Methylprednisolone prevents bacterial translocation in thioacetamide-induced liver failure in rats

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ABSTRACT

Background/Aims: Steroids have been shown to prevent intestinal oxidative stress. We investigated the effects of methylprednisolone on intestinal oxidative damage and bacterial translocation in thioacetamide-induced liver failure in rats.

Materials and Methods: Group 1 (n=8) was the control group. In group 2 (n=8), the thioacetamide group, rats received 300 mg/kg intraperitoneal thioacetamide daily for 2 days. In group 3 (n=8), the thioacetamide+methylprednisolone group, treatment with methylprednisolone (30 mg/kg intraperitoneal) was commenced 48 h before the first dose of thioacetamide. In group 4 (n=8), the methylprednisolone group, the rats received only methylprednisolone (30 mg/kg intraperitoneal).

Results: Serious hepatic and intestinal oxidative damage and high bacterial translocation frequencies were observed in the thioacetamide group compared with those of the controls. Bacterial translocation frequency in the thioacetamide+methylprednisolone group was significantly lower than that in the thioacetamide group ($p<0.05$). Intestinal thiobarbituric acid-reactive substances and myeloperoxidase levels and tissue damage scores for the intestines in the thioacetamide+methylprednisolone group were lower than those in the thioacetamide group ($p<0.01$, $p<0.01$, and $p<0.0001$, respectively).

Conclusion: Our findings suggest that methylprednisolone reduces bacterial translocation by preventing intestinal oxidative damage in this model of acute liver failure in rats.

Keywords: Methylprednisolone, liver failure, bacterial translocation

INTRODUCTION

A large body of work has implicated the translocation of enteric bacterial infections in many serious conditions such as liver failure, hemorrhagic shock, burns, and acute pancreatitis (1-4). Many experimental studies have reported that intestinal oxidative stress plays a key role in the disruption of intestinal barrier function (5-7).

Glucocorticoids are well-known anti-inflammatory agents and are commonly used as therapeutic agents for many acute and chronic inflammatory illnesses such as ulcer-ative colitis, Crohn's disease, and autoimmune hepatitis. Steroids inhibit the expression of a variety of genes involved in the inflammation process, including cytokines and cellular adhesion molecules, and steroids have been reported to protect the intestine from oxidative stress in different models of intestinal damage (8-10).

In this study, we investigated the effects of methylprednisolone (MP) on intestinal oxidative damage and bacterial translocation (BT) in thioacetamide (TAA)-induced liver failure in rats.

MATERIALS AND METHODS

Animals

Healthy male Wistar rats weighing 300-350 g were obtained from Inonu University Animal Laboratory (Malatya, Turkey). They were housed under strictly controlled conditions of humidity, temperature (about 23°C), and illumination (12 h dark:12 h light cycle) and were given food and water ad libitum. The study protocol was in accordance with the guidelines for animal research and was approved by the Ethics Committee of Inonu University Medical School.

Induction of Liver Failure

Liver failure was induced by intraperitoneal (ip) injections of 300 mg/kg TAA (Merck, Darmstadt, Germany) daily for 2 days (11,12). Subcutaneous 5% dextrose (25 mL/kg) and 0.9% NaCl with potassium (20 mEq/L) were given every 6 h as supportive therapy to prevent weight loss, hypoglycemia, and renal failure as described previously (5).

Experimental Design

Thirty-two male Wistar rats were divided into four groups. Group 1 (n=8), the control group, received 0.1 mL of NaCl (0.9%) ip for four consecutive days. In group 2 (n=8), the TAA group, the rats received 300 mg/kg ip TAA daily for two days at intervals of 24 h. In group 3 (n=8), the TAA+MP (Methylprednisolone sodium succinate; Mustafa Nevzat Drug Company, Istanbul, Turkey) group, MP was commenced 48 h before the first dose of TAA, at 30 mg/kg ip daily, and was maintained for 4 consecutive days (13,14). In group 4 (n=8), the MP group, the rats received only ip MP at 30 mg/kg daily for 4 consecutive days.

