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Original Article

Protective effect of probiotics and ascorbic acid on bile duct ligation-induced chronic hepatic encephalopathy in rats

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Abstract

Background and purpose: Hepatic encephalopathy (HE) is a brain dysfunction caused by acute and chronic hepatic failure. The pathogenesis of HE is unknown, although small intestinal bacterial overgrowth associated with chronic liver damage, hyperammonemia, and oxidative stress are considered major factors for HE. Effective lowering of circulating ammonia and neuroinflammation is the main strategy for preventing and treating HE in cirrhosis. In the present study, the protective effect of probiotics (*Lactobacillus plantarum* and *Bacillus clausii*) and ascorbic acid in combination was assessed in bile duct ligation (BDL)-induced chronic HE in rats.

Experimental approach: Sprague Dawley rats were divided into five groups (n = 6). All groups were subjected to double ligation of the bile duct and fed a hyperammonemia diet, except group I (normal control). Groups III and IV were treated with a low and high dose of combination therapy, respectively, while group V was given lactulose. Four weeks post ligation, behavioral, biochemical, and neurochemical parameters were measured. The liver and brain were dissected for histopathology and protein analyses.

Findings / Results: Combination therapy reduced plasma AST, ALT, ALP, and ammonia levels and attenuated hepatic inflammation/fibrosis in cirrhotic rats. Furthermore, combination therapy significantly improved behavioral parameters and restored the antioxidant enzyme activity. Histological changes were observed in the brain and liver of BDL animals.

Conclusion and implications: The additive impact of probiotics and ascorbic acid on BDL-induced chronic HE in rats was mediated by a reduction in ammonia and oxidative stress, implying the therapeutic potential of combination therapy in HE.

Keywords: *Bacillus clausii*; Bile duct ligation; Hepatic Encephalopathy; *Lactobacillus plantarum*; Probiotics.

INTRODUCTION

Hepatic encephalopathy (HE) is a brain dysfunction caused by acute and chronic hepatic failure, and it produces a wide range of neurological or mental conditions (1). Despite over a century of effort, the pathophysiology of HE remains unknown. The most common hypotheses include the role of neurotoxins (i.e. ammonia), disordered neurotransmission as a result of metabolic changes in the liver, changes in brain energetic disturbances, oxidative stress, neuroinflammation, and alterations of the blood-brain barrier (1). Ammonia is a well-studied neurotoxin associated with HE.

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The gastrointestinal tract is a primary source of ammonia. Much of the ammonia in the portal vein is cleared by the liver, which converts ammonia to glutamine and thus prevents its entry into the systemic circulation. However, conditions such as damaged liver, small intestinal bacterial overgrowth, and dysbiosis of the gut microbiota raise blood ammonia levels (2). Excess ammonia reaches the brain, causing oxidative stress in the brain and astrocytic neuroinflammation through the nuclear factor kappa-B pathway.

Ascorbic acid is an essential vitamin and a powerful antioxidant. Ascorbic acid is linked to tissue repair that improves immunocompetence by modulating functioning enzymes. Clinical and fundamental research has demonstrated that ascorbic acid can protect against oxidative stress and liver/brain disease in a rat model of acute HE elicited by thioacetamide (TAA). Furthermore, the pharmacological actions of ascorbic acid are mostly implicated in pathways such as the inflammation-associated tumor necrosis factor (TNF) signaling pathway and the nuclear factor kappa-B signaling system, which contribute to the suppression of liver disease progression. Ascorbic acid is also reported to show synergistic action with other agents such as vitamin E (3).

A growing body of research suggests the role of an altered gut microbiota profile in complications, such as HE. Modification of the intestinal microbiota is an essential component of current HE therapy. Alteration in the intestinal microbiota changes the colonic lumen pH, which contributes to increased production and absorption of ammonia from the gut microbiota. Treatment of HE including nutritional intervention and pharmacological therapy mainly aims at reducing ammonia production and favors its excretion. For example, antibiotics such as rifaximin target deaminating bacteria that produce nitrogenous compounds, while the osmotic effect of lactulose facilitates the removal of harmful metabolites such as ammonia (4, 5). Probiotics are living microorganisms, when administered in sufficient quantity, may provide several health benefits to the host, and are usually regarded as safe (5). Among others, one justification for using probiotics for HE is to lower the predominance of toxic ammonia-producing bacteria in the gastrointestinal tract (4, 6). Probiotics are considered to lower blood ammonia levels through a variety of processes, including reduced bacterial urease activity, lowered ammonia absorption *via* decreasing pH, decreased intestinal permeability, and improved nutritional condition of the gut epithelium (4, 6).

