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The Effect of Caffeic Acid Phenethyl Ester (CAPE) Against Cholestatic Liver Injury in Rats

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Objectives. **Caffeic acid phenethyl ester (CAPE) has been subjected to considerable investigations that have revealed its antioxidant and anti-inflammatory activities in different conditions. But there is not a previous investigation about its effect on cholestatic liver injury. The aim of this study was to investigate the effect of CAPE in rat liver against cholestatic liver injury induced by bile duct ligation.**

Methods. **Swiss-albino rats were recruited in the study as follows; Group 1 rats subjected to simple laparotomy known as the sham group; Group 2 rats subjected to bile duct ligation (BDL); Group 3 bile duct ligated rats treated with CAPE. The third group received CAPE (10 μmol/kg) intraperitoneally daily throughout 14 d.**

Results. **Data showed a decrease in γ glutamyl transferase (GGT), aspartate aminotransferase (AST), and alanine aminotransferase levels (ALT) of the CAPE treated rats, compared with BDL group ($P < 0.001$, $P < 0.01$, and $P < 0.02$, respectively). In the CAPE treated rats, tissue levels of malondialdehyde (MDA) and myeloperoxidase (MPO) were significantly lower than that of the BDL group ($P < 0.001$). The levels of glutathione (GSH) in CAPE treated rats were significantly higher than that of BDL group ($P < 0.001$). In CAPE treated group, the levels of interleukin-1alpha (IL-1α) and interleukin-6 (IL-6) were significantly lower than that of BDL group ($P < 0.03$, $P < 0.02$, respectively). Administration of CAPE in the rats with biliary obstruction resulted in inhibition of necro-inflammation.**

Conclusion. **These results suggest that treatment of CAPE maintains antioxidant defenses, reduces oxidative liver injury, cytokine damage, and necro-inflammation in bile duct ligated rats. Thus, CAPE seems to be a promising agent for the attenuation of cholestatic liver injury.**

Key Words: **caffeic acid phenethyl ester (CAPE); bile duct ligation; cholestatic liver injury.**

INTRODUCTION

The pathogenesis of liver injury induced by bile duct ligation (BDL) is poorly understood. There are many experimental and clinical data that show the important role of reactive oxygen species (ROS) in the pathogenesis of hepatic damage and cholestasis produced by biliary obstruction [1–3]. The bile acid concentrations increase in rats after BDL and induce lipid peroxidation, which is probably related to the stimulation of phagocytic activity in polymorphonuclear phagocytes and inflammatory cells [4]. A recent study showed that hepatic neutrophil infiltration is closely related to an increase in hepatic lipid peroxidation in bile duct ligated rats [5].

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 have been shown to inhibit lipo-oxygenase activities as well as to suppress lipid peroxidation [6–9]. Also, it has been previously shown that intraperitoneal administration of caffeic acid phenethyl ester in bile duct-ligated rats

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reduces intestinal oxidative [10]. To date, there is no reported study regarding the effect of CAPE on cholestatic liver damage. The aim of this study was to evaluate whether CAPE administration would protect against cholestatic liver injury in rats with BDL .To assess the effect of CAPE in bile duct ligated rats, we measured the activities of glutathione (GSH), malondialdehyde (MDA), myeloperoxidase (MPO), interleukin-1alpha (IL-1α), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α). Also we examined histopathological changes in liver.

MATERIALS AND METHODS

Experimental Conditions

Three-month-old male Swiss-albino rats, weighing 300 to 350 g, were included in the study. The study was approved by Inonu University Ethics Committee.

Rats were kept in stainless steel cages, allowed access to food and water *ad libitum,* and quarantined 7 d before surgery. Food was withheld 8 h prior to surgery, but free access to water was allowed. Rats were subjected to a controlled environment regarding temperature and humidity and a 12-h light-dark cycle. All surgical procedures were performed while the rats were under intraperitoneal ketamine (50 mg/kg) and xylazine HCl (10 mg/kg) anesthesia.

