Liver-Intestine Axis: Effects of Physical Exercise and Melatonin on Intestinal Implications in Secondary Biliary Cirrhosis

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Abstract

Introduction Modulation of the liver-gut axis, including interventions that reduce oxidative stress, is essential in the management of cirrhosis and in the prevention of its complications. Physical exercise and the use of antioxidants such as melatonin (MLT) improve oxidative stress by increasing the production of endogenous antioxidants, resulting in a better ability of the body to neutralize free radicals.

Objective To investigate the effects of EX and the use of MLT, the possible biological markers and mechanisms involved in the disease process.

Materials and Methods Twenty-six male Wistar rats were used, divided into 4 groups: Control (CO), Bile duct ligation (BDL), BDL + EX, and BDL + MLT. Physical exercise (swimming) was conducted daily, starting on the 15th day after BDL surgery, as well as MLT, intraperitoneally at a dose of 20 mg/kg, lasting until the 28th day, the end of the experiment. Blood, liver, and intestine were collected. Statistical analysis was performed using One-Way ANOVA followed by the student-Newman-Keuls test ($p < 0.05$).

Results The treatments were effective in preserving the tissue architecture of the liver and intestine. Hepatic integrity enzymes showed a significant reduction in the EX and MLT groups. The treatments reduced lipid peroxidation (LPO) levels, restored antioxidant activity and total antioxidant capacity, as well as lowered the levels of nitric oxide metabolites. It was found that the treatments activated the Nrf2 pathway and reduced inflammatory mediators.

Conclusion The treatments (EX and MLT) were effective against the damage assessed in this experimental model, suggesting that these interventions may be promising for future clinical use.

Keywords
- Cirrhosis
- intestine
- oxidative stress
- physical exercise
- melatonin

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Introduction

Liver cirrhosis is characterized by chronic, progressive, and continuous alterations to the liver, evidenced by the replacement of liver tissue by fibrous scar tissue which modifies hepatic blood flow and causes hepatocyte dysfunction, triggering clinical metabolic events due to the inability to perform their essential functions.[1]

The liver-gut axis is fundamental to the regulation of digestion and absorption of nutrients, which are transported to the liver via the hepatic portal vein. Through this structural connection, the liver receives nutrients and substances derived from the intestine, such as bacterial products, environmental toxins, and food antigens.[2] Cirrhosis triggers changes in the intestinal microbiota that potentially exacerbate intestinal inflammation and contribute to the progression of liver disease.[3]

The experimental model of secondary biliary cirrhosis, via bile duct ligation consists of total obstruction of bile flow, which leads to oxidative stress, edema, acute inflammatory reactions, and development of periportal fibrosis due to alterations in the synthesis and degradation of proteins, such as collagen, causing hepatic parenchyma disorganization.[4,5]

Inflammation is a process strongly linked to the generation of reactive oxygen species (ROS). In inflammatory bowel diseases, (IBD) a significant influx of inflammatory cells into the affected intestinal mucosa is observed. The production of ROS compromises the integrity of the mucosa and leads to increased intestinal permeability.[6] Physical exercise has been researched for its beneficial effects on gastrointestinal health, lowering the production of ROS, reducing inflammation and fibrosis, as well as contributing to the composition of the intestinal microbiota.[7,8]

Melatonin (MLT), N-acetyl-5-methoxytryptamine, is the main hormone secreted by the pineal gland. It acts as a potent antioxidant, neutralizing ROS and improving the organism's antioxidant defense, reducing the inflammatory process, and potentially improving liver function.[5,9] In experimental models, MLT shows potential in preserving the integrity of the intestinal mucosa, lowering intestinal permeability, and protecting against damage associated with inflammation.[10]

This study aimed to analyze the intestinal changes resulting from secondary biliary cirrhosis and investigate the effects of cirrhosis on the intestinal mucosa, as well as evaluate the beneficial effects of physical exercise and the antioxidant MLT in improving the damage caused by the disease.

Materials and Methods

Ethical Aspects

The research project was approved by the Ethics Committee on the Use of Animals of the Hospital de Clínicas de Porto Alegre (CEUA/HCPA) under No. 2021-0642. All procedures were performed by Federal Law No. 11.794 of October 8, 2008, which regulates the use of animals in scientific research.

