CHOLESTASIS IN A MURINE EXPERIMENTAL MODEL: LESIONS INCLUDE HEPATOCYTE ISCHEMIC NECROSIS

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OBJECTIVE: To establish a murine experimental model of bile duct obstruction that would enable controlled observations of the acute and subacute phases of cholestasis.

METHODOLOGY: Adult male isogenic BALB/c mice underwent a bile duct ligation (22 animals) or a sham operation (10 animals). Fifteen days after surgery, or immediately after the animal's death, macroscopic findings were noted and histological study of the liver, biliary tree, and pancreas was performed (hematoxylin-eosin and Masson trichromic staining).

RESULTS: Beginning 24 hours after surgery, all animals from the bile duct ligation group presented progressive generalized malaise. All animals presented jaundice in the parietal and visceral peritoneum, turgid and enlarged liver, and accentuated dilatation of gallbladder and common bile duct. Microscopic findings included marked dilatation and proliferation of bile ducts with accentuated collagen deposits, frequent areas of ischemic necrosis, hepatic microabscesses, and purulent cholangitis. Animals from the sham operation group presented no alterations.

CONCLUSION: We established a murine experimental model of induced cholestasis, which made it possible to study acute and subacute tissue lesions. Our data suggests that in cholestasis, hepatic functional ischemia plays an important role in inducing hepatic lesions, and it also suggests that the infectious process is an important factor in morbidity and mortality.

DESCRIPTORS: **Cholestasis. Ischemic necrosis. Fibrosis. Experimental pathology. Bile duct.**

INTRODUCTION

Cholestasis is an impairment in bile secretion that occurs in many human liver diseases[1]. It can be extra-hepatic when there is a mechanical obstacle in the extra-hepatic biliary ducts, or it can be intra-hepatic when the cholestatic process is due to an intra-hepatic mechanical obstacle or to metabolic changes in the hepatic cell without any obstruction[2].

Although the pathogenic events culminating in cholestasis differ in each disease, hepatocellular injury is an invariant feature of cholestasis, leading to liver dysfunction, promoting fibrinogenesis, and ultimately resulting in liver failure. Retention and accumulation of toxic hydrophobic bile salts within the hepatocytes is partially responsible for the hepatocellular injury during cholestasis[3-5]. Other mechanisms have also been suggested to be involved.

The purpose of the present study was to establish a murine experimental model for the study of the changes occurring in acute and subacute phases of cholestasis and to correlate them with human cholestatic disease. A better understanding of the disease can enable the study of potential drugs that could delay, or even prevent, development of cirrhosis under irreversible cholestatic conditions.

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METHODS

Animals

Thirty-two adult male isogenic BALB/c mice weighing 24 to 28 g were used. The animals were obtained at DTAEP (*Technical Directorate of Support to the Education and Research*) of our institution.

Animals were housed under continuous observation in appropriate cages and allowed free access to a commercial standard diet (Nuvilabâ) and water *ad libitum*.

Animal experiments were performed in accordance with the US National Institute of Health – Guidelines for Care and Use of Laboratory Animals (publication NIH n° 85-25, revised in 1985).

Surgery Procedure

Mice were anesthetized by intraperitoneal administration of 2% thiazine chlorohydrate (0.02 mL) and ketamine chlorohydrate 0.5% (0.02 mL). After laparotomy, animals underwent a sham operation (10 animals, control group) or a common bile duct ligation (22 animals, study group): the common bile duct was isolated, doubly ligated, and resected between the ligatures.

The animals that died within the first week after surgery were observed immediately after death.

The surviving mice were sacrificed 15 days after surgery. In both conditions, macroscopic findings were noted, and the liver and pancreas were promptly removed for study.

Liver and pancreatic tissue was 10% formalin-fixed and paraffin-embedded. Four mm slides were hematoxylin-eosin stained for morphological evaluation, and in the case of liver tissue, Masson stained for collagen detection.

RESULTS

Survival following surgery

All animals recovered completely after surgery.

