The effects of *Gingko biloba*, vitamin E and melatonin on bacterial translocation in thioacetamide-induced fulminant hepatic failure in rats

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Abstract

Background and study aims : **Bacterial translocation (BT) has been implicated in the development of infectious complications in many serious clinical conditions such as fulminant hepatic failure (FHF). We aimed to investigate the effects of *Gingko biloba* (GB), vitamin E (Vit E) and melatonin on intestinal oxidative damage and BT in thioacetamide (TAA)-induced FHF in rats.**

Materials and methods : **A total of 42 rats were divided into five groups. Group 1 (n = 8) was the control group. Group 2 (n = 10) was the TAA group, in which rats received 350 mg/kg TAA daily by the intraperitoneal (ip) route for 3 days. Oral 100 mg/kg GB per day was administered to group 3 (n = 8), oral 200 mg/kg Vit E per day to group 4 (n = 8) and ip 3 mg/kg melatonin per day to group 5 (n = 8) 48 h prior to the first TAA injection and was continued for 5 consecutive days.**

Results : **When compared with the control group, serious hepatic and intestinal oxidative damage, increased *Escherichia coli* counts in ileal aspirates and high BT frequencies were observed in the TAA group (all $p < 0.0001$). Only GB treatment attenuated hepatic oxidative damage ($p < 0.0001$). There was no difference in intestinal oxidative damage, *E. coli* counts in ileal aspirates and BT frequency between TAA and the other antioxidant treatment groups ($p > 0.05$).**

Conclusion : **Our results suggest that intestinal oxidative damage plays a major role in the development of BT by disrupting the barrier function of intestinal mucosa.** (Acta gastroenterol. belg., **2006**, 69, **268-275**).

Key words : bacterial translocation, fulminant hepatic failure, oxidative stress, thioacetamide, antioxidants, Platelet-activating factor.

Introduction

In critically ill patients, bacteremia, sepsis, or multiple organ failure syndrome (MOFS) are frequently present. No identifiable focal point of infection has been detected in these conditions (1). Infection causes in these cases are commonly Gram-negative bacteria originating from patients' own intestinal flora (2,3). Translocation of bacteria and their products from the gut to extraintestinal sites may explain infectious complications frequently observed in many serious clinical conditions, including fulminant hepatic failure (FHF) (4).

A common feature in clinical conditions such as burns, surgical stress and MOFS is intestinal hypoperfusion, which increases the production of reactive oxygen species (ROS) in the intestine. This leads to overproduction of nitric oxide, direct lipid peroxidation and the release of proinflammatory cytokines, causing leukocyte infiltration in the intestinal wall. All these effects lead to intestinal barrier dysfunction by creating intestinal damage (1,5-7). It has been shown that intestinal oxidative stress plays an important role in bacterial translocation (BT) by causing intestinal barrier dysfunction via the same mechanism in experimental portal hypertension (8). Kasravi *et al.* reported that intestinal hypoperfusion facilitates BT by causing intestinal barrier dysfunction in D-galactosamine-induced liver failure (9). Platelet-activating factor (PAF) is another factor that plays a role in intestinal inflammation and damage. It has been shown that PAF is an important mediator in inflammation and tissue damage in liver and intestine, and this inflammation can be reduced with PAF antagonists (10,11). It has also been shown that PAF antagonists reduce BT by preventing intestinal barrier dysfunction in acute pancreatitis and intestinal ischemia/reperfusion models (12,13).

Gingko biloba (GB) is a well-tolerated plant extract used in traditional Chinese medicine. It has antioxidant and PAF antagonistic effects (14). It has been reported that GB prevents oxidative damage in liver and intestine via its antioxidant effect (15,16).

Vitamin E (Vit E) is the name given to a family of eight molecules, each consisting of a chromanol ring with an aliphatic side chain. The a-isoform (a-tocopherol) is predominant in Vit E supplements. a-Tocopherol functions as a very powerful scavenger of ROS and reactive nitrogen species (RNS) (17). Melatonin, which is synthesized in the pineal gland and other tissues, has a variety of physiological, immunological, and biochemical functions. It is a direct scavenger of free radicals and has indirect antioxidant effects owing to its stimulation of the expression and activity of antioxidative enzymes such as glutathione peroxidase, superoxide dismutase and catalase, and nitric oxide synthase (18).