Tissue Samples

On day 5 of the study, a laparotomy was performed under strictly sterile conditions after the rats had been anesthetized with 50 mg/kg ketamine (Ketolar; Parke-Davis) and 10 mg/kg xylazine HCl (Alfazyne 2%; Alfasan, The Netherlands). After the skin had been sterilized with iodine and shaved, the abdomen was opened widely. The ileocecal mesenteric lymph nodes (MLN), spleen, and left lobe of the liver were aseptically dissected, removed, weighed, and liquefied in sterile saline for bacterial culture. We aspirated 0.1 mL of jejunal fluid with a sterile needle for the assessment of intestinal bacterial overgrowth (IBO). Blood was taken from the right ventricle under sterile conditions for biochemical tests. The liver and ileum were excised for histopathological and biochemical tests.

Liver and Intestinal Histology

For liver histopathological analysis, the right lobes of the livers were processed for light microscopy. This processing consisted of fixing the specimen in a 5% neutral formol solution, embedding it in paraffin, slicing sections of 5 mm thickness, and staining the sections with hematoxylin and eosin and Masson trichrome. The tissue slices were scanned and scored by two ex-

pert pathologists who were blinded to the group assignment. The degree of inflammation and necrosis was expressed as the mean of 10 different fields within each slide and were classified on a scale of 0-3 (normal, 0; mild, 1; moderate, 2; severe, 3) (12). The ileum samples taken from the rats were fixed for 24 h in 5% neutral formol solution. The intestinal segments were divided into pieces of 0.5x0.5x0.5 cm^3 and were processed for routine examination. Intestinal tissues from each animal were obtained in separate blocks. Sections of 6-7 mm were prepared from all tissue samples and examined by light microscopy. A scoring system ranging from 0 (slight) to 3 (severe) was used for intestinal histopathological evaluations. Mucosal integrity, edema, inflammatory cellular infiltration of the lamina propria, and vessel vasodilation were scored accordingly (2).

Biochemical Analyses

Preparation of Serum and Tissue Homogenates

Blood samples of approximately 4 mL were taken from each rat. Each blood sample was used for the separation of serum after transfer into marked centrifuge tubes. These samples were kept at room temperature for 30 min and centrifuged at 3000´g for 10 min. The serum samples obtained in this way were aliquoted for biochemical analysis and were stored at -20°C until use. Liver and intestinal tissues were removed from the rats and washed three times with cold physiological saline. They were then weighed and the wet weight was recorded. The tissues were sliced into approximately 0.5-1 g portions using a lancet, placed in covered plastic cups wrapped with aluminum foil, and stored at -20°C until analysis. Tissue samples taken from the freezer on the day of analysis were weighed after they had been thawed at ambient temperature and then homogenized in a glass-Teflon homogenizer (Tempest Virtishear, model 278069; Virtis, Gardiner, NY, USA) at 5000 rpm for 2 min after the addition of 10 volumes of cold KCL (150 mM). The homogenates were used for thiobarbituric acid-reactive substances (TBARS) analysis on the same day.

Determination of Lipid Peroxidation

The TBARS and other products of lipid peroxidation considered to be indicators of oxidative stress were analyzed using the method described by Buege and Aust in 1978 (15). Briefly, 250 µL of tissue or serum homogenate, 500 µL of thiobarbituric acid (TBA) reactant (3.7 g/L TBA dissolved in 0.25 mol/L HCl), and 1.5 mL of 15% trichloroacetic acid were added to screw-topped Pyrex centrifuge tubes (~10 mL) and mixed. The tubes were placed in a hot water bath at 95°C for 30 min and then immediately cooled under tap water. *n*-Butanol (3 mL) was added to each tube and mixed so that the pink chromogen separated into the butanol phase. The absorbance of the colored organic phase was read against a blank at 535 nm. A calibration curve was prepared with 1,1,3,3-Tetramethoxypropane and was used to calculate the serum and tissue levels of TBARS. Serum levels were expressed as nmol/L and tissue levels as nmol/g wet tissue.