Experimental Model

For this experiment, 26 male Wistar rats, weighing ± 250g were split into 4 groups: control (CO), bile duct ligation (BDL), bile duct ligation + physical exercise (BDL + EX), bile duct ligation + melatonin (BDL + MLT). The animals were kept in plastic enclosures measuring 47 × 34 × 18cm, lined with wood shavings, in a 12h light/dark cycle, at a temperature of 18-22°C. Water and food were freely distributed.

Before the BDL surgery, the animals were weighed, anesthetized, with isoflurane diluted in 100% O_2 (O_2 flow = 0.5 L/min) for anesthetic induction (4-5%) and maintenance (2-3%) and positioned for the procedure. Trichotomy and antiseptic cleaning of the abdominal region were performed, followed by mid-ventral laparotomy with subsequent identification and dissection of the bile duct, which was ligated using two knots (made with 3-0 silk thread) and sectioned. Afterward, the abdominal wall and skin were sutured. For animals in the CO group, a simulated surgery was performed.[4,5]

Exercise and Melatonin Administration

Treatments with EX and MLT began on the 15th day after the experiment and were administered until the 28th day. To conduct the physical exercise, the animals were placed to swim in pairs in a 40 × 100 cm tank, with a water temperature of ± 30°C. Animals in the BDL + EX group swam for 10 minutes at a depth of 50 cm. Animals in the CO, BDL, and BDL + MLT groups were placed in a 5 cm deep water tank, for approximately 5 minutes, to simulate similar conditions. After the exercise, the animals were dried individually using towels and a hairdryer.[11,12]

The MLT solution (Sigma Chemical®, St. Louis, M) was prepared before administration, and shielded from light sources, always at 6 p.m. 20 mg/kg of MLT was administered via intraperitoneal injetion (IP) with a vehicle consisting of 500 µL of 0.9% NaCl and 5 µL of 1% EtOH. In the CO, BDL, and BDL + EX groups, 500 µL of saline solution (0.9% NaCl) and 5 µL of ethanol (1% EtOH) were administered IP.[5,8,12]

Analyses

Histological Analysis

For histological evaluation, approximately 2 cm of tissue (liver and intestinal large bowel) were removed from each animal, and placed in 10% formalin for fixation for 24h, followed by histological processing. Afterward, the resulting paraffin blocks were fixed on a microtome and 3 µm slices were obtained. These samples were placed in a histological bath at 50 °C. The slides were stained with hematoxylin-eosin (HE) and periodic acid-Schiff (PAS).

Assessment of Liver Integrity

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined via an ultraviolet kinetic test. Alkaline phosphatase (ALP) was quantified by colorimetric kinetic test, using an automated enzymatic method (Siemens Advia 1800 Chemistry system).

Biochemical Evaluations

A fragment of the large intestine was homogenized in ULTRA-TURRAX and placed in a refrigerated centrifuge. The supernatant was used for biochemical analyses. The protein concentration in the homogenate was determined by the Bradford method[13]

Lipoperoxidation

Lipoperoxidation was measured using the thiobarbituric acid reactive substances (TBARS) assay. The method consists of heating the homogenate with thiobarbituric acid which results in the formation of a colored product. Coloration occurs due to the presence of malondialdehyde, and other substances derived from lipid peroxidation in the biological material. The TBARS measurements obtained were expressed in nmol/mg of protein.[14]

Antioxidant Enzyme Activity

The activity of the enzyme superoxide dismutase (SOD) was measured by evaluating the ability of the enzyme to inhibit the reaction of the superoxide radical with adrenaline. The reading was performed at 480nm. The data were expressed in USOD units/min/mg of protein.[15]

Catalase (CAT) was determined by measuring the decrease in absorption in an action medium containing 50 mmol/L of phosphate-buffered saline (pH 7.2) and 0.3 mol/L of hydrogen peroxide. The enzyme activity was analyzed spectrophotometrically at 240nm and expressed as pmol/mg of protein.[16]

The activity of the antioxidant enzyme glutathione peroxidase (GPx) was evaluated by the rate of NADPH oxidation in the presence of reduced glutathione and glutathione reductase. Sodium azide was added to inhibit catalase activity. The GPx activity was measured in a spectrophotometer at 340nm, and its activity was expressed in mmol/min/mg of protein.[17]

Total Antioxidant Capacity (TAC) Analysis

Total antioxidant capacity (TAC) was measured using the ABTS method. ABTS is oxidized to form a free radical (ABTS•+) in a reaction with hydrogen peroxide or other oxidants. The sample homogenate was added to the diluted radical. TAC was analyzed spectrophotometrically at 734nm and expressed in µmol/g.[18]