A total of 21 rats were divided in to three groups: Group 1, BDL ($n = 7$); Group 2, BDL plus CAPE ($n = 7$), and Group 3, sham-operated group ($n = 7$), subjected to simple laparotomy. Acute cholestatic liver injury was induced in animals by double ligation and division of the bile duct [11]. Abdominal layers were closed with appropriate suture materials. All animals were maintained under the same conditions after surgery. The CAPE was synthesized by the standard method of Grunberger [12], and administered intraperitoneally once a day at a dose of 10 μmol/kg, from 25 μmol/mL solution in 1% ethanol.

Animals in CAPE group were treated with CAPE (10 μmol/kg/d, i.p) throughout 14 d. To eliminate complications arising from the diurnal effects, all rats were sacrificed under anesthesia at the same time of the day and liver was rapidly removed. Blood samples were taken by vena cava inferior puncture. A part of the liver was preserved in formalin for histological examination and the remainder was stored at –30°C for the analyses of MDA, MPO, GSH, and cytokines (IL-1α, IL-6, TNF-α) levels.

Biochemical Analyses

The plasma was used to measure total bilirubin to reflect mechanical obstruction, AST and ALT to reflect the degree of hepatocellular injury, and ALP and GGT to reflect the degree of cholestasis. The plasma activities of total bilirubin, AST, ALT, ALP, and GGT were estimated by commercially available kits (Olympus Diagnostica GmbH, Dublin, Ireland). The liver tissues were homogenized and the MDA contents of homogenates were determined spectrophotometrically [13].

Myeloperoxidase 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 [14]. One unit of MPO activity was defined as the amount of protein that degrades 1 μmol H_2O_2 min^{-1} at 25 °C. Results were presented as mUg^{-1} protein.

Results were expressed as umol/g tissue. GSH was determined by the spectrophotometric method, which was based on the use of Elman's reagent [15]. Results were expressed as nmol/g tissue.

Tissue homogenates were prepared using a homogenizer IKA Ultra-Turnax (2 × 45 s, 0°C) in 0.5 m TRIS/1.5m NaCL/50mM $CaCL_2$/2 mM sodium azide buffer at pH = 7. The homogenates were then centrifuged at 15,000 *g* for 15 min at a temperature +4°C and the supernatants were used for ELISA. Rat IL-1α, Rat IL-6, and Rat TNF-α (Biosource Immunoassay Kit, Camarillo, CA) levels were measured using a Sandwich enzyme-linked immunosorbent assay (ELISA) protocol supplied by the manufacturer of the antibodies and resultant optical density determined using a microplate reader at 450 nm. Results were expressed as pg/g tissue.

Histopathologic Evaluation

After the macroscopic findings were noted, the livers were promptly removed and processed for histologic and biochemical examination. The right lobe of the liver was divided into two pieces. The first samples were placed in 10% formalin for histopathologic examination by light microscopy. Liver tissues were fixed in 10% formalin and embedded in paraffin. For histopathologic evaluation, 4 mm slides were stained with hematoxylin-eosin. Sections were scored by an independent observer blinded to the experimental protocol. The following lesions were scored according to the modified histological activity index (HAI) [16, 17]: portal inflammation, focal necrosis, confluent necrosis, piecemeal necrosis, apoptosis, and focal inflammation.

Statistical Analysis

The results were statistically analyzed by the Kruskal-Wallis H test. The differences between groups were evaluated by the Mann-Whitney U test followed by *t*-test with Bonferroni correction when indicated. The results were given in the text as mean ± STD for all comparisons; statistical significance was defined as $P < 0.05$.

RESULTS

All of the animals survived until the end of the experiment. Jaundice was observed in the visceral and parietal peritoneum of all animals except those of the sham group. The livers were enlarged and the bile ducts above the obstruction point were dilated. Animals of the sham group did not show any alteration after surgery.