Twenty four-hours after operation, the clinical condition of all animals (100%) of study group worsened, with weight loss, decreasing activity in cages, pile bristling, yellowed ears, and darkened and yellow urine.

From the study group, 18 animals died an average of 5 days after surgery (3 to 10 days). The other 4 survived more than 10 days, being sacrificed on day 15.

Animals from the control group showed no alteration after surgery, being sacrificed on day 15.

Macroscopic findings

Study group (Fig. 1)

Jaundice was observed in parietal and visceral peritoneum of 100% of the animals.

The biliary bladder and common bile duct were dilated, becoming as wide as or even wider than the intestinal loops.

The liver was turgid and enlarged in all animals, with multiple pointed pale areas in 8 of them.

Microscopic findings

Study group:

- The hematoxylin-eosin staining of liver tissue showed high degree of dilatation and proliferation of the bile ducts with fibrotic reaction in the periphery of the majority of the portal space areas (Figs. 2A and 2C). There were numerous nodular small areas of ischemic necrosis with fibrosis, randomly distributed (Fig. 2A). A dense infiltration of inflammatory cells was observed, usually surrounding the biliary ducts, being comprised mainly of PMN cells, with the presence of intra-ductal purulent collection (Fig. 2B) and sometimes the development of microabscesses (Fig. 2C). The Masson staining showed an intense deposit of collagen fibers on the wall of the dilated portal and intralobular ducts, usually accompanied by inflammatory cells (Fig. 2D).
- Hematoxylin-eosin staining of pancreatic tissue revealed a normal pattern in all animals (not shown).
- Control group: Histological study showed no alteration in any animals (not shown).

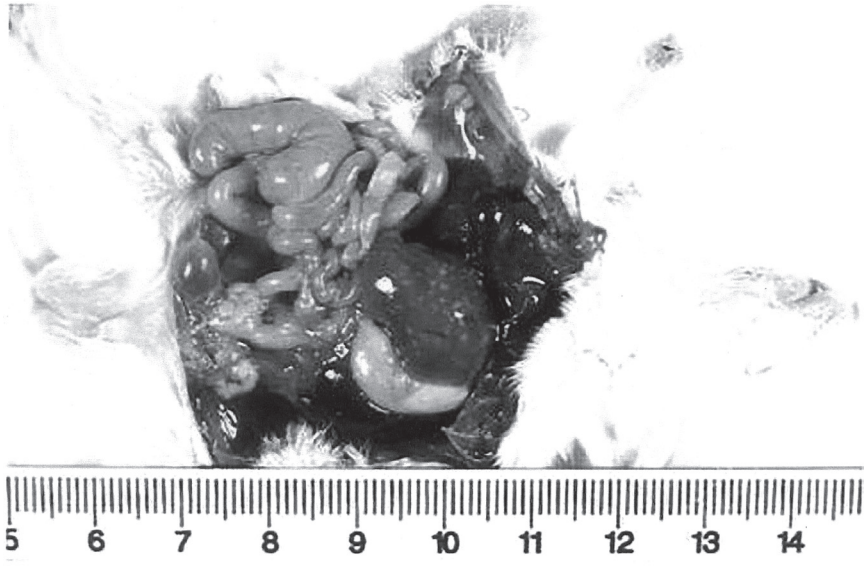

Figure 1 - Macroscopic aspects: yellowed peritoneum, significant dilatation of the gallbladder and the common bile duct, and congested liver with pale areas.