Evaluation of Nitric Oxide Metabolites

The production of nitric oxide was measured indirectly through a quantitative colorimetric test using the Griess reaction. This method is based on the enzymatic reduction of nitrates to nitrites when in the presence of nitrate reductase and NADPH, and the subsequent reaction of the nitrites formed (or initially present in the samples) with the Griess reagent (which combines sulfanilamide and naphthylethylenediamine, specific for NO_2). The reading was conducted in a microplate reader at 540nm, and the results were expressed in mmol of NO_2/NO_3.[19]

Protein Expression by Western Blot

The samples were loaded onto a polyacrylamide gel and electrophoresis was performed to separate the proteins based on their molecular weight. By electrotransfer, the proteins from the gel were transferred to a PVDF membrane which was blocked with a solution of TTBS and powdered milk to prevent non-specific binding. The primary antibody specific to the protein of interest (Nrf2 and Keap1) was incubated, followed by the secondary antibody. Revealing was performed using a developer to detect the protein of interest and quantification of the band intensity was performed using ImageJ®. Control proteins (β-actin or GAPDH) were used as normalizers to correct variations in protein loading.

Immunohistochemistry

Tissue samples were fixed in 10% formalin and embedded in paraffin. The samples were deparaffinized with xylene and rehydrated with different degrees of ethanol. The specimens were then embedded and the blocks cooled, shaped, and connected to a microtome (Leitz® 1512) obtaining 4 µm slices for placement on the slides. They were incubated with (NFkβ and TNFα) antibodies (Santa Cruz Biotechnology®). The results were evaluated using a binocular microscope equipped with a digital camera for image to capture, images at 200x (NFkβ) and 100x (TNFα)) magnification. Quantification was determined by averaging image density by the percentage of the area stained positively using image analysis software (ImageJ®).

Statistical Analysis

Data were presented as the mean and standard error of the mean and calculated using GraphPad Software Instat, version 3.0 for Windows®. Analysis of variance (ANOVA – One-Way) was performed followed by the student-Newman-Keuls test, with a significance level of 5% ($p < 0.05$).

Results

Histology with HE

In liver histology, the CO group displayed normal parenchyma with clearly defined hepatocyte cords. In the BDL group, tissue disorganization with loss of hepatocyte cords and inflammatory infiltration was observed. The groups that underwent treatment with physical exercise and melatonin (BDL + EX and BDL + MLT) showed restructuring of these patterns, with the formation of hepatocyte cords originating from a centrilobular vein (►**Fig. 1**).

The histological analysis of the intestinal tissue (large bowel) revealed a well-preserved mucosa, in the CO group, with well-defined and regular Lieberkühn crypts, as well as organized muscle layers. In the BDL group, tissue disruption occurred, and the mucosa displayed atrophy of the columnar epithelium with fewer goblet cells, disorganized crypts, inflammatory infiltration in the lamina propria, and edema in the submucosal layer. The BDL + EX