Bacillus clausii (UBBC07) is a gram-positive, rod-shaped, non-pathogenic, spore-forming bacteria that are tolerant to acids, bile salts, and bile salt hydrolase activity. *B. clausii* can colonize in the intestine even in the presence of antibiotics. *B. clausii* are intended to change the gut microbiota and encourage the development of non-urease generating organisms. Furthermore, the presence of *B. clausii* in the stomach may lower ammonia, as these bacteria may use ammonia. *Lactobacillus plantarum* (UBLP40) is derived from milk products and fermented meals. It is beneficial in managing gut acidity. *L. plantarum*-induced acidic medium traps ammonium ions and thus reduces hyperammonemia. In addition, *L. plantarum* is bacteriostatic against the urease-producing gut microbiota. These qualities indicate that *B. clausii* UBBC07 and *L. plantarum* UBLP40 may be useful in reducing ammonia levels in patients with HE (7).

In our previous study, we reported the effect of both probiotics (*B. clausii* UBBC07 and *L. plantarum* UBLP40) in acute HE induced by TAA (7). Furthermore, the individual effect of ascorbic acid against TAA-induced acute HE has also been reported (8). Ascorbic acid and probiotics act through different pharmacological mechanisms. While ascorbic acid has antioxidant and anti-inflammatory properties, probiotics act by the removal of ammonia. Hence, it would be beneficial to understand the synergistic effect of probiotics and ascorbic acid in HE treatment. The present study was conducted to evaluate the effect of ascorbic acid and probiotic combination on bile duct ligation (BDL)-induced chronic HE in rats.

MATERIALS AND METHODS

Probiotic

The strain of *B. clausii* UBBC07 and *L. plantarum* UBLP40 were procured from Unique Biotech Ltd, India. The strains were inoculated in sterilized MRS broth solution and incubated in the bacteriological incubator at 37 °C. Bacteria were pelleted at 3000 rpm in a centrifuge, then resuspended in 500 mL phosphate buffer saline (PBS, pH 7.4) and administered to rats every day *via* oral gavage.

Selection of animals

Sprague Dawley rats weighing 180-200 g, male or female, were obtained from the Department of Pharmacology and Toxicology, Bombay Veterinary College, India, and divided into 5 groups (n = 6) for inducing the type C (chronic) HE model. The rats used in this experiment were randomly chosen, and tail markings were done to identify them. The animal cages were kept in a room with constant humidity and temperature of 30-70% and 23 °C, respectively. In the room where the animals were housed, 12/12-h light/dark cycles were maintained. The experimental procedures involving animals were approved by the Institutional Animal Ethics Committee under Ethic No. SSR/IAEC/2019/05.

Experimental design and BDL

Rats were divided into five groups after a seven-day acclimatization period as shown in Fig. 1:

Group I (negative normal control): water

Group II (induced control): BDL + hyperammonemia diet for 1 week

Group III low dose of probiotics (*L. plantarum* UBLP40, 10^5 CFU/day, p.o for 2 weeks + *B. clausii* UBBC07, 10^5 CFU/day, p.o for 2 weeks) and ascorbic acid (250 mg/kg, p.o for 2 weeks) combination after BDL and hyperammonemia diet (1 week)

Group IV high dose of probiotics (*L. plantarum* UBLP40, 10^7 CFU/day, p.o for 2 weeks + *B. clausii* UBBC07, 10^7 CFU/day, p.o for 2 weeks) and ascorbic acid (250 mg/kg, p.o for 2 weeks) combination after BDL and hyperammonemia diet (1 week)

Group V was given standard treatment lactulose (2.5 mL/kg in 3 divided doses, p.o for 2 weeks) after BDL and a hyperammonemia diet (1 week).

The dose of probiotics was selected based on our previous work (7) on the effect of probiotics in TAA-induced acute HE, while the dose of ascorbic acid was decided on the basis of inhibitory interaction between probiotics (*L. plantarum* and *B. clausii*) and ascorbic acid *via* minimum inhibitory concentration (MIC) method (data not shown). In the MIC test of probiotics with different concentrations of ascorbic acid, inhibition was observed in wells containing 300 mg/mL and higher concentrations. Therefore, the ascorbic acid dose was finalized at 250 mg for combining with probiotics.