Thiobarbituric acid-reactive substances (TBARS) are the end products of lipid peroxidation processes and are widely used for the assessment of oxidative stress (19).

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In our study, serum, liver and intestine TBARS levels were measured to assess oxidative stress. Thioacetamide (TAA) is a hepatotoxin frequently used to induce FHF in rats. TAA inflicts tissue damage by increasing ROS, thus directly promoting the peroxidation of membrane lipids (20).

No study to date has reported the effects of GB, Vit E and melatonin on BT in TAA-induced FHF in rats. We aimed to investigate the effects of GB, Vit E and melatonin on intestinal oxidative damage and BT in TAA-induced FHF in rats.

Materials and methods

Animals

Healthy male Wistar rats weighing 200-250 g were used in this study. Animals were housed on a 12-h light/dark cycle (lights on from 08 :00 h) at a constant ambient temperature (24 ± 1 °C) with normal rat chow and water available ad libitum. The study protocol was in accordance with the guidelines for animal research and was approved by the Ethics Committee of our hospital.

Induction of fulminant hepatic failure

As previously described, FHF was induced by intraperitoneal (ip) injection of 350 mg/kg TAA (Merck, Germany) daily for 3 days. Supportive therapy of subcutaneous administration of 5% dextrose (25 ml/kg) and NaCl 0.9% with potassium (20 mEq/l) every 6 h was given to avoid weight loss, hypoglycemia and renal failure, as previously described (21).

Experimental design

A total of 42 male Wistar rats were divided into five groups. Group 1 (n = 8) was the control group, which received 0.1 ml of NaCl (0.9%) ip daily for 5 days. Group 2 (n = 10) was the TAA group, in which rats received 350 mg/kg TAA ip daily for 3 days at intervals of 24 h. Group 3 (n = 8) was the TAA+GB group. GB (supplied as a dry powder, obtained from TriPharma Drug Company, Turkey) was orally administered at 100 mg/kg daily 48 h before the first dose of TAA and was maintained for 5 consecutive days. The dose and treatment duration were chosen according to previous studies on GB (22,23). Group 4 (n = 8) was the TAA+Vit E group. Vit E (a-tocopheryl acetate ; Sigma, St. Louis, MO, USA) was orally administered at 200 mg/kg daily 48 h before the first dose of TAA and was maintained for 5 consecutive days. The dose and treatment duration were chosen according to a previous study on Vit E (24). Group 5 (n = 8) was the TAA+melatonin group. Melatonin (Sigma) was dissolved in ethanol and diluted in saline to give a final concentration of 5% ethanol. It was administered by the ip route at 3 mg/kg daily 48 h prior to the first TAA injection and was continued for 5 consecutive days. The dose and treatment duration were chosen according to a previous study on melatonin (21).

Tissue samples

On day 6 of the study, laparotomy was performed under anesthesia with 50 mg/kg ketamine (Ketolar, Parke-Davis) and 10 mg/kg xylazine HCl (Alfazyne 2%, Alfasan, Netherlands) under strictly sterile conditions. After the skin was sterilized with iodine and shaved, the abdomen was opened wide. The mesenteric lymph nodes (MLN) from the ileo-cecal area and the spleen were aseptically dissected, removed, weighed, and then liquefied in sterile saline for bacterial culture. The liver was aseptically dissected and the left lobe was used for bacterial cultures. The right lobe was excised for histopathological and biochemical examinations (TBARS). We aspirated 0.1 ml of ileal fluid with a sterile needle for assessment of intestinal bacterial overgrowth (IBO). Blood was taken from the right ventricle for biochemical determinations (ammonia, aspartate transaminase (AST), alanine transaminase (ALT) and TBARS). The ileum was removed for histopathological and biochemical examinations (TBARS).

Liver and intestinal histology

For liver histopathological analysis, midsections of the right lobes of the liver were processed for light microscopy. This processing consisted of fixing the specimen in a 5% neutral formol solution, embedding the specimens in paraffin, slicing sections of 5 mm in thickness, and staining the sections with hematoxylin and eosin. The tissue slices were scanned and scored by two expert pathologists who were not aware of sample assignment to experimental groups. The degree of inflammation and necrosis is expressed as the mean of 10 different fields within each slide, classified on a scale of 0-3 (normal, 0 ; mild, 1 ; moderate, 2 ; severe, 3) (21).