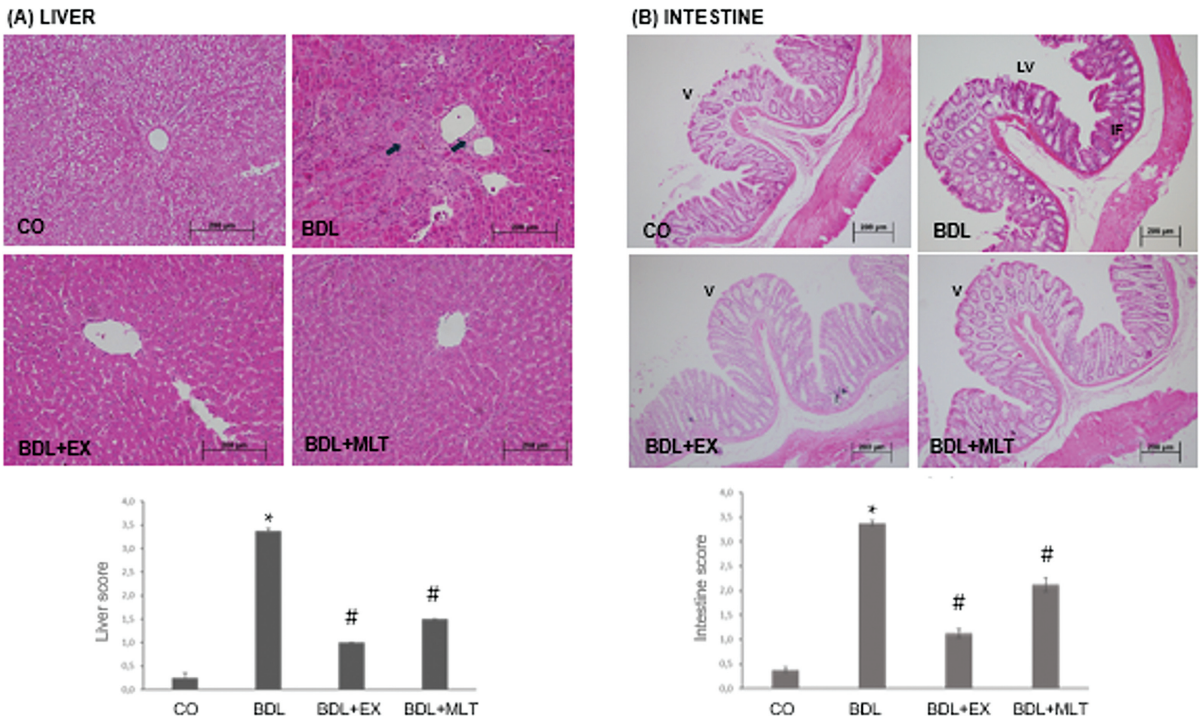

Fig. 1 **Histology (HE)** - (A) liver histology: (200x). Black arrows indicate the presence of inflammatory infiltrate. (B) Intestinal epithelial histology: (100x). Legend: viii (V). viii loss (LV). inflammatory infiltrate (IF). Data are expressed as mean ± standard error. * = $p < 0.001$; # = $p < 0.001$. CO: control; BDL: bile duct ligation; BDL + EX: bile duct ligation + exercise; BDL + MLT: bile duct ligation + melatonin.

and BDL + MLT groups presented a more preserved epithelium, with crypt reorganization, a reduction in inflammatory infiltrate, reduced edema in the submucosal layer, and preserved muscular layer when compared to the BDL group (►**Fig. 1**).

Histology by PAS

The BDL group exhibited a significant stain uptake, indicating an increase in mucus secretion. The presence of inflammatory infiltrate in the mucous layer was also observed. Groups treated with EX and MLT displayed decreased staining in the crypts and there was no inflammatory infiltrate evidence (►**Fig. 2**).

Liver Integrity Enzymes (AST, ALT, and ALP)

In the evaluation of the AST, ALT and ALP enzymes, performed in plasma, a significant increase was observed in the BDL group when compared to the CO group, and a significant

