Microsurgical Extrahepatic Cholestasis in the Rat: A Histopathological Liver Study

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Abstract: Fibrosis, bile duct proliferation and mast cell hyperplasia characterize extrahepatic cholestasis. We studied these liver alterations in a new microsurgical model of extrahepatic cholestasis in the rat. Male Wistar rats: Sham-operated (n=9) and Microsurgical Cholestasis (n=10). After 4 weeks, a liver morphometric study was carried out using an image analysis system to assess bile proliferation and the fibrosis. The liver expression of alpha-smooth muscle actin was assayed by an immunohistochemical technique and mast cells were also counted. Cholestatic-rats presented portal hypertension, portosystemic circulation, and increased (p=0.0001) plasma bilirubin, alkaline phosphatase, Aspartate-aminotransferase, Alanine-aminotransferase, and Lactate-dehydrogenase. Plasma levels of Albumin decreased (p=0.001). Cholestatic-rats showed intense biliary duct proliferation (p=0.0001) and fibrosis (p=0.0001). Mast cells accumulate (p=0.0001) around proliferating bile ducts and fibrous septa. The microsurgical cholestasis in the rat induces fibrosis and hyperplasia of bile ducts and mast cells. Mast cell liver infiltration could be an etiopathogenic remodeling factor in experimental cholestasis.

Keywords: Cholestasis, microsurgery, mast cell, fibrosis, rat.

INTRODUCTION

The experimental model most widely used to study the extrahepatic cholestasis is based on common bile duct ligation (BDL). In essence, the bile duct is isolated, doubly ligated and resected between the two ligatures [1-7].

However, this technique can evolve bile recanalization as well as a bile pseudocyst, derived from the proximal bile-duct. This bile cyst can therefore produce hepatopulmonary abscesses and sepsis [8, 9]. We have proposed a microsurgical technique that consists in resectioning the extrahepatic bile *via* in order to avoid these severe and frequent complications [10].

The extrahepatic cholestasis models in the rat are very useful to study antifibrogenic therapies [11-13] and that is why we measure the fibrosis degree related to microsurgical extrahepatic in this study in order to assay future alternative therapeutics for biliary fibrosis.

The models of extrahepatic cholestasis in the rat are not only useful for evaluation of drugs and their effects on the secondary biliary fibrosis, but also to characterize this pathology [1]. Since mast cells are reported to participate in the development of liver fibrosis induced by bile duct resection [1], we have studied the existence of a relationship between the liver fibrosis grade and the mast cell number infiltrating the liver parenchyma in this new experimental model of microsurgical extrahepatic cholestasis.

MATERIAL AND METHODOLOGY

Male Wistar rats, with weights ranging between 230g and 270g from the *vivarium* of the Complutense University of Madrid were used. The animals were fed a standard laboratory rodent diet (rat/mouse A04 maintenance diet, Panlab, Spain) and water *ad libitum*. They were housed in a light/dark-controlled room (light cycles: 7:30a.m. -7:30p.m.), with an average temperature (22±2 ºC) and humidity (65-70%) in groups of three to four animals.

The experimental procedures employed in this study agree with the principles and practices of the 1986 *European Guide for the Care and Use of Laboratory Animals* in accordance with Ethical Guidelines from European Community Council Directive (*86/609/EEC*) and published in Spanish Royal Decree 1201/2005.

EXPERIMENTAL DESIGN

The animals were divided into two groups: Sham-operated (SO) (Group I: n= 9), in which the common bile duct was dissected, and microsurgical cholestasis (MC) (Group II: n=10) in which the resection of the extrahepatic biliary tract was performed [10, 14]

SURGICAL PROCEDURES

Rats were anesthetized with Ketamine hydrochloride (100 mg/kg) and Xylazine (12 mg/Kg) i.m. Surgery was performed under aseptic but not sterile conditions. An abdominal midline incision was made and the intestinal loops

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were covered with wet gauze and shifted toward the left side of the animal to expose the hilar structures.

