ROS

RESEARCH ARTICLE

Methylene Blue Protects against Acidified Sodium Taurocholate-Induced Gastric Mucosal Damage

Omar M.E. Abdel-Salam[1], Amany Ameen Sleem[2], Dalia Medhat[3], Rania A.A. Salama[1], Fatma A. Morsy[4], Abdel Razik H. Farrag[4], and Noha N. Yassen[4]

[1]Department of Toxicology and Narcotics, National Research Centre, Cairo, Egypt; [2]Department of Pharmacology, National Research Centre, Cairo, Egypt; [3]Department of Medical Biochemistry, National Research Centre, Cairo, Egypt; [4]Department of Pathology, National Research Centre, Cairo, Egypt

Correspondence: omasalam@hotmail.com (O.M.A-S.)

Abdel-Salam OM et al. Reactive Oxygen Species 7(20):93–105, 2019; ©2019 Cell Med Press
http://dx.doi.org/10.20455/ros.2019.815
(Received: October 2, 2018; Revised: November 1, 2018; Accepted: November 2, 2018)

ABSTRACT | The effect of methylene blue (MethyB) on the development of gastric mucosal injury caused by orally given acidified sodium taurocholate (80 mM in 2 ml of 0.15 N HCl) in pylorus-ligated rats was studied. MethyB was intraperitoneally given at doses of 20 and 40 mg/kg at time of pylorus-ligation, and animals were euthanized 90 min later, when gastric secretory responses and the number and severity of mucosal lesions were determined. Lipid peroxidation (malondialdehyde), nitric oxide, reduced glutathione (GSH), and paraoxonase-1 (PON-1) activity in gastric homogenates were measured. Gastric mucosal histopathology and histochemical staining for mucopolysaccharides were also done. Results indicated that acidified sodium taurocholate (Na^+-taurocholate) caused severe gastric lesions. It also increased gastric malondialdehyde by 70% and decreased GSH levels by 36.4% compared with the corresponding control values. Additionally, nitric oxide decreased by 49.3% and PON-1 activity fell by 54.2% in gastric tissue of Na^+-taurocholate-treated rats. The administration of MethyB reduced the number and severity of gastric mucosal lesions but had no effect on gastric acid secretion in Na^+-taurocholate-treated rats. MethyB resulted in decreased malondialdehyde and increased GSH, nitric oxide, and PON-1 activity in a dose-dependent manner. Na^+-taurocholate caused massive sloughing and hemorrhagic erosions of the superficial parts of gastric epithelium, lamina propria, and sloughing of gastric glands. These changes were markedly attenuated by MethyB at 40 mg/kg. MethyB also restored gastric mucus as indicated by the increase in apical epithelial cells positively stained with periodic acid Schiff. These data suggest a protective effect for MethyB against gastric mucosal damage caused by Na^+-taurocholate which is likely to be due to decreased oxidative stress.

KEYWORDS | Gastric acid; Gastric mucosa; Glutathione; Hydroxyproline; Lipid peroxidation; Methylene blue; Oxidative stress; Paraoxonase; Sodium taurocholate

ABBREVIATIONS | GSH, reduced glutathione; H&E, hematoxylin and eosin; MDA, malondialdehyde; MethyB, methylene blue; NSAID, non-steroidal anti-inflammatory drug; PAS, periodic acid-Schiff; PON-1, paraoxonase-1

©2019 AIMSCI Inc. All Rights Reserved.

ROS

RESEARCH ARTICLE

CONTENTS

1. INTRODUCTION

The gastric mucosa is constantly exposed to high concentrations of intraluminal acid and the proteolytic enzymes, pepsins. In addition, reflux of bile acids, intake of non-steroidal anti-inflammatory drugs (NSAIDs), and ethanol consumption constitute a serious threat to the gastric mucosa [1]. The latter owes its remarkable resistance, to acid-pepsins and other noxious agents in the lumen, to a group of defense mechanisms that collectively constitute the so called "the gastric mucosal barrier" [2]. These include the supply of bicarbonate and mucus by the surface epithelial cells constituting the "mucus-bicarbonate barrier" which, in addition to surface phospholipids, prevents the back-diffusion of H^+ from the lumen. Maintaining adequate gastric mucosal blood flow and in particular the microcirculation is also crucial to the integrity of the gastric mucosa, by providing adequate bicarbonate for buffering and disposal of back-diffused H^+ that has permeated the mucosa and by supplying oxygen and nutrients to cells [2–4]. In the gastric mucosa, sensory nerves, prostaglandins, and nitric oxide orchestrate many aspects of the gastric mucosal barrier and play a fundamental role in maintaining the integrity of the gastric mucosa [5–7]. Damage to the gastric mucosa ensues when the above defense mechanisms become overwhelmed by excessive back-diffused H^+ resulting from the action of barrier breakers, including NSAIDs, bile salts, and ethanol, among others [1, 4].

In humans, methylene blue (MethyB) has a number of important clinical applications. It is well known for a role in the treatment of methemoglobinemia [8], and cyanide poisoning [9, 10]. It is also employed in the management of severe sepsis [11], and hypotension that is refractory to vasopressor agents and intravenous fluids [12]. The dye is also an effective remedy for encephalopathy caused by the alkylating agent ifosfamide [13, 14]. Recent interest in MethyB focuses on its neuroprotective potential that has been demonstrated in various in vitro and in vivo models of Parkinson's disease [15–17], Huntington's disease

©2019 AIMSCI Inc. All Rights Reserved.

[18], and Alzheimer's disease [19], and in mitigating neurotoxicity caused by organophosphates [20]. It also protected against brain and liver damage during endotoxemia [21]. MethyB showed antioxidant properties which prevent the formation of mitochondrial oxygen free radicals [16, 22]. It was shown that MethyB could increase brain cytochrome c oxidase activity [23], activity of mitochondrial complexes I–III, and brain oxygen consumption [24].

In this study, the effect of MethyB on the gastric mucosa after the administration of the barrier breaker Na^+-taurocholate was studied. Reflux of bile acids into the stomach has been implicated in the development of gastritis and gastric ulcer. Na^+-taurocholate instilled into the human stomach was shown to cause biochemical and endoscopic damage to the mucosa [25]. Acidified Na^+-taurocholate introduced into the rat stomach has been shown to cause marked H^+ back-diffusion and extensive gastric mucosal lesions and has been used as a model to study the pathogenetic mechanisms involved in bile-reflux disease and likely therapeutic interventions [26–28].