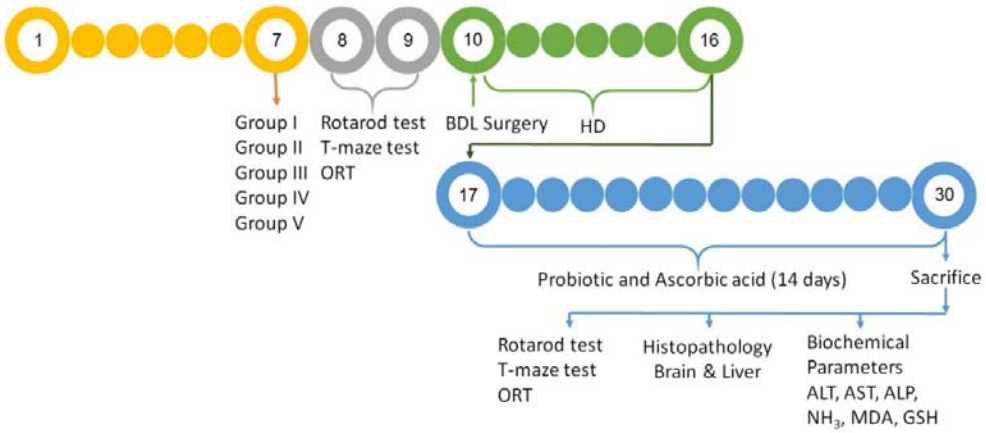

Fig. 1. Experimental design in chronic hepatic encephalopathy model. ALP, Alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BDL, bile duct ligation; HD, hyperammonemic diet; GSH, reduced glutathione; MDA, malondialdehyde; ORT, object recognition test.

The BDL procedure was performed on all the rats except the negative control group. A horizontal laparotomy was performed and the abdomen was closed in the negative control group. The rats in the BDL group, as well as all other treatment groups (low dose and high dose), had their chests shaved, a horizontal laparotomy was performed, and the common bile duct was ligated in two spots. Then, after tying the knot, a cut in the middle of the knot was made. Sutures were used to seal the abdominal incision. To avoid infection, a proper bandage and antiseptics were used. Surgeries were performed under a mix of ketamine (90 mg/kg) and midazolam (5 mg/kg) anesthesia. The rats were returned to their home cages and fed a hyperammonemia diet (ammonium acetate 20% w/w) while being kept at a constant temperature and humidity until they were sacrificed. Rats were assessed for behavioral functions at the end of treatment (after 2 h of treatment). Rats were sacrificed the next day, and blood samples were taken to assess biochemical parameters. Liver and brain samples were collected to determine neurochemical parameters and histological examinations.

Behavioral tests

Rotarod test

The Rotarod test was used to record motor coordination using an accelerating rotarod device (9). The basal falling latency of rats was measured after they were trained. Before beginning the treatment, three training sessions at a steady speed of 4 rpm were done for three days in a row until the performance was stable. The rats were placed on a revolving rod again on the fourth day, and the rod speed was slowly increased from 4 to 40 rpm in 300 s, while basal falling latency was observed. Rats were placed on rotating rods again after the treatment was completed to capture the final falling latency. The rat's motor coordination had a time limit of 300 s.

T-maze test

The cognitive ability of rats was investigated using a T-shaped maze (10). The rats were first exposed to habituation periods. This was critical in instilling learning in animals. Later, they were appropriately rewarded and instructed based on how they acted and the path they choose during the habituation process. The basic functioning principle is spontaneous alternation. The rats were initially placed at the T-maze, then the barrier was raised after 10 s, and the animals were allowed to travel through the maze for 30 s. They were given the option to choose. The arm was blocked for 30 s when the rat entered one of the arms to inspect the site. Later, the rats were returned to the starting point. If the rats did not choose one of the arms at the end of the 30 s, the session was resumed. The correct response is "an alternating choice between the two arms". The response time (in seconds) was also recorded. The lag time in entering the goal arm was also noted.

Object recognition test

Object recognition test (ORT), a behavioral assay, was used to assess cognitive functions like memory and learning in rats (11). Two trials, T1 and T2, were carried out for a duration of 2 min each. In trial T1, two identical objects were used, while in trial T2, one of the identical objects was replaced with a novel object. The objects were placed at the opposite corners and the rat was placed in the middle. The rat was permitted to investigate the objects after being placed in the ORT apparatus. Trial T2 was conducted for 1 h following trial T1. Exploration was defined as follows: directing the nose toward the object at a distance of not more than 2 cm and/or touching the object with the nose. The time spent exploring the novel object against the familiar object was compared, and the difference in time spent was used to interpret the memory deficit.

Biochemical parameters

Evaluation of the serum level of alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase

The blood sample was centrifuged at 3000 rpm at 4 °C for 15 min. Serum was isolated and used for further testing. A commercially available kit (Span Diagnostics Limited, India) was used to determine the serum levels of alanine aminotransferase (ALT; DNPH Assay

kit), aspartate aminotransferase (AST; DNPH Assay kit), and alkaline phosphatase (ALP; pNPP-A.M.P. kinetic assay technique kit) according to the manufacturer's instructions.

