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Effects of Nebivolol on Liver Fibrosis Induced by Bile Duct Ligation in Wistar Rats

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Abstract. *Aim: To study the effect of nebivolol on liver fibrosis induced by common bile duct ligation (BDL) in rats. Materials and Methods: After BDL, Wistar rats were divided into three groups (n=24): SO, sham-operated animals; BDL, BDL rats without treatment; BDL+N, BDL rats treated with nebivolol for five weeks. Alanine aminotransferase, aspartate aminotransferase, gamma glutamyltransferase, total bilirubin and albumin levels were assessed. Liver samples collected were stained with hematoxylin-eosin, Masson's trichrom and reticulin. Results: Mortality reached 37.5% in the BDL group, whereas no deaths were observed in the SO and BDL+N groups. The BDL group showed hepatic damage as evidenced by elevation in serum biochemical parameters and fibrosis scores. These pathophysiological changes were attenuated in the BDL+N group. However, there was no significant difference between these two groups. Conclusion: Nebivolol improved the survival rate of animals with BDL, but was unable to significantly improve liver function or reduce liver fibrosis.*

Liver fibrosis represents an important medical problem with significant morbility and mortality worldwide (1, 2). It is a consequence of continued wound-healing response to chronic liver injury from a diversity of causes, including chronic viral hepatitis, drugs, autoimmune response, alcoholism, parasitic diseases, metabolic disorders and cholestasis, which contributes significantly to the deleterious outcome of chronic liver diseases (1, 3). The deposition of extracellular matrix (ECM) in the space of Disse, the generation of subendothelial basement membranes, and the strangulation of hepatocytes by a surrounding matrix, impair not only the blood flow through the organ, but also the biosynthetic function of hepatocytes and the clearance capability of these and other hepatic cells (4). Current pharmacological approaches for the treatment of liver fibrosis are not effective. Removal of the underlying cause of chronic liver injury and liver transplantation are the only currently available therapeutic interventions capable of modifying the natural history of liver fibrosis. However, lack of available donors, immune rejection and overall cost of the procedure warrants new therapies for liver fibrosis (5).

Nebivolol is a highly selective β1-adrenergic receptor antagonist, with additional vasodilating (6-8), antioxidant (9, 10) and antiproliferative properties (11-13) related to increased nitric oxide (NO) bioavailability. We have found that in rats with renal mass reduction, the administration of nebivolol delayed the progression of renal fibrosis (14). The effects of nebivolol in that study appeared to be mediated, in part, through its ability to increase NO bioavailability. NO has antiproliferative and antifibrogenic activities (15, 16) that may be relevant in chronic liver disease. Some studies suggest that NO donors may exert a direct antifibrogenic action by inhibiting proliferation, motility and contractility of hepatic stellate cells (HSCs), and reducing ECM deposition (17-19). Thus, by increasing the bioavailability of NO, nebivolol may exert a beneficial effect on liver fibrosis. No experimental data on the efficacy of nebivolol on liver fibrosis are available. Hence, we proposed to study the effect of nebivolol treatment on liver fibrosis induced by bile duct ligation (BDL), a model of secondary biliary cirrhosis.

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Materials and Methods

Animals and experimental conditions. Thirty-two male Wistar rats (*Rattus norvegicus*) (Harlan Interfauna Iberica SL, Barcelona, Spain) with an initial body weight of 390±34 g, 15 weeks of age, were used. Animals were housed in appropriate cages under controlled conditions of temperature (22±2˚C), humidity (55±5%), lighting (12/12 dark-light cycle) and air system filtration (10-20 ventilations/hour). All animals had free access to rat chow diet (Teklad Global Diet®; Barcelona, Spain) and bottled water. The experimental procedures were performed according to ordinance no. 1005/92 and EU Directive 2010/63/EU on the use of laboratory animals.

BDL and experimental protocol. Liver fibrosis was induced by BDL in 24 rats. Each rat was weighed and anesthetized by intraperitoneal administration of ketamine (Imalgene®, 75 mg/kg; Merial SAS, Lyon, France) and medetomidine (Dormitor®, 0.5 mg/kg; Orion Pharma, Espoo, Finland). An abdominal was performed trichotomy (AESCULAP, Isis GT420, Braun®) and antisepsis with 10% povidone iodine (Betadine®) followed by a midline laparotomy. The common bile duct was exposed and ligated at two points with 4.0 silk. To avoid re-permeabilization, the bile duct between the double ligatures was cut, followed by careful suturing of the peritoneum and muscle layers, as well as the skin wound. In sham-operated (SO) rats, the bile duct was dissected and similarly manipulated, but no ligation was made.