2. MATERIALS AND METHODS

2.1. Animals

Sprague-Dawley rats of both sexes, 140–150 g, were used in the study. The animals were provided by the Animal House of the National Research Centre (Cairo, Egypt). The rats were group-housed under temperature- and light-controlled conditions and allowed standard laboratory rodent chow and water ad libitum. Animal procedures followed the recommendations of the Ethics Committee of the National Research Centre (Cairo, Egypt) and the United States National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Drugs and chemicals

MethyB, Na^+-taurocholate, and other chemicals and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.3. Gastric Ulcerogenic Studies

The rats were divided into four equal groups (six rats each). Gastric mucosal damage was induced in pylorus-ligated by the orogastric administration of acidified sodium taurocholate (80 mM in 2 ml of 0.15 N HCl) [27]. The effect of MethyB (20 and 40 mg/kg, via intraperitoneal injection) given at the time of Na^+-taurocholate administration was studied. The rats were euthanized 90 min after the above treatment. The stomachs were then removed, opened along the greater curvature, rinsed with normal saline, extended on a plastic board, and examined for the presence of mucosal lesions. The number and severity of mucosal lesions were examined and lesions were scaled according to Mózsik et al. [29] as follows: petechial lesions = 1; lesions smaller than 1 mm = 2; lesions between 1 and 2 mm = 3; lesions between 2 and 4 mm = 4; and lesions bigger than 4 mm = 5. A total lesion score for each animal is calculated as the total number of lesions multiplied by the respective severity scores and results are expressed as the severity of lesions/rat.

2.4. Gastric Secretory Studies

The volume of gastric contents was measured, and the acid output was determined by titration with 0.1 N NaOH to pH 7.0. The H^+ output is expressed in µEq/rat/90 min.

2.5. Determination of Oxidative Stress Markers

2.5.1. Determination of Lipid Peroxidation

Lipid peroxidation was measured by determining malondialdehyde (MDA) according to Ruiz-Larrea et al. [30]. In this assay, 2-thiobarbituric acid reacts with MDA at 25°C to yield a red colored complex which is measured spectrophotometrically at 532 nm.

2.5.2. Determination of Reduced Glutathione

Reduced glutathione (GSH) was determined according to Ellman et al. [31]. The Ellman's reagent 5,5′-dithiobis (2-nitrobenzoic acid) reacts with the free thiol group of GSH to form 2-nitro-5-mercaptobenzoic acid. The chromophore has yellow color and is determined spectrophotometrically at 412 nm.

2.5.3. Determination of Nitric Oxide

Nitric oxide was determined using the Griess reagent. Nitrate is converted to nitrite by the enzyme nitrate reductase. Nitrite then reacts with the Griess reagent

©2019 AIMSCI Inc. All Rights Reserved.

to form a purple azo compound, and its absorbance is measured spectrophotometrically at 540 nm [32].

2.5.4. Determination of Paraoxonase-1 Activity

Paraoxonase-1 (PON-1) arylesterase activity was measured using phenylacetate as a substrate and the formation of phenol was measured by monitoring the increase in absorbance at 270 nm at 25°C. One unit of arylesterase activity is defined as 1 µmol of phenol formed per min. Enzyme activity was calculated based on the extinction coefficient of phenol of 1310 $M^{-1}cm^{-1}$ at 270 nm, pH 8.0 and 25°C, and expressed as kilo international unit/liter (kU/L) [33].

2.6. Determination of Hydroxyproline

Gastric hydroxyproline content was determined using the colorimetric method described by Bergman and Loxley [21].

2.7. Histopathological Studies

For histopathologic assessment, the stomachs were pinned flat on a cardboard and immersed in a 10% formalin solution and later embedded in paraffin. From the paraffin-embedded tissue blocks, hematoxylin and eosin (H&E)-stained sections were coded. Sections were evaluated qualitatively under light microscopy. Morphometric measurement of the mean value of mucosal thickness was done by image analysis (Leica Qwin 500, Cambridge, UK).

2.8. Histochemical Studies

Sections of 5 µm thickness of the glandular portion of the rat stomach of each group were stained with periodic acid-Schiff (PAS) stain to observe for mucus production and to note the changes in both acidic and basic glycoproteins. Morphometric analysis of PAS stained sections was done using image analyzer (Leica Qwin 500) for detection of the PAS intensity. Images were captured with a 10x magnification objective. Five fields were randomly selected that did not include large non-connective tissue elements. A color image of positive spots of PAS could be made into a single linear scale of pixel intensities by converting the image to grayscale, using monochromatic incident light. The image was transformed into a gray image [a grid of pixels each representing the intensity or brightness at that point by a range of numbers, typically from 0 (black) to 255 (white)]. A gray scale image is a color mode that displays image using 256 shades of gray, referred to as 8-bit gray scale image. Each color was defined as a value between 0 and 255, where 0 is the darkest (black) and 255 is the lightest (white). The mean and the median intensity of PAS ranged from 0 (black) to 255 (total white). The final PAS intensity was calculated according to the formula f = 255 – i, where f denotes final PAS intensity, and i denotes mean PAS intensity obtained from the software; i ranges from 0 (zero = deep brown, highest expression) to 255 (total white) [34].

2.9. Statistical Analysis

Values are presented as means ± standard error (SEM). Results of the ulcer number and severity were analyzed using Kruskal–Wallis non-parametric one-way analysis of variance (ANOVA) followed by Mann–Whitney multiple comparisons test. The results of the biochemical analysis and histopathology were analyzed using one-way ANOVA followed by Duncan's multiple range test. Statistical analysis of results was done using SPSS software (SAS Institute, Cary, NC). A probability value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Effect of MethyB on Gastric Acid Secretion

Following orogastric administration of Na^+-taurocholate, the gastric secretory volume and acid content were elevated by 28.0% and 40.5%, respectively, compared with the saline control value. MethyB given intraperitoneally at the time of pylorus-ligation at doses of 20 and 40 mg/kg did not affect gastric secretory volume or gastric acid output in response to Na^+-taurocholate (**Table 1**).

3.2. Effect of MethyB on Gastric Mucosal Lesions

MethyB (20 and 40 mg/kg) given at the time of Na^+-taurocholate administration prevented the development of gastric mucosal lesions in a dose-dependent manner. The severity of lesions caused by Na^+-taurocholate was reduced from a control value of 38.7 ± 2.5 to 23.7 ± 1.9 and 15.4 ± 1.4 by MethyB at 20 and

©2019 AIMSCI Inc. All Rights Reserved.