Serum ammonia

This approach consists of two steps: blood treatment and color development. Blood samples were processed in the following way: an aliquot of ice-cold 10% trichloroacetic acid was placed in a centrifuge tube and an equivalent volume of freshly collected blood was added to it. Centrifugation was used to separate the precipitated protein from the treated blood. An aliquot of supernatant fluid was transferred to a test tube and neutralized with a nearly equivalent volume of 0.1 N NaOH. The solution was then brought up to a volume of 1.0 mL with water, and the amount of ammonia was determined. To determine ammonia, color was developed by mixing 1.0 mL of sample, 2 mL of alkaline solution (1 L of 0.1 M Na_2HPO_4 + 80 mL of 1 N NaOH), 0.5 mL of 3% phenol, 0.25 mL of 0.05% sodium nitroprusside in a stoppered test tube followed by the addition of 0.25 mL of 2% antiform in solution. The glass stopper on the tube was closed, and the tube was placed in a water bath at 80 °C for 10 min before cooling in tap water for 5 min. The optical density of the resulting color was measured at 610 nm. A reagent blank with water was used instead of a sample. The difference in optical density between experimental and blank samples was determined, and the ammonia content in the model was calculated using a standard curve generated from working standard ammonium sulfate.

Neurochemical parameters

Brain tissue homogenization

The brain tissue was homogenized in 20% PBS, centrifuged for 15 min at 3000 rpm at 4 °C, and the supernatant was used for direct measurement of oxidative stress parameters.

Lipid peroxidation

The thiobarbituric acid reactive substance method was used to determine lipid peroxides in rat brains (12). In a 10 mL centrifuge tube, an aliquot of 0.5 mL brain tissue homogenate was added to 1 mL 10% ice-cold trichloroacetic acid solution, and the mixture was centrifuged at 4000 rpm for 10 min (Eppendorf Centrifuge 5810, Norway). Thiobarbituric acid reactive substance (0.1 mL) was added to 0.2 mL supernatant. The mixture was heated in a water bath (100 °C) for 15 min and subsequently cooled to room temperature before being measured for absorbance at 532 nm against a blank. The equation employed was $A = \varepsilon bc$, where A is absorbance, ε is an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}, b is path length, and c is molar concentration.

Reduced glutathione

Ellman's reagent (5,5-dithio-bis-(2-nitrobenzoic acid) was used to determine the amount of reduced glutathione in brain tissues (13). The mixture was centrifuged for 20 minutes to separate the supernatant [equal amounts of tissue homogenate and T.C.A. (10%)]. 3ml of phosphate buffer (0.2M, pH 8) and 0.5ml of DTNB reagent (0.6mM in 0.2M phosphate buffer) were mixed into 1ml of the supernatant, and the absorbance was measured at 412 nm. The $A = \varepsilon bc$ formula and the extinction coefficient of 1.36×10^3 M^{-1} cm^{-1} were used for the rest of the calculations.

Histopathological study of brain and liver

The rats were euthanized after collecting the blood samples. The liver and brain of rats from various groups were separated and fixed in 10% neutral buffered formalin. These samples were sent to Sakshi Histopathology Works for histopathology slides and paraffin wax preparation. The prepared slides were examined under a microscope and photographs were taken. The damage to liver cells and injury/inflammation in brain cells was investigated. The histology activity index (HAI) system was used to classify histologic findings in the liver. The HAI was created by assigning weighted numeric values to liver lesions, which resulted in a score (14).

Statistical analysis

Graph Pad Prism version 8 was used for statistical analysis. The data were expressed as a mean ± SEM. ANOVA followed by the Tukey post hoc test was used to examine all data. *P* values < 0.05 were considered significant.

RESULTS

Rotarod test

In the first week of testing (day 8 and day 9, prior to surgery), the mean latency to fall time for rats was normalized. No significant differences were observed in basal falling latency between different groups. Also, lactulose (group V) and high dose probiotics and ascorbic acid combination treatment (group IV) groups did not show significant differences in falling latency compared to the negative control group (group I). (Fig. 2). The upward trend in latency time indicates a learning effect. As demonstrated in Fig. 2B, BDL was able to produce a significant motor imbalance (significant decrease in rotarod retention time) in comparison with the normal control rats. The results also indicated that both doses of probiotic and ascorbic acid combination significantly improved motor balance as shown by increasing the latency time in BDL rats in a dose-dependent manner with respect to BDL-induced rats (group II) (Fig. 2).

