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# Single versus double experimental bile duct ligation model for inducing bacterial translocation

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ABSTRACT

*Background:* Double common bile duct ligation plus section in rats is used as a model for bacterial translocation, a phenomenon that has been correlated with the degree of liver damage. This study analyzes whether a simpler variant of the technique is also a valid model to study bacterial translocation.
*Methods:* Fifty-six male Sprague Dawley rats underwent one of three surgical interventions: a) proximal double ligation and section of the common bile duct; b) proximal simple ligation of the bile duct; and c) sham operation. Bacterial translocation was measured by cultures of mesenteric lymph nodes, blood, spleen and liver. Stool culture and histological analysis of liver damage were also performed.
*Results:* The incidence of bacterial translocation in SBL and DBDL groups was 23,5% and 25% respectively. Mortality was similar between ligation groups (11.2% versus 10%). Liver cirrhosis developed in the group of double ligation and section (100% of the animals at 4 weeks), while portal hypertension appeared starting at week 3. None of the animals submitted to simple ligation developed liver cirrhosis.
*Conclusions:* Simple bile duct ligation is associated with a similar incidence of bacterial translocation as double ligation, but without cirrhosis or portal hypertension.



## Introduction

Bacterial translocation (BT) is the microbiological isolation of viable bacteria in mesenteric lymph nodes (MLN)[1] and other extranodal compartments, forming the pathogenic basis for spontaneous bacterial peritonitis. Research in the field uses experimental models of liver cirrhosis, whether induced by carbon tetrachloride ($CCl_4$) or by double common bile duct ligation (CBDL).[2–12] The CBDL model is associated with high mortality,[13] although not as high as the $CCl_4$ model, and it incurs a risk of acute pancreatitis due to the accidental ligation of the pancreatic duct. In this experimental model, the animals experience important structural damage in their livers, developing cirrhosis in 2–4 weeks.[14,15] However, the literature rarely describes the level at which the ligatures are performed, nor is it clear whether a subsequent transection of the common bile duct (CBD) is carried out

*Abbreviations:* BT, bacterial translocation; CBD, common bile duct; CFU, colony forming units; DBDL, double bile duct ligation plus section between ligatures; MLN, mesenteric lymph nodes; SBL, simple bile ligation; SBP, Spontaneous Bacterial Peritonitis.

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between the ligatures.[14,16–20] Evidence suggests that BT occurs soon after ligation and obstruction of the bile duct, even before the development of clear liver damage.[21] Therefore, it seems reasonable to use a simpler surgical procedure, such as a proximal, high, simple bile ligature (SBL) as a new model of study for development of BT decreasing the risk of mortality associated to the more complex surgical procedure.

Several physiopathological mechanisms have been implied in the appearance of BT and the development of Spontaneous Bacterial Peritonitis (SBP)[36]: a) integrity of the intestinal epithelium, B) immunological defenses of the host, since the isolation in MLN of bacteria from the intestine after crossing the intestinal barrier produces a local and systemic inflammatory stimulus, with secretion of cytokines and mediators of the inflammation, and c) the balance and stability of the intestinal flora, since the magnitude of BT is likely to be closely related to the bacterial density in the intestine. BT represents the breakdown of normal balance in these 3 mainstays. We have also analyzed intestinal bacterial growth and characterized the inflammatory pattern in both types of biliary ligation.

## Materials and methods

### *Rats*

We used 56 Sprague-Dawley male adult rats weighing 230–446 g. The animals were kept in individual wire cages in order to minimize coprophagy and the consumption of litter substrate. The temperature remained constant at 21 °C, and there were light-dark cycles of 17-7 h. Rats received standard feed (no later than 10:00 am on experimentation days) and free access to water, and were cared for according to the criteria outlined in Spanish regulations, which is consistent with the *Guide for the Care and Use of Laboratory Animals*. The design of the study was approved by the Animal Research Committee of Universidad Miguel Hernandez (Alicante, Spain).