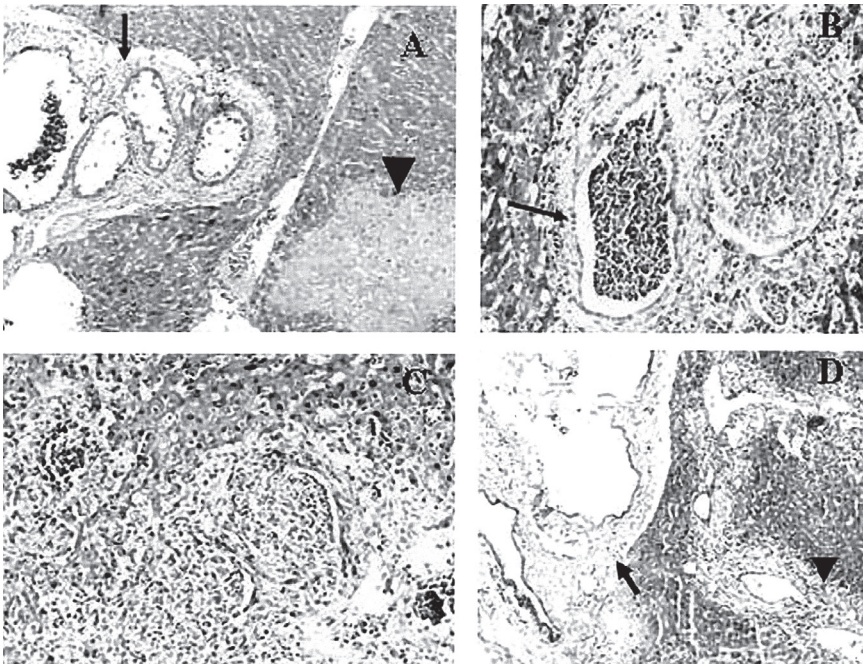

Figure 2 - A: Dilatation and proliferation of the bile ducts surrounded by increased fibrosis (arrow). Focus of healed necrosis (head arrow). (H & E, 100X magnification). **B:** Periductal fibrosis and intraductal purulent cholangitis. (Masson´s technique, 200X magnification). **C:** Microabscesses in portal spaces associated with purulent cholangitis. (H & E, 200X magnification). **D:** Severe dilatation of bile ducts surrounded by increased collagen tissue (arrow). Periductal acute inflammation (head arrow). (Masson´s technique, 100X magnification).

DISCUSSION

The experimental induction of extra-hepatic cholestasis and secondary biliary cirrhosis is not always successful. The recanalization of the hepatic duct after ligation with or without section of biliary tree can occur in up to 50% of animals and can be observed in the first 5 to 7 days after surgery[6]. The recanalization process can occur through the internalization of the hepatic duct, from below the ligation site up to the dilated part of the biliary tree[7]. There is proliferation of the biliary epithelium of the hepatic duct below the ligation point that, through time, connects itself to the dilated proximal portion of the biliary tree[8]. In our experiment, there was no recanalization of the duct. The cholestasis induction endeavor was successful. In all test animals, the common biliary duct remained totally obstructed until the end of the experiment. This maintenance of obstruction was probably due to the fact that, in our study, the common bile duct was doubly ligated and resected between the ligatures. The obstructive jaundice condition was demonstrated by the coloration of the parietal and visceral peritoneum, the auricle, and urine; the accentuated dilatation of the common biliary duct and gallbladder above the obstruction point (Fig. 1); and by the microscopic findings (Fig. 2A).

One possible complication of surgery of the biliary tree in mice is the induction of traumatic pancreatitis. In these animals, the pancreatic tissue was organized in a distributed pattern around terminal choledochus. In our model, the histological study showed no pancreatic alteration, probably due to careful manipulation of viscera during surgery.

Animals from the control group presented no clinical, macroscopic, or microscopic alteration. This observation excludes the anaesthetic procedure as well as the surgical trauma as possible causes of injury.

In our study, the very early mortality was low, with an average survival time of 5 days. Survival time was long enough to allow observation of the histological alterations occurring in acute and subacute cholestasis.

Our data strongly suggested that an infectious event, such as cholangitis and/or septicemia, was the most frequent cause of death of the animals. Although we did not perform liver tissue or bile culture, animals developed highly suspicious signs of infection, exhibiting a worsening of the clinical condition including, weight loss, pile bristling and decreased activity. In addition, multiple microabscesses were demonstrated in the histological exam.