TABLE 1. Effect of MethyB on gastric acid secretion in acidified Na^+-taurocholate-treated pyloric-ligated rats

Treatment	Gastric volume (ml)	Gastric acid secretion (μEq/90 min)
Saline	3.75 ± 0.1	190.0 ± 8.3
Na^+-taurocholate	4.80 ± 0.32* (28.0%)	267.0 ± 11.0* (40.5%)
Na^+-taurocholate + MethyB 20 mg/kg	4.75 ± 0.42* (26.7%)	297.2 ± 15.8* (56.4%)
Na^+-taurocholate + MethyB 40 mg/kg	4.63 ± 0.23* (23.5%)	295.1 ± 10.3* (55.3%)

Note: Values are means ± SEM (n = 6). *, $p < 0.05$ vs. saline. The percent change from the saline only group is shown in parentheses.

TABLE 2. Effect of MethyB on number and severity of gastric mucosal lesions in acidified Na^+-taurocholate-treated pyloric-ligated rats

Treatment	Number of lesions per rat	Severity of lesions per rat
Saline	0.00 ± 0.00	0.00 ± 0.00
Na^+-taurocholate	14.5 ± 1.6*	38.7 ± 2.5*
Na^+-taurocholate + MethyB 20 mg/kg	8.25 ± 1.0*+ (–43.1%)	23.7 ± 1.9*+ (–38.7%)
Na^+-taurocholate + MethyB 40 mg/kg	6.4 ± 0.81*+ (–55.7%)	15.4 ± 1.4*+# (–60.2%)

Note: Values are means ± SEM (n = 6) and percent inhibition (%) compared to the Na^+-taurocholate control group. *, $p < 0.05$ compared to the saline control group. +, $p < 0.05$ compared to the Na^+-taurocholate control. #, $p < 0.05$ compared to the Na^+-taurocholate + MethyB 20 mg/kg treatment group.

40 mg/kg, respectively. The number of lesions was also decreased by MethyB at 20 and 40 mg/kg from a control value of 14.5 ± 1.6 to 8.25 ± 1.0 and 6.4 ± 0.81, respectively (**Table 2** and **Figure 1**).

3.3. Effect of MethyB on Gastric Tissue Oxidative Stress

The administration of Na^+-taurocholate resulted in a significant increase in gastric tissue MDA by 70% as compared to the saline control value. There were also significant decrements in gastric tissue nitric oxide and GSH by 49.4% and 36.4%, respectively, compared with corresponding control values. In rats treated with Na^+-taurocholate, MethyB given at 20 and 40 mg/kg resulted in a significant decrease in tissue MDA by 48.8% and 52.2% and an increase in GSH content by 24.5% and 54.7%, respectively. In addition, tissue nitric oxide showed a 25.6% increment upon treating with the higher dose of MethyB (**Table 3**).

3.4. Effect of MethyB on Gastric Tissue PON-1

PON-1 activity in gastric tissue decreased by 54.2% in rats treated with only Na^+-taurocholate as compared to the saline control value. PON-1 activity increased by 57% and 85.1% in rats treated with Na^+-taurocholate and MethyB at 20 and 40 mg/kg, respectively, compared with the Na^+-taurocholate control group (**Table 3**).

3.5. Effect of MethyB on Gastric Tissue Hydroxyproline

A significant increase in gastric hydroxyproline content by 186.5% was observed in rats treated with only Na^+-taurocholate as compared to the saline control

©2019 AIMSCI Inc. All Rights Reserved.

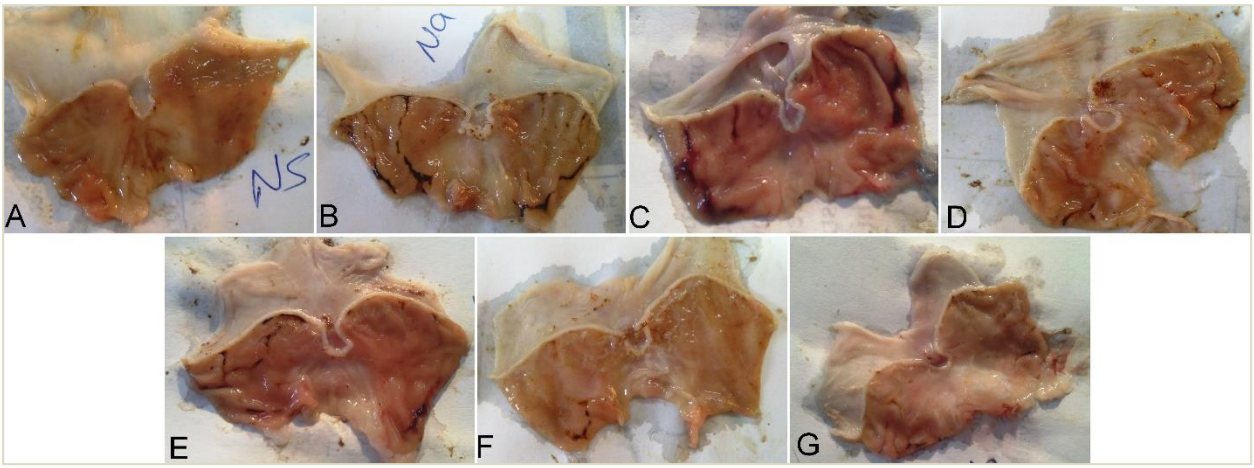

FIGURE 1. Gross appearance of rat gastric mucosa. Shown are the representative photos of the gross appearance of gastric mucosa from rats treated with saline (A), acidified Na^+-taurocholate alone (B and C), or acidified Na^+-taurocholate + MethyB at 20 mg/kg (D and E) or 40 mg/kg (F and G).

value. MethyB given at 20 and 40 mg/kg resulted in a significant decrease of hydroxyproline content by 17.2% and 25.2%, respectively, as compared with the Na^+-taurocholate control value (**Table 3**).

3.6. Histopathological Results

By light microscopy, the histological examination of the stomach wall of the saline control group showed the normal structure being formed of mucosa, submucosa, and muscle layer; the mucosa was separated from the submucosa by a layer of smooth muscle, called muscuolaris mucosa. The mucosa consists of surface epithelium that invaginated forming the gastric pits **(Figure 2A** and **2B)**. Sections of the stomach from rats treated with Na^+-taurocholate showed massive sloughing and erosions of the superficial parts of gastric epithelium, lamina propria, and sloughing in gastric glands. Congestion and edema were seen **(Figure 2A)**. There were also severe congestion and multiple hemorrhagic erosions in the stomach tissue, particularly in mucus-secreting cells **(Figure 2C)**, signs of degeneration in the lining of gastric glands in the form of pyknosis, and some cells exhibiting eosinophilic cytoplasm with dark nuclei **(Figure 2D** and **2E)**.