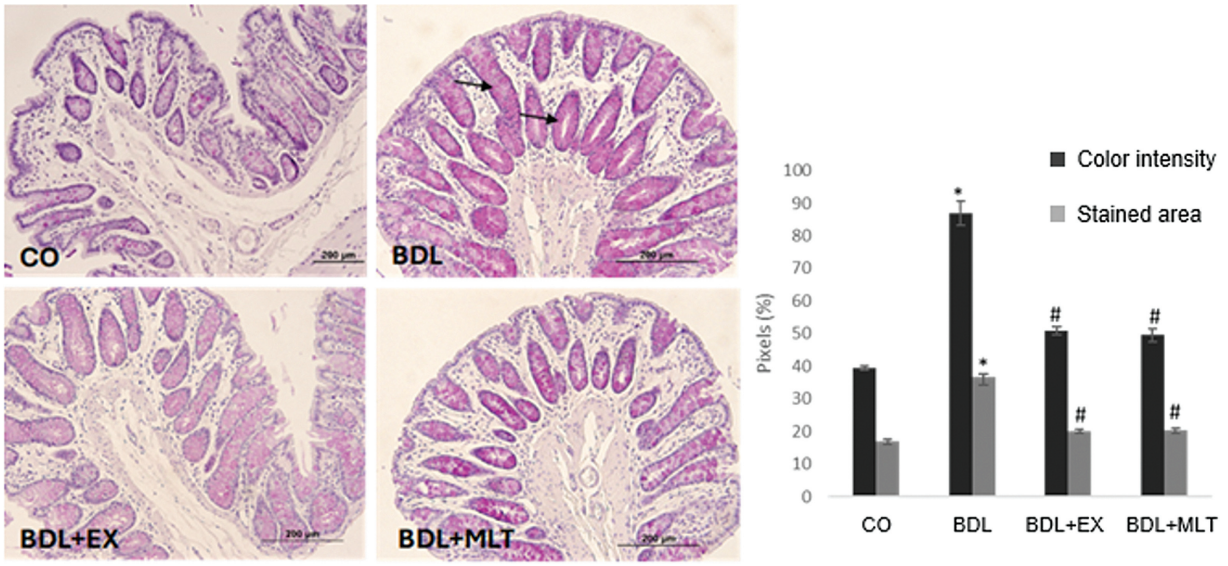

Fig. 2 **PAS histology** - (200x). Quantitative parameter evaluated, staining intensity and percentage of stained area. Black arrows indicate intense staining of mucins. Data are expressed as mean ± standard error. * = $p < 0.001$; # = $p < 0.001$. CO: control; BDL: bile duct ligation; BDL + EX: bile duct ligation + exercise BDL + MLT: bile duct ligation + melatonin.

	CO	BDL	BDL+EX	BDL+MLT
AST (U/L)	64.5±3.88	197.75±64.99*	75.5±4.03#	85.83±3.26##
ALT (U/L)	36.67±3.25	56±7.91*	35.5±2.78#	32.33±1.93##
ALP (U/L)	114.33±6.33	344±22.27*	131±15.53##	116.8±8.45#
TBARS (nMol/mgProt)	0.31±0.02	0.53±0.03*	0.34±0.03#	0.21±0.01##
SOD (USOD/mg prot)	2.39±1.43	20.7±3.03*	3.17±0.80#	4.88±0.93#
CAT (pmoles/min/mg Prot)	2.80±0.15	6.90±0.41*	4.23±0.17#	4.89±0.19#
GPX (nmoles/min/ mg Prot)	2.56±0.17	1.59±0.050*	2.46±0.31##	2.57±0.44#
TAC (μmol/g)	381.38±21.22	194.02±25.89*	375.15±20.47##	279.16±12.01#
NO_2/NO_3 (nMol/L)	0.09±0.01	0.55±0.13*	0.11±0.02#	0.20±0.08#

Fig. 3 **Biochemical analysis performed in plasma (AST, ALT and ALP) and intestine (TBARS, SOD, CAT, GPx, TAC and *NO 2* / *NO 3*).** Data are expressed as Mean Standard Error. Significant increase in the BDL group compared to controls ($p < 0.001$). # Significant decrease in the BDL + EX and BDL + MLT groups compared to the BDL group; #($p < 0.01$), ##($p < 0.001$). CO (Control); BDL (Bile duct ligation); BDL + EX (Bile duct ligation + exercise); BDL + MLT (Bile duct ligation + melatonin).

decrease in these values was observed after administering EX and MLT treatments when compared to the BDL group (►**Fig. 3**).

Assessment of Lipoperoxidation (LPO)

The LPO analysis indicated that TBARS levels were significantly increased in the BDL group when compared to the CO group. Groups that received treatment with EX and MLT presented lower levels when compared to the BDL group (►**Fig. 3**).

Activity of Antioxidant Enzymes (SOD, CAT and GPx)

The enzymatic activity of SOD and CAT in the BDL group increased significantly when compared to the CO group. Groups undergoing treatment (BDL + EX and BDL +MLT) displayed a significant reduction in enzymatic activity when compared to the BDL group. In the GPx enzyme, a significant decrease was observed in the BDL group when compared to the CO group, and when treated with EX and MLT, a significant increase in the activity of this enzyme was shown when compared to the BDL group (►**Fig. 3**).

Total Antioxidant Capacity (TAC)

A significant reduction in TAC was observed in the BDL group when compared to the CO group. Groups that underwent treatment with EX and MLT showed a significant increase compared to the BDL group (►**Fig. 3**).

Nitric Oxide Metabolites

The levels of nitric oxide metabolites (NO_2/NO_3) were significantly increased in the BDL group when compared to the CO group. After treatment with EX and MLT, these levels significantly lowered when compared to the BDL group (►**Fig. 3**).

Expression of Nrf2 and Keap1

The nuclear expression of Nrf2 was significantly lower in the BDL group compared to the CO group and significantly higher in the BDL + EX and BDL + MLT groups when compared to the BDL group. The cytoplasmic expression of Keap1 was significantly higher in the BDL compared to the CO group, and significantly lower in the EX and MLT-treated groups when compared to the BDL group (►**Fig. 4**).