The ileum samples taken from rats were fixed for 24 h in 5% neutral formol solution. The intestinal segments were divided into pieces of 0.5 × 0.5 × 0.5 cm 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. For intestinal histopathological evaluations, a scoring system ranging from 0 (slight) to 3 (severe) was used. Mucosal integrity, edema, inflammatory cellular infiltration in the lamina propria and vessel vasodilation were scored accordingly (25).

Biochemical analyses

Preparation of serum and tissue homogenates

Blood samples of approximately 4 ml were taken from each rat. A 1 ml aliquot from each blood sample was transferred to centrifuge tubes containing 1 ml of 10% TCA for deproteinization and centrifuged at 3000 *g*

for 10 min following mixing. The clear supernatant at the top of the tube was used for ammonia analysis. The remaining 3 ml of each blood sample was used for separation of serum following transfer into marked centrifuge tubes. These samples were kept at room temperature for 30 min and centrifuged at 3000 *g* for 10 min. Serum samples obtained in this way were aliquoted for use for biochemical analyses and were stored at -20 °C until analysis. Liver and ileum were removed from rats following sacrifice and washed with cold physiological saline (0.9% NaCl) three times. Then they were weighed and the wet weight was recorded. Tissues were sliced to 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 out of the freezer on the day of analysis were weighed after they had been thawed at ambient temperature and were homogenized in a glass-Teflon homogenizer (Tempest Virtishear, model 278069 ; Virtis, Gardiner, NY, USA) at 5000 rev/min for 2 min after adding 10 volumes of cold KCl (150 mM). The homogenates were used for TBARS analysis on the same day.

Blood ammonia analysis

Blood levels of ammonia were analyzed using the colorimetric Berthelot (indophenol) reaction (26). In this reaction, free ammonia in previously deproteinized samples generates a blue-colored indophenol molecule by reacting with sodium nitroprusside in an alkaline environment containing phenol and hypochlorite ions. The color intensity of the chromogen, spectrophotometrically measured against blank samples at 625 nm, increases in direct proportion to the concentration of ammonia. $(NH_4)_2SO_4$ was used as a standard. Results are expressed in mg ammonia nitrogen/dl.

Determination of lipid peroxidation

Analysis of TBARS and other products of lipid peroxidation considered to be indicators of oxidative stress was carried out using the method defined by Buege and Aust in 1978 (27). Briefly, 250 ml of tissue or serum homogenate, 500 ml of TBA reactant (3.7 g/l thiobarbituric acid dissolved in 0.25 mol/l HCl) and 1.5 ml of 15% TCA were added into screw-topped Pyrex centrifuge tubes (~10 ml) and mixed. Tubes were placed in a hot-water bath at 95 °C for 30 min and were then immediately cooled in 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. 1,1,3,3-tetramethoxypropane (TMOP) was used to prepare a calibration curve from which serum and tissue levels of TBARS were calculated. Serum levels are expressed as nmol/l and tissue levels as nmol/g wet tissue.

Serum AST and ALT levels

Serum levels of AST and ALT were measured using a kinetic UV method defined by the International Federation of Clinical Chemistry (IFCC) using pyridoxal phosphate and NADH as co-factors. An Olympus auto-analyzer and commercial kits of the same brand were used for two analyses in the routine biochemistry laboratories of Turgut Ozal Medical Center. AST and ALT levels are expressed as U/l.

Microbiological studies

Bacterial translocation

Bacterial translocation was defined as the positivity of cultures of mesenteric lymph nodes, liver or spleen. From each animal group, 1 g tissue samples (liver, spleen, and mesenteric lymph nodes) were homogenized in 1 ml of normal saline. Then 100 ml was aspirated and inoculated into blood agar, eosin methylene blue (EMB) agar, and Sabouraud agar and incubated at 37 °C for 24 h. On the following day, bacterial growth was checked and colonies were counted. Gram staining was carried out for each colony. According to the Gram stain results, specific biochemical tests, including catalase, oxidase, coagulase, indole, citrate reduction, urease, sugar fermentation on triple sugar iron agar, were carried out for primary identification of the bacteria isolated. An Api 20 E test (bio-Merieux, Marcy L'Etoile, France) was used for confirmation of identification. For growing yeasts, a Remel Rapid™ Yeast plus System 8311007 (Remel Inc., Lenex, KS, USA) was used.