Serum Aspartate Transaminase (AST) and Alanine Transaminase (ALT) Levels

Serum levels of AST and ALT were measured using a kinetic ultraviolet method as defined by the International Federation of Clinical Chemistry, with pyridoxal phosphate and NADH as the cofactors. An Olympus autoanalyzer and commercial kits of the same brand were used for the two analyses in the routine biochemistry laboratories of Turgut Ozal Medical Center (Malatya, Turkey). AST and ALT levels were expressed as U/L.

Determination of Methylperoxidase (MPO)

The tissue samples from the liver and small intestine, previously prepared and stored at -20°C, were thawed, weighed, suspended (10% w/v) in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, and homogenized. The homogenate was sonicated three times for 10 s each (Bandelin Electronic, Sonopuls GM 750, Berlin, Germany). After sonication, the specimens were freeze-thawed three times and microcentrifuged at 14,000´g for 15 min at 4°C. The supernatants were used for MPO analysis.

The MPO assay was commenced by mixing 0.1 mL of tissue material with 2.9 mL of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/mL o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide (16). The reaction was carried out at room temperature for about 5 min. The absorbance change at 460 nm was recorded kinetically with an LKB-UV spectrophotometer (LKB Biochrom Ultraspec Plus, Cambridge, England) at 1 min intervals. One unit of MPO activity was defined as that required to degrade one micromole of peroxide per minute at 25°C. Protein concentrations in the supernatants were measured using Bradford's method (17). MPO activity was expressed as U/g protein.

Reduced Glutathione (GSH) Assay

Reduced glutathione was measured in the tissue homogenates with the method of Elman (18). A total volume of 250 mL of 10% trichloroacetic acid was added to 250 mL of tissue homogenate containing 6 mM disodium EDTA. The mixture was vortexed vigorously for 2 min, followed by centrifugation at 3000´g for 10 min. An aliquot (0.2 mL) of the supernatant was mixed with 1.7 mL of 0.1 M potassium phosphate buffer (pH 7.5), and 0.1 mL of 1 mM Ellman's reagent (5,5¢-dithio-bis-[2-nitrobenzoic acid]) was added to each tube. After 5 min incubation at room temperature, the optical density was measured spectrophotometrically at 412 nm against a reagent blank. The results were expressed as mmol/g wet tissue.

Microbiological Studies

Bacterial translocation and Intestinal Bacterial Overgrowth

Bacterial translocation was defined as positive bacterial cultures in MLN, liver, or spleen samples. From each animal group, 1 g tissue samples (liver, spleen, and MLN) were

homogenized in 1 mL of saline. Aliquots of this homogenized solution were then inoculated onto blood agar, eosin methylene blue agar (EMB agar), and Sabouraud agar and incubated at 37°C for 24 h. On the following day, the bacterial growth was checked and the colonies were counted. Gram staining was carried out for each colony. According to the Gram stain results, specific biochemical tests, including those for catalase, oxidase, coagulase, indole, citrate reduction, urease, and sugar fermentation on triple sugar iron agar, were carried out for primary identification of the isolated bacteria. An Api 20 E test (bioMereux, Marcy L'Etoile, France) was used to confirm the identification. To grow yeasts, the Remel RapID™ Yeast Plus System 8311007 (Remel Inc.; Lenex, KS, USA) was used.

The IBO of a specific organism was defined as a bacterial count in the ileal aspirate above the mean+two standard deviations (SD) for the same organism in normal rats. Aliquots of the ileal aspirate (0.1 mL) were inoculated onto blood agar, EMB agar, and Sabouraud agar and incubated at 37 °C for 24 h. The bacterial growth was checked on the following day and the colonies were counted. Colony counts for ileal aspirates are presented as ´10^3 colony-forming units per mL of ileal aspirate (19).