The invasive manipulation of patients with obstructive jaundice can frequently lead to sepsis or infection-related complications[9-11]. Intestinal-derived endotoxemia is implicated in the pathophysiology of these events. An impaired endocytic function of the mononuclear phagocytic system is suggested as a mechanism of endotoxemia in the biliary obstruction[12, 13]. In addition, absence or a low level of bile in the small intestine impairs the emulsification and anti-endotoxic effects of the biliary salts, resulting in elevated levels of endotoxins in the large intestine, which are absorbed through portal circulation[14,15]. The exact mechanism of bacterial translocation is not well established, but the internalization of bacteria by enterocytes seems to be one of the initial events. It has been demonstrated *in vitro* that bile-treated bacteria are less likely to be internalized by enterocytes and more susceptible to intracellular lysis by the same cells[16], compared to non-treated bacteria. This finding demonstrates the pro-

tector role of the bile in the intestine. Additionally, Kupffer cells exposed to endotoxins in portal blood produce higher amounts of PAF (platelet activator factor), an important inductor of hepatocellular lesions.

Biochemical changes that occur in obstructive jaundice can also impair immunologic system function. Scott-Conner, et al.[17] isolated a serum factor with immune suppressive action in animals with obstructive jaundice.

Febrile patients with obstruction of the bile duct frequently present septicemia, especially due to Gram-negative organisms. Patients with a partial obstruction of the bile duct or cholangitis have a greater amount of bacteria in bile, sometime as high as that existing in the colon. The occurrence or not of systemic sepsis also depends on the pressure within the biliary tree, i.e., on the degree[18] of obstruction.

The morphologic features of cholestasis frequently include bile pigment accumulation within the hepatic parenchyma, dilated bile canaliculi, and rupture of canaliculi, which leads to bile extravasation that is phagocytosed by Kupffer cells. Kupffer cells filled with bile can also be seen in long-term cholestasis. Droplets of bile pigment also accumulate within hepatocytes, which can take on a wispy appearance (feathery or foamy degeneration)[19].

With sustained severe obstruction disease, bile can accumulate inside biliary ducts, ductules, and even in the parenchyma, leading to formation of "bile lakes". Bile accumulation in hepatocytes can cause cell degeneration: hepatocellular cytoplasm becomes translucent and the cells lose turgidity. The presence of bile lakes is considered the hallmark of destruction of large biliary ducts. On the other hand, the detection of bile accumulation inside bile ducts can also be seen in sepsis, in some drug reactions, and in graft rejection.

Although cholestasis was fully demonstrated in this experiment, we observed no bile lakes. This aspect had already been reported once before, i.e., that in rodents there is no bile lake accumulation in cholestasis[1]. Although this finding has not been fully elucidated, it is probably due to some anatomic or metabolic particularity of rodents.

However, although we could not identify local accumulation of biliary salts, we cannot exclude a hepatotoxic role by these products.

In this study, we observed a high death rate of hepatocytes. As we have said, this finding may be due to the great concentration of biliary salts above the obstruction[20,21] or to a functional ischemia caused by the decrease of portal flow that may accompany cholestatic diseases[22, 23].

Experimental models have shown a decrease of the portal blood flow in biliary extra-hepatic obstruction. Studies with rats and dogs have shown that this decrease can be as high as 40% to 50%[24-26]. The decrease of the portal flow seems not to be counterbalanced by a corresponding increase of the arterial hepatic flow. Therefore, a net reduction of the total hepatic blood flow results. Other researchers have shown that the dilatation of the intra-hepatic biliary ducts increases the portal pressure level after the sinusoids[27]. In 1991, experimental studies showed that portal blood flow is reduced during the extra-hepatic biliary obstruction and that portal blood flow increased significantly after 3 minutes of drainage of the biliary tree, confirming the cause-effect relationship between the ductal obstruction and the decrease of portal blood flow.