Assessment of intestinal bacterial overgrowth

Intestinal aerobic bacterial overgrowth of a specific organism was defined as a bacterial count in ileal aspirate higher than mean + 2SD for the same organism in normal rats. Ileal aspirate (0.1 ml) was inoculated into blood agar, EMB agar, and Sabouraud agar and incubated at 37 °C for 24 h. On the following day, bacterial growth was checked and colonies were counted. Colony counts for ileal aspirates are presented as 10^3 colony-forming units (CFU) per ml of ileal aspirate (median and range) (28).

Survival rates

Mortality counts in groups were recorded daily following the first dose of TAA. Survival rates were determined according to the number of live rats on the day of sacrifice.

Statistical analyses

Results are expressed as mean ± SD. Comparisons of quantitative variables among groups were made with the one-way ANOVA or the corresponding non-parametric test (Kruskal-Wallis), as required. Post-hoc comparisons were performed with the LSD test. Proportion comparisons were carried out using the χ^2 or Fisher 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 on the day of sacrifice was lower than in the control group, but this difference was not significant ($p > 0.05$). When TAA+GB, TAA+Vit E and TAA+Mel groups were compared with TAA group, no significant difference was found for survival rates ($p > 0.05$).

Liver enzymes

Serum ammonia, AST and ALT levels in the TAA group were significantly higher than in the control group (all $p < 0.0001$) (Table 1). Serum ammonia, AST and ALT levels in the TAA+GB group were significantly lower than in the TAA group (all $p < 0.0001$). No significant difference was observed in the TAA+Vit E and TAA+Mel groups compared with the TAA group for serum ammonia, AST and ALT levels ($p > 0.05$).

Oxidative stress results

Serum, liver and intestine TBARS levels in the TAA group were significantly higher than in the control group (all $p < 0.0001$) (Table 1). Serum and liver TBARS levels in the TAA+GB group were significantly lower than in the TAA group (both $p < 0.001$) (Fig. 1), but there was no significant difference for intestine TBARS levels ($p > 0.05$). No significant difference was found for serum, liver and intestine TBARS levels between the TAA group and the other groups (TAA + Vit E and TAA + Mel) ($p > 0.05$).

Liver and intestine histology

Liver necrosis and inflammation scores were significantly higher in the TAA group than in controls (both $p < 0.0001$) (Table 2). Liver necrosis and inflammation scores were significantly lower in the TAA + GB group than in the TAA group (both $p < 0.0001$). No significant

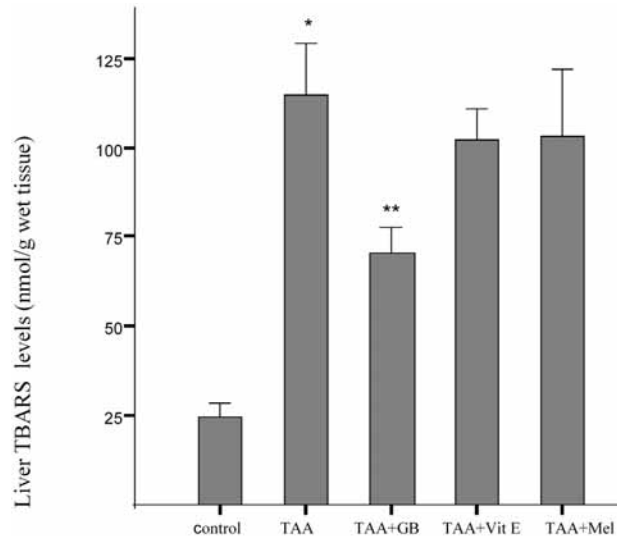

Fig. 1. — Liver TBARS levels by group

difference was observed for these scores between the TAA group and the other groups (TAA+Vit E and TAA+Mel) ($p > 0.05$). Scores for mucosal integrity, edema, inflammatory cellular infiltration in the lamina propria, and vessel vasodilation were significantly higher in the TAA group than in the control group (all $p < 0.0001$) (Table 2). There were no significant differences for these scores between the TAA group and the antioxidant treatment groups ($p > 0.05$).

IBO and BT

Counts of *E. coli* grown in ileal aspirate were significantly higher in the TAA group than in controls ($p < 0.0001$) (Table 3). There was no significant difference for *E. coli* counts between the TAA group and the antioxidant treatment groups ($p > 0.05$).