Survival Rates

Mortality counts in the four groups of rats were recorded daily following the first dose of TAA. The survival rates were determined based on the numbers of live rats on the day of sacrifice.

Statistical Analyses

Results are expressed as means±SD. Comparisons of quantitative variables among the groups were made with one-way analysis of variance or the corresponding nonparametric test (Kruskal-Wallis test) as required. Post-hoc comparisons were made with the LSD test. Proportion comparisons were made using the χ^2 test or Fisher's exact test. For all comparisons, a statistically significant difference was defined as $p < 0.05$.

RESULTS

Mortality and Survival Rates

The survival rate in the TAA group (87.5%) on the day of sacrifice was lower than in the control, TAA+MP, and MP groups (all 100%), but this difference was not statistically significant ($p > 0.05$).

Serum AST, ALT, and TBARS Levels

Serum AST and ALT levels in the TAA group were significantly higher than those in the control and MP groups (all $p < 0.01$; Table 1). No significant differences were observed in the serum AST or ALT levels of the TAA+MP group and TAA group ($p > 0.05$). The mean serum TBARS level in the TAA group was significantly higher than the levels in the other groups (all $p < 0.0001$; Table 1).

Tissue Oxidative Stress Results

Liver and intestinal TBARS levels in the TAA group were significantly higher than the levels in the control and MP groups (p<0.05 and p<0.0001, respectively; Table 2). No significant difference was observed in the liver TBARS levels of the TAA and TAA+MP groups (p>0.05), but the mean intestinal TBARS level in the TAA group was significantly higher than that in the TAA+MP group (p<0.01; Table 2). Liver and intestinal GSH levels in the TAA group were significantly lower than those in the control and MP groups (all p<0.0001; Table 2). No significant difference was observed in the liver GSH levels of the TAA and TAA+MP groups (p>0.05), but the mean intestinal GSH level of the TAA group was significantly lower than that of the TAA+MP

group (p<0.05; Table 2). Liver and intestinal MPO levels in the TAA group were significantly higher than those in the control and MP groups (p<0.0001 and p<0.01, respectively; Table 2). Liver and intestinal MPO levels in the TAA+MP group were significantly lower than those in the TAA group (p<0.05 and p<0.01, respectively; Table 2).

Liver and Intestinal Histology

Liver necrosis and inflammation scores were significantly higher in the TAA group than those in the control and MP groups (all p<0.0001; Figure 1a, b). No significant differences were observed in these scores between the TAA and TAA+MP groups (p>0.05; Figure 1c, Table 3). In the evaluations of intestinal histology, the scores for edema, inflammatory cellular infiltration of the lamina propria, and vessel vasodilation were significantly higher in the TAA group than those in the other groups (all p<0.0001; Table 3, Figure 2a-c). No significant differences were observed in the mucosal integrity score of the TAA group and the scores of the other groups (p>0.05).