The results of MDA, GSH, and MPO are shown in Table 1. In the CAPE treated rats, the MDA and MPO levels were significantly lower, and the levels of GSH were significantly higher than that of the BDL group ($P < 0.001$ and $P < 0.001$, respectively). Total bilirubin,

TABLE 1

Tissue Levels of MDA, GSH, and MPO Activities in the Groups

Groups	MDA (nmol/g tissue)	GSH (nmol/g tissue)	MPO (mUg^{-1} protein)
I. Sham (n = 7)	68.35 ± 12.9	1.37 ± 0.8	6.21 ± 1.1
II. BDL (n = 7)	155.42 ± 9.58	1.31 ± 0.1	17.29 ± 2.3
III. BDL + CAPE (n = 7)	119.38 ± 16.7	2.75 ± 0.5	8.79.1 ± 1.4
P values*			
I*versus*	0.001	0.53	0.001
II *versus* III	0.001	0.001	0.001

BDL=bile duct ligation; CAPE=caffeic acid phenethyl ester; MDA=malondialdehyde; GSH=reduced glutathione; MPO=myeloperoxidase.

*$P < 0.05$ was considered to be statistically significant.

TABLE 2

Plasma Total Bilirubin, AST, ALT, AP, and GGT Levels in the Groups

Groups	Total bilirubin (mg/dL)	AST (U/L)	ALT (U/L)	ALP (U/L)	GGT (U/L)
Sham ($n = 7$)	0.18 ± 0.17	122.8 ± 13.9	54.4 ± 7.5	353.5 ± 27.9	2.5 ± 0.9
BDL ($n = 7$)	9.5 ± 3.5	498.00 ± 121.4	157.5 ± 22.2	1663.2 ± 401.3	12.1 ± 2.7
BDL+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*					
I *versus II*	0.001	0.001	0.001	0.01	0.02
II *versus* III	0.12	0.01	0.02	0.31	0.001

BDL=bile duct ligation; CAPE=caffeic acid phenethyl ester; AST=aspartate aminotransferase; ALT=alanine aminotransferase; ALP=alkaline phosphatase; GGT=Gama glutamyl transferase.

*$P < 0.05$ was considered to be statistically significant.

AST, ALT, ALP, and GGT levels in rats with BDL were significantly higher than that of sham group (Table 2). In the CAPE treated rats, the levels of AST, ALT, and GGT were significantly lower than that of the BDL group ($P < 0.01$, $P < 0.02$, and $P < 0.001$, respectively). Decreases of total bilirubin and ALP levels was not statistically significant in CAPE treated group compared with BDL group ($P = 0.12$, $P = 0.31$, respectively). The results of IL-1α, IL-6, and TNF-α are shown in Table 3. In the CAPE treated rats, the liver levels of IL-1α, IL-6, and were found to be significantly lower when compared to the BDL group ($P < 0.03$, $P < 0.02$, respectively). Although levels of TNF-α were decreased in CAPE injected rats compared to BDL group, the difference was not statistically significant ($P = 0.053$).

Microscopic Findings

Animals of the sham group did not present any histological alterations. The liver specimens of the BDL group showed prominent lobular and portal changes. The degree of necro-inflammation (focal necrosis, confluent necrosis, piecemeal necrosis, focal and portal inflammation, and apoptosis) showed significant difference between the BDL and BDL+CAPE groups ($P < 0.05$). HAI scores of the groups are summarized in Table 4. In the BDL group, the histopathological changes including necrosis (Fig. 1), bile duct proliferation, fibrosis, periportal and parenchymal polymorphonuclear leucocyte (PNL), and lymphocyte infiltration were prominent (Figs. 2 and 3). There were numerous large areas of coagulation necrosis, randomly distributed (Fig.1). Fibrotic reaction was usually accompanied with inflammatory cells in the periphery of the majority of the portal space areas. In some of the portal areas, cholangitis was observed. Bile ducts were filled with pus cells, which also infiltrated their walls (Fig. 3). In the BDL+CAPE group, histopathological evidence of parenchymatous injury was markedly reduced (Figs. 4 and 5). Relatively small, scattered necrotic hepatocyte groups between normal-appearing parenchymal cells were observed (Fig. 4). Portal fibrosis was not evident. Bile duct proliferation was still prominent (Fig. 5).

