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ORIGINAL PAPER

The Effect of Caffeic Acid Phenethyl Ester on Bacterial Translocation and Intestinal Damage in Cholestatic Rats

Cengiz Ara · Mukaddes Esrefoglu · Alattin Polat · Burak Isik · Murat Aladag · Mehmet Gul · Selma Ay · M. Sait Tekerleklioglu · Sezai Yilmaz

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Abstract We investigated the effect of caffeic acid phenethyl ester in rat ileum injury induced by chronic biliary obstruction. Swiss albino rats were divided into three groups: Group 1, sham ($n = 7$); Group 2, common bile duct ligation ($n = 7$); and Group 3, common bile duct ligation plus caffeic acid phenethyl ester ($n = 7$). In the caffeic acid phenethyl ester-treated rats, ileum tissue levels of malondialdehyde and myeloperoxidase were significantly lower than those of the bile duct-ligated rats ($P < 0.001$). The levels of tumor necrosis factor-α, interleukin-6, and interleukin-1α in the caffeic acid phenethyl ester group were significantly lower than those in the bile duct ligation group ($P < 0.03$, $P < 0.01$, and $P < 0.02$ respectively). The present study demonstrates that intraperitoneal administration of caffeic acid phenethyl ester in bile duct-ligated rats reduces intestinal oxidative stress. This effect may be useful in the preservation of intestinal damage in cholestasis.

Keywords Common bile duct ligation · Bacterial translocation · Caffeic acid phenethyl ester · Oxidative stress · Cytokines

C. Ara (✉) · B. Isik · S. Yilmaz
Department of General Surgery, Inonu University School of Medicine,
44069 Malatya, Turkey
e-mail: cara@inonu.edu.tr

M. Esrefoglu · M. Gul
Department of Histology and Embryology, Inonu University School of Medicine,
Malatya, Turkey

A. Polat
Department of Physiology, Inonu University School of Medicine,
Malatya, Turkey

M. Aladag
Departments of Gastroenterology and Medicine Microbiology, Inonu University School of Medicine,
Malatya, Turkey

S. Ay · M. S. Tekerleklioglu
Department of Medicine Microbiology, Inonu University School of Medicine,
Malatya, Turkey

Introduction

Despite the improvement in surgical procedures and the development of powerful antibiotics, septic complication after surgical operation is still a major cause of the high mortality rate in patients with obstructive jaundice [1, 2]. Clinical and experimental works suggest that bacterial translocation is the major source of bacteria implicated in the pathogenesis of endotoxemia, sepsis, and multiorgan failure during cholestasis [3, 4]. Bacterial translocation is described as the passage of viable bacteria from the gastrointestinal tract to extraintestinal sites without apparent rupture of the intestinal wall [5]. Although the pathogenesis of bacterial translocation is still unknown, disturbance of the homeostasis between the intestinal microflora and host defense mechanisms such as the mucosal barrier, immunologic defense, gastric acidity, and gastrointestinal motility can lead to bacterial translocation [6]. Most researchers believe that the absence of bile in the intestine in patients with obstructive jaundice and increased absorption of endotoxin can cause bacterial translocation. The administration of antibiotics to suppress aerobic intestinal flora has proven effective in the prevention of bacterial infections in rats with experimental cirrhosis [7]. However, long-term administration of antibiotics has been noted to be associated with the emergence of bacterial resistance to

antibiotics [8]. Thus nonantibiotic drugs need to be evaluated in the treatment and prevention of bacterial and endotoxin translocation in cirrhotic rats [9].

Caffeic acid phenethyl ester (CAPE) is an active component of honeybee propolis extracts and has been used for many years as a folk medicine. It has anti-inflammatory, immunomodulatory, antiproliferative, and antioxidant properties and has been shown to inhibit lipooxygenase activities as well as suppress lipid peroxidation [10–13]. To date, there is no study in the literature regarding the effect of CAPE on bacterial translocation and intestinal damage in common bile duct-ligated (CBDL) rats.