Three days after surgery, rats were divided into three groups: SO: sham-operated rats (n=8); BDL: BDL rats without treatment (n=8); BDL+N: BDL rats treated with nebivolol (Nebilet®, 8 mg/kg per day; Menarini, Florence, Italy) (n=8). Treatment was administered in drinking water for five weeks and the concentration of nebivolol was adjusted weekly according to water intake and animal body weight. The dosage used in this study was based on dosing experiments of our previous study (14). Animals' body weight was measured weekly and weight gain (WG) was calculated according to the formula: WG=Wf–Wi/Wf×100 where Wf final weight and Wi initial weight (20). Mean arterial pressure (MAP) and heart rate were measured at the end of the five-week study by the tail-cuff method using an LE 5001 Pressure Meter (Letica®, Panlab, Barcelona, Spain). The MAP value for each rat was calculated as the average of six separate measurements not differing from one another by 10 mmHg.

Animals were then sacrificed with pentobarbital sodium overdose. Blood samples were collected from the abdominal aorta into heparinised capillaries and tubes without anticoagulant. A complete necropsy was performed and liver tissue was collected and fixed in 10% buffered formalin solution and processed for histopathological analyses.

Heparinised capillaries were centrifuged (6000 ×*g* for 5 min) to determine the haematocrit. Serum was obtained by centrifugation at 656 ×*g* for 15 min and used to evaluate the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyltransferase (GGT), and the concentration of total bilirubin (TB) and albumin by automated standardized procedures (RX Daytona Randox Laboratories, Dublin, Ireland).

Histopathological evaluation. For the histopathological evaluation, representative liver fragments fixed in 10% buffered formalin were paraffin-embedded. Two-micrometer tissue sections were processed and stained with haematoxylin and eosin, reticulin and Masson's trichrome. The liver samples were analyzed by optical microscopy to identify liver lesions. The following four categories were evaluated according to the Modified Histological Activity Index (HAI): piecemeal necrosis, confluent necrosis, focal necrosis and portal inflammation, and scores added together for the activity grade (21, 22). The mean±SE of the score for each group was calculated. The degree of liver fibrosis was evaluated semiquantitatively under a bright-field microscope with a ×10 objective, by means of two scoring systems that have already been applied in the BDL model, namely, the Knodell and METAVIR systems (23). The Knodell scoring system was applied as follows: level 0, indicating absence of fibrosis; level 1, fibrous portal expansion; level 3, bridging fibrosis (portal–portal or portal–central vein linkage); level 4, cirrhosis (24). The METAVIR scoring system was applied as follows: level 0 indicating the absence of fibrosis; level 1 indicated enlargement of portal area; level 2 was assigned to a lobule with only one septa forming between two adjacent portal areas; level 3 was assigned to lobules with two or three well-formed septa, and level 4 was assigned to lobules completely encircled by ductular reaction (cirrhotic nodule) (23). The fibrosis scoring was performed by a pathologist blinded to the experimental protocol. The mean of the scores taken from 10 non-overlapping random fields per section was used to generate a single score for each specimen.

Statistical analysis. The differences between groups were evaluated by one-way analysis of variance (ANOVA) when indicated, followed by Bonferroni *post hoc* tests. In other cases, the results were statistically analyzed by the Kruskal–Wallis test followed by multiple comparisons by Dunn procedure. All data are presented as the mean±SE and were considered significant at $p<0.05$.

Results

Survival and macroscopic findings. During this experimental protocol, animals were observed daily to evaluate their clinical condition. Animals of BDL groups presented a decrease of activity, weight loss, icteric skin and mucosa, dark urine and hippo/acholic faeces. Three animals from the BDL group died and were not included in the final data analysis. After sacrifice, all animals from BDL and BDL+N groups exhibited hepatomegaly and bile duct dilatation before the obstruction point. Some of these animals also presented ascites. Animals from the SO group showed no alteration after surgery.