Intestinal Bacterial Overgrowth Results and BT Frequency

The BT frequency in the TAA group was significantly higher than that in the control and MP group (χ^2 test, p<0.01), and the BT frequency in the TAA+MP group was significantly lower than that in the TAA group (Fisher test, p<0.05; Figure 3). The counts of *Escherichia coli* grown in the ileal aspirate were sig-
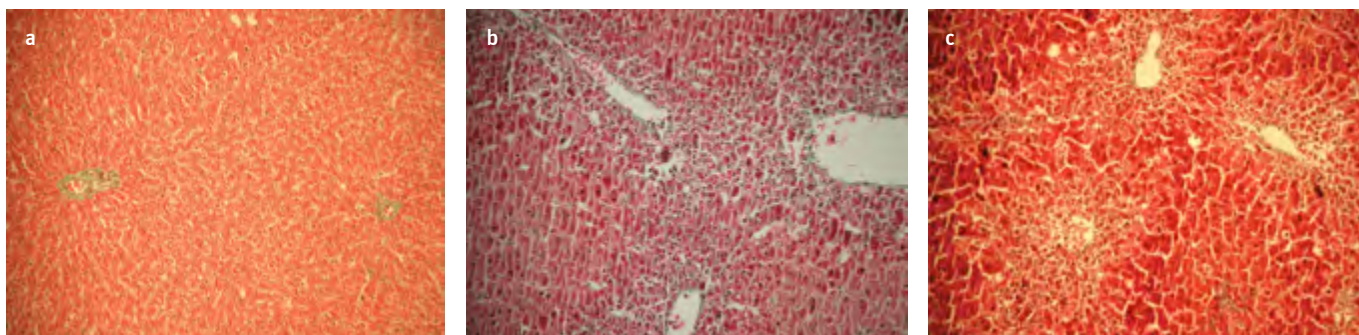
Table 1. Mean±SD values for serum aspartate transaminase (AST), alanine transaminase (ALT), and thiobarbituric acid-reactive substances (sTBARS) by group

Group	ALT (U/L)	AST (U/L)	sTBARS (nmol/L)
Control (n=8)	63.38±10.96	141.63±25.36	137.88±15.33
TAA (n=7)	6231.20±1921.15*	11876.00±2442.62*	199.57±36.45**
TAA+MP (n=8)	5451.13±1352.10	9275.63±5060.47	130.63±32.11
MP (n=8)	53.13±10.09	98.63±25.12	126.13±16.98

TAA: thioacetamide; TAA+MP: thioacetamide+methylprednisolone; MP: methylprednisolone
*p<0.01 compared with the control and MP groups
**p<0.0001 compared with the other groups

Table 2. Mean±SD values for liver thiobarbituric acid-reactive substances (lTBARS), reduced glutathione (lGSH), and myeloperoxidase (lMPO) and intestinal thiobarbituric acid-reactive substances (iTBARS), reduced glutathione (iGSH), and myeloperoxidase (iMPO) by group

Group	lTBARS (nmol/g wet tissue)	lGSH (μmol/g wet tissue)	lMPO (units/g protein)	iTBARS (nmol/g wet tissue)	iGSH (μmol/g wet tissue)	iMPO (units/g protein)
Control (n=8)	188.38±27.93	5.73±0.30	1.52±0.58	208.00±88.01	2.03±0.45	74.86±37.73
TAA (n=7)	335.14±143.27*	3.65±0.54**	31.40±12.32**	490.29±136.58**	0.81±0.15**	165.71±72.90*****
TAA+MP (n=8)	296.63±145.75	3.93±0.67	22.65±7.41***	342.38±100.51****	1.25±0.30***	75.75±28.83
MP (n=8)	199.13±40.97	5.12±0.58	3.38±1.66	256±60.63	1.45±0.21	77.57±47.28

TAA: thioacetamide; TAA+ MP: thioacetamide+methylprednisolone; MP: methylprednisolone
*p<0.05 compared with the control and MP groups
**p<0.0001 compared with the control and MP groups
***p<0.05 compared with the TAA group
****p<0.01 compared with the TAA group
*****p<0.01 compared with the other groups

Table 3. Mean±SD scores for hepatic necrosis and inflammation, mucosal integrity, edema, inflammatory cellular infiltration of the lamina propria, and vessel vasodilation in the intestine by group

Group	Liver			Intestines		
	Necrosis	Inflammation	MI	Edema	INF	VV
Control (n=8)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
TAA (n=7)	2.86±0.37*	3.14±0.69*	0.00±0.00	1.29±0.48**	1.14±0.37**	1.14±0.37**
TAA+MP (n=8)	2.50±0.53	3.13±0.64	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
MP (n=8)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