DISCUSSION

The results of the present study demonstrate that treatment of CAPE markedly ameliorates the liver injury in rats subjected to BDL. Several reports showed that oxidative stress associated with lipid peroxidation is involved in the development of cholestatic liver injury in bile duct ligated rats [3, 18, 19]. The present study indicates that intraperitoneal administration of CAPE at

TABLE 3

Tissue Levels of TNF-α, IL-6, and IL- 1α Activities in the Groups

Groups	TNF-α (pg/g tissue)	IL-6 (pg/g tissue)	IL-1α (pg/g tissue)
I. Sham ($n = 7$)	15314.00 ± 4245.57	4941.14 ± 1111.90	1834.28 ± 578.57
II. BDL ($n = 7$)	35112.85 ± 17142.95	11678.71 ± 6477.56	4493.85 ± 2158.76
III. BDL+CAPE ($n = 7$)	19284.00 ± 5540.03	6104.57 ± 1632.42	2609.42 ± 780.41
P values*			
I *versus* II	0.01	0.01	0.004
II *versus* III	0.053	0.02	0.03

BDL=bile duct ligation; CAPE=caffeic acid phenethyl ester; IL-1α=interleukin-1 alpha; IL-6=interleukin-6; TNF-α=tumor necrosis factor-alpha.

*$P < 0.05$ was considered to be statistically significant.

TABLE 4

Scores for Necro-Inflammation (Focal Necrosis, Confluent Necrosis, Piecemeal Necrosis, Focal and Portal Inflammation, and Apoptosis) of Groups Using Modified HIA Grading System (Mean ± SE)

Groups	Scores for necro-inflammation
Sham ($n = 7$)	0.285 ± 0.184
BDL ($n = 7$)	8.714 ± 1.523*
BDL+CAPE ($n = 7$)	5.285 ± 0.860**

BDL=bile duct ligation; CAPE=caffeic acid phenethyl ester; HAI=histological activity index.
*$P < 0.005$ *versus* sham.
**$P < 0.05$ *versus* BDL.

a dose of 10 μmol/kg/d reduced the already increased levels of plasma AST, ALT, and GGT after bile duct ligation. Additionally, CAPE decreased oxidative stress and the increased levels of IL-1α, IL-6, and inhibited the occurrence of necro-inflammation (focal necrosis, confluent necrosis, piecemeal necrosis, focal and portal inflammation, and apoptosis), seen in bile duct ligated rats.

In the current study, an increase in neutrophil infiltration in liver tissues, which was assessed by measuring hepatic activity, was established [20]. Neutrophil infiltration and activation are involved in the development of cholestatic liver injury in bile duct ligated rats [21, 22]. Our results are in agreement with previous works reporting high levels of MPO after the ligation of bile duct [20, 23, 24]. In the present study, levels of MPO in the CAPE treated rats were significantly lower than that of the BDL group. Reductions in MPO levels in the CAPE treated rats may probably be due to its antioxidant, free-radical-scavenging effect [8, 9], and inhibition of neutrophil infiltration [25]. This finding shows that CAPE exerts a preventive effect on cholestatic liver injury by inhibiting enhanced neutrophil infiltration into the liver tissue.

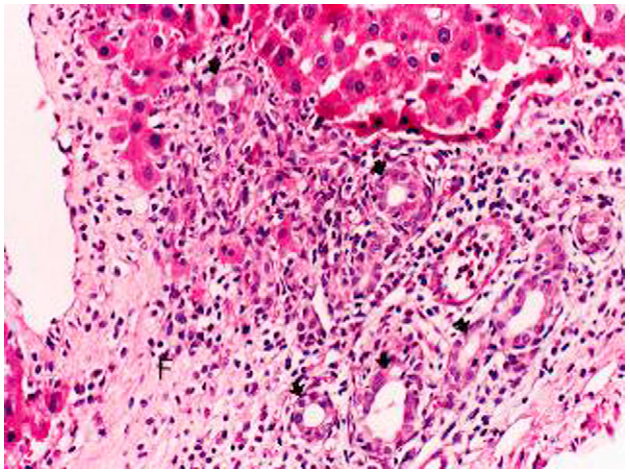

FIG. 2. BDL group. Portal fibrosis (F) with bile duct proliferation (arrows) and cell infiltration are observed. H and E; ×20. (Color version of figure is available online.)