In Group I (SO), the bile duct and its lobular branches were dissected. In Group II (MC), the extrahepatic bile tract was resected using a binocular operatory microscope (Zeiss, OPMI 1-FR). First, the common bile duct was ligated (silk 4/0) and sectioned close to the beginning of its intrapancreatic portion. This maneuver, which produces dilation of the extrahepatic biliary tract, facilitates the posterior dissection of the bile duct and the biliary branches of the four hepatic lobes, which make up the liver of the rat. Once the bile duct is sectioned, it is shifted upwards. The dissection and section between ligatures of all the biliary branches that drain the hepatic lobes is possible by using a binocular operatory microscope (Zeiss, OPMI 1-FR). First, the biliary branch (sometimes there are two branches) of the caudate lobe and then the two biliary branches of the right lateral lobe are dissected, ligated and sectioned close to the hepatic parenchyma. The upward dissection of the extrahepatic biliary tract makes it possible to individualize, ligate and section the biliary branches draining the middle lobe and, finally, the same procedure is followed with the biliary branch of the left lateral lobe. The dissection and section of the bile ducts from the four liver lobes of the rat must be done without injuring either the portal and, most especially, the arterial vascularization of these lobes [10, 14]. The abdomen was closed in two layers by continuous running sutures with an absorbable suture (3/0 Polyglycolic acid) and silk (3/0).

Portal Vein Pressure Measurement

Splenic pulp pressure, an indirect measurement of portal pressure (PP) was measured by inserting a fluid-filled 20-gauge needle into the splenic parenchyma [15]. The needle was joined to a PE-50 tube, then connected to a pressure recorder (PowerLab 200 ML 201) and transducer (Sensonor SN-844) with a Chart V 4.0 computer program (ADI Instruments) and finally was calibrated before each experiment. The pressure reading was considered satisfactory when a stable recording was produced and respiratory variations were observed. The external zero reference point was placed at the midpoint of the animal, 1 cm above the operating table.

Portosystemic Collateral Circulation Method

Portosystemic collateral circulation was studied as follows. First, a midline abdominal incision with a large bilateral subcostal extension was performed and then the areas in which the collateral venous circulation developed, i.e. the splenorenal, gastroesophageal, colorectal and hepatic hilum, were carefully studied for the presence of increased collateral veins [16].

Gross Mesenteric Vein Study

Three grades of mesenteric venous vasculopathy were considered: grade 0: normal aspect of the branches of the superior mesenteric vein; without dilation and tortuosity related to the superior mesenteric vein compression for 30sg; grade I: dilation and tortuosity of these branches related to this maneuver and grade II; in which the dilation and tortuosity of the branches of the upper mesenteric vein were spontaneous.

Histopathological Examinations

The animals were sacrificed by exsanguination and representative samples of the right lateral and the middle hepatic lobes were collected and fixed in phosphate-buffered neutral formalin (4%) and embedded in paraffin. 5μ-thick sections were stained with Hematoxylin & eosine (HE), Masson's trichromic and Giemsa dyes to specifically stain fibrosis and mast cells. Furthermore, an immunohistochemical technique to measure the expression of alpha-smooth muscle actin (SMA) with an automatic staining machine (Techmate, DakoCytomation) was used. A mouse monoclonal antibody anti-actin (ref. Number MS-1295-P1, Westinghouse, LabVision/Neomarker), which has reactivity for different animal species including the rat, was used at 1:100 dilution as pretreatment for antigenic exposure, in a pressure cooker with citrate buffer. The binding with the primary antibody was revealed with diaminobencidine and the samples were contrated with hematoxilin & eosin [17].

The morphometric study was carried out using an image analysis semi-automatic system (Microimage, Olympus) to objectively assess bile proliferation and the fibrosis content of the hepatic parenchyma. The mast cells stained with Giemsa, per mm^2 of portal space and/or fibrotic tracts, were identified by the presence of multiple intacytoplasmic granules [18]. A minimum area of hepatic tissue was digitalized to $15mm^2$ through microscopic images with a 10x lens.

Statistical Analysis

Statistical analyses were performed using SPSS software (Statistical Package for the Social Sciences, versión 14.00). The results are expressed as the mean ± the standard deviation. Student t test for unpaired data were used for the statistical comparison of the variables between the two groups. The relationship between the number of mast cells, bile proliferation and actin expression were verified using the Spearman coefficient correlation. The results are considered significant if p<0.05.

RESULTS

Portal Hypertension Signs

In rats with cholestasis, the existence of portal hypertension was confirmed by the increase in portal pressure (15.72 ± 2.28 *vs* 7.84 ± 1.92 mmHg; p< 0.001) and the development of mesenteric venous vasculopathy, whether of grade II (70%) or grade I (30%), as well as of portosystemic collateral circulation (100%) through splenorenal, paraesophageal and hemorrhoidal vessels.