Expression of NFkβ and TNFα

The animals in the BDL group had a significant increase in the expression of NFkβ and TNFα, observed by the pronounced marking in the group when compared to the CO group. Groups that received the treatments (BDL + EX, BDL + MLT) displayed significantly lower expression of these proteins compared to the BDL group (►**Fig. 5**).

Discussion

Oxidative stress is a key factor in the pathogenesis of cirrhosis, contributing to hepatocellular damage and the progression of fibrosis. In the intestine, it is relevant in several health conditions, including inflammatory bowel diseases. The incorporation of physical exercise in experimental models becomes increasingly important and necessary to understand how exercise can benefit intestinal health.

Changes in the liver parenchyma, with the formation of fibrotic septa and necrosis, are frequently associated with the cirrhotic process. Ferrari et al[20] demonstrated that rats with cirrhosis presented fibrotic nodules, inflammatory infiltrate, and cellular alterations. In studies with cirrhotic rats, physical exercise and antioxidants showed the potential to reduce inflammation and improve liver tissue regeneration.[5,8]

Liver dysfunction leads to a series of changes in the intestinal environment, including dysbiosis and reduced mucus production, which may be related to changes in the intestinal barrier by increasing permeability and causing alterations in the architecture of the intestinal tissue, shortening of the crypts, inflammatory infiltrate, edema in the submucosa and decreased muscular layer.[21,22] These changes were observed in this study through intestinal histology of the large bowel by HE and PAS staining in the