The aim of this study was to evaluate whether CAPE administration protects CBDL rats against bacterial translocation and intestinal damage. To assess the protective ability of CAPE in CBDL rats, we measured the activities of tissue reduced glutathione (GSH), malondialdehyde (MDA), myeloperoxidase (MPO), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1α (IL-1α); we also examined histopathological changes in the small bowel.

Materials and methods

Experimental conditions

A total of 21 3-month-old male Wistar albino rats weighing 300–350 g were included in this study. Experiments were done at the Inonu University Experimental Research Center. Animal experiments were performed in accordance with the guidelines for animal research from the National Institutes of Health and were approved by the Committee of Animal Research at Inonu University, Malatya, Turkey.

Animals were housed under continuous observation in appropriate cages in a quiet temperature ($21^\circ \pm 2^\circ$C)- and humidity ($60\% \pm 5\%$)-controlled room in which a 12/12-hr light/dark cycle was maintained. They were allowed free access to a commercial standard diet and water ad libitum. The 21 rats were divided into three equal groups: Group 1, sham ($n = 7$); Group 2, CBDL ($n = 7$); and Group 3, CBDL plus CAPE ($n = 7$).

All surgical procedures were performed while the rats were under intraperitoneal ketamine (50 mg/kg) and xylazine HCl (10 mg/ kg) anesthesia. All operations were performed under sterile conditions. Sham-operated animals were mobilized without ligation. CBDL was performed by ligation of the common bile duct with a 4-0 silk ligature. Afterward, the abdominal wall was closed with 2-0 silk continuous sutures. For the second control group, 1 ml of dilution vehicle (NaCI) containing 5% ethanol was given for 14 consecutive days after surgery.

CAPE was synthesized by the standard method of Grunberger [14] and administered intraperitoneally once a day at a dose of 10 μmol/kg (25 μmol/ml solution in 5% ethanol). This treatment was continued for 14 days. The animals were sacrificed under aseptic conditions on the 14th day and blood was drawn from the inferior vena cava to measure serum total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), and γ-glutamyl transferase (GGT). A part of the ileum was preserved in formalin for histological examination and the remainder was stored at -85°C until determination of MDA, MPO, GSH, and cytokines (IL-1α, IL-6, TNF-α).

Mesenteric lymph nodes (MLNs) in the terminal ileum and one liver lobe were plated on MacConkey's agar to culture gram-negative enteric bacilli, on blood agar to culture gram-positive cocci, and on selective agar to culture lactobacilli. All agar plates were incubated aerobically for 48 hr at 37 °C. The culture results were determined by the number of colony-forming units per gram of tissue (CFU/g) calculated from the dilutions of organ homogenates.

Biochemical analysis

One hundred milligrams of ileum tissues was homogenized in 1.5% KCl and phosphate-buffered solution (1:9, w/v) using a manual glass homogenizer for approximately 5 min and flushed by centrifugation for approximately 10 sec to remove large debris. The supernatant was used for analysis.

MDA content of homogenates was determined spectrophotometrically by measuring the presence of thiobarbituric acid-reactive substances (TBARS) [15]. Results are expressed as nanomoles per gram of tissue.

GSH was determined by the spectrophotometric method, which is based on the use of Elman's reagent [16]. Results are expressed as nanomoles per gram of tissue. MPO activity was determined using a 4-aminoantipyrine/phenol solution as the substrate for MPO- mediated oxidation by H_2O_2 and changes in absorbance at 510 nm (A_{510}) were recorded [17]. One unit of MPO activity was defined as the amount of protein that degrades 1 μmol of H_2O_2 min^{-1} at 25°C. Results are presented as milliunits per gram of protein.