Serum Hepatobiliary Injury Parameters

The rats with micosurgical cholestasis showed a significant increase (p=0.0001), in the serum levels of direct bilirubin, alkaline phosphatase (AP), aspartate-aminotransferase (AST), alanine-aminotransferase (ALT), AST/ALT ratio and lactate-dehydrogenase (LDH) compared to sham-operated animals. On the contrary, plasma albumin levels decreased (p=0.001) in rats with cholestasis (Table **1**).

Histopathologic Examination

The SO animals did not show any histopathological change. On the contrary, in the animal with cholestasis, the

Table 1.   **Direct Bilirubin (DB), Alkaline Phosphatase (AP), Aspartate Aminotransferase (AST), Alanin Aminotransferase (ALT), AST/ALT Ratio, Lactate Dehydrogenase (LDH), and Albumin in Sham-Operated (SO) Rats and in Rats with Microsurgical Cholestasis (MC) 4 Weeks After the Intervention**

Group	SO (n=9)	MC (n=10)
DB (mg.dl^{-1})	0.21 ± 0.26	6.81 ± 1.76***
AP (U.L^{-1})	129.28 ± 32.53	298.37 ± 78.27***
AST (U.L^{-1})	65.56 ± 10.07	388.65 ± 73.67***
ALT (U.L^{-1})	40.46 ± 6.78	89.87 ± 20.12***
AST/ALT	1.45 ± 0.19	4.78 ± 1.34***
LDH (U.L^{-1})	384.80 ± 98.93	689.99 ± 76.23***
Albumin (g/dl)	2.97 ± 0.12	2.73 ± 0.14g/dl***

Mean $\pm$ SD; ***p<0.001: statitically significant value in relation to SO-rats.

hepatic parenchyma showed intense biliary duct proliferation (p=0.0001) in relationship with SO-rats (Table **2**, Fig. **1**). Masson´s staining and immunohistochemical expression of actin showed an intense fibrosis that was quantified by digital image analysis (p=0.0001) (Table **2**, Fig. **1**). Results obtained from mast cell counting are presented in Table **2**. The statistical analyses of the number of mast cell showed that in cholestatic livers mast cell infiltration was significantly (p=0.0001) higher than those in the SO samples. Besides, the number of mast cells was superior in the right lateral lobe in relation to the middle lobe (Fig. **2**). In the liver of cholestatic rats there was an accumulation of mast cells, particularly in portal spaces and in the fibrous septa, close to both, bile proliferation and fibrosis (Fig. **3**). However, the number of mast cells was not correlated with bile proliferation (r= -1.40; p= 0.56) neither actin (r=-0.07; p= 0.77) in microsugical cholestatic livers.

Table 2.   **Liver Bile Proliferation, α-Smooth Muscle Actin (SMA) Positive Cells and Mast Cell Infiltration in Sham-Operated (SO) Rats and in Rats with Microsurgical Cholestasis (MC) 4 Weeks After the Intervention**

Group	SO (n=9)	MC (n=10)	P
Bile proliferation (%)	4.02 ± 3.04	29.14 ± 13.12	p= 0.0001
(α-SMA) n/mm^2	320.35 ± 97.88	1261.44 ± 202.42	p= 0.0001
Mast cell n/mm^2	69.47 ± 49.66	293.58 ± 145.33	P= 0.0001

Mean $\pm$ SD. (The value of each rat is the mean of the middle and the right lateral lobe).

DISCUSSION

The microsurgical extrahepatic cholestasis in the rat makes it possible to obtain a model of biliary fibrosis 4 weeks after the intervention. Moreover, the hepatic histomorphometric study shows the coexistence of bile duct proliferation and mast cell accumulation in this experimental model. Fibrosis together with bile duct proliferation and mast cell accumulation in the liver is a characteristic change in experimental cholestatic liver disease [19, 20].