MDA is a secondary product of oxidative stress formed during lipid peroxidation and it is released as a result of toxic effect of reactive oxygen species in rats after BDL [26]. There are several reports indicating that levels of MDA increases after BDL in rats [19, 27, 28]. Results of the present study are consistent with previous works reporting high levels of MDA. In the present study, levels of MDA in the CAPE treated rats were significantly lower than in the BDL group. Although tissue MDA levels were clearly decreased by CAPE, its exact mechanism is not clear. A recent study reported by Kus *et al.* [29] showed that administration of CAPE decreased levels of MDA in rats exposed to carbon tetrachloride (CCI4) induced hepatotoxicity. It is possible that the interference of CAPE with free radical

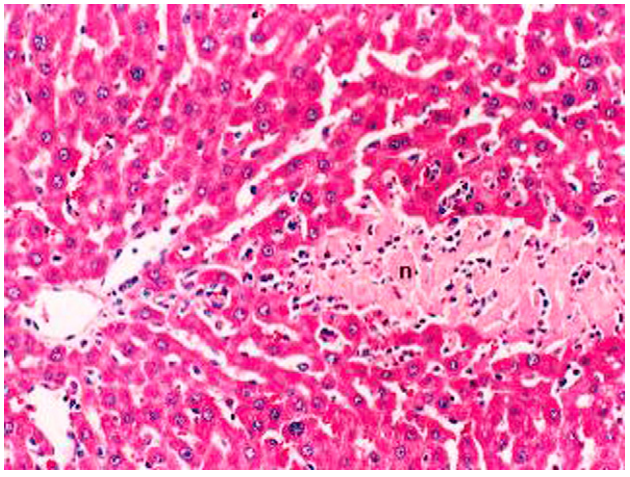

FIG. 1. BDL group. A large area of coagulation necrosis (n) is observed. H and E; ×20. (Color version of figure is available online.)

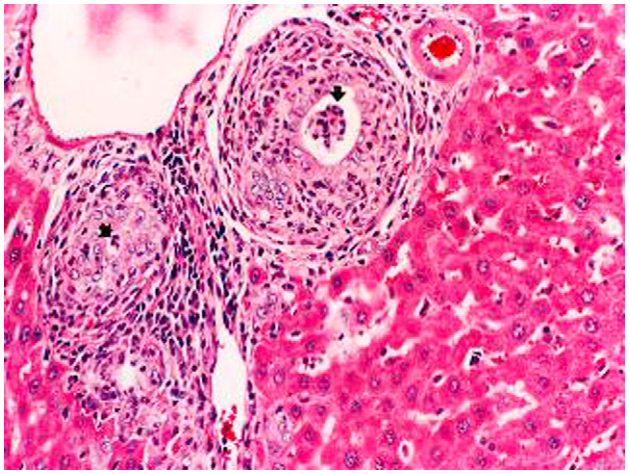

FIG. 3. BDL group. Periportal cell infiltration and cholangitis are observed. Bile ducts are filled with pus cells (arrows). H and E; ×20. (Color version of figure is available online.)

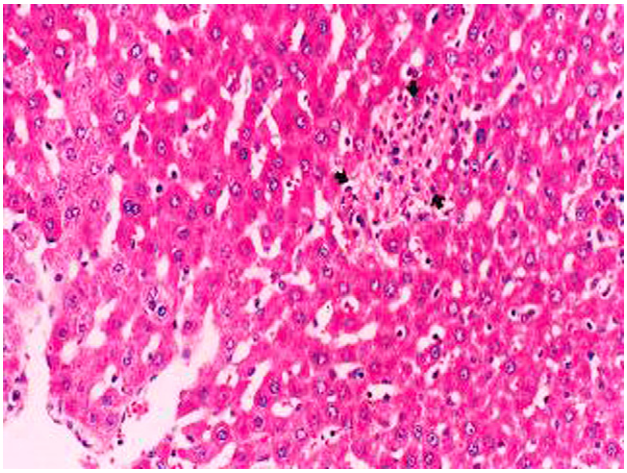

FIG. 4. BDL+CAPE group. A small necrotic hepatocyte group (arrows) between normal-appearing parenchymal cells are observed. H and E; ×20. (Color version of figure is available online.)

generation is related to a decline oxidative stress in cholestatic rats.