Tissue homogenates were prepared using an IKA Ultra-Turnax homogenizer (2×45 sec, 0°C) in 0.5 M Tris/1.5 M NaCl/50 mM $CaCl_2$/2 mM sodium azide buffer at pH 7. The homogenates were then centrifuged at 15,000 pg/g tissue for 15 min at a temperature of $+4^\circ$C and the supernatants were used for ELISA. Rat IL-1α, IL-6, and TNF-α (Biosource Immunoassay Kit; USA) levels were measured using a sandwich ELISA protocol supplied by the manufacturer of the antibodies and the resultant optical density was determined using a microplate reader at 450 nm. Results are expressed as picograms per gram of tissue.

Table 1 Histological grading system developed by Chiu *et al.*

Grade	
0	Normal villi
1	Development of subepithelial space, usually at the apex of villus, capillary congestion
2	Extension of the subepithelial space with moderate seperation of epithelial layer from lamina propria
3	Extensive epithelial seperation from lamina propria down the sides of the villi; ulceration at villus tip
4	Denuded villi; dilated capillaries, increased cellularity of lamina propria
5	Disintegration of lamina propria, hemorrhage, and ulceration

Histopathologic evaluation

Portions 1 cm long were harvested from the terminal ileum. Intestinal lumen was carefully cannulated and gently washed with 10% formalin. Samples were fixed in 10% formalin for histopathological examination by light microscopy. Sections of 4 μm were cut and stained with hematoxylin and eosin (H&E). Sections were scored by an independent observer blinded to the experimental protocol. Mucosal lesions were graded according to a system described by Chiu *et al*. [18) (Table 1).

Statistical analysis

Data are expressed as the arithmetic mean $\pm$ SD of the number (n) of experiments; differences were considered to be statistically significant at $P < 0.05$. Data were analyzed statistically using the SPSS statistical program. Results were statistically analyzed by the Kruskal-Wallis H and Fisher's exact chi-square test. Differences between groups were evaluated by the Mann-Whitney U test followed by t test with Bonferroni correction when indicated.

Results

The results of bacterial translocation in the groups are reported in Table 2. The most common bacteria cultured from the liver and MLNs of these animals were *Escherichia coli, Staphylococcus aureus*, and *Proteus mirabilis*. The results for MDA, GSH, and MPO are given in Table 3. In CAPE-treated rats MDA and MPO levels were significantly lower than in CBDL rats ($P < 0.001$). Although the levels of GSH in CAPE-injected rats were higher than in the CBDL group, the difference was not statistically significant ($P > 0.05$). Total bilirubin, AST, ALT, AP, and GGT levels in rats with bile duct ligations were higher than those in the sham group (Table 4). The results of for TNF, IL-6, and IL-1 are listed in Table 5. In CAPE-treated rats, ileal levels of TNF-α, IL-6, and IL-1α were found to be significantly lower compared to those in the BDL group ($P < 0.03$, $P < 0.01$, and $P < 0.02$ respectively).

Table 2 Bacterial translocation number and rates in study groups

Group	MLNs	Liver
1. Sham ($n = 7$)	—	—
2. CBDL ($n = 7$)	5 (71.4%)	2 (66.7%)
3. CBDL + CAPE ($n = 7$)	2 (28.6%)	1 (33.3%)
P value:*		
2 vs 3	0.28	0.12

Note. CBDL, common bile duct ligation; CAPE, caffeic acid phenethyl ester; MLN, mesenteric lymph nodes. $P < 0.05$ considered statistically significant.