The extensive positive areas of alpha-SMA in rat livers of microresected extrahepatic bile *via* are principally located in the portal tracts and they are associated with proliferating bile ducts (Fig. **1**). It has been demonstrated that the level of liver fibrosis can correspond to alpha-SMA and that alpha-SMA is in turn a marker of activated hepatic stellate cells (HSC), a cell that represents the pivot of the fibrotic process [6, 21, 22]. Therefore, the obtained results related to the extension of alpha-SMA marker in the liver of rats with microsurgical extrahepatic cholestasis support the use of this experimental model to study, both the pathophysiological mechanisms involved in the liver fibrosis development and new anti-fibrogenic therapies.

HSC undergo a transition into alpha-SMA, expressing myofibroblastic-like cells in response to extrahepatic cholestasis [22]. Furthermore HCS activation is associated with stellate cell proliferation, increased contractility and enhanced extracellular matrix production [11, 22, 23]. Therefore, HSCs play a crucial role in cellular and molecular events that lead to hepatic fibrosis [6, 22, 23].

Mast cells are widely distributed through virtually all vascularized tissues where they normally reside in close proximity to blood vessels and nerves [24]. They are specially numerous in anatomical sites that are directly exposed to the environment, including the gastrointestinal tract, airways and skin [24, 25]. It has been recognized that mast cells are present in normal and pathologic livers in both humans and rats [26, 27]. A number of studies have found that mast cells are involved in various liver diseases [26, 28-30], thus showing that they play a broader role than originally thought [31].

Mast cells are reported to participate in the development of liver fibrosis in rodent and human models [28, 32]. Experimental studies have shown that bile duct ligation in rat, a model of liver fibrosis [1], caused an increase in intrahepatic mast cells [19, 27]. However, it is not yet known how the mast cells participate in liver fibrosis. Although given that bile duct ligation in the rat causes liver inflammation [33] and many mast cell mediators are considered to be pro-inflammatory [24, 25], it could be supposed that mast cell activation will influence liver fibrosis within the context of a chronic inflammatory response. In this way, fibrosis associated to tissue remodeling, both mediated by mast cells, would be one of the major consequences of the chronic liver inflammation [25, 34]. Thus, it has been suggested that liver fibrogenesis resembles a wound-healing process leading to scar formation [33, 35].