TAA: thioacetamide; TAA+MP: thioacetamide+methylprednisolone; MP: methylprednisolone; MI: mucosal integrity; INF: inflammatory cellular infiltration of the lamina propria; VV: vessel vasodilation
*p<0.0001 compared with the control and MP groups
**p<0.0001 compared with the other groups

Figure 1. a-c. Liver histology in the control group (a), TAA group (b), and TAA+MP group (c) (Masson trichrome, ×100). Widespread severe zonal necrosis was noted in Figure 1b and 1c
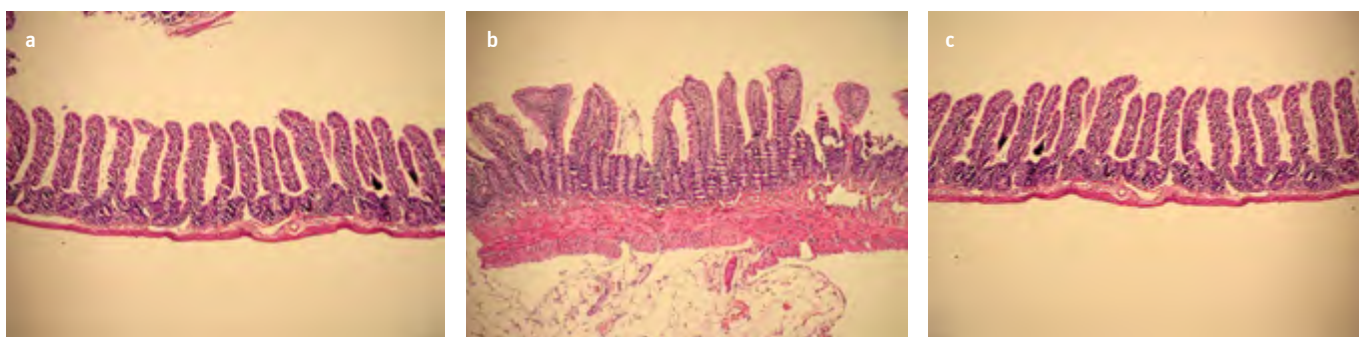

Figure 2. a-c. Intestinal histology of the control group (a), the TAA group (b), and the TAA+MP group (c) (hematoxylin and eosin, × 100). Edema and inflammatory cellular infiltration were noted in Figure 2b
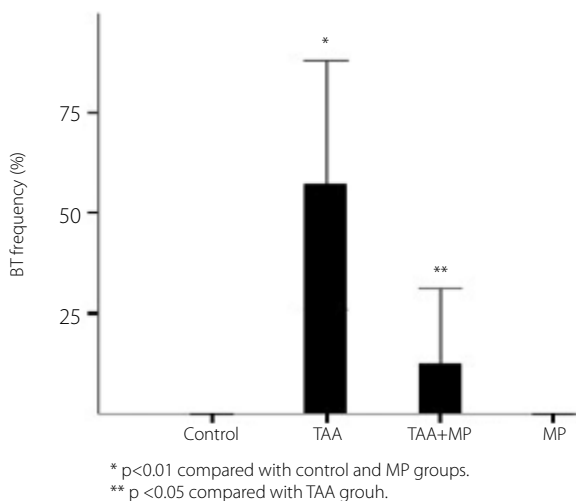

* p<0.01 compared with control and MP groups.
** p <0.05 compared with TAA grouh.

Figure 3. Bacterial translocation frequency (%) by group

Table 4. *Escherichia coli* (×10³ CFU/mL) in ileal aspirates and frequency of bacterial translocation (BT) by group

Group	*Escherichia coli* count	BT frequency (%)
Control (n=8)	2.87±3.09	0
TAA[†] (n=7)	35.71±43.91*	57.1*
TAA+MP [‡] (n=8)	32.50±41.91	12.5**
MP[§] (n=8)	6.87±6.85	0

TAA: Thioacetamide; TAA+ MP: thioacetamide+methylprednisolone; MP: methylpredniso-lone; BT: bacterial translocation
*p<0.01 compared with the control
**p<0.05 compared with the TAA group

nificantly higher in the TAA group than those in the control and MP groups (p<0.01; Table 4). There were no significant differences in the *E. coli* counts of the TAA and TAA+MP groups (p>0.05).