The grading of mucosal injury in groups is listed in Table 6. Animals from the SO group presented no histological changes (Fig. 1). The specimens obtained from the CBDL group presented many histopathological alterations. Partial or subtotal villous atrophy (Fig. 2) and apparently pronounced increases in lymphocytes and neutrophils in the lamina propria (Fig. 2) and lymphocytes in the villous epithelium (Fig. 3) were observed. The villi were reduced in height (Fig 2). Intestinal mucosa showed areas of villous epithelial damage. Sometimes the villous tip was denuded (Fig 4). Subepithelial edema and widespread dilatation of the mucosal lymphatics (Figs. 3 and 4) were seen. There were many bacteria on the surface of the epithelium. While many of them were embedded in the epithelium (Figs. 3 and 5), some of them were located in the lymphatic capillaries (Fig. 4). The CBDL + CAPE group generally revealed normal histology. In some areas, partial villous atrophy (Fig. 6), subepithelial edema, dilatation of the mucosal lymphatics, and intraepithelial lymphocyte migrations were observed (Fig. 6). Epithelium was always intact. A bacterium located on the surface of the epithelium was rarely detected.

Discussion

Obstructive jaundice has been defined as a risk factor for translocation of bacteria and their endotoxins from the gut microflora [19, 20]. Biliary sepsis, wound infections, intra-abdominal abscess formation, and renal failure frequently occur during obstructive jaundice [21, 22].

The present study indicates that intraperitoneal administration of CAPE at a dose of 10 μmol/kg day reduced tissue levels of MDA and MPO but increased levels of GSH in the ileum after bile duct ligation. Additionally, CAPE decreased IL-1α, IL-6, TNF-α, and intestinal mucosal injury but the effect of CAPE on bacterial translocation was not revealed.

In the present study, although bacterial translocation in CAPE-treated rats was lower than in the CBDL group, the difference was not statistically significant ($P > 0.05$). The

Table 3 Tissue levels of MDA, GSH, and MPO activities

Group	MDA (nmol/g tissue)	GSH (nmol/g tissue)	MPO (MUg-1 protein)
1. Sham ($n=7$)	47.6 ± 11.3	3.2 ± 0.7	120.3 ± 28.2
2. CBDL ($n=7$)	87.1 ± 20.3	1.8 ± 0.3	168.1 ± 42.3
3. CBDL + CAPE ($n=7$)	51.2 ± 7.3	2.1 ± 0.6	80.1 ± 20.3
P values: *			
1 vs 2	0.001	0.001	0.09
2 vs 3	0.001	0.12	0.001

Note. CBDL, common bile duct ligation; CAPE, caffeic acid phenethyl ester; MDA, malondialdehyde; GSH, reduced glutathione; MPO, myeloperoxidase.
*$P < 0.05$ considered statistically significant.

Table 4 Plasma total bilirubin, AST, ALT, AP, and GGT levels

Group	Total bilirubin (mg/dl)	AST (U/L)	ALT (U/L)	AP (U/L)	GGT (U/L)
1. Sham ($n=7$)	0.18 ± 0.17	122.8 ± 13.9	54.4 ± 7.5	353.5 ± 27.9	2.5 ± 0.9
2. CBDL ($n=7$)	9.5 ± 3.5	498.00 ± 121.4	157.5 ± 22.2	1663.2 ± 401.3	12.1 ± 2.7
3. CBDL + CAPE ($n=7$)	6.8 ± 1.6	315.7 ± 96.6	98.7 ± 45.7	1564.2 ± 913.4	4.7 ± 2.8
P values:*					
1 vs 2	0.001	0.001	0.001	0.01	0.02
2 vs 3	0.12	0.01	0.02	0.31	0.001

Note. CBDL, common bile duct ligation; CAPE, caffeic acid phenethyl ester; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AP, alkaline phosphatase; GGT, γ-glutamyl transferase.
*$P < 0.05$ considered statistically significant.