Interactions between mast cells and fibroblasts are paramount in the genesis of fibrosis [36, 37]. It has been demonstrated that mast cells can communicate with fibroblast in a manner that leads to fibroblast activation and subsequent modulation of some aspects of extracellular fibrosis [37, 38]. However, the results of a study using mast cell-deficient Ws/Ws rats suggested that an increase of mast cells in the liver may be associated with fibrosis, but was not a cause of fibrosis [39]. Moreover, recently it has also been shown that mast cells might participate in fibrolysis by producing matrix metalloproteinases (MMP)-1 throughout the late stages of experimental fibrosis and cirrhosis [40]. Since improved therapies of liver cirrhosis must be based on the understand-
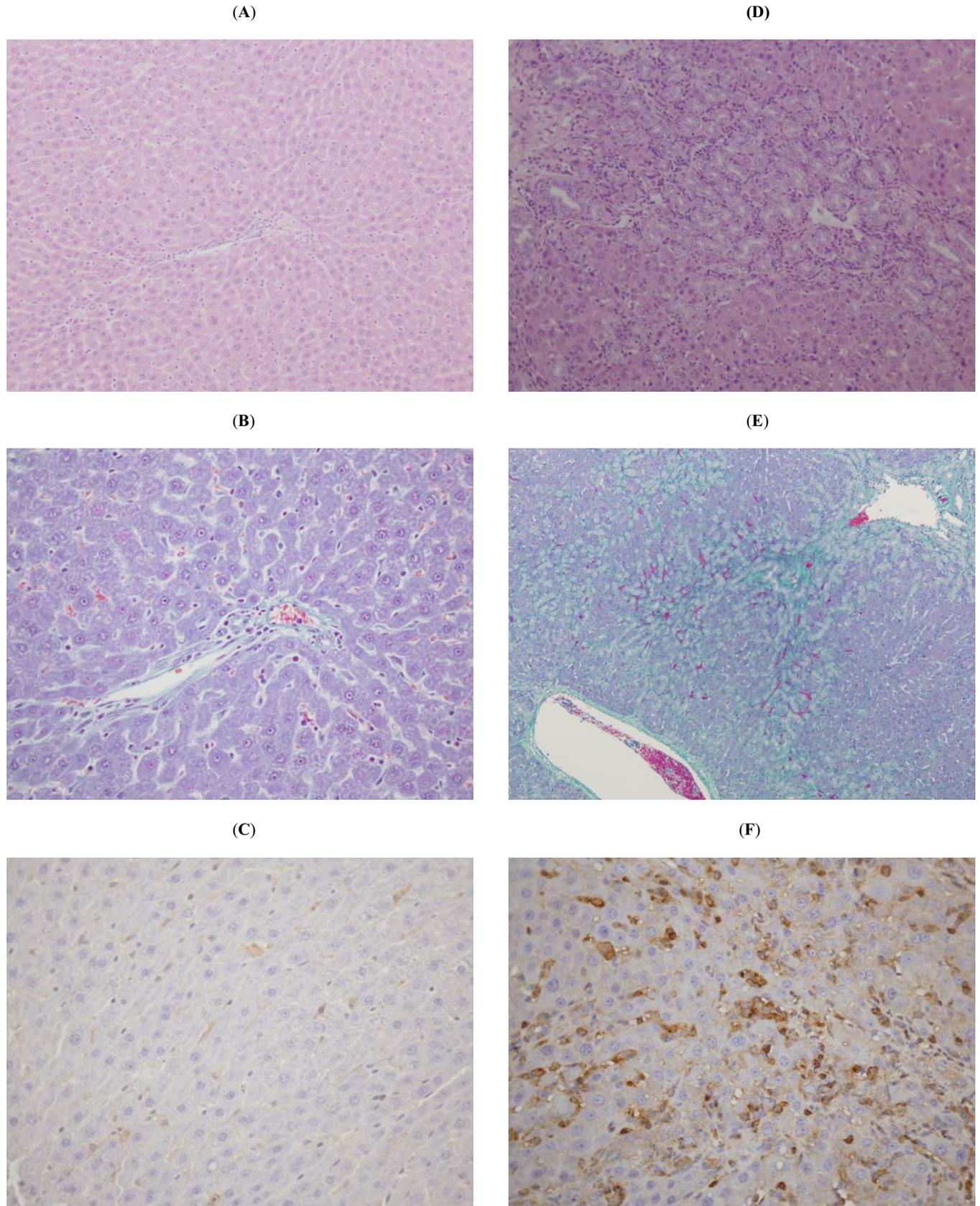
(A)

(D)

(B)

(E)

(C)

(F)

Fig. (1). Representative photomicrographs of liver from sham-operated (**A**, **B** and **C**) and microsurgical cholestatic-rats (**D**, **E** and **F**). Paraffin embedded sections were processed for H&E (**A** and **D**); Masson trichromic (**B** and **E**) and immunohistochemical expression of alpha-smooth muscle actin (SMA) (**C** and **F**). Extensive bile duct proliferation and peribiliary fibrosis (arrows) with bridging fibrosis was observed (original magnifications x 100, X 50 and x 200, respectively).

(A)

(B)