Body weight, WG and MAP. As shown in Table I, at the end of the experimental protocol, animals from the BDL and BDL+N groups presented a lower body weight than animals from the SO group; however, this difference was significant only between the SO and BDL groups. The WG was significantly lower in the BDL and BDL+N groups when compared with the SO group ($p<0.01$). BDL+N animals had significantly lower MAP ($p<0.01$ compared to the SO group and $p<0.05$ compared to the BDL group) and heart rate ($p<0.01$ *vs*. the SO group and $p<0.05$ *vs*. the BDL group), whereas no significant differences were noted in MAP and heart rate between the SO and BDL groups (Table I).

Table I. *Effect of nebivolol on weight, weight gain, mean arterial pressure, heart rate and liver biochemical parameters (mean±SE).*

Parameters	SO (n=8)	BDL (n=5)	BDL+N (n=8)	Difference between groups
Weight (g)	415.88±12.78	351.80±16.96	388.00±7.89	SO *vs*. BDL **
Weight gain (%)	5.74±0.59	−5.71±6.23	−4.08±1.70	SO *vs*. BDL**
				SO *vs*. BDL+N**
MAP (mmHg)	108.13±1.46	101.80±3.40	89.75±3.09	SO *vs*. BDL+N**
				BDL *vs*. BDL+N*
HR (bpm)	442.63±13.92	420.00±7.20	368.38±7.86	SO *vs*. BDL+N**
				BDL *vs*. BDL+N*
Ht (%)	47.06±0.88	34.00±4.23	43.25±2.66	SO *vs*. BDL**
Albumin (g/dl)	1.44±0.05	0.98±0.22	1.20±0.10	SO *vs*. BDL*
ALT (UI/l)	52.71±4.35	60.00±10.58	42.63±2.43	NS
AST (UI/l)	83.29±4.50	233.60±91.98	129.88±24.11	NS
GGT (UI/l)	1.29±0.25	17.20±8.41	11.75±6.83	NS
TB (mg/dl)	0.31±0.03	5.42±2.50	2.84±1.18	SO *vs*. BDL *

SO: Sham operation; BDL: bile duct ligation; BDL+N: BDL rats treated with nebivolol; MAP: mean arterial pressure; HR: heart rate; Ht: haematocrit; ALT: alanine transaminase; AST: aspartate transaminase; GGT: gamma glutamyltransferase; TB: total bilirubin; NS: not significant. *$p<0.05$; **$p<0.01$.

Table II. *Effect of nebivolol on necroinflammatory and fibrosis scores (mean±SE).*

Scores	SO (n=8)	BDL (n=5)	BDL+N (n=8)	Difference between groups
HAI	0.63±0.18	8.80±1.98	7.13±1.44	SO *vs*. BDL **
				SO *vs*. BDL+N **
Knodell	0±0	3.52±0.35	3.31±0.26	SO *vs*. BDL **
				SO *vs*. BDL+N **
METAVIR	0±0	3.56±0.29	3.33±0.25	SO *vs*. BDL **
				SO *vs*. BDL+N **

SO: Sham operation; BDL: bile duct ligation; BDL+N: BDL rats treated with nebivolol; HAI: histological activity index. **$p<0.01$.

Haematocrit and serum biochemical parameters. Animals from the BDL and BDL+N groups had lower haematocrit values compared with animals from the SO group (Table I). However, this decrease was statistically significant only for the BDL group ($p<0.01$). Albumin levels were decreased in the BDL and BDL+N groups compared with the SO group, being statistically significant only for the BDL group ($p<0.05$). Serum activity of ALT, AST, GGT, and total bilirubin, common biochemical indices of hepatocellular injury, were elevated in BDL animals (Table I). However, these biochemical parameters were lower in BDL animals treated with nebivolol, but no significant difference between the BDL and BDL+N groups was found.

Microscopic findings. Animals from the SO group presented minimal liver histological changes: liver tissue displayed a normal parenchymal morphology, with intact hepatocytes, sinusoids and portal areas (Figure 1 A-C), although some animals exhibited mild portal inflammation (Table II). The liver samples of the BDL and BDL+N groups showed similar histopathological changes (Figure 1 D-I), with no significant differences found in necroinflammatory and fibrosis scores (Table II). With regard to the HAI score, in both groups, liver tissue was frequently characterized by piecemeal necrosis, focal and/or portal inflammation. In addition, fibrosis evaluation by the Knoddel scoring system revealed that cirrhotic nodules and bridging fibrosis were commonly observed in examined liver sections, associated with severe, often diffuse, ductular reaction. The hepatocytes exhibited several morphological changes, such as cellular and nuclear size variation, and ocasionally presented a ductular phenotype. We also detected frequent mitotic figures. Semiquantitative analysis performed by adopting the METAVIR scoring system confirmed the above findings.