GSH is an essential component of the cellular defense mechanism against oxidative stress that induced by ROS in rats with bile duct ligation [30]. GSH plays a pivotal role in the defense against oxidative stress, as a cofactor of glutathione peroxidases (selenium dependent and independent) participates in the elimination of hydrogen peroxide and lipid hydroperoxides [31]. In accordance with previous reports, our results showed depleted tissue GSH levels attributable to BDL [3, 32, 33]. Administration of CAPE restored GSH levels significantly. Restoration of tissue GSH levels by CAPE treatment could be partly related to its antioxidant and free-radical scavenging effect. Another explanation of this statistically significant increase in GSH levels seen in the CAPE treated rats compared with the BDL rats is the effect of CAPE upon the enzymes involved in GSH synthesis, whereby CAPE may maintain the levels of glutathione during oxidative stress [34]. This increased GSH is consistent with the protective effects of CAPE against oxidative damage in cholestasis, and that depletion of tissue GSH content enhances cellular injury caused by oxidative stress [35].

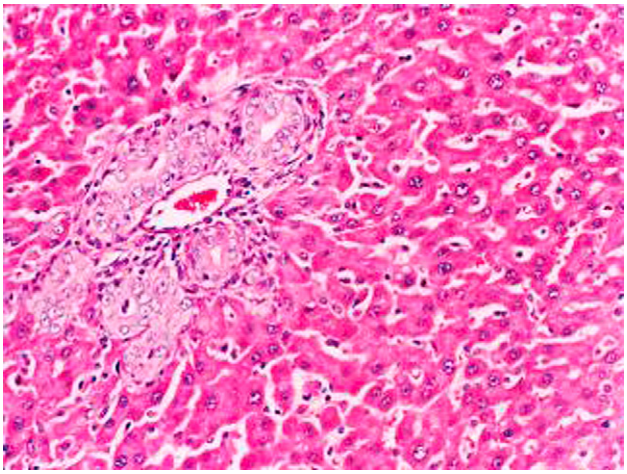

FIG. 5. BDL+CAPE group. Portal fibrosis is not evident. Bile duct proliferation is prominent. H and E; ×20. (Color version of figure is available online.)

In the present study, we have observed many histopathological features of cholestasis such as focal necrosis, confluent necrosis, piecemeal necrosis, focal and portal inflammation, fibrosis, and apoptosis. HAI scores of the BDL group were significantly higher than that of the sham group. The degree of necro-inflammation showed significant difference between the BDL and CAPE treated groups. Portal fibrosis was not evident and histopathological evidence of parenchymatous injury was significantly reduced in the CAPE treated group.

In bile duct ligated rats; phospholipase is activated as a result of retention of bile salts, which are toxic for cells. Arachidonic acid products trigger ductal proliferation that is the initial phase of hepatic inflammation [36, 37]. As a result, several cytokines such as TNF-α from Kupffer cells and hepatocytes, IL-6, and IL-1α are secreted [38, 39]. Both TNF-α and IL-6 are proinflammatory and have been studied specifically with respect to the pathophysiology of liver disease because many of their biological effects parallel the clinical and biochemical abnormalities found in patients with chronic liver disease [40]. The inhibitory effects of CAPE on IL-6 [41], IL-1α, and TNF-α production [42] have been reported previously. In the current study, the levels of IL-6, IL-1α, and TNF-α were significantly elevated due to BDL, while CAPE treatment markedly reduced these cytokine levels. Increased cytokine levels due to cholestasis, which tended to decrease with the treatment of CAPE, also supports the notion that CAPE ameliorates cholestatic liver injury through its antioxidant effect.

CONCLUSIONS

The present study demonstrates that intraperitoneal administration of CAPE maintains antioxidant defenses and reduces liver oxidative and cytokine damage, necro-inflammation (focal necrosis, confluent necrosis, piecemeal necrosis, focal and portal inflammation, and apoptosis) in bile ligated rats. This effect of Cape may be useful to preserve liver function in patients with biliary obstruction. However, more investigations are required to evaluate CAPE's antioxidant, anti-inflammatory, and hepatoprotective effect in clinical and experimental models.

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