(C)
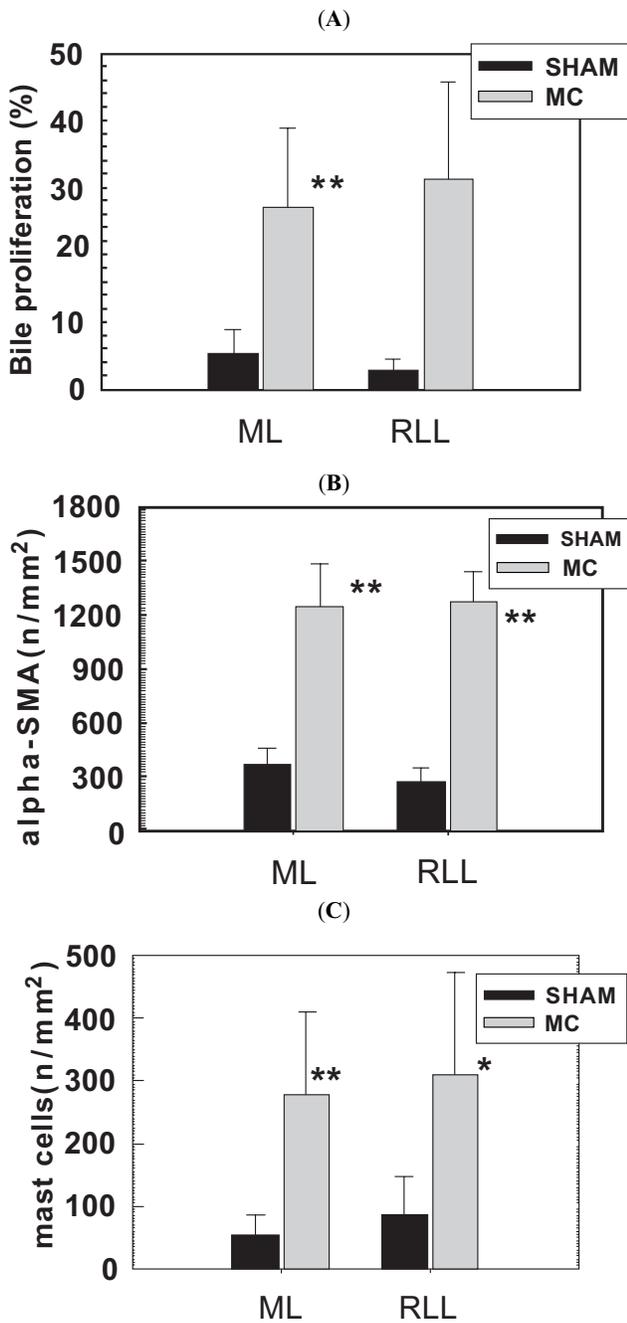
Fig. (2). Bile proliferation (**A**), alpha-smooth muscle actin (SMA) positive cells (**B**) and mast cell infiltration (**C**) in the liver of sham-operated rats (SO) and in microsurgical cholestatic rats (MC) 4 weeks after the intervention.

ing of its pathogenesis [41] a better understanding of this dual action, pro- and anti-fibrogenic of mast cells in the experimental related biliary fibrosis, would be of great interest [42, 43]. In addition, chymase which is expressed by mast cells is also known to be an Angiotensin II-forming enzyme and Angiotensin II is a potent profibrogenic factor. Therefore, mast cells may contribute to liver fibrogenesis through chymase-dependent Angiotensin II formation [44].

Mast cell hyperplasia is associated with the proliferation of bile ductules during microsurgical extrahepatic cholestasis. These findings suggest that mast cells accumulating in the portal triads may be involved in bile duct proliferation.

However, the recanalization of the ligated common bile duct in the rat leads to an abrupt increase in liver mast cells and a rapid decrease in proliferated bile ductules [20]. That is why it has been suggested that the accumulation of mast cells during cholestasis resolution would be related to the induction of apoptosis of the biliary epithelial cells [20]. Apoptosis of the biliary epithelial cells may also be caused, among other factors, by hypoxia [45]. Thus, a pathological relationship between the bile pathway blood flow and its injury is established [46, 47].
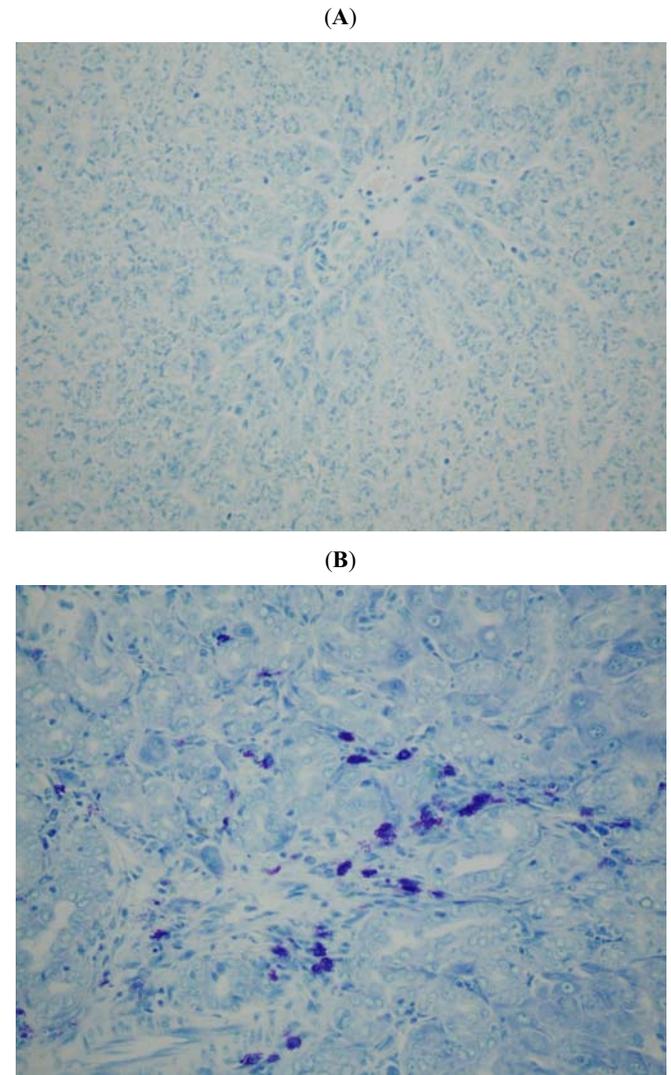
(A)