DISCUSSION

In this study, liver failure was successfully induced with the administration of TAA. Increased intestinal oxidative damage and increased BT frequency accompanied this liver failure in the TAA group. MP treatment clearly ameliorated this intestinal oxidative damage and lowered the BT frequency.

It has been proposed that intestinal oxidative stress plays an important role in the BT process by disrupting the intestinal barrier function, and several studies have reported that antioxidant treatments reduce BT by preventing intestinal oxidative injury (6,20,21). It has also been reported that steroids prevent intestinal oxidative damage (9,10). However, little work has been performed to investigate the effects of steroids on BT. Kirimlioglu et al. (9) reported that MP treatment improved intestinal oxidative biochemical parameters (malondialdehyde and GSH) but increased intestinal histological damage and BT rates in a temporary liver inflow occlusion model in rats (9). Alverdy et al. (22) also reported that glucocorticoids might promote BT by the impairment of mucosal IgA synthesis. Choudhry et al. (23) reported that endogenous corticosterone does not directly alter the intestinal barrier function, but does upregulate interleukin-18 expression, which then directly or indirectly contributes to an impaired intestinal barrier function in a rodent

model of acute alcohol intoxication and burn injury. In contrast to these studies, Wenzl et al. (24) reported that prenatal cortisone treatment reduced BT in neonatal rats. Wan et al. (25) reported that MP pretreatment reduced blood endotoxin levels during cardiopulmonary bypass.

Until now, there has been no study of the effects of MP on intestinal oxidative damage and BT in a TAA-induced model of liver failure. In this study, we observed that MP treatment reduced the intestinal oxidative damage and BT frequency in TAA-induced liver failure. It has been postulated that proinflammatory cytokines, oxidants, and reactive nitrogen moieties play important roles in the process of BT by causing the disruption of the gut barrier (26). These mediators are released by inflammatory cells into the milieu secondary to inflammation. Steroids reduce the migration of inflammatory cells to the sites of injury, and glucocorticoids are believed to inhibit arachidonic acid metabolism and the activation of complement and vasoactive substances and to prevent neutrophilic chemotaxis by affecting intercellular adhesion molecules (27). In this study, MP reduced intestinal MPO levels (p<0.01), which are used as an index of neutrophil infiltration. MP treatment also significantly reduced the levels of intestinal TBARS (p<0.01).

Reduced glutathione is an important intracellular protective mechanism against various noxious stimuli, including oxidative stress, and we found that MP treatment restored the intestinal GSH levels after TAA-induced liver failure. Intestinal damage scores were significantly lower in the TAA+MP group compared to the TAA group (p<0.0001), and our results are consistent with studies demonstrating that steroids prevent intestinal oxidative damage (9,10,28). These findings suggest that the amelioration of intestinal oxidative damage played an important role in reducing the BT frequency in the TAA+MP group in this model of acute liver failure in rats.

Many cytokines act as oxidants and have direct toxic effects on the intestinal barrier, and recent studies have suggested that glucocorticoids reduce the expression of the cytokines that are released by inflammatory cells into the milieu secondary to inflammation (27,29). We did not evaluate cytokine levels in the intestinal wall in this study; however, we observed that MP treatment reduced intestinal inflammation and MPO levels. Therefore, our findings suggest that MP treatment probably protects the intestine against the direct toxic effects of the cytokines produced by inflammatory cells. In conclusion, our findings suggest that MP reduces BT by preventing intestinal oxidative damage in this model of acute liver failure in rats.

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