In rats treated with Na^+-taurocholate, MethyB given at 20 mg/kg considerably attenuated but did not completely prevent the severity of the above histopathological changes, with some erosions in superficial gastric epithelial being detected. Marked vascular congestion in submucosal layer and an increase in inflammatory cells were seen. Thickening of muscularis mucosa could also be observed **(Figure 2F** and **2G)**. The stomach from rats treated with Na^+-taurocholate and MethyB at 40 mg/kg exhibited normal architecture with inflammatory infiltrate and congestion of blood vessels **(Figure 2H** and **2I)**.

3.7. Morphometric Results

In rats treated with only Na^+-taurocholate, morphometric results showed a significance decrease ($p < 0.05$) in the thickness of stomach mucosa by 60.8% as compared with the saline-treated control group. Rats given methylene blue at doses of 20 and 40 mg/kg along with Na^+-taurocholate showed significant increase ($p < 0.05$) in the thickness of the gastric mucosa as compared with Na^+-taurocholate control group and the percentages of increase were 20.4% and 38.3%, respectively **(Table 4).**

3.8. Histochemical Results

Sections of the stomach from saline-treated rats showed normal distribution of mucin. The magenta color in the apical epithelial cells showed glycoprotein accumulation in the gastric glands **(Figure 3A)**. Rats

©2019 AIMSCI Inc. All Rights Reserved.

TABLE 3. Effect of MethyB on gastric tissue content of MDA, NO, GSH, PON-1, and hydroxyproline (HOP) in the gastric tissue of pyloric-ligated rats treated with acidified Na^+-taurocholate

Treatment	MDA (nmol/g.Ts)	NO (μmol/g.Ts)	GSH (μmol/g.Ts)	PON1 activity (kU/l)	HOP (μg/g.Ts)
Saline	17.00 ± 0.74	17.60 ± 1.30	25.00 ± 1.90	24.90 ± 1.80	29.20 ± 2.70
Na^+-taurocholate	28.90 ± 1.70*	8.90 ± 0.14*	15.90 ± 1.10*	11.40 ± 0.60*	80.80 ± 5.30*
Na^+-taurocholate + MethyB 20 mg/kg	14.8 ± 0.16^{*+} (–48.8%)	8.70 ± 0.47^{*+}	19.60 ± 0.74* (24.5%)	17.90 ± 1.50* (57.0%)	66.90 ± 2.30* (–17.2%)
Na^+-taurocholate + MethyB 40 mg/kg	13.60 ± 1.4^{*+} (–52.2%)	13.10 ± 1.40$^+$ (47.2%)	24.60 ± 0.65$^+$ (54.7%)	21.10 ± 0.48$^{*+\#}$ (85.1%)	60.10 ± 3.60$^{*+\#}$ (–25.2%)

Note: Values are means ± SEM (n = 6). *, $p < 0.05$ vs. saline. $^+$, $p < 0.05$ vs. Na^+-taurocholate only. $^\#$, $p < 0.05$ compared to the Na^+-taurocholate + MethyB 20 mg/kg treatment group. The percent change from the Na^+-taurocholate only group is shown in parentheses. NO, nitric oxide; Ts, tissue.

treated with only Na^+-taurocholate showed complete depletion of PAS-stained granules in the erosive area of the apical epithelium, with a thin layer of mucin in other areas, i.e., degeneration of surface mucus **(Figure 3B)**. The gray level of PAS staining of this group was extensively decreased ($p < 0.05$) as compared with the saline control group **(Table 5)**. MethyB given at doses of 20 and 40 mg/kg along with Na^+-taurocholate resulted in restoring the amount of mucus production demonstrated by more positively stained cells with PAS as compared to the group treated with Na^+-taurocholate only **(Figure 3C** and **3D)**. A significant increase by 71.0% and 86.6%, in the grey level of PAS staining ($p < 0.05$) was found in the groups treated with Na^+-taurocholate and MethyB at doses of 20 and 40 mg/kg, respectively, compared with that treated with Na^+-taurocholate only. Moreover, there was no significant difference in grey level of PAS staining between the groups treated with Na^+-taurocholate and MethyB (20 or 40 mg/kg) and the saline-treated group **(Table 5)**.

4. DISCUSSION

Previous studies demonstrated that extensive injury to the gastric mucosa could be evoked by the combined effect of bile salt and acid introduced into the human stomach [25] or that of rats [28, 35, 36]. The present findings are in accordance with these early studies. The orogastric administration of Na^+-taurocholate in HCl resulted in severe gastric necrotic lesions. Histopathological examination of gastric tissue revealed sloughing and necrosis of the superficial epithelium extending into the gastric glands and lamina propria, as well as decreased mucosal thickness. Sodium taurocholate increases H^+ back-diffusion and, in the presence of exogenously given acid, results in severe damage to the gastric mucosa [27, 28]. Our results indicated increased gastric tissue oxidative stress by Na^+-taurocholate evidenced by the increase in gastric MDA and the decrease in GSH levels. Moreover, there was a significant and marked decrease in nitric oxide in gastric tissue of Na^+-taurocholate-treated rats. Nitric oxide is synthesized from L-arginine via the enzyme nitric oxide synthase (NOS), an enzyme that exists in two constitutively expressed isoforms, namely, endothelial and neuronal NOS. There is also a third inducible form of NOS (iNOS) which is not expressed under normal conditions. In the stomach, the constitutively released nitric oxide of endothelial origin is important in maintaining the gastric mucosal microcirculation and consequently mucosal integrity [37, 38]. Phillipson et al. [39], however, found that iNOS was also constitutively expressed in the gastric mucosa and was involved in acid-induced hyperemia. The increase in mucosal blood flow in response to installation of intraluminal acid was not observed after non-selective inhibition of NOS or in iNOS-deficient mice. Thus, nitric oxide from iNOS could also be involved in gastric mucosal protection. Nitric oxide also inhibits gastric acid secretion, a major threat to the gastric mucosa [40]. The decrease in nitric oxide would therefore impair the ability of the stomach to withstand the combined effect of the exogenously given acid and the barrier breaker Na^+-taurocholate

©2019 AIMSCI Inc. All Rights Reserved.