BT frequency in the TAA group was significantly higher than in the control group (χ^2 test, $p < 0.0001$). BT frequencies in the TAA+GB and TAA+Mel groups were

Table 1. — **Mean ± SD values for serum ammonia, AST, ALT, serum (sTBARS), liver (lTBARS) and intestine (iTBARS) TBARS by group**

Group	Ammonia[1]	AST[2]	ALT[2]	sTBARS[3]	lTBARS[4]	iTBARS[4]
Control (n = 8)	21,75 ± 6,02	150,75 ± 40,93	58,00 ± 12,05	21,00 ± 3,42	24,75 ± 4,62	41,38 ± 9,53
TAA (n = 9)	145,22 ± 14,49[a]	1767,44 ± 174,91[a]	1165,67 ± 167,40[a]	76,56 ± 19,93[a]	114,78 ± 18,46[a]	127,33 ± 16,49[a]
TAA + GB (n = 7)	87,57 ± 12,03[b]	1018,57 ± 201,81[b]	687,00 ± 153,48[b]	27,00 ± 2,16[c]	70,43 ± 7,80[c]	117,86 ± 11,00
TAA + Vit E (n = 7)	137,29 ± 15,83	1602,71 ± 295,73	1065,86 ± 60,42	68,71 ± 7,29	102,29 ± 9,19	122,86 ± 11,53
TAA + Mel (n = 8)	135,13 ± 12,23	1601,88 ± 227,68	1068,38 ± 121,36	68,13 ± 8,70	103,00 ± 22,67	122,88 ± 10,42

[1] Serum ammonia is expressed in mg ammonia nitrogen/dl.
[2] AST and ALT are expressed in U/l.
[3] Serum TBARS (sTBARS) level is expressed in nmol/l.
[4] Liver TBARS (lTBARS) and intestine TBARS (iTBARS) levels are expressed in nmol/gr wet tissue.
[a] $p < 0.0001$ compared with control.
[b] $p < 0.0001$ compared with TAA group.
[c] $p < 0.001$ compared with TAA group.

Table 2. — **Mean ± SD scores for hepatic necrosis and inflammation, and mucosal integrity, edema, inflammatory cellular infiltration in the lamina propria, and vessel vasodilation in intestines by group**

	Liver		Intestines			
Group	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 = 9)	2,78 ± 0,44[a]	2,78 ± 0,44[a]	2,67 ± 0,50[a]	2,89 ± 0,33[a]	2,89 ± 0,33[a]	2,67 ± 0,50[a]
TAA + GB (n = 7)	1,57 ± 0,53[b]	1,57 ± 0,53[b]	2,57 ± 0,53	2,57 ± 053	2,71 ± 0,48	2,43 ± 0,78
TAA + Vit E (n = 7)	2,43 ± 0,53	2,29 ± 0,48	2,43 ± 0,53	2,86 ± 0,37	2,71 ± 0,48	2,29 ± 0,48
TAA + Mel (n = 8)	2,50 ± 0,53	2,38 ± 0,51	2,50 ± 0,53	2,75 ± 0,46	2,75 ± 0,46	2,38 ± 0,51

[a] $p < 0.0001$ compared with control.
[b] $p < 0.0001$ compared with TAA group.

MI : Mucosal integrity, INF : Inflammatory cellular infiltration in the lamina propria, VV : Vessel vasodilation.

Table 3. — ***E. coli*** **(as 10^3 CFU/ml) in ileal aspirates and frequency of bacterial translocation by group**

Group	*Eschericia coli* count	BT frequency (%)
Control(n = 8)	9,06 ± 2,52	0 (%0)
TAA (n = 9)	177,77 ± 44,09[a]	9 (%100)[a]
TAA + GB (n = 7)	164,28 ± 47,55	5 (%71.4)
TAA + Vit E (n = 7)	171,42 ± 39,33	7 (%100)
TAA + Mel (n = 8)	168,75 ± 26,42	6 (%75)

[a] $p < 0.0001$ compared with control.

lower than in the TAA group, but these differences were not significant (Fisher test, $p > 0.05$). There was no difference in BT frequency between the TAA and TAA + Vit E groups. In all groups, the bacterial species translocated to MLN, spleen and liver was *E. coli*.