All surgical interventions were performed after 4:00 pm. Animals were weighed using the standard scale, calibrated prior to each session. They were anaesthetized with ketamine 100 mg/kg (Ketolar, Parke Davis S.A., Barcelona, Spain) and diazepam 3 mg/kg (Valium, Roche S.A., Basil, Switzerland) via intraperitoneal injection, adjusting the volume of injection to 1 mL per 300 g. A reinforcement of $^1/_3$ to ½ of the initial doses was administered as needed via subcutaneous or intramuscular injection to minimize problems related to absorption stemming from losses caused by the opening of the peritoneal cavity.

### *Study groups*

The animals were divided equally between the control (n = 19) and the two intervention groups (single ligation: n = 18; double ligation plus transection: n = 19), and these groups were further divided for sample collection at one, two, three, and four weeks.

### *First surgical procedure: control (sham), simple bile ligation (SBL), or double bile duct ligation plus transection between ligatures (DBDL)*

Once the animal was anesthetized, the abdomen was shaved with an electric razor, sterilized with povidone, and isolated with green sterile drapes. A mid-line laparotomy was carried out using a cold scalpel with 3 cm blade. In the control group, the CBD was only identified and referenced with surgical tweezers. The SBL was performed at a proximal, high level (after the confluence of both hepatic ducts) with 3/0 silk suture. The DBDL consisted of a high, double ligature with 3/0 silk suture (uniting both hepatic ducts), with the distal ligature at 5 mm from the proximal one to minimize the accidental ligation of the pancreatic duct, plus transection between ligatures. The abdominal wall was closed with 2/0 silk suture, and the skin with 3/0 silk suture. The animal that had been intervened was placed in its individual cage and moved to the animal facility at baseline feeding conditions (See Fig. 1) .

### *Second surgical procedure: sample collection*

For the second surgical intervention, and in accordance with the group and week assigned to each animal, we performed the same perioperative procedures described above (isolation, feeding restrictions, anaesthesia). Following the laparotomy (5–6 cm in the re-interventions), we obtained the samples in the following sequence:

a) Peritoneal swab taken for culture (quality measure for asepsis in order to rule out any accidental contamination during the procedure).
b) Ileocecal MLN (minimum 6) identified, dissected, resected, harvested, and inoculated in thioglycollate (Scharlab, Barcelona, Spain).
c) Portal vein and inferior vena cava were identified for direct venipuncture of 0.5–1.0 cc of blood via 2 cc sterile syringe with subcutaneous needle (blood culture) following by haemostasis of the venipuncture sites through direct compression with sterile gauze for 1–2 min.
d) Two liver samples obtained via excision with cold scalpel for microbiological and histological study.
e) Splenectomy performed, determining the weight on a calibrated scale and subjecting it to microbiological study.
f) Clamping performed with two haemostats at the level of the terminal ileum (2–3 cm from the cecum) and then the section was taken from between the two clamps with a new, cold scalpel blade. The section was removed in a whole to collect stool from the cecum and for the study of intestinal bacterial overgrowth.
g) Median sternotomy and incision on the left hemidiaphragm, for optimal cardiac exposure. Cardiac puncture and extraction of 4–5 cc of blood was performed with 5 cc heparinised syringe and intramuscular needle, for subsequent study of the inflammatory response through determination of the circulating levels of cytokines, tumour necrosis factor alpha (TNF-α), and gamma interferon (IFN). Exitus of the animal due to direct intracardiac exsanguination.

### *Processing of samples*

1. Bacterial translocation. Prior to the animal's death, we assessed BT via bacteriological studies of MLN, portal and cava blood, and abdominal viscera (liver and spleen), defining extranodal growth as the presence of viable bacterial cultures (>100 CFU/g) in these compartments, separate from the regional MLN. Immediately after sample collection, we transferred the MLN homogenates as well as the liver and spleen tissue to grinding tubes with **s**terile brain-heart infusion (0.1 ml). Specimens were homogenized in brain-heart infusion medium and plated on colistin-nalidixic acid (CNA) and selective MacConkey's agar to detect gram-negative bacteria and enteric bacilli; selective lysine-binding site agar to detect lactobacilli; and blood agar to detect other bacteria (e.g. gram-positive cocci). At 24 and 48 h of incubation at 37 °C, the hospital clinical microbiology laboratory