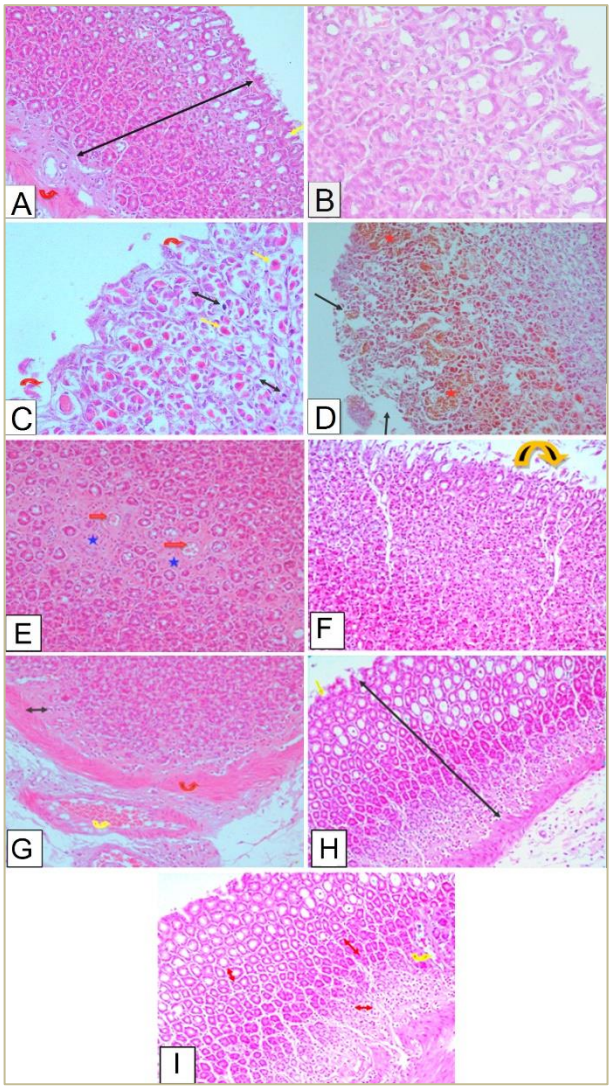

FIGURE 2. Representative hematoxylin and eosin (H&E)-stained sections of the gastric mucosa in rats from various groups. (A) Saline group, showing the normal histological structure (long black arrow), gastric pits (yellow arrow), and gastric glands, divided into isthmus, neck, and base. Muscularis mucosa (red curved arrow) is also seen (×200). (B) High power image of the same saline group as in (A), showing normal architecture of gastric mucosa (×400). (C) Na^+-taurocholate only group, showing massive sloughing and eroded superficial parts of gastric epithelium (red arrow) and lamina propria. Sings of degeneration in the lining of gastric gland in the form of pyknosis (black arrow) and some cells appeared with easinophilic cytoplasm and dark nuclei (yellow arrow) (×400). (D) Na^+-taurocholate only group, showing severe congestion and multiple hemorrhagic erosions in the stomach tissue, particularly in mucus-secreting cells (×200). (E) Na^+-taurocholate only group, showing congestion (red arrow) and edema in mucosa (star) (×100). (F) Na^+-taurocholate + MethyB 20 mg/kg group, showing some erosions in superficial gastric epithelial (orange arrow) (×100). (G) Na^+-taurocholate + MethyB 20 mg/kg group, showing marked vascular congestion in submucosa layer (yellow arrow) and increase in inflammatory cells (black arrow). Thickening of muscularis mucosa is observed (red arrow) (×100). (H) Na^+-taurocholate + MethyB 40 mg/kg group, showing normal gastric gland architecture (×200). (I) Na^+-taurocholate + MethyB 40 mg/kg group, showing inflammatory infiltrate in mucosal layer (red arrow) and congestion of a blood vessel (yellow arrow) (×200).

and to result in extensive mucosal damage as shown in this study.

In this study, the effect of the redox dye MethyB on the Na^+-taurocholate-induced mucosal damage was investigated. Our findings provide the first evidence for a gastric protective effect of MethyB in this model of gastric mucosal damage. MethyB given via the intraperitoneal route was able to decrease the extent of the necrotic lesions caused by the acidified bile salt. The gastric protective effect of MethyB was confirmed by quantitative assessment of the extent of the histologic damage. It should be noted that the dye was given intraperitoneally and thus a direct topical action is not involved in the observed protection. Moreover, MethyB at the doses used in the study showed no inhibitory action on gastric acid secretion in Na^+-taurocholate-treated rats. These observations thus suggest a cytoprotective property for MethyB. Cytoprotection is the term introduced by Robert and coworkers [41] to indicate the ability of prostaglandins to protect the gastric mucosa from such necrotizing agents as 0.6 M HCl and 100% ethanol, despite no inhibitory effect on gastric acid secretion. In this context, Utley et al. [42] have shown that the histamine H_2 receptor blocker cimetidine was able to inhibit gastric mucosal damage due to Na^+-taurocholate in HCl at doses devoid of anti-secretory activity. In contrast, anti-secretory doses of cimetidine or the proton pump

©2019 AIMSCI Inc. All Rights Reserved.

TABLE 4. Thickness of gastric mucosa in different treated groups

Treatment	Thickness of mucosa
Saline	112.11 ± 1.88
Na^+-taurocholate	69.7 ± 1.83*
Na^+-taurocholate + MethyB 20 mg/kg	83.95 ± 1.45*+ (20.4%)
Na^+-taurocholate + MethyB 40 mg/kg	96.39 ± 3.23+ (38.3%)

Note: Values are means ± SEM (n = 5). *, $p < 0.05$ vs. saline. +, $p < 0.05$ vs. Na^+-taurocholate only. The percent change from the Na^+-taurocholate only group is shown in parentheses.