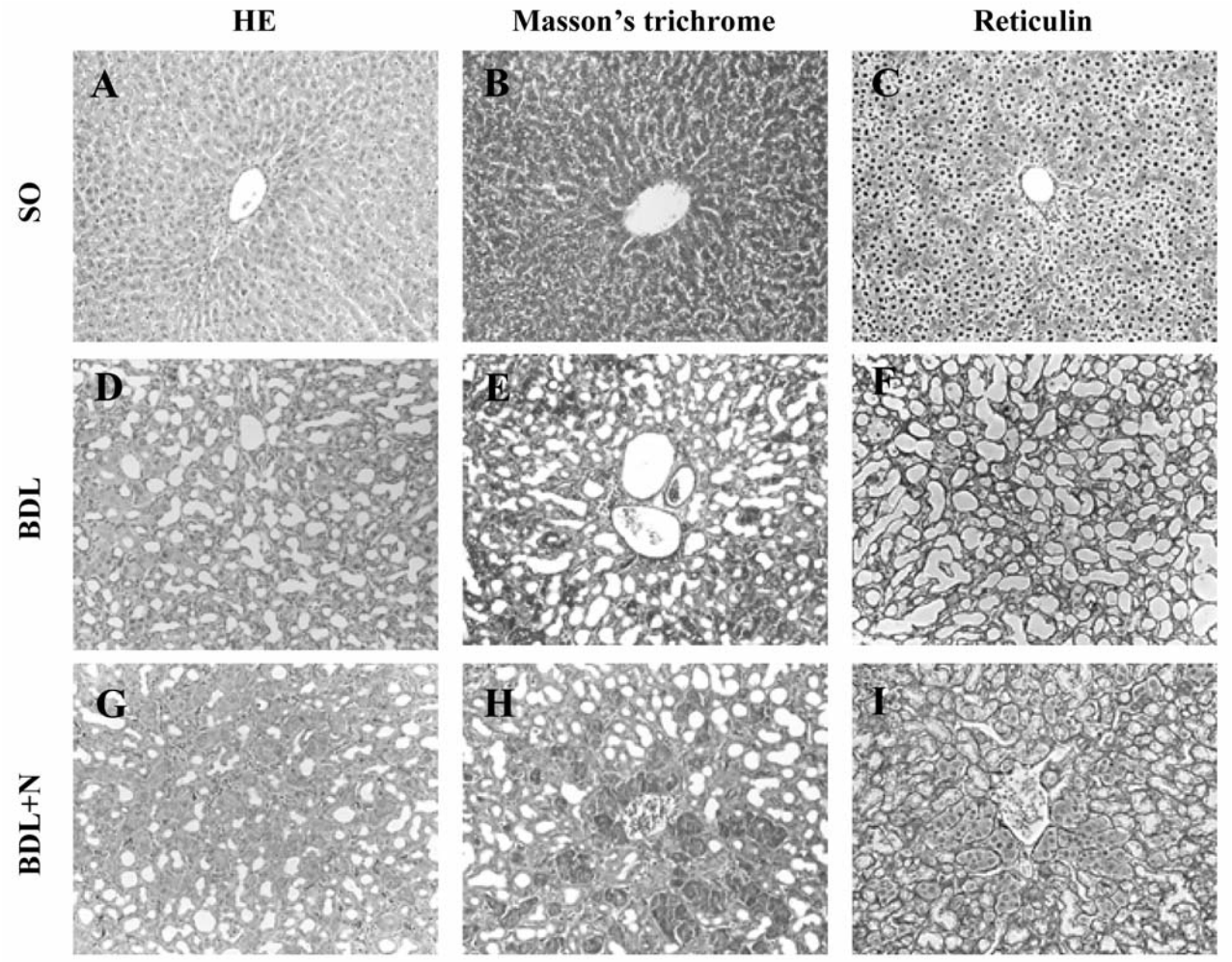

Figure 1. *Liver tissue sections from groups stained with HE, Masson's trichrome and reticulin stain. In the SO group, normal liver morphology was found (A-C); in both BDL (D-F) and BDL+N (G-I) groups, similar histological changes were found, characterized by collagen deposition associated with severe, diffuse, ductular reaction. Original magnification: ×200.*