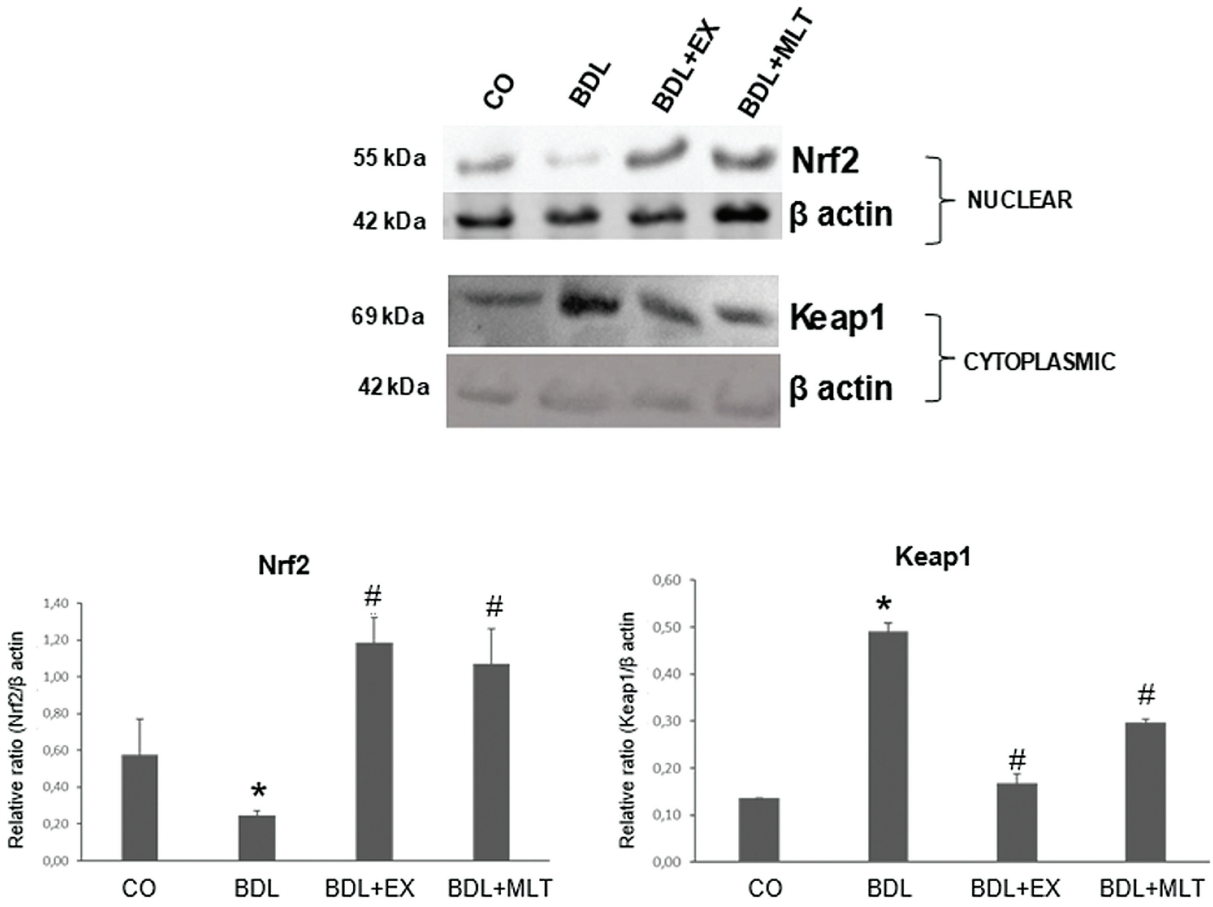

Fig. 4 **The expression of Nrf2/Keap1 in the intestine** - Data are expressed as mean ± standard error. * = $p < 0.001$; # = $p < 0.001$. CO: control; BDL: bile duct ligation; BDL + EX: bile duct ligation + exercise; BDL + MLT: bile duct ligation + melatonin.

BDL group. Treatments with EX and MLT showed significant reductions in histological damage and increased mucin production. Positive staining for PAS in goblet cells reflects the presence of carbohydrate-rich glycoproteins, which support the protective function of the intestinal barrier.

The BDL model induced significant changes in AST, ALT and ALP enzymes. The results presented in this study indicate that administering EX and MLT led to significant improvements in those enzymes. The results corroborate the findings of Rosa et al[12] who determined a reduction in these enzymes following physical exercise through swimming. The study by Colares et al[5] presented similar findings with the use of MLT.

When evaluating intestinal lipid peroxidation with the TBARS method, a significant increase was observed in the BDL group when compared to the CO group. The BDL + EX and BDL + MLT groups displayed a significant decrease compared to the BDL group, thus suggesting a beneficial effect of moderate physical exercise, and a protective role of MLT, in reducing lipid oxidation and decreasing the formation of toxic products. A study performed with rats with nonalcoholic fatty liver disease (NAFLD) that underwent treatment with physical exercise and an antioxidant (resveratrol) demonstrated a reduction in LPO levels, which corroborates the findings in this study.[23]

Systemic inflammation and the release of inflammatory mediators generated by cirrhosis increase oxidative stress, compromising the intestinal barrier and, therefore, intestinal function.[24] With the results obtained, it is possible to infer that some of the beneficial effects of exercise are due to the reduction of oxidative stress. This study supports current literature, in which physical exercise and melatonin were able to induce greater antioxidant defense.[5,25,26] In addition to lowering ROS, treatments with EX and MLT significantly reduced NO_2/NO_3 levels in the intestine. In experimental cirrhosis models, there is significant data about the beneficial effects of physical exercise and MLT in reducing NO_2/NO_3 levels in the muscle[12] and liver of cirrhotic rats.[8]

Intestinal damage resulting from cirrhosis triggers the initiation of several metabolic pathways, such as the Nrf2/Keap1, which has a protective effect against oxidative damage. Under stress conditions, Nrf2 is separated from Keap1, and it is then translocated to the nucleus, thus activating the expression of genes encoding several antioxidant enzymes.[27] This study evaluated Nrf2 expression, which was significantly reduced in the BDL group when compared to the CO group, as well as the increased cytoplasmic expression of Keap1 in the BDL group. In the treatment groups (BDL + EX and BDL + MLT) oxidative stress was lowered in the intestine through modulation of the Nrf2/Keap1 pathway. Similar findings