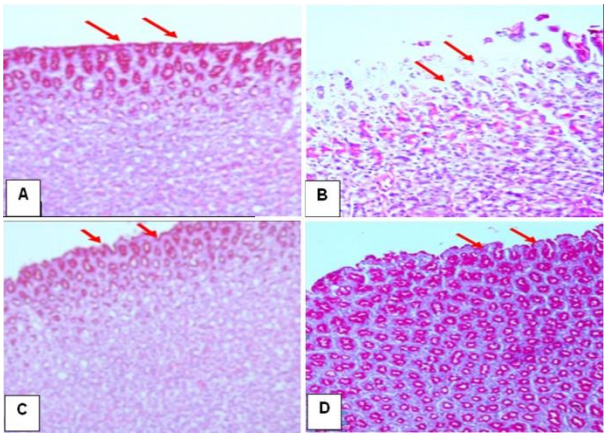

FIGURE 3. Representative PAS-stained sections of the gastric mucosa in rats from various groups. (A) Saline group, showing the normal distribution of mucopolysaccharides in the upper surface and in the cells lining the gastric pits. (B) Na^+-taurocholate only group, revealing extensive decrease in PAS^+ surface epithelial cells and gastric glands. (C) Na^+-taurocholate + MethyB at 20 mg/kg group, showing an increase in the PAS positivity of the gastric mucosa in comparison with group treated with Na^+-taurocholate only. (D) Na^+-taurocholate + MethyB at 40 mg/kg group, showing restoration of the normal distribution of mucopolysaccharides in the gastric mucosa (×200).

inhibitor omeprazole were not protective. Other researchers reported inhibition of Na^+-taurocholate mucosal lesions by non-anti-secretory doses of prostaglandin E2 and the muscarinic M_1 receptor antagonist pirenzepine [28]. Prevention of Na^+-taurocholate-induced gastric damage thus does not depend on inhibition of gastric acid secretion. On the other hand, damage could be exacerbated by inhibitors of prostaglandins synthesis [43] but could be prevented by a topically applied selective 5-lipoxygenase inhibitor, thereby, suggesting a role for inflammatory mediators [35].

The mechanism by which MethyB protects the gastric mucosa against Na^+-taurocholate is likely to involve decreased oxidative stress. Our results showed increased lipid peroxidation as indicated by the rise in the MDA level in the gastric tissue of Na^+-taurocholate-treated rats. There was also a significant decrease in gastric GSH. The increase in lipid peroxidation and the decrease in GSH were attenuated by MethyB. The tripeptide GSH (L-γ-glutamyl-L-cysteinyl-glycine) is the most abundant non-protein thiol in the cell. GSH is an important antioxidant which scavenges free radicals and other reactive oxygen metabolites, such as hydroxyl radical, lipid peroxyl radical, peroxynitrite, and hypochlorous acid, both directly, and through enzymatic reactions involving glutathione reductase and glutathione peroxidase [44]. GSH is important in gastric mucosal defense. It has been shown that gastric mucosal GSH, glutathione peroxidase and glutathione *S*-transferase activities were lower in the patients with gastric ulcer compared with normal controls [45]. Gastric mucosal levels of GSH were also significantly lower and MDA levels were higher in patients with *Helicobacter pylori*-positive peptic ulcer or gastritis compared with controls, suggesting a role for increased free radicals [46]. On the other hand, oral supplementation with GSH was able to protect against gastritis and epithelial proliferation caused by *Helicobacter suis* in Mongolian gerbils [47]. In healthy human volunteers, ethanol introduced into the stomach evoked hyperemia and submucosal hemorrhagic lesions. Parenteral GSH prevented the depletion of gastric sulfhydryls and decreased the extent of gastric mucosal damage [48]. Studies suggested inhibition of NOS by MethyB [49]. In this study, however, nitric oxide, which was decreased in gastric tissue of Na^+-

©2019 AIMSCI Inc. All Rights Reserved.

TABLE 5. Grey level of mucus in gastric mucosa in different treated groups

Treatment	Grey level of mucus
Saline	95.88 ± 4.9
Na^+-taurocholate	52.44 ± 3.01*
Na^+-taurocholate + MethyB 20 mg/kg	89.67 ± 5.20+ (71%)
Na^+-taurocholate + MethyB 40 mg/kg	97.86 ± 3.39+ (86.6%)

Note: Values are means ± SEM (n = 5). *, $p < 0.05$ vs. saline. +, $p < 0.05$ vs. Na^+-taurocholate only. The percent change from the Na^+-taurocholate only group is shown in parentheses.

taurocholate-treated rats, showed an increase after the higher dose of MethyB, most probably as a consequence of a gastric protective effect of the dye.

Paraoxonases are a group of detoxifying enzymes comprising three members [50], and among them PON-1 has been of considerable interest in view of studies linking the enzyme to atherogenesis [51], liver diseases [52], and neurological disorders [53]. PON-1 possesses esterase and lactonase activities, and catalyzes the hydrolysis of the active metabolites, i.e., "oxons" of a number of organophosphates and many xenobiotics [50, 54]. The enzyme has an antioxidative activity preventing the oxidation of high-density lipoproteins in plasma and displays an anti-inflammatory action [51, 55]. It is inactivated by increasing levels of oxidative stress [56]. In this study, the arylesterase activity of the enzyme was measured to determine PON-1 status in gastric tissue. Our results showed for the first time that the activity of the enzyme was markedly inhibited following the development of mucosal damage by Na^+-taurocholate. PON-1 activity increased in rats treated with MethyB at 20 and 40 mg/kg, most likely reflecting a decrease in oxidative stress and mucosal protection.

In this study, a marked increase in gastric tissue level of hydroxyproline was observed in the stomachs from Na^+-taurocholate-treated rats. This increase in gastric hydroxyproline was reduced but not normalized by MethyB. Other researchers found increased hydroxyproline in gastric mucosa 2 and 4 h after intragastric administration of 50% ethanol-0.15 M HCl to rats compared with the untreated gastric mucosa [57]. We also showed that Na^+-taurocholate resulted in extensive decrease in PAS^+ surface epithelial cells and gastric glands, indicating depletion of gastric mucus. The latter is an important component of the gastric mucosal barrier which retards the back-diffusion of luminal H^+ and thus protects the surface epithelial cells [58]. The administration of MethyB was able to prevent the Na^+-taurocholate-induced depletion of gastric mucus, which could be another mechanism that underlies its gastric protective effect.

In summary, the present study indicated that MethyB administered via the intraperitoneal route protected against gastric mucosal damage caused by acidified bile salt. This action of MethyB involved a decrease in gastric tissue oxidative stress and increased mucus secretion.

ACKNOWLEDGMENTS.

This works was not supported by research grants. The authors declare no conflicts of interest.

REFERENCES

1. Miller TA. Gastroduodenal mucosal defense: factors responsible for the ability of the stomach and duodenum to resist injury. *Surgery* 1988; 103(4):389–97.
2. Werther JL. The gastric mucosal barrier. *Mt Sinai J Med* 2000; 67(1):41–53.
3. Holzer P. Gastroduodenal mucosal defense. *Curr Opin Gastroenterol* 2000; 16(6):469–78.
4. Abdel-Salam OM, Czimmer J, Debreceni A, Szolcsanyi J, Mozsik G. Gastric mucosal integrity: gastric mucosal blood flow and microcirculation. An overview. *J Physiol Paris* 2001; 95(1–6):105–27.
5. Gyires K. Gastric mucosal protection: from prostaglandins to gene-therapy. *Curr Med Chem* 2005; 12(2):203–15.
6. Abdel-Salam OME, Mózsik GY, Szolcsányi J. The role of afferent sensory nerve in gastric mucosal protection. In: *Twenty Five Years of Peptic Ulcer Research in Hungary: From Basic*

©2019 AIMSCI Inc. All Rights Reserved.