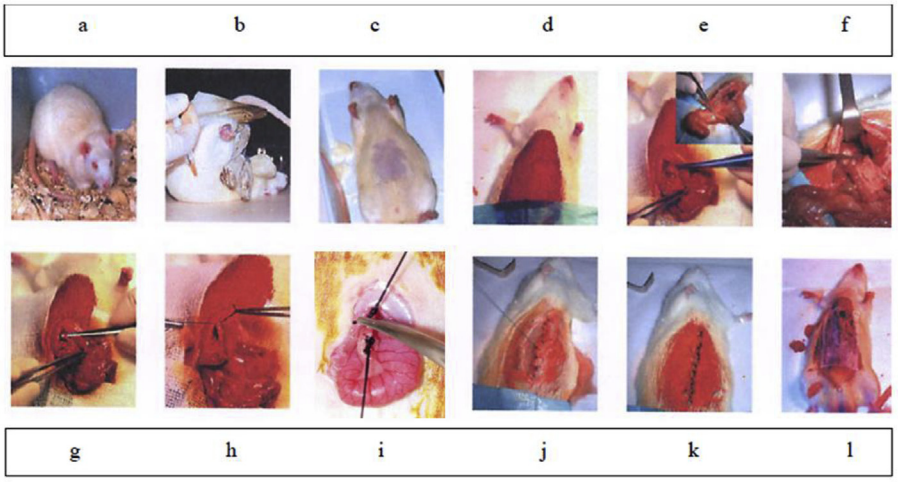


**Fig. 1.** First Surgical Procedure: Panel Summary of the anesthesia and surgery procedure in the first intervention following the temporal sequence: a, b and c (Adult male Sprague Dawley rat, with intraperitoneal administration of the anesthetic and shaving with conventional electric razor); d (surgical field sterilization after surgical table fixation); e (MLN identification and its posterior excision); f (CBD identification); g (Tunneling of CBD in Sham Group); h (single ligation without section in the SBL group); i (Double pre-section ligation in the DBDL Group); j and k (closure with loose points of the preperitoneal plane and of the abdominal cavity); l protective dressing placement.

analyzed the plates, expressing results as colony-forming units per gram of organ tissue (CFU/g). Blood cultures were performed via inoculation into blood culture bottles (Bactec Plus/aerobic) and subsequent processing in the Bactec 9240 system (Becton-Dickinson, MD, USA).

2. Intestinal bacterial overgrowth. After collection, homogenization, and dilution of cecal and ileum stool samples in brain-heart medium with normal saline, we carried out cultures of appropriate dilution volumes (0.1 mL) in aerobic media and incubated them at 37 °C in the same CNA and MacConkey plates. We considered total intestinal aerobic bacterial flora to be the sum of all aerobic bacteria detected in the stool, and IBO of a specific organism to be a stool bacterial count greater than the mean value plus two standard deviations of the organism in normal rats.[3,22] We expressed colony stool counts as $\log_{10}$ CFU/g of stool (medians and ranges) and identified microorganisms by standard bacteriological methods: API 20E (Bio Mérieux SA. Mercy l'Etolite. France) and MicroScan (Baxter Healthcare, West Sacramento, CA, USA).
3. Liver histology. We fixed the morphological liver tissue for light microscopy in formal saline, staining it with haematoxylin and eosin for reticulin fibres and using Masson's Trichromic method for collagen. An experienced pathologist examined histological samples by light microscopy, classifying liver disease according to the five stages described by Kountouras[14]: 0, normal histology; I, minimal or subtle ductal proliferation, limited to portal space; II, mild-moderate ductal proliferation in portal space and parenchyma; III, severe ductal proliferation with incomplete nodules, causing disorganization of liver architecture; and IV, ductal proliferation delineating clear well-defined nodules, indicative of cirrhosis.
4. Indirect evaluation of portal hypertension. The animals' spleens were weighed at 3–4 weeks post-ligation, as an indirect indicator of portal hypertension.
5. Inflammatory status. To determine the level of cytokines, we performed an enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions for quantitative measurement of TNF-α and interferon in serum samples. All samples were tested in duplicate and read using a ThermoMax microplate reader (Molecular Devices, Sunnyvale, California, USA).