Discussion

Nebivolol is a β1-selective adrenergic receptor antagonist with vasodilating properties attributable to its ability to increase NO bioavailability (7). To our knowledge, these are the first data on the effects of nebivolol on liver fibrosis obtained from a rat model of liver fibrosis. After five weeks of treatment, we assessed the effects of nebivolol on hepatic function and liver fibrosis. Although increased hepatic NO bioavailability represents an attractive pharmacological approach for the treatment of hepatic fibrosis, nebivolol (8 mg/kg/day) did not seem to have a beneficial effect on liver fibrosis. However, nebivolol did improve the survival rate of BDL rats.

In this study, we used a model known to induce liver fibrosis, a cholestatic BDL model (25). The yellow colouration of the skin and mucosa, dark urine, hippo/acholic faeces, dilatation of the common bile duct above the obstruction point, serum biochemical markers, histological observations and fibrosis score measurements, proved that BDL had been successfully performed.

According to Abraldes *et al*. (25), the mortality of Wistar rat submitted to BDL is 20% at five weeks after the surgery. In the present study, mortality reached 37.5% in the BDL group five weeks after surgery, whereas no deaths were observed in the SO and BDL+N groups, suggesting a beneficial effect of the treatment with nebivolol.

As expected, β1-selective receptor blockade with nebivolol significantly decreased MAP and heart rate in the BDL+N group. In patients with advanced cirrhosis, the use of this drug may be dangerous by enhancing the already existing hypotension, which may lead to water and sodium

retention, and renal failure (26). However, in this study, treatment with nebivolol (8 mg/kg/day) was well-tolerated without any signs of hypotension.

The level of serum albumin is regarded as an important index of liver function (27). Serum albumin decreased significantly in the BDL group when compared with the SO group. This change may reflect a reduction in hepatic synthesis associated with bile duct obstruction. Animals submitted to BDL had marked biochemical cholestasis as assessed by serum bilirubin and GGT activity when compared with SO animals. The liver transaminases are released into the bloodstream when liver cells are damaged (27, 28). In the present study, AST activity was elevated in BDL rats. In general, nebivolol reduced all liver function parameters elevated by BDL including total bilirubin, ALT, AST and GGT, although this decrease was not significant. This was in agreement with the survival rate and the increase in haematocrit with the nebivolol treatment.

Next, we examined the extent of necroinflamation and liver fibrosis, of surviving rats after five weeks of treatment. Deposition of ECM components, including collagen, is a commom phenomenon in BDL (1). Activated HSCs are the predominant source of the ECM (29). Endothelin (ET)-1 is markedly overexpressed within the cirrhotic liver, particularly in sinusoidal endothelial cells and myofibroblasts, and the process of HSC activation seems to be closely related to an increased expression of this peptide (30). NO donors can exert antifibrogenic action as NO has negative regulatory properties specifically on migration of activated HSCs, contraction and proliferation, inducing apoptosis of these cells (17-19). Nebivolol increases the bioavailability of NO and inhibits secretion of ET1, and these effects seem to be related with its potent antioxidant property (12, 13). Antiproliferative activity of nebivolol was demonstrated in smooth muscle cells of human aortic artery (11) and in endothelial cells and smooth muscle cells of human coronary artery (12, 13). Furthermore, nebivolol reduced renal expression of type I collagen in rats with reduced renal mass (14). Despite this evidence, we did not observe significant differences between the BDL and BDL+N groups in fibrosis scores, although the fibrosis scores of the livers from rats treated with nebivolol were slightly reduced in comparison with those of the BDL rats without treatment.

Depending on its concentration, NO can have pro- or antiapoptotic properties (31). High NO concentrations promote apoptosis in most cases, whereas low NO concentrations can result in resistance to apoptosis (18, 32). Therefore, an explanation for our results might be a low NO concentration obtained with the dose of 8 mg/kg of nebivolol that was insufficient to induce apoptosis, and instead induced proliferation of HSCs.

Therefore, under our experimental conditions, nebivolol (8 mg/kg/day) improved the survival rate of BDL animals, but was unable to significantly improve liver function or reduce fibrosis in a rat model of liver fibrosis. To better understand the effects of nebivolol on the BDL model, future studies with other drug concentrations are necessary.

Conflicts of Interest

Nothing to disclose.

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