Discussion

In our study, hepatic failure was successfully induced with ip TAA administration (350 mg/kg daily for 3 days). We observed serious oxidative tissue damage (Figs. 2b and 3b), with high hepatic and intestinal TBARS levels in the TAA group. In addition, high BT frequencies accompanied the severe oxidative tissue damage. GB treatment significantly improved hepatic biochemical and histological parameters. There was no improvement in intestinal biochemical and histological parameters with the administration of GB, Vit E or melatonin. Also in all three groups, high BT frequencies were observed along with serious intestinal oxidative injury, similar to the findings in the TAA group.

Under normal conditions, the intestinal mucosa functions as a local defense barrier to prevent bacteria and their products contained within the intestinal lumen from escaping and spreading to extraluminal tissues. It has been reported that normal intestinal barrier function is disrupted in serious clinical conditions such as burns, surgical stress, sepsis and multi-organ failure (1,5-7). It has been proposed that intestinal oxidative stress plays an important role in the development of BT process (8). Increased intestinal oxidative stress impairs cellular homeostasis and leads to the release of many proinflammatory cytokines, causing leukocyte infiltration in the intestinal wall. All these effects lead to intestinal barrier dysfunction by creating damage in the intestine (1,8,29). In our study, severe intestinal oxidative damage developed in the TAA group and was accompanied by high BT frequency. These findings are in agreement with previous studies (8,9) suggesting that intestinal oxidative stress plays an important role in the development of the BT process.

GB treatment (100 mg/kg daily) decreased BT frequency from 100% (TAA group) to 71.4%, but this difference was not statistically significant. In contrast to Vit E and melatonin treatments, GB treatment clearly improved hepatic damage (Fig. 2c) and significantly decreased liver TBARS levels. GB has antioxidant and PAF antagonistic effects because it contains flavone glucosides and terpenoids, and it is a well-tolerated plant extract (14). It has been reported that GB prevents oxidative tissue damage in many tissues such as liver, intestine and brain by its antioxidant actions (15,16,22,23). Our results suggest that the antioxidant effects of GB play an important role in its hepatoprotective effect, in agreement with the above studies.

The most important reason for choosing GB in this study was that it has PAF antagonistic properties in addition to its antioxidant effect. PAF is an important mediator in neutrophil activation, causing chemotaxis, aggregation, superoxide release, and degranulation, and it plays a crucial role in acute inflammation (30). PAF is also implicated in acute liver injury after extended hepatectomy, cirrhosis, lethal hepatitis, ischemia/reperfusion hepatic and renal injury, and intestinal inflammation (31). It has been shown that the PAF antagonist BN52021 prevents liver damage in an acetaminophen-induced liver injury model (32). We did not use any parameter to reflect the contribution of the PAF antagonist effect of GB on its hepatoprotective effects. However, in contrast to the other antioxidant treatments, GB treatment clearly attenuated TAA-induced inflammatory cell infiltration in liver. This finding suggests that the PAF antagonist effect of GB may represent an important contribution to its hepatoprotective effect, in addition to its antioxidant effect.

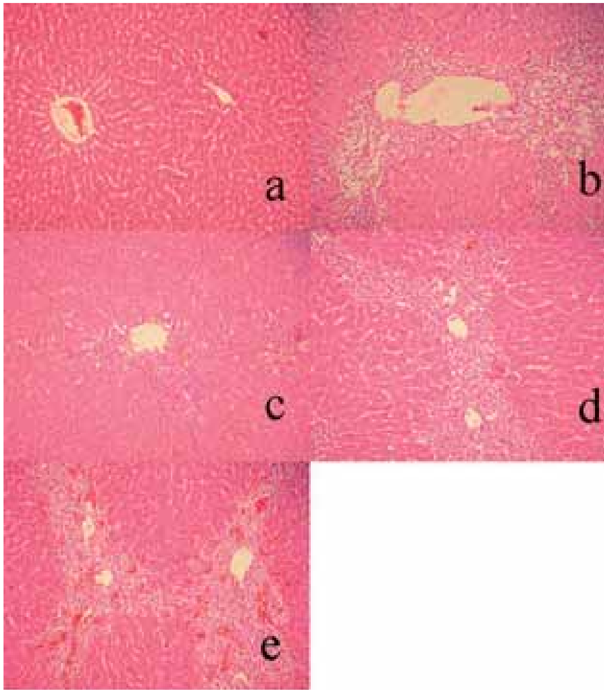

Fig. 2. — Liver histology in the control animals (a), TAA-induced hepatic injury (b), TAA + GB group (c), TAA + Vit E group (d), and TAA + melatonin (e) (HE, 200×). Less inflammation and necrosis were noted in TAA + GB group.