### *Statistical analysis*

Quantitative variables were expressed as medians and interquartile ranges (IQR); comparisons between two groups were carried out using the U Mann-Whitney test, while multiple comparisons were done with the Kruskal-Wallis test with Bonferroni correction. Categorical and qualitative data were described as percentages (frequencies) and compared using the Chi-squared test and Fisher's exact test, as required. P values of less than 0.05 were considered statistically significant. All statistical analyses were performed with the SPSS statistical package (SPSS Inc. version 19.0, Chicago, IL, USA).

## Results

### *Bacterial translocation*

Eighteen bacterial isolates were identified in 11 animals: three isolates from 2 animals in the sham group, seven isolates from 5 animals in the SBL group, and eight isolates from 4 animals in the DBDL group (Table 1). Overall, BT occurred spontaneously in two (11.76%) control animals at weeks 1 and 4. In the ligature groups, BT was present in 29.4% (SBL) and 25% (DBDL) of rats, usually within the first three weeks. Differences in BT between the SBL and sham groups only reached statistical significance at week 3 ($p = 0.038$). There were no significant differences between the ligature groups ($p > 0.05$). Likewise, we did not observe differences in survival between animals with and without BT (95% and 89% survival, respectively; $p = 0.128$).

At the MLN level, we isolated 12 positive cultures: 9 with monomicrobial growth and 1 with polymicrobial bacteria, both gram-positive and -negative. Nine gram-negative bacteria were found in seven animals: *Escherichia coli* ($n = 4$), *Klebsiella pneumoniae* ($n = 2$), *Proteus mirabilis* ($n = 1$), and *Pseudomona sp* ($n = 2$). Isolates were found most frequently at week 1. There was no gram-positive growth in animals without ligature, nor did we find anaerobic bacteria in any of the groups.

At the extranodal level, we identified six isolates: four in the liver, one in the spleen, and one in the cava blood. We did not detect viable bacteria in the peritoneal cavity nor in the portal blood.

**Table 1**
BT by number of isolates, study group, location and time point (weeks). There was no bacterial growth in any of the rats in the peritoneal cavity or portal blood. The lower part of the table shows the number of isolates per week. *p < 0.05 (SBL vs sham).

| Groups | BT | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | MLN | Cava | Liver | Spleen | Total isolates | Nº rats with isolates | GRAM | | Total BT N (%) |
| | | | | | | | + | - | |
| **SHAM** | **2** | **-** | **1** | – | **3** | **2** | **3** | **0** | **2/17 (11.76%)** |
| Week 1 | *E coli* | – | – | – | | | | | |
| Week 2 | – | – | – | – | | | | | |
| Week 3 | – | – | – | – | | | | | |
| Week 4 | *P aerug* | – | *Ps aerug* | – | | | | | |
| **SBL** | **4** | **1** | **2** | – | **7** | **5** | **5** | **2** | **5/17 (29.4%)** |
| Week 1 | *E coli* | – | *E coli* | – | | | | | |
| | *E coli* | – | – | – | | | | | |
| Week 2 | – | – | – | – | | | | | |
| Week 3* | – | *E coli* | – | – | | | | | |
| | *S faecalis* | – | – | – | | | | | |
| | *E coli* | – | *S faecalis* | – | | | | | |
| Week 4 | – | – | – | – | | | | | |
| **DBDL** | **6** | **-** | **1** | **1** | **8** | **4** | **6** | **2** | **4/16 (25%)** |
| Week 1 | *Klebsiella* | – | – | – | | | | | |
| | *P aeruginosa* | | | | | | | | |
| | *S faecalis* | | | | | | | | |
| | *Klebsiella* | – | – | – | | | | | |
| Week 2 | *Proteus* | – | *Proteus* | *Proteus* | | | | | |
| Week 3 | *S faecalis* | – | – | – | | | | | |
| Week 4 | – | – | – | – | | | | | |
| **TOTAL** | **12** | **1** | **4** | **1** | **18** | **11** | **14** | **4** | **11/50 (22%)** |

Coexistence with BT occurred in four animals: one in the sham group (week 4 in the liver), two in the SBL group (weeks 1 and 3, also in the liver), and one in the DBDL group (week 2, with concomitant growth in the liver and spleen). All were gram-negative except in the SBL animal at week 3.