(B)

Fig. (3). Mast cells accumulate around proliferating bile ducts and fibrous septa in the liver of cholestatic-rats (**B**) compared with sham-operated animals (**A**) (Giemsa, X 200).

The intrahepatic and extrahepatic biliary tract is supplied and nourished by a network of fine vessels called the peribiliary vascular plexus derived from the hepatic arterial branches [47]. In extrahepatic cholestasis the injury to the peribiliary arterial plexus can result in ischemia and oxidative stress of the biliary tract [9, 48]. After common bile duct ligation in the rat the enormous extension of the peribiliary vascular plexus suggests the important role of the arterial blood supply of the biliary tree in conditions of cholangiocyte proliferation, such as it occurs during chronic cholestasis [47]. Proliferation of the peribiliary arterial plexus by

angiogenesis follows cholangiocyte proliferation. In both, the vascular endothelial growth factor (VEGF) plays a role [47, 49].

Mast cells in fibrotic livers can also be involved in hepatic arterialization. During the establishment of liver fibrosis the altered structure of the sinusoids resembles that of capillaries [50]. This change is known as "sinusoidal capillarization" and mast cells can be considered key elements in this process of transforming sinusoidal endothelial cells into capillary-type endothelial cells [31, 50]. Capillarization hinders normal exchanges between plasma and hepatocytes and is the main cause of worsening liver function [50].

Therefore, it could be considered that the role developed by mast cells in the experimental extrahepatic cholestasis is dual, that is, it changes along the evolution of the cholestatic liver [42, 43]. If so, in early evolutive phases mast cells would participate in the pro-inflammatory process that induces biliary proliferation and fibrogenesis [27]. However, the prolonged cholestasis would favor the change of the mast cell environment and induce a switch in their phenotype, promoting fibrolysis by producing metalloproteinases and matrix degradation [40]. In this way, interferon-alpha and steroids may have antifibrogenic properties in limiting the actuation of stellate cells and by increasing the production of collagenase and metalloproteinase; steroids share similar antifibrogenic properties [51].

This supposed protective and antifibrotic role of mast cells in chronic cholestasis would explain the persistent increased number of mast cells in the development of both fibrosis and liver cirrhosis associated with the markedly decrease of myofibroblasts and macrophages in periportal regions [40]. Mast cells, owing to their periportal location, would induce bile epithelium apoptosis with attenuation of hepatocyte apoptosis and, therefore this may be another mechanism by which mast cells could promote an antifibrogenic activity as well as hepatocyte preservation [52, 53].

CONCLUSION

In summary, by correcting the epithelial imbalance produced by cholestasis between the two main types of hepatic epithelial cells, namely biliary (proliferation) and hepatocytic (with apoptosis), the mast cells would try to limit fibrosis characteristic of chronic liver inflammation. One protective role that in some circumstances mast cell assume to orchestrate inflammation [54].

ABBREVIATIONS

ALT   =   Alanine-aminotransferase

AP   =   Alkaline phosphatase

AST   =   Aspartate-aminotransferase

BDL   =   Bile duct ligation

HSC   =   Hepatic stellate cells

LDH   =   Lactate-dehydrogenase

MMP   =   Matrix metalloproteinases

MC   =   Microsurgical cholestasis

PP   =   Portal pressure

SO   =   Sham-operated

SMA   =   Smooth muscle actin

SPSS   =   Statistical Package for the Social Sciences

VEGF   =   Vascular endothelial growth factor

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