Table 5 Tissue levels of TNF-α, IL-6, and IL-1α activities (pg/g tissue)

Group	TNF- α	IL-6	IL-1α
1. Sham ($n=7$)	$3{,}639.00 \pm 1{,}399.7$	758.2 ± 265.2	723.9 ± 341.4
2. CBDL ($n=7$)	$19{,}284.1 \pm 10{,}744.3$	$4{,}199.7 \pm 5{,}188.7$	$2{,}535.0 \pm 955.8$
3. CBDL + CAPE ($n=7$)	$7{,}889.2 \pm 3{,}023.7$	$1{,}342.9 \pm 499.8$	$1{,}548.5 \pm 348.9$
P values:*			
1 vs 2	0.001	0.001	0.001
2 vs 3	0.03	0.01	0.02

Note. CBDL, common bile duct ligation; CAPE, caffeic acid phenethyl ester; TNF-α, tumor necrosis factor-α; IL, interleukin.
*$P < 0.05$ considered statistically significant.

mechanism of bacterial translocation is not completely understood. Luminal flow of bile salts has antibacterial effects and the direct detergent effect on endotoxin and increased absorption of endotoxin in rats with obstructive jaundice has been suggested to be associated with the absence of bile salts [19, 23]. Alteration of enteric flora, physical or functional injury of the intestinal barrier, and impaired immune function can all induce bacterial translocation [24]. Intestinal bacterial overgrowth can also cause intestinal oxidative damage [25]. Increased intestinal oxidative damage and bacterial translocation have been revealed in rats with CBDL and chronic portal hypertension [26, 27]. An increased concentration of MDA reflects the level of lipid peroxidation in tissues and it is considered a marker of tissue injury [28]. Several reports indicate increases in ileum MDA levels in rats with bile duct ligation and cirrhosis induced by CCl_4 [25–27, 29]. Our results are in agreement with previous works reporting high levels of MDA. In addition, we measured the abundance of neutrophil infiltration to the ileal tissue as MPO activity; the infiltration of neutrophils in intestinal mucosa seems to be responsible for the increased mucosal lipid peroxidation. In

Table 6 Mucosal injury grades

Group	Histological grade
Sham ($n=7$)	0.00 ± 0.00
CBDL ($n=7$)	2.85 ± 0.40^a
CBDL + CAPE ($n=7$)	1.14 ± 0.40^b

Note. Values expressed as mean ± SE. CBDL, common bile duct ligation; CAPE, caffeic acid phenethyl ester.
[a] $P = 0.001$ vs sham.
[b] $P = 0.018$ vs CBDL.

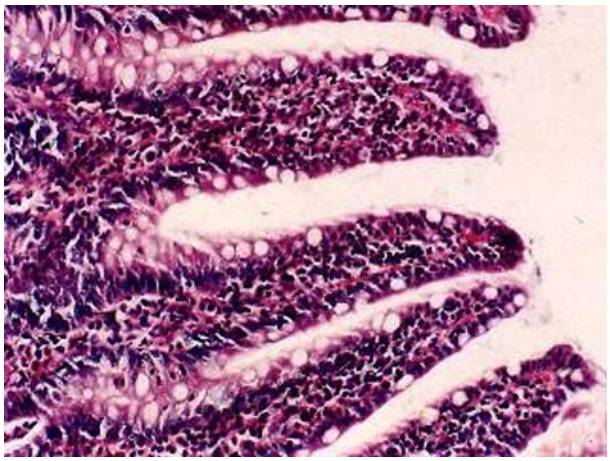

Fig. 1 Sham group. Mucosa is normal in histological appearance. (H&E; original magnification, × 20.)

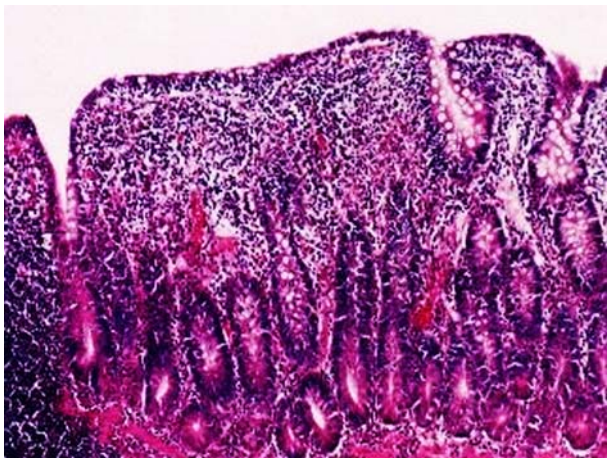

Fig. 2 CBDL group. Subtotal villous atrophy is evident. Grade 1 mucosal damage. Development of subepithelial space and capillary congestion are seen. The lamina propria is infiltrated by lymphocytes. (H&E; original magnification, × 20.)