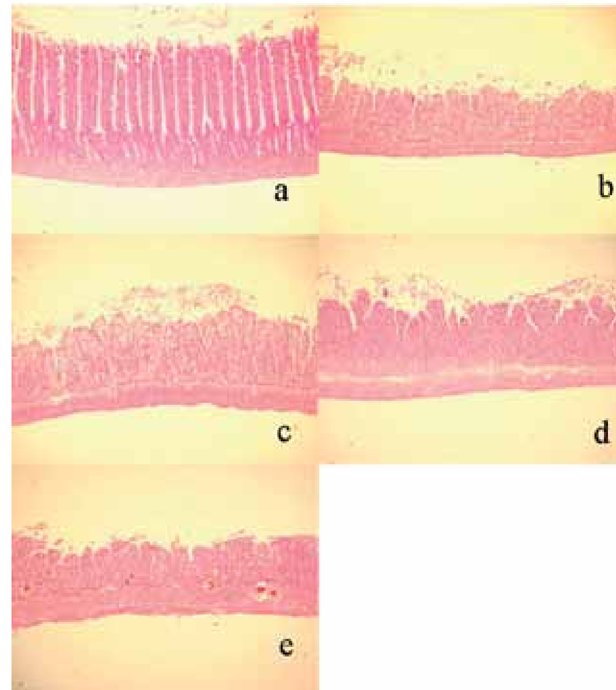

Fig. 3. — Intestinal histology of the control animals (a), TAA group (b), TAA + GB group (c), TAA + Vit E group (d), and TAA + melatonin (e) (HE, 100×). Severe inflammation and extensive erosions were noted in all intestinal specimens except in the control animals.

The GB dose applied did not prevent oxidative stress and inflammation in the intestine (Fig. 3c), contrary to what was observed in the liver. Although it has been shown that PAF antagonists reduce BT in pancreatitis and intestinal ischemia/reperfusion models (12,13), GB treatment did not reduce the BT frequency in our study. This lack of GB protective effect on the intestine may be due to the dose applied, since it has been reported that GB exhibits protective effects in a dose-dependent manner (33,34). The high BT frequencies observed in the TAA + GB group are probably attributable to a lack of improvement in intestinal oxidative damage.

Vit E treatment (200 mg/kg daily) did not reduce the BT frequency in this study. Vit E is a potent fat-soluble antioxidant that scavenges free radicals and protects membrane integrity. It protects cells and cellular components against oxidative stress by reducing lipid peroxidation products (17). It has been suggested that vitamin E treatment might improve fibrosis in patients with steatohepatitis (35) We did not observe any protective effect of Vit E treatment on either liver or intestinal tissue (Figs. 2d and 3d). Tunez *et al.* (36) and Sarkar *et al.* (both using a single dose of TAA at 150 mg/kg) (24) reported that Vit E treatment reduced liver oxidative injury in TAA-induced liver damage models. In our study, TAA was used at a dose of 350 mg/kg daily for 3 days. This is much higher than the TAA doses in above studies. Therefore, the lack of antioxidant activity of the Vit E treatment in liver may be due to the high TAA dose and duration. It has been reported that Vit E treatment shows a protective effect in intestinal mucosa and, even if statistically insignificant, reduces BT in an intestinal obstruction model in rats (37). In addition, it has been reported that Vit E and vitamin C combination treatment reduces ileal mucosal lipid peroxidation and BT in chronic portal hypertensive and common bile duct-ligated rats (38). Until now, no study has considered the effects of Vit E on intestinal oxidative damage and BT in TAA-induced liver failure. Therefore, we cannot explain the reason for the lack of Vit E effects on intestinal oxidative damage and BT in our model.