### *Weight evolution*

Median baseline weight of the study rats was 330 g (IQR 231–430 g). During week 1, control animals gained an average of 34 g; thereafter their weight stabilized. The SBL group lost a significant amount of weight in the first week (median −27 g) and then began to gain again by week 2. By contrast, there was a notable decrease in weight in the DBDL group (−22 g and −1 g at weeks 1 and 2, respectively); these animals began to gain weight again only at week 3.

### *Mortality*

Mortality rates were as follows: sham, 9.5% (n = 2, at weeks 2 and 4); SBL, 5.3% (n = 1, at week 2); and DBDL, 13.6% (n = 3, at weeks 1, 3, and 4). In the Kaplan-Meier survival curves, the accumulated survival between ligation and no ligation groups was not statistically different, overall nor in the comparative analysis by groups (log rank-test; p = 0.550).

### *Spleen weight*

During the laparatomy at weeks 3 and 4 in the DBDL group, there was a notable increase in the size of the spleen with respect to findings at week 1 and 2. At week 3, the median spleen weight was significantly higher in the animals with SBL (1.27 g) and DBDL (1.68 g) compared to the control (0.68 g; p < 0.05). At week 4, the increase was only significantly different in the DBDL group (2.64 g) compared to the values observed in the other groups (SBL: 0.90 g; control: 0.66 g) and the values recorded at week 3.

### *Intestinal bacterial overgrowth*

The cecal growth of gram-negative bacteria (log CFU per gram of stool) was steady across groups and time points; there were no significant differences between the SBL group (median 10.65 CFU/g, range 9.51–11.72) and the control (median 10.78 CFU/g, range 10.56–11.37, p > 0.05) or DBDL group (median 11.44 CFU/g, range 9.92–12.19, p > 0.05). However, the growth in gram-positive bacteria did see a significant increase in the DBDL group (median 12.06 CFU/g, range 11.82–12.31) versus SBL (median 7.5, range 6.7–8; p < 0.05) and control (median 6.34, range 3.42–11.14, p < 0.05). This gram-positive bacterial overgrowth was apparent from week 1 and remained so throughout the study period.

### *Liver histology (Fig. 2)*

In animals without ligature there was no histological lesion. The clearest and most representative histological changes in the SBL animals occurred during week 2 (grade II lesions in 4 animals: enlarged portal space, dilated biliary ducts, and ductal proliferation with distinct border reaching hepatic lobe). At weeks 3 and 4, however, there was negligible evidence of lesions (n = 4, grade 0; n = 1, grade I) was similar to that detected at week 1 and in animals without ligature. In the DBDL group, severe histological changes developed from week 1 (greater ductal proliferation, notable fibrosis with regenerative nodules, and non-visible centrolobular vein). All of the animals undergoing DBDL had developed cirrhosis at 4 weeks.(See Fig. 3)

### *Inflammatory response*

Overall, the serum concentrations of TNF-α were similar across all study groups. In the sham group, serum values remained stable at roughly 100 pg/mL; in the SBL group, there was a non-significant decrease at week 3. In the DBDL group, there was a significant decrease at week 1 (p < 0.05), but the levels registered thereafter showed recovery and stabilization.