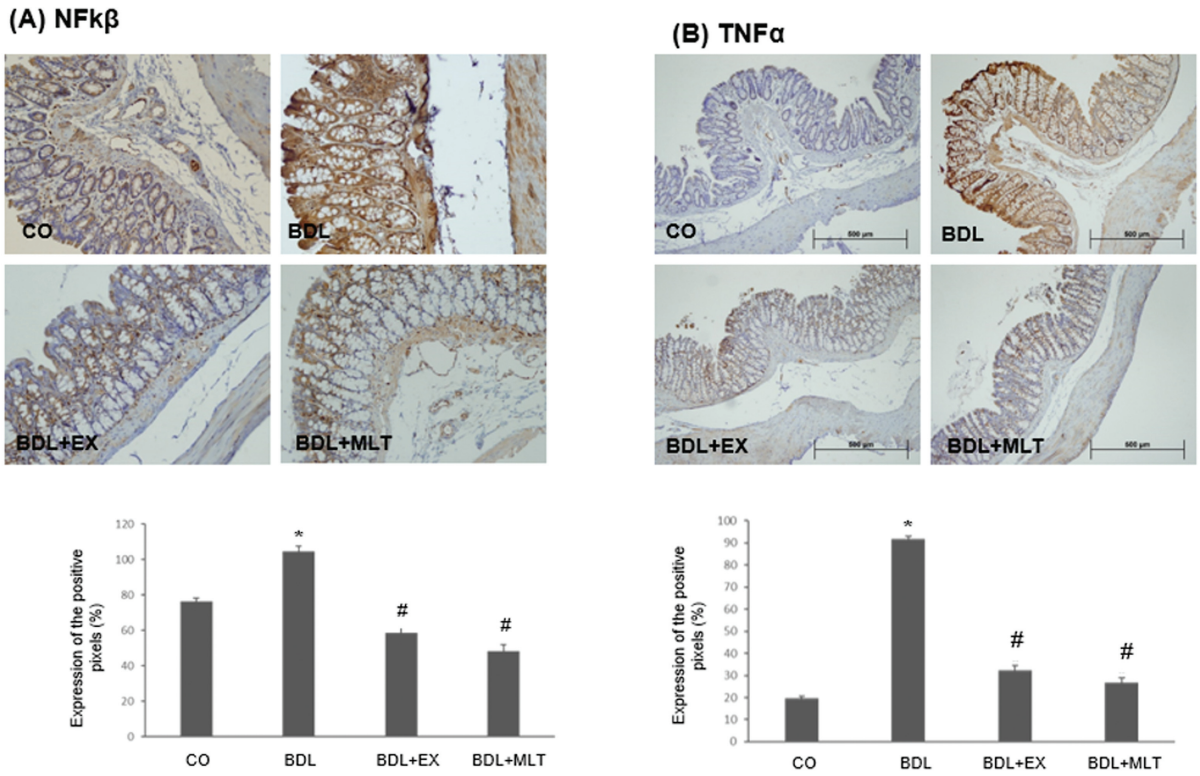

Fig. 5 Immunohistochemistry - **(A)** The expression of NFkβ in intestine (200X); **(B)** The expression of TNFα in intestine (100x). Data are expressed as mean ± standard error. * = $p < 0.001$; # = $p < 0.001$. CO: control; BDL: bile duct ligation; BDL + EX: bile duct ligation + exercise; BDL + MLT: bile duct ligation + melatonin.

were found in a study with rats where the authors demonstrated the protective effect of glutamine in the liver and intestine damaged by ischemia-reperfusion.[28]

The increased expression of NFkβ and TNFα in the BDL group was likely triggered by factors such as oxidative stress, inflammatory cytokines, and bacterial products crossing the compromised intestinal barrier. The EX and MLT treatments reduced the expression of NFkβ and TNFα, suppressing inflammatory processes. Physical exercise is able to support the immune system and help promote an anti-inflammatory state to improve health, especially regarding chronic diseases.[29] Studies have demonstrated that exercise interventions can significantly reduce IL-6 and TNFα levels in obese individuals.[30] This study's treatments with EX and MLT improved the liver-gut axis homeostasis by modulating the NFkβ and TNF-α pathway while increasing antioxidant defenses with Nrf2/Keap1 pathway, activation, thus alleviating the negative effects of systemic inflammation induced by cirrhosis.

Conclusion

The liver-gut axis is important in the pathogenesis and progression of liver diseases such as cirrhosis. Liver dysfunction caused by cirrhosis leads to a series of alterations in the gastrointestinal tract, activating pro-inflammatory pathways that aggravate the systemic inflammatory condition.

In this study, treatments with EX and MLT demonstrated promising results in cirrhotic rats, such as improvement in intestinal histology, reduction of oxidative and nitrosative stress, activation of antioxidant pathways and modulation of the inflammatory process, which led to reduction of chronic intestinal inflammation and improvement of homeostasis of the liver-intestinal axis.

These findings highlight the therapeutic potential of moderate physical exercise and the beneficial effects of melatonin on intestinal complications associated with cirrhosis, suggesting that these interventions may be promising for future clinical use.

Authors' Contribution

Martins GS, Schemitt EG, Brasil M, Fonseca SRB, Marroni NP participated in the production and development of the experimental model; Martins GS, Schemitti EG, Brasil MS, Fonseca SRB, Engeroff MO participated in the application of analysis techniques; Martins GS, Schemitt EG, Fillmann HS, Pavorato MA, Marroni NP participated in data analysis and manuscript review.

Conflict of Interest

None declared.

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