This decrease in hepatic blood flow predisposes the organ to a state of anoxia or even to a functional ischemic situation[28,26] that may be the trigger factor for the cellular lesions. This could initiate an oxidative stress condition that would make hepatocytes and its organelles more susceptible to the toxic effects of the biliary salts and bilirubins that would not ordinarily injury the hepatic cells. In addition, other studies have shown that the decrease of the portal blood flow that occurs in obstructive jaundice makes the liver functionally dependent on the arterial hepatic blood[29-31]. In our experiment, we observed many areas of ischemic necrosis of hepatic tissue, suggesting that the reduction in portal blood flow may have played an important role in causing the death of hepatocytes.

Obstruction of the biliary tree induces distention of upstream bile ducts. The bile stasis and back pressure induce proliferation and reduplication of ducts, termed bile duct proliferation. The labyrinthine ducts further slow the bile flow and favor the formation of concrements, which obstruct the ductal lumens. Additional factors that hamper bile flow develop as follows: portal tract edema with periductular infiltrates of neutrophils and bile lake formation containing cellular debris and bile pigment. Unrelieved obstruction leads to portal tract fibrosis, which initially extends into and subdivides the parenchyma with relative preservation of hepatic architecture. Ultimately, an end-stage, bile-stained, cirrhotic liver develops (biliary cirrhosis)[19]. In our study, we observed great dilatation and accentuated proliferation of bile ducts with peri-ductular infiltration of polymorphonucleocytes and accentuated deposits of collagen surrounding dilated bile ducts. Our results demonstrate that the characteristic changes of fibrosis are already present in the acute phase of cholestasis.

In conclusion, we were successful in establishing a murine experimental model of cholestasis induction, enabling the study of the morphologic alterations occurring in the acute and

subacute phases of cholestasis. Our data suggests that in cholestasis, hepatic functional ischemia plays an important role in inducing hepatic lesions, and it also suggests that the infectious process is an important factor in morbidity and mortality. Additional studies are in progress that are designed to evaluate the participation of apoptosis/necrosis in the pathology of cholestasis, as well as to compare histological alterations produced in this animal model with those that occur in humans.

RESUMO

PRADO IB e col. - Colestase em modelo experimental em murinos: lesões incluem necrose isquêmica dos hepatócitos. **Rev. Hosp. Clín. Fac. Med. S. Paulo 58**(1):27-32, 2003.

OBJETIVO: Realizar um modelo experimental de obstrução do ducto biliar que permita uma observação controlada das fases aguda e subaguda da colestase.

MÉTODOS: Submeteram-se camundongos BALB/c, adultos, machos, a ligadura do ducto biliar (22 animais) ou a cirurgia-controle (10 animais). Quinze dias após a cirurgia, ou imediatamente após a morte do animal, foram observados os achados macroscópicos e realizado o estudo histológico do fígado, árvore biliar e pâncreas (haematoxylina-eosina e tricrômico de Masson).

RESULTADOS: Vinte e quatro horas após cirurgia, todos os animais do grupo ligadura do ducto biliar apresentaram estado progressivo de mal estar. Todos apresentaram icterícia no peritônio parietal e visceral, fígado aumentado e congesto, dilatação acentuada da vesícula biliar e do ducto biliar comum. Os achados microscópicos incluíram: marcada dilatação e proliferação dos ductos biliares com acentuada deposição de colágeno, áreas freqüentes de necrose isquêmica de hepatócitos, microabcessos hepáticos e colangite purulenta. Animais do grupo cirurgia-controle não apresentaram alterações macro ou microscópicas.

CONCLUSÃO: Este estudo conseguiu estabelecer um modelo experimental de indução de colestase, possibilitando a observação das lesões teciduais agudas e subagudas. Nossos resultados apontaram a necrose isquêmica dos hepatócitos como um importante fator contribuinte para o distúrbio hepático na colestase, sugerindo uma participação da isquemia funcional na lesão do tecido. Esse estudo também sugere o quadro infeccioso como causa determinante de morte.

DESCRITORES: **Colestase. Necrose isquêmica. Fibrose. Patologia experimental. Ducto biliar.**

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