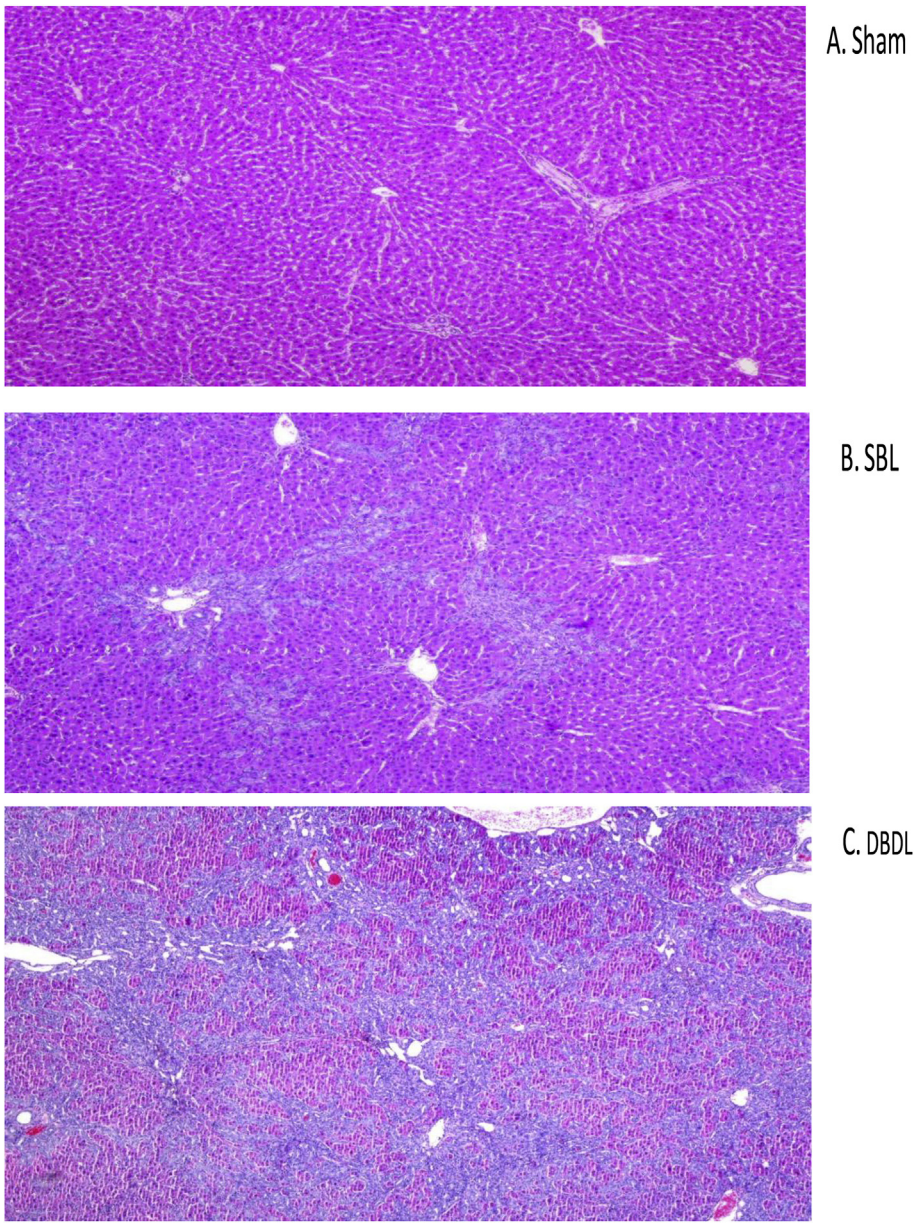


**Fig. 2.** Image from light microscope (× 40) of liver tissue from different study groups, stained with haematoxylin and eosin. Panel A: intact hepatic architecture (Sham group and SLB at 3 and 4 weeks). Panel B (SLB at week 2): enlarged portal space, dilatation and ductal proliferation reaching the hepatic lobe. Panel C (DBDL from 1 to 4 weeks); maximum ductal proliferation with fibrosis and regenerative nodules; centrilobular vein is not visible.

Levels of IFB were also relatively constant around 200 pg/mL, showing no significant difference between groups overall or at specific time points ($p > 0.05$).

## Discussion

The DBDL rat model has been widely used to study events that are traditionally associated with cirrhosis and portal hypertension, as is the case of BT. In a review of 30 papers comparing the experimental model for cirrhosis induced by double bile duct ligation versus $CCl_4$, investigators reported that both the ligation and $CCl_4$ models were capable of inducing advanced fibrosis in rats by 2–3 weeks.[23]

Both the bile ligation and $CCl_4$ models induce BT to MLN but the $CCl_4$ model is associated with relevant animal mortality. Single ligation is easier and faster to perform than DBDL and one of the aims of our investigation has been to compare the rate of BT and complications in animals subjected to classic DBDL *vs* single ligation model. Also, we have considered different post-ligation time points for evaluation of BT. As shown earlier, we have confirmed that BT is a phenomenon that occurs spontaneously in healthy rats, making necessary to include control groups for adequate estimation of pathological translocation. As expected, the incidence of BT increases after ligation, even prior to development of cirrhosis or portal hypertension. This finding is consistent with previous studies from other groups, who report the development of BT despite the absence of a clear liver lesion.[21] It is unknown whether these early phenomena are different or respond to a distinct physiopathology compared to that from cirrhotic animals, but the time point chosen for sample collection (MLN, liver, spleen) stands out as clearly relevant for the study of BT.

