116–125.
- Kontouras J, Billing BH, Scheuer PJ. Prolonged bile duct obstruction: a new experimental model for cirrhosis in the rat. *Br J Exp Pathol* 1984; **65**:305–311.
- Krähenbühl S, Stucki J, Reichen J. Reduced activity of the electron transport chain in liver mitochondria isolated from rats with secondary biliary cirrhosis. *Hepatology* 1992; **15**:1160–1166.
- Zimmermann H, Fellay M, Zimmermann A. Hepatic stellate cells (Ito cells) but not collagen IV may partly be responsible for lower portal pressure after reversing secondary biliary cirrhosis in the rat. *J Hepatol* 1997; **26**:158–166.
- Johnstone JMS, Lee EG. A quantitative assessment of the structural changes in the rat's liver following obstruction of the common bile duct. *Br J Exp Pathol* 1976; **57**:85–94.
- Gross JB, Reichen J, Zeltner T, Zimmermann A. The evolution of changes in quantitative liver function tests in rat model of cirrhosis. *Hepatology* 1987; **7**:57–63.
- Krähenbühl L, Schafner M, Krähenbühl S. Reversibility of hepatic mitochondrial damage in rats with long-term cholestasis. *J Hepatol* 1998; **28**:1000–1007.
- Santos JS, Ceneviva R, Castro e Silva Junior O, Zucoloto S, Cabrera AME. Evolution of secondary bile cirrhosis by comparing the effects of bili-duodenal and bili-jejunal shunts. *Digestion* 1998; **59**:554.
- Kemp R, de Araújo WM, de Castro AA, Ardengh JC, Neder L, dos Santos JS. Influence of biliary drainage on the repair of hepatic lesions in biliary fibrosis. *J Surg Res* 2011; **169**:127136.
- Sakoda K, Atik M. Influence of common bile duct ligation on hepatic blood flow. *Am Surg* 1970; **36**:731–736.
- Roselino JE, Castro e Silva Jr. O, Ceneviva R. Lack of control of liver gluconeogenesis in cholestatic rats with reduced portal blood flow. *Hepatology* 1992; **16**:1055–1060.
- Ferreira MA, Santos JS, Dutra RA, Salgado W Jr., Kemp R, Domiciano C, *et al.* Bilio-duodenal anastomosis in rats with extra-hepatic biliary obstructions followed by lesions ischemia and reperfusion-induced. *Acta Cir Bras* 2008; **23**:47–52.
- Hashimoto N, Ohyanagi H. Hepatobiliary scintigraphy after biliary reconstruction – a comparative study on Roux-Y and ESCD. *Hepatogastroenterology* 2000; **47**:1210–1212.
- Madden JL, Gruwez JA, Tan PY. Obstructive (surgical) jaundice: a analysis of 140 consecutive cases and a consideration of choledochoduodenostomy in its treatment. *Am J Surg* 1965; **109**:89–99.
- Madden JL, Shun JY, Kandalaft S, Parekh M. Choledochoduodenostomy – an unjustly maligned surgical procedure? *Am J Surg* 1970; **119**:45–54.
- Morgenstern L, Shore JM. Selection of an optimal procedure for decompression of the obstructed common bile duct. Experimental and clinical observations. *Am J Surg* 1970; **119**:38–44.

- 43 Miedema BW, Kelly KA, Camilleri M, Hanson RB, Zinsmeister AR, O'Connor MK, *et al.* Human gastric and jejunal transit and motility after Roux gastrojejunostomy. *Gastroenterology* 1992; **103**:1133–1143.
- 44 Vantrappen G, Coremans G, Janssens J, Mantides A, Van den Borre F. Inversion of the slow wave frequency gradient in symptomatic patients with Roux-en-Y anastomosis. *Gastroenterology* 1991; **101**:1282–1288.
- 45 Matthews JB, Baer HU, Schweizer WP. Recurrent cholangitis with and without anastomotic stricture after biliary-enteric bypass. *Arch Surg* 1993; **128**:269–272.
- 46 Ducrotte P, Peillon C, Guillemot F, Testart J, Denis P. Could recurrent cholangitis after Roux-em-Y hepaticojejunostomy be explained by motor intestinal anomalies? A manometric study. *Am J Gastroenterol* 1991; **86**:1255–1258.
- 47 Goldman LD, Steer ML, Silen WS. Recurrent cholangitis after biliary surgery. *Am J Surg* 1983; **145**:450–454.
- 48 Santos JS, Salgado W Jr., Módena JLP, Brunaldi JE, Ceneviva R. Effect of preoperative endoscopic decompression on malignant biliary obstruction and postoperative infection. *Hepatogastroenterology* 2005; **52**:45–47.
- 49 Diamond T, Dolan S, Thompson RL, Rowlands BJ. Development and reversal of endotoxemia and endotoxin-related death in obstructive jaundice. *Surgery* 1990; **108**:370–375.
- 50 Chuang JH, Lee SY, Chen WJ, Hsieh CS, Chang NK, Lo SK. Changes in bacterial concentration in the liver correlate with that in the hepaticojejunostomy after bile duct reconstruction: implication in the pathogenesis of postoperative cholangitis. *World J Surg* 2001; **25**:1512–1518.
- 51 Friedman SL. Hepatic fibrosis – overview. *Toxicology* 2008; **254**:120–129.
- 52 Iredale JP. Cirrhosis: new research provides a basis for rational and targeted treatments. *BMJ* 2003; **327**:143–147.
- 53 Rockey DC. Hepatic blood flow regulation by stellate cells in normal and injured liver. *Semin Liver Dis* 2001; **21**:337–349.