Melatonin treatment did not decrease the BT frequency. In this group, severe oxidative damage in liver and intestine was accompanied by high BT rates, as in the Vit E group. Melatonin (*N*-acetyl-5-methoxytryptamine) is one of the main secretory products of the pineal gland. It is a direct scavenger of free radicals and has indirect antioxidant effects owing to its stimulation of the expression and activity of many antioxidative enzymes (18). It easily crosses biological barriers and reaches all components of the cell owing to its small size and high lipophilic property (39). Tunez *et al.* reported that melatonin treatment prevents oxidative stress and liver injury in TAA-induced liver failure (36,40). Cruz *et al.* observed that melatonin treatment reduced oxidative stress, hepatocyte injury and liver fibrosis in TAA-induced cirrhosis in rats (41). In addition, Bruck *et al.* showed that melatonin treatment reduces oxidative stress and liver damage by inhibiting nuclear factor-kB activation and improves survival in TAA-induced liver

damage in rats (21). Although it has been reported in the above studies that melatonin clearly prevents oxidative liver damage in TAA-induced hepatotoxicity models, Karabay *et al.* reported that melatonin treatment (10 mg/kg daily) does not alter oxidative stress parameters (hepatic malondialdehyde, reduced glutathione and hepatic catalase activity) and cannot prevent liver damage in TAA-induced (200 mg/kg daily for 2 days) hepatotoxicity (42). They proposed the following explanations for the lack of melatonin activity. Melatonin scavenges ROS and RNS in a dose-dependent manner, but since melatonin did not prevent inflammatory cell infiltration, this condition led to excessive production of ROS and RNS. The authors also stated that generation of reactive TAA products may contribute to this damage. In the present study, melatonin treatment (3 mg/kg daily) did not reduce liver damage and high liver TBARS levels in TAA-induced liver damage. As shown in Fig. 2e, there was severe liver inflammation in the melatonin group. Compared with the study of Karabay *et al.*, our study used a higher TAA dose and a lower melatonin dose. In light of these findings, the lack of protective effects of melatonin on liver in our study is possibly due to overproduction of reactive TAA products because of the high TAA dose, and inadequacy of the melatonin dose used in preventing inflammatory cell infiltration in liver.

Furthermore, melatonin treatment did not attenuate tissue damage (Fig. 3e) or reduce TBARS levels in intestine. It has been reported that melatonin shows protective effects in different models of intestinal oxidative damage (43,44). Sileri *et al.* reported that melatonin reduced intestinal tissue damage and BT in an intestinal ischemia/reperfusion model (45). Although no study has considered the effects of melatonin on intestinal oxidative injury and BT in TAA-induced FHF, Cetinkaya *et al.* reported that melatonin reduced BT without showing a protective effect on intestine in liver failure induced by major liver resection, although the authors could not explain the mechanism of reduced BT incidence (25). In our study, we evaluated intestinal oxidative stress in addition to intestinal histology. The fact that high BT levels were observed together with intestinal oxidative injury in the melatonin group suggests that intestinal oxidative injury may be a key factor in BT in TAA-induced FHF. It has been shown in many studies that melatonin treatment decreases intestinal oxidative injury in a dose-dependent manner similar to that in liver (46,47). Remarkable intestinal tissue inflammation was observed in the melatonin group, similar to that observed in hepatic tissue. Based on these findings, the same explanations previously proposed to explain the insufficient antioxidant effect of melatonin on liver may also be valid for explaining the lack of effect on intestine.

It has been shown that IBO plays an important role in BT in cirrhosis (28) and TAA-induced hepatic failure (48) models. In our study, *E. coli* counts in ileal aspirate in the TAA group were significantly higher than in controls ($p < 0.001$). In the TAA group, the BT rate was 100% and the species of bacteria translocated to MLN, liver and spleen was *E. coli*. These findings support the notion that IBO plays an important role in the BT process. It has been reported that intestinal oxidative damage decreases intestinal motility in experimental studies. Decreased intestinal motility is one of the main mechanisms of IBO in cirrhosis (8). Although Chiva *et al.* reported that antioxidants (vitamin C and glutamate) decreased Enterococci and Enterobacter counts in ileum and cecum and prevented BT by decreasing intestinal oxidative stress in experimental cirrhosis (8), none of antioxidant treatments (GB, Vit E and melatonin) decreased *E. coli* counts and BT in our study. We did not evaluate intestinal motility in this study. However, our findings suggest that intestinal oxidative damage contributes to IBO and facilitates BT by altering intestinal motility similar to cirrhosis models.

In conclusion, GB, Vit E and melatonin treatments did not decrease intestinal oxidative injury, IBO or BT frequencies in a TAA-induced FHF model. These results suggest that intestinal oxidative injury plays a major role in the development of BT by disrupting the barrier function of the intestinal mucosa and increasing enteric bacteria counts, even though the mechanism is not completely understood.

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