In the literature it is rare to find an exact description of the bile

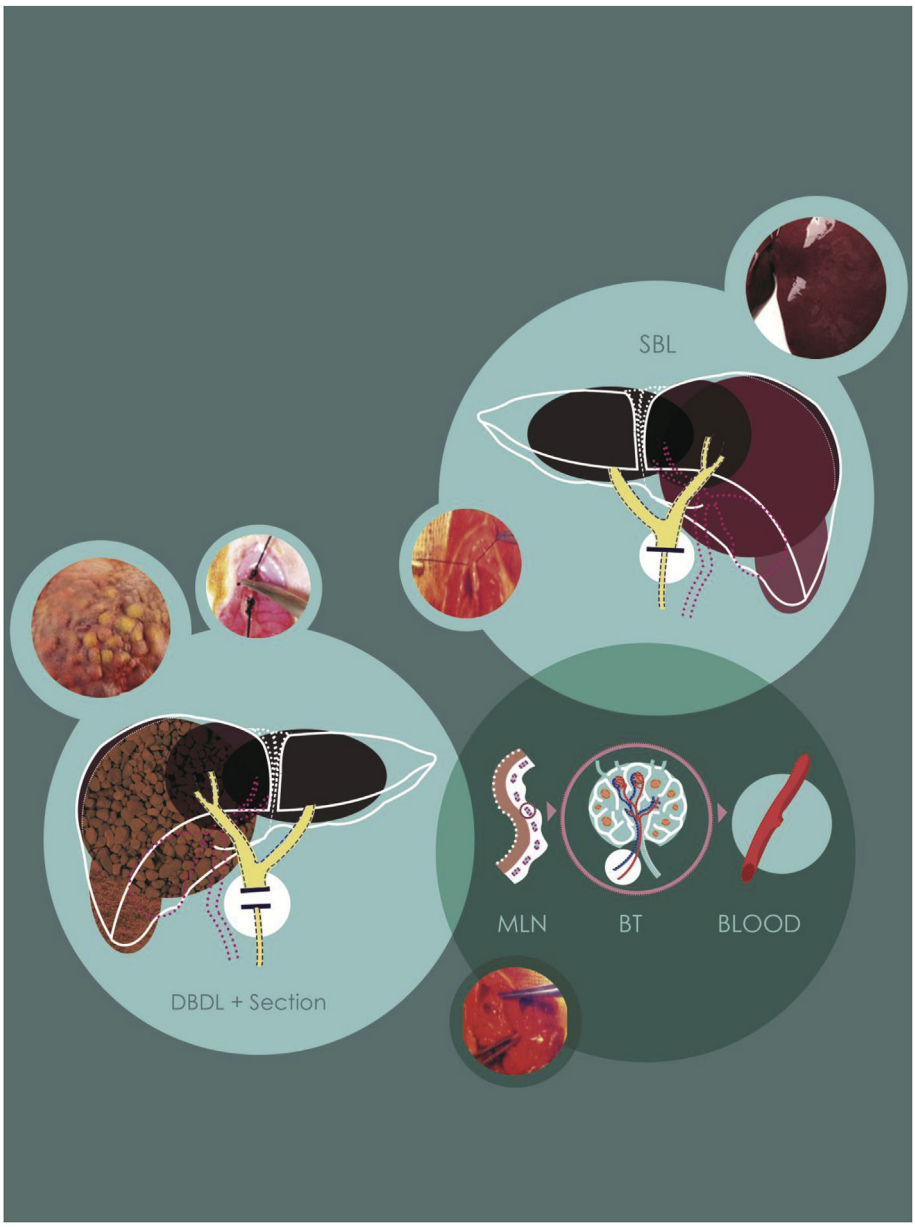


**Fig. 3.** This draw summarizes two experimental bile duct ligation models for the study of Bacterial Translocation and liver histology.