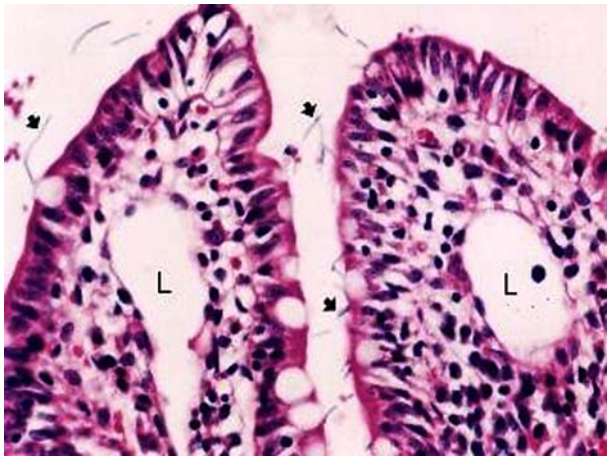

Fig. 3 CBDL group. Lymphocyte migration to the epithelium and dilatation of the mucosal lymphatics (L) are observed. There are many bacteria on the surface of the epithelium (arrows). (H&E; original magnification, × 40.)

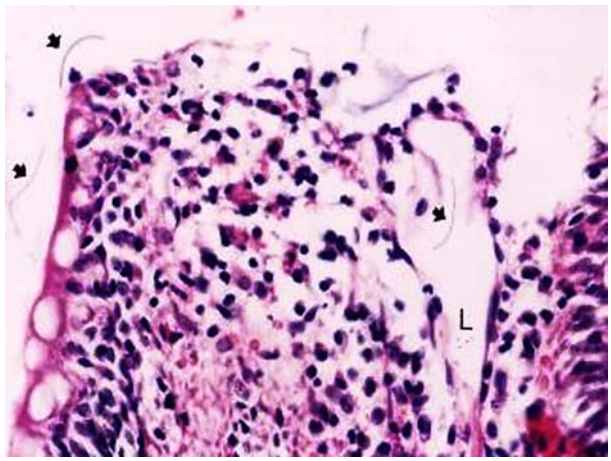

Fig. 4 CBDL group. Grade 4 mucosal damage. The villous tip is denuded. Bacteria on the surface of the epithelium and in the lumen of the lymphatic capillary (L) are obvious (arrows). (H&E; original magnification, × 40.)

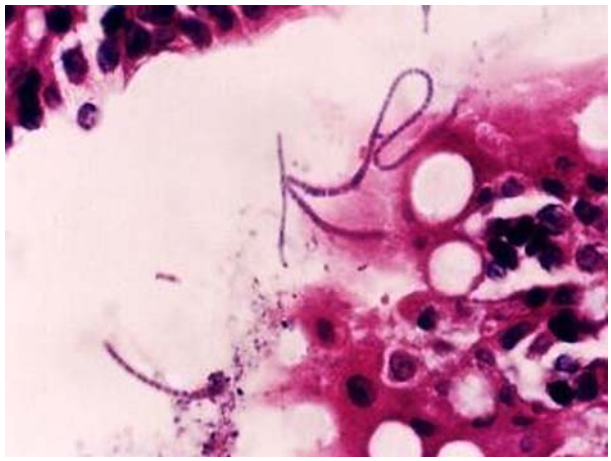

Fig. 5 CBDL group. Bacteria embedded in the epithelium are visible. (H&E; original magnification, × 100.)