Sciences to Clinical Perspectives (N Mozsik, G Mozsik, LNagy). Akadémiai Kiadó, Budapest, Hungary. 1997, pp. 295–308.
7. Laine L, Takeuchi K, Tarnawski A. Gastric mucosal defense and cytoprotection: bench to bedside. *Gastroenterology* 2008; 135(1):41–60. doi: 10.1053/j.gastro.2008.05.030.
8. Bradberry SM. Occupational methaemoglobinaemia. Mechanisms of production, features, diagnosis and management including the use of methylene blue. *Toxicol Rev* 2003; 22(1):13–27.
9. Clifton J, 2nd, Leikin JB. Methylene blue. *Am J Ther* 2003; 10(4):289–91.
10. Betten DP, Vohra RB, Cook MD, Matteucci MJ, Clark RF. Antidote use in the critically ill poisoned patient. *J Intensive Care Med* 2006; 21(5):255–77. doi: 10.1177/0885066606290386.
11. Kwok ES, Howes D. Use of methylene blue in sepsis: a systematic review. *J Intensive Care Med* 2006; 21(6):359–63. doi: 10.1177/0885066606290671.
12. Rutledge C, Brown B, Benner K, Prabhakaran P, Hayes L. A novel use of methylene blue in the pediatric ICU. *Pediatrics* 2015; 136(4):e1030–4. doi: 10.1542/peds.2014-3722.
13. Zulian GB, Tullen E, Maton B. Methylene blue for ifosfamide-associated encephalopathy. *N Engl J Med* 1995; 332(18):1239–40. doi: 10.1056/NEJM199505043321817.
14. Sánchez-Muñoz A, García Tapiador AM, Martínez Ortega E, Dueñas García R, Ortega Granados AL, Sánchez Rovira P. Treatment and prophylaxis of ifosfamide-induced encephalopathy with intravenous methylene blue. *Oncología* 2006; 29(3):140–1.
15. Zhang X, Rojas JC, Gonzalez-Lima F. Methylene blue prevents neurodegeneration caused by rotenone in the retina. *Neurotox Res* 2006; 9(1):47–57.
16. Poteet E, Winters A, Yan LJ, Shufelt K, Green KN, Simpkins JW, et al. Neuroprotective actions of methylene blue and its derivatives. *PLoS One* 2012; 7(10):e48279. doi: 10.1371/journal.pone.0048279.
17. Abdel-Salam OM, Omara EA, Youness ER, Khadrawy YA, Mohammed NA, Sleem AA. Rotenone-induced nigrostriatal toxicity is reduced by methylene blue. *J Neurorestoratol* 2014; 2:65–80. doi: 10.2147/JN.S49207.
18. Sontag EM, Lotz GP, Agrawal N, Tran A, Aron R, Yang G, et al. Methylene blue modulates huntingtin aggregation intermediates and is protective in Huntington's disease models. *J Neurosci* 2012; 32(32):11109–19. doi: 10.1523/JNEUROSCI.0895-12.2012.
19. Medina DX, Caccamo A, Oddo S. Methylene blue reduces abeta levels and rescues early cognitive deficit by increasing proteasome activity. *Brain Pathol* 2011; 21(2):140–9. doi: 10.1111/j.1750-3639.2010.00430.x.
20. Abdel-Salam OME, Youness ER, Esmail RSE, Mohammed NA, Khadrawy YA, Sleem AA. Methylene blue as a novel neuroprotectant in acute malathion intoxication. *React Oxyg Species (Apex)* 2016; 1(2):165–77. doi: 10.20455/ros.2016.821.
21. Abdel-Salam OME, Sleem AA, Youness ER, Mohammed NA, Shaffie N, Yassen NN. Neuro- and hepatoprotective effects of methylene blue in rats treated with lipopolysaccharide endotoxin. *React Oxyg Species (Apex)* 2018; 6(17):325–37. doi: 10.20455/ros.2018.849.
22. Wen Y, Li W, Poteet EC, Xie L, Tan C, Yan LJ, et al. Alternative mitochondrial electron transfer as a novel strategy for neuroprotection. *J Biol Chem* 2011; 286(18):16504–15. doi: 10.1074/jbc.M110.208447.
23. Callaway NL, Riha PD, Bruchey AK, Munshi Z, Gonzalez-Lima F. Methylene blue improves brain oxidative metabolism and memory retention in rats. *Pharmacol Biochem Behav* 2004; 77(1):175–81.
24. Lin AL, Poteet E, Du F, Gourav RC, Liu R, Wen Y, et al. Methylene blue as a cerebral metabolic and hemodynamic enhancer. *PLoS One* 2012; 7(10):e46585. doi: 10.1371/journal.pone.0046585.
25. Stern AI, Hogan DL, Isenberg JI. Effect of sodium taurocholate on the human gastric mucosa at acid and neutral pH's. *Gastroenterology* 1984; 87(6):1272–6.
26. Whittle BJ. Mechanisms underlying gastric mucosal damage induced by indomethacin and bile-salts, and the actions of prostaglandins. *Br J Pharmacol* 1977; 60(3):455–60.
27. Chaudhury TK, Robert A. Prevention by mild irritants of gastric necrosis produced in rats by sodium taurocholate. *Dig Dis Sci* 1980; 25(11):830–6.

©2019 AIMSCI Inc. All Rights Reserved.