T-maze test

In the T-maze test, a significantly lower percentage of spontaneous alternations and increased response time in BDL-induced rats (group II) compared to normal control rats (Fig. 3), indicating an impairment in working memory. The treatment groups (groups III-V) showed a significant decrease in response time compared to BDL-induced rats (group II) (Fig. 3A). The results also indicated that probiotic and ascorbic acid combination treatment significantly increased the percentage of correct spontaneous alternations compared to BDL-induced rats (group II) (Fig. 3B), indicating an improvement in working memory.

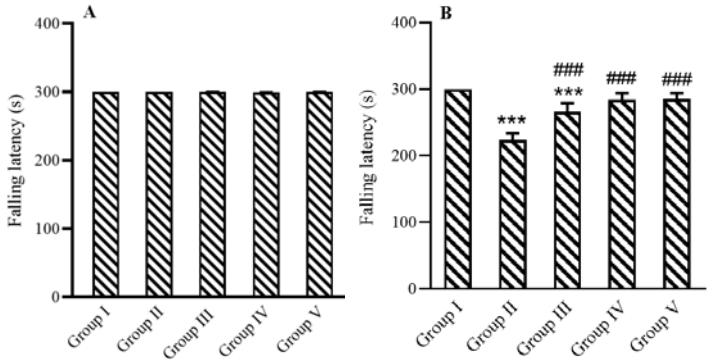

Fig. 2. The evaluation of the effects of probiotic and ascorbic acid combination and lactulose treatment on (A) basal and (B) final falling latency in BDL-induced rats by rotarod test. The values are expressed as mean ± SD, n = 6. ***$P < 0.001$ Indicates significant differences compared to negative control (group I); ###$P < 0.001$ relative to the BDL-induced control group. BDL, Bile duct ligation.

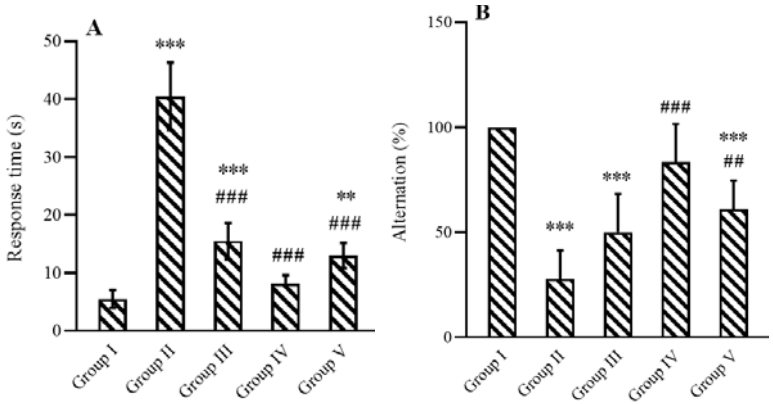

Fig. 3. The T-maze test. The effect of probiotic and ascorbic acid combination and lactulose treatment on the (A) response time and (B) the percentage of correct alteration in BDL-induced rats. The values are expressed as mean ± SD, n = 6. **$P < 0.01$ and ***$P < 0.001$ indicate significant differences compared to negative control (group I); ##$P < 0.01$ and ###$P < 0.001$ relative to the BDL-induced control group. BDL, Bile duct ligation.

ORT

In the ORT, the discrimination index was low for BDL-induced (group II) rats compared to the negative control group, which indicated BDL-induced (group II) rats were not able to distinguish new and familiar objects (Fig. 4A). However, as shown in Fig. 4B, after high dose probiotic and ascorbic acid combination treatment (group IV), rats distinguished between new and familiar objects (significantly reduced discrimination index compared to BDL-induced control rats).

Biochemical assessment

Serum AST, ALT, and ALP

The levels of AST, ALT, and ALP are shown in Table 1. The serum levels of AST, ALT, and ALP significantly increased in the BDL-induced (group II) rats compared to the normal controls. In contrast, the BDL-induced group treated with lactulose, probiotics, and the ascorbic acid combination had significantly lower levels of AST, ALT, and ALP as compared to the BDL-induced control group. Furthermore, when compared between the treatment groups, a high dose of probiotic and ascorbic acid combination (2 weeks) produced the highest improvement in liver function parameters (AST, ALT, and ALP).

Serum ammonia

The serum ammonia level was significantly increased in BDL-induced control rats (group II) compared to group I rats (Fig. 5). Oral treatment of lactulose, probiotics, and ascorbic acid combination in BDL-induced rats diminished serum ammonia levels significantly in a dose-dependent manner when compared to the BDL-induced control rats.