duct ligation model used in rats to study BT, or an explanation of all the steps followed when doing so (type of ligature, anatomical level at which it is done, whether a transection is performed between ligatures, or even variations of the traditional common bile duct ligation procedure with or without transection between ligatures). In fact, in most studies using the DBDL experimental model for cirrhosis, authors do not describe the exact location of the ligature,[13–15,24–26] or they refer to a double ligation but do not perform the transection between them.[20,21,27,28] Of the studies that reported performing a transection, we found only one that specified its exact location.[29] In our study, we observed that a simple ligature without transection of the bile duct generates a totally different model, with minimal liver damage but with the presence of BT at short term. These findings suggest that aspects such as performing (or not) a transection of the bile duct, or the number of ligatures carried out, may produce non-comparable experimental models.

Most studies using the bile duct ligation model schedule the second surgical intervention and sample collection for the BT study four weeks later, with the assumption that most animals will have developed advanced fibrosis or fully stablished cirrhosis. This is not the rule though. Kountouras et al.[14] reported the presence of cirrhosis in 80% of the rats 28 days after ligature while Slocum et al. did not detect cirrhosis in any of the 15 rats with ligature undergoing a second intervention at 5, 10, or 15 days.[21]

In our study we have analysed the characteristics of two experimental bile duct ligation models, simple and double with transection, studying BT and development of liver disease at different time points after surgery. We observed that both models rapidly induce BT, but liver damage is almost invariable induced by DBDL and transection, while single ligation is frequently followed by bile duct recanalization, precluding development of liver disease.[30,31] Animals in the single ligation group developed fibrosis, although did not reach the degree of cirrhosis. According to the

Kontouras scoring system[14] that we followed in our experiments, in the 1st week after ligation the degree of histological lesion was 0-I, progressing to grade II in week 2 and regressing to grade 0-I in weeks 3 and 4th. This behavior was just the opposite of what we found in animals with double ligation that progressed to grade IV in week 4. Also, visual observation of the bile duct in the single ligation group disclosed a reduced caliber when comparing to animals with double ligation. Therefore, and although we have no cholangiography in our animals, and therefore the patency of the bile duct in the single ligation group was not formally assessed, we have several objective data that suggest recanalization. First, the evolution of fibrosis and biliary proliferation as we discussed above, and second, the visual observation of the caliber of the bile duct, clearly less in animals of the single ligation group. Unfortunately we have no biochemical data on the degree of cholestasis after surgery. We believe this set of observations can be only explained by recanalization.

Further, SBL was not associated with bacterial overgrowth or portal hypertension, suggesting that the development of BT in this model is not caused by fibrosis or portal hypertension, but probably by the sudden lack of bile in the intestinal lumen. This is supported by the known development of bacterial infections shortly after bile lock for different reasons in patients.[37,38]

With regard to the inflammatory response and in contrast with other reports in the literature,[25,32] we did not detect an increase in blood cytokine levels in the DBDL group. Several reasons may explain this effect.[33] First, inflammation could be controlled locally (the liver is an organ that is very active immunologically), without reaching concentrations at a systemic level that are measurably different. There may be an early migration (before week 1) via the bloodstream of cells from the immune system to the liver, where important histological damage is beginning to take place. This could occur despite concomitant phenomena related to BT and intestinal bacterial overgrowth. The limited number of rats used in these experiments might also impede the statistical observation of small differences in blood cytokine concentrations.[34] Secondly, our findings may be due to the participation of the liver as an organ involved in defending the host, capable of producing powerful proinflammatory cytokines through the Kupffer cells following a bacterial infection.[35]

The SBL model in rats does not cause cirrhosis, portal hypertension, increased inflammation or bacterial overgrowth to the same extent as the DBDL model. However, the rate of BT episodes is comparable, making the SBL model a good method to study BT at short term with little or no liver damage.

## Conflicts of interest

All authors have contributed to the review and submission on this manuscript.

None of the authors have personal conflict of interest nor have they received payments for the performance of this work.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.amjsurg.2018.09.034.

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