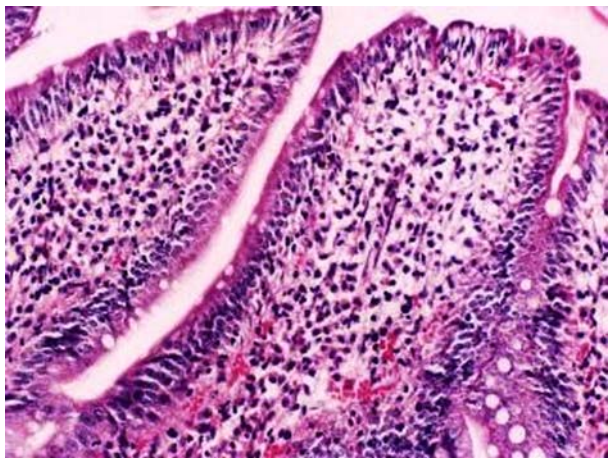

Fig. 6 CBDL + CAPE group. Partial villous atrophy is observed. The epithelium is intact. There are no bacteria on the surface of the epithelium. (H&E; original magnification, × 20.)

the present study, intestinal MPO activity in the CBDL group was significantly higher than in the CAPE-treated group. Our results are in accordance with other studies reporting high MPO activity [26, 29]. In another study, in experimental cirrhosis induced by CCl_4 no significant differences were observed in MPO activity between normal and cirrhotic rats in ileal mucosa [30]. Although tissue MDA and MPO levels were clearly decreased by CAPE, its exact mechanism is not clear. Reports that CAPE directly scavenges hydroxy radicals and thereby inhibits lipid peroxidation are well documented [10–12]. The reduction in MDA and MPO levels in the CAPE-treated group was probably due to CAPE's antioxidant and free radical scavenging effects. Also, the decrease in MPO activity in the CAPE-treated group may be due to CAPE's anti-inflammatory effect [31]. It is possible that CAPE interferes with free radical generation and may be related to the decline in intestinal oxidative stress in rats with CBDL.

Histologically, in the CBDL group, villous atrophy, subepithelial edema, epithelial damage, and the number of bacteria on the surface of the epithelium were more pronounced compared to those in the CAPE-treated group. Histopathological studies have shown translocation of bacteria such as *Candida albicans* and *Escherichia coli* by direct penetration of enterocytes associated with disturbance of the basement membrane [32]. We observed bacteria embedded in the cytoplasm of enterocytes and in the lumen of lacteals. In our study, the morphologic changes of the ileal mucosa in the CBDL group are in accordance with previous works [19, 33, 34]. Although the mechanism of morphologic change of the ileal mucosa during obstructive jaundice is not completely understood, different mechanisms may contribute to the mucosal morphologic changes. For instance, experimental obstructive jaundice resulting in increased endotoxemia, bacterial traslocation, and production of proinflammatory cytokines (TNF, IL-1, IL-6) may cause mucosal damage [35, 36]. In the present study, in the CBDL group, levels of TNF-α, IL-1α, IL-6, and IL-1α were significantly higher than in the CAPE-treated group. Our results are similar to previous studies. Also, the absence of bile salts from the intestinal lumen may have a detrimental effect on normal epithelial growth and maintenance [37]. The depression of reticuloendothelial system function in obstructive jaundice may cause epithelial damage and bacterial translocation [38]. The protective effect of CAPE against intestinal damage in cholestatic rats may be due to anti-inflammatory action [31], inhibition of TNF-α [39], and immunomodulatory action [10].

The present study demonstrates that intraperitoneal administration of CAPE maintains antioxidant defenses and reduces intestinal mucosal injury and oxidative damage in the ileum in cholestatic rats. This effect of CAPE may be useful for preserving intestinal injury in patients with biliary obstruction. However, more investigations are required to evaluate CAPE's antioxidant and anti-inflammatory effect in clinical and experimental models.

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