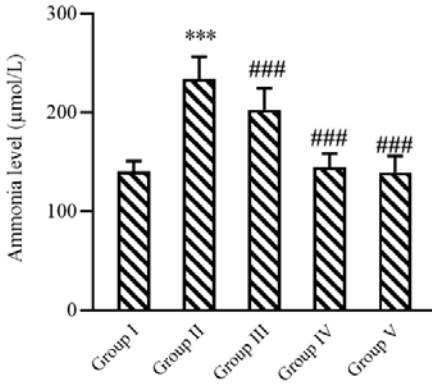

Fig. 5. The effect of probiotic and ascorbic acid combination and lactulose treatment on serum level of ammonia in BDL-induced rats. The values are expressed as mean ± SD, n = 6. ***P < 0.001 Indicates significant differences compared to negative control (group I); ###P < 0.001 relative to the BDL-induced control group. BDL, Bile duct ligation.

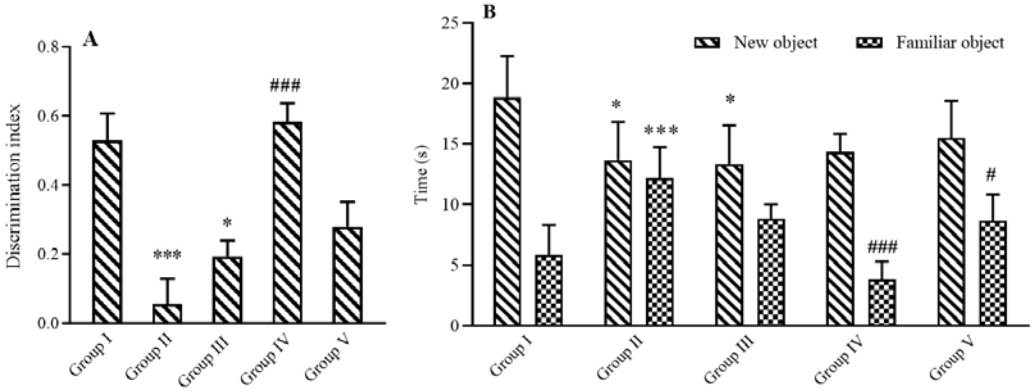

Fig. 4. The object recognition test. The effect of probiotic and ascorbic acid combination and lactulose treatment on discrimination in BDL-induced rats. The values are expressed as mean ± SD, n = 6. *P < 0.05 and ***P < 0.001 indicate significant differences compared to negative control (group I); #P < 0.05 and ###P < 0.001 relative to the BDL-induced control group. BDL, Bile duct ligation.

Neurochemical assessment

Lipid peroxidation and GSH level

In brain homogenates of BDL-induced control, rats increased lipid peroxidation and decreased GSH levels were observed compared to group I rats (Table 1). Oral administration of probiotics and the ascorbic acid combination resulted in lower MDA levels (prevent lipid peroxidation) in brain homogenates of BDL-induced rats, which were similar to the animals of the normal group. GSH levels in probiotic and ascorbic acid combination groups (III and IV) also improved and maintained normal levels of GSH in brain homogenates of BDL-induced rats.

Histopathological assessment

The liver of rats in group I showed a hepatic lobular architecture integrated with no signs of inflammation or necrosis (Fig. 6A$_2$). On the other hand, the livers of BDL rats appeared turgid with a finely nodular surface, which is characteristic of liver cirrhosis (Fig. 6B$_1$). The presence of severe tissue fibrosis, inflammation, and localized necrosis in BDL rats indicated liver injury (Fig. 6B$_2$). The liver sinusoids narrowed or disappeared due to compression. The liver cells of group IV (high dose probiotic) and the normal group were similar (Fig. 6D$_2$). The liver histopathology of group III (Fig. 6C$_2$) and group V rats (Fig. 6E$_2$) showed only slight inflammation that was less obvious than in the BDL-induced control rats. In the brain histopathology study, the hippocampus region was observed. Normal control rats did not show any signs of neuronal damage and normal neuroglial cells (Fig. 7A$_2$). Whereas, the hippocampus region of BDL rats showed Alzheimer's type II astrocytes (Fig. 7B$_2$). The rat hippocampus region of groups IV (Fig. 6D$_2$) and V (Fig. 6E$_2$) showed no lesions of neuronal damage or Alzheimer's type II astrocytes. However, the hippocampus region of group III rats showed mild lesions of neuronal damage with Alzheimer's type II astrocytes (Fig. 6C$_2$).

Table 1. The effect of probiotic and ascorbic acid combination and lactulose treatment on biochemical parameters (serum AST, ALT, and ALP) and neurochemical parameters (GSH and MDA) in bile duct ligation-induced rats. The values are expressed as mean ± SD, n = 6

Parameters	Groups				
	I	II	III	IV	V
AST	69.7 ± 11.23	306.33 ± 27.47***	183.83 ± 12.70$^{\#\#\#}$	197.5 ± 17.67$^{\#\#\#}$	243.66 ± 18.95$^{\#\#\#}$
ALT	36 ± 7.79	96.66 ± 5.50***	81.16 ± 5.11$^{\#\#}$	65.33 ± 2.33$^{\#\#\#}$	74 ± 5.09$^{\#\#\#}$
ALP	90.83 ± 8.06	219.66 ± 12.59***	187.83 ± 7.167$^{\#\#}$	177.33 ± 11.60$^{\#\#\#}$	174.33 ± 22.84$^{\#\#\#}$
MDA	133.5 ± 3.93	177.66 ± 2.73***	152.16 ± 2.78$^{\#\#\#}$	145.16 ± 3.18$^{\#\#\#}$	141 ± 4.81$^{\#\#\#}$
GSH	3.18 ± 0.04	2.19 ± 0.03***	3.03 ± 0.04$^{\#\#\#}$	3.23 ± 0.03$^{\#\#\#}$	3.28 ± 0.07$^{\#\#\#}$

$^{***}P < 0.001$ Indicates significant differences compared to negative control (group I); $^{\#\#\#}P < 0.001$ relative to the BDL-induced control group. AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, Alkaline phosphatase; MDA, malondialdehyde; GSH, reduced glutathione.

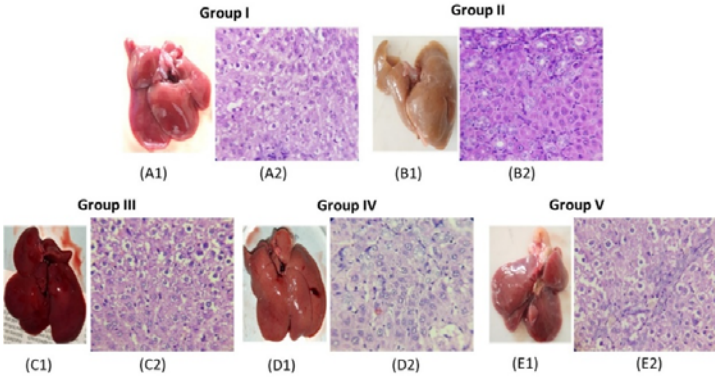

Fig. 6. The effect of probiotic and ascorbic acid combination and lactulose treatment on liver tissue histopathological alterations in bile duct ligated rats. Hematoxylin and eosin staining; × 400 magnification.

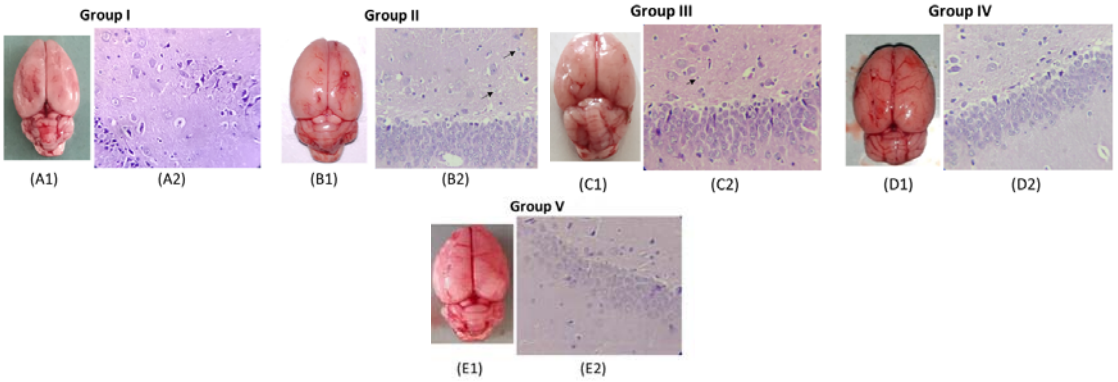

Fig. 7. Brain tissue histopathological alterations in bile duct ligated rats. Arrows show Alzheimer's type II astrocytes. Hematoxylin and eosin staining; × 400 magnification.

DISCUSSION

Type-C HE (chronic) is a complex neuropsychiatric disease associated with cirrhosis and portal-systemic shunts (15). In the present study, we have assessed the effect of probiotics and vitamin C combination in BDL-induced experimental model of chronic HE (type C). The BDL experimental model for inducing liver fibrosis and type C HE is widely acknowledged and utilized in hundreds of laboratories across the world. In this study, double ligation of the common bile duct resulted in a high percentage of cirrhosis in rats, with morphological abnormalities comparable to those seen in human biliary cirrhosis (16).

In the present study, chronic HE was induced by double BDL in rats. The histological examination of the liver in BDL-induced rats showed severe tissue fibrosis, inflammation, and focal necrosis indicating liver injury (Fig. 6). The liver morphology of BDL-induced rats also appeared turgid with a finely nodular surface. Furthermore, BDL also elevated liver dysfunction indicators, such as ALT, AST, and APK in serum. These results are in accordance with several investigations on BDL-induced liver fibrosis and cirrhosis in experimental animals (17). The BDL-induced fibrosis results from an obstruction in the flow of bile juice from the common bile duct leading to acute obstructive jaundice that progresses further into cirrhosis and fibrosis (18). Stagnant hydrophobic bile acids can harm the mitochondrial electron transport pathway and cause the liver to create reactive oxygen species (ROS). ROS triggers an inflammatory response and disrupts the balance of antioxidant and pro-oxidant activity (19). Fourteen-day administration of low- or high-dose probiotics (*L. plantarum* and *B. clausii*) and ascorbic acid combination reversed BDL-induced liver fibrosis and focal necrosis in rats (Fig. 6). Furthermore, the combination also ameliorated the elevated liver enzyme concentration (AST, ALT, and ALP) in the serum of BDL rats, indicating the hepatoprotective effect of the combination in chronic HE. The possible protective mechanisms of this combination include the antioxidant activity of ascorbic acid along with sustaining the intracellular antioxidant system (20). Moreover, ascorbic acid has an anti-inflammatory function through the eradication of ROS, which stimulates pro-inflammatory cytokines in diverse inflammatory illnesses and liver fibrosis (21). These results are in accordance with a study reported by Mustafa *et al*. wherein vitamin C and vitamin E and/or their combination protected the liver and brain against TAA-induced damage (8). However, Ho *et al*. reported that ascorbate failed to influence ALT, AST, hepatic hydroxyproline content, liver fibrosis ratio, hepatic protein expressions, and liver histology in rats with common BDL-induced chronic liver injury (22). The difference in results may be partly attributed to the synergistic effect of ascorbic acid and probiotics. Furthermore, probiotics also provide anti-inflammatory effects and stabilize the gut barrier (both inflammasomes and increased gut permeability have been involved in the development of hepatic fibrosis) (23). Therefore, ascorbic acid and probiotic supplementation might improve liver function and prevent fibrosis.

BDL-induced liver damage fails to adequately metabolize ammonia, resulting in its accumulation in the blood (4, 24). In this study, elevated serum ammonia level was observed in BDL-induced rats. The probiotic and ascorbic acid combination significantly decreased the elevated blood ammonia levels in BDL-induced rats. Alzheimer's type II astrocytes are primarily observed in diseases resulting from hyperammonemia (25). The presence of abnormal Alzheimer's type II astrocytes is a major neuropathological characteristic feature of BDL-induced chronic HE (26-28). Numerous studies have shown that ammonia-induced synaptic defect and astrocytic dysfunction in chronic HE is implicated in the development of cognitive and motor alterations in chronic HE (28-33). In the current study, we observed the presence of Alzheimer's type II astrocytosis in brain histology in BDL-induced rats. In addition, significantly impaired cognition (rats were unable to differentiate between the novel object and familiar object), motor coordination (decrease in retention time on rotarod apparatus), and working memory (lower percentage of correct spontaneous alternations and increased response time) were observed in BDL-induced rats. The current findings are consistent with prior reports of cognitive, motor, and memory deficits in rodents caused by BDL. Fourteen-day administration of high dose probiotic and ascorbic acid combination significantly protected the brain and improved cognition, motor coordination, and working memory. Furthermore, the rats given combination therapy showed a significant reduction in BDL-induced lipid peroxidation and an increase in GSH content, indicating a significant reduction in oxidative stress in brain tissues. Ascorbic acid exerts its activity in HE through reducing oxidative stress and non-antioxidant effect, which is mainly through modulation of binding of neurotransmitters to receptors as well as regulating their release and thus preventing neurodegeneration and psychiatric disorders (8).

CONCLUSION

The results of this study suggested that ascorbic acid and probiotic combination offers better protection against liver fibrosis and chronic HE induced by bile duct ligation in rats, similar to standard treatment with lactulose. Further clinical studies are needed for considering ascorbic acid and probiotic combinations in HE management.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contribution

C. Patel contributed to the conceptualization, design of the study, analysis, and interpretation of the data, drafting of the article, final editing; L. Shagond contributed to the design of the study, methodology, acquisition of the data, and analysis of the data; S. Acharya contributed to supervision, conceptualization, revising the article critically for important intellectual content; S. HS. Boddu contributed to drafting, resources, data analysis, and final editing; K. Ranch contributed to supervision, formal analysis, drafting, and revising of the article. The final version of the article was approved by all the authors.

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