28. Takeda F, Kitagawa H, Kohei H. Gastric cytoprotection by pirenzepine in rats: evaluating method for cytoprotective activity by antisecretory agents. *Jpn J Pharmacol* 1985; 38(4):337–46.
29. Mozsik G, Moron F, Javor T. Cellular mechanisms of the development of gastric mucosal damage and of gastrocytoprotection induced by prostacyclin in rats: a pharmacological study. *Prostaglandins Leukot Med* 1982; 9(1):71–84.
30. Ruiz-Larrea MB, Leal AM, Liza M, Lacort M, de Groot H. Antioxidant effects of estradiol and 2-hydroxyestradiol on iron-induced lipid peroxidation of rat liver microsomes. *Steroids* 1994; 59(6):383–8.
31. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959; 82(1):70–7.
32. Moshage H, Kok B, Huizenga JR, Jansen PL. Nitrite and nitrate determinations in plasma: a critical evaluation. *Clin Chem* 1995; 41(6 Pt 1):892–6.
33. Higashino K, Takahashi Y, Yamamura Y. Release of phenyl acetate esterase from liver microsomes by carbon tetrachloride. *Clin Chim Acta* 1972; 41:313–20.
34. Fuhrich DG, Lessey BA, Savaris RF. Comparison of HSCORE assessment of endometrial beta3 integrin subunit expression with digital HSCORE using computerized image analysis (ImageJ). *Anal Quant Cytopathol Histpathol* 2013; 35(4):210–6.
35. Sullivan TR, Jr., Cordero JA, Jr., Mercer DW, Ritchie WP, Jr., Dempsey DT. Selective lipoxygenase inhibitor reduces bile acid-induced gastric mucosal injury. *J Surg Res* 1992; 53(6):568–71.
36. Yu BP, Sun J, Li MQ, Luo HS, Yu JP. Preventive effect of hydrotalcite on gastric mucosal injury in rats induced by taurocholate. *World J Gastroenterol* 2003; 9(7):1427–30.
37. Quintero E, Guth PH. Nitric oxide-mediated gastric hyperemia decreases ethanol-induced gastric mucosal injury in uremic rats. *Dig Dis Sci* 1992; 37(9):1324–8.
38. Wallace JL, Miller MJ. Nitric oxide in mucosal defense: a little goes a long way. *Gastroenterology* 2000; 119(2):512–20. doi: 10.1053/gast.2000.9304.
39. Phillipson M, Henriksnas J, Holstad M, Sandler S, Holm L. Inducible nitric oxide synthase is involved in acid-induced gastric hyperemia in rats and mice. *Am J Physiol Gastrointest Liver Physiol* 2003; 285(1):G154–62. doi: 10.1152/ajpgi.00432.2002.
40. Kato S, Kitamura M, Korolkiewicz RP, Takeuchi K. Role of nitric oxide in regulation of gastric acid secretion in rats: effects of NO donors and NO synthase inhibitor. *Br J Pharmacol* 1998; 123(5):839–46. doi: 10.1038/sj.bjp.0701691.
41. Robert A, Nezamis JE, Lancaster C, Davis JP, Field SO, Hanchar AJ. Mild irritants prevent gastric necrosis through "adaptive cytoprotection" mediated by prostaglandins. *Am J Physiol* 1983; 245(1):G113–21. doi: 10.1152/ajpgi.1983.245.1.G113.
42. Utley RJ, Salim AS, Carter DC. Effect of cimetidine and omeprazole on aspirin- and taurocholate-induced gastric mucosal damage in the rat. *Gut* 1985; 26(8):770–5.
43. Whittle BJ. The potentiation of taurocholate-induced rat gastric erosions following parenteral administration of cyclo-oxygenase inhibitors. *Br J Pharmacol* 1983; 80(3):545–51.
44. Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. *J Nutr* 2004; 134(3):489–92. doi: 10.1093/jn/134.3.489.
45. Hirokawa K, Kawasaki H. Changes in glutathione in gastric mucosa of gastric ulcer patients. *Res Commun Mol Pathol Pharmacol* 1995; 88(2):163–76.
46. Demir S, Yilmaz M, Koseoglu M, Akalin N, Aslan D, Aydin A. Role of free radicals in peptic ulcer and gastritis. *Turk J Gastroenterol* 2003; 14(1):39–43.
47. De Bruyne E, Ducatelle R, Foss D, Sanchez M, Joosten M, Zhang G, et al. Oral glutathione supplementation drastically reduces Helicobacter-induced gastric pathologies. *Sci Rep* 2016; 6:20169. doi: 10.1038/srep20169.
48. Loguercio C, Taranto D, Beneduce F, del Vecchio Blanco C, de Vincentiis A, Nardi G, et al. Glutathione prevents ethanol induced gastric mucosal damage and depletion of sulfhydryl compounds in humans. *Gut* 1993; 34(2):161–5.
49. Mayer B, Brunner F, Schmidt K. Inhibition of nitric oxide synthesis by methylene blue. *Biochem Pharmacol* 1993; 45(2):367–74.

©2019 AIMSCI Inc. All Rights Reserved.

50. Primo-Parmo SL, Sorenson RC, Teiber J, La Du BN. The human serum paraoxonase/arylesterase gene (PON1) is one member of a multigene family. *Genomics* 1996; 33(3):498–507.
51. Mackness M, Mackness B. Targeting paraoxonase-1 in atherosclerosis. *Expert Opin Ther Targets* 2013; 17(7):829–37. doi: 10.1517/14728222.2013.790367.
52. Camps J, Marsillach J, Rull A, Alonso-Villaverde C, Joven J. Interrelationships between paraoxonase-1 and monocyte chemoattractant protein-1 in the regulation of hepatic inflammation. In: *Paraoxonases in Inflammation, Infection, and Toxicology; Advances in Experimental Medicine and Biology* (ST Reddy). Humana Press, Springer, Berlin, Germany. 2010, pp. 5–18.
53. Menini T, Gugliucci A. Paraoxonase 1 in neurological disorders. *Redox Rep* 2014; 19(2):49–58. doi: 10.1179/1351000213Y.0000000071.
54. Richter RJ, Jarvik GP, Furlong CE. Paraoxonase 1 (PON1) status and substrate hydrolysis. *Toxicol Appl Pharmacol* 2009; 235(1):1–9. doi: 10.1016/j.taap.2008.11.001.
55. Aharoni S, Aviram M, Fuhrman B. Paraoxonase 1 (PON1) reduces macrophage inflammatory responses. *Atherosclerosis* 2013; 228(2):353–61. doi: 10.1016/j.atherosclerosis.2013.03.005.
56. Nguyen SD, Sok DE. Preferential inhibition of paraoxonase activity of human paraoxonase 1 by negatively charged lipids. *J Lipid Res* 2004; 45(12):2211–20. doi: 10.1194/jlr.M400144-JLR200.
57. Arisawa T, Shibata T, Kamiya Y, Nagasaka M, Nakamura M, Fujita H, et al. Effects of sucralfate, cimetidine and rabeprazole on mucosal hydroxyproline content in healing of ethanol-hcl-induced gastric lesions. *Clin Exp Pharmacol Physiol* 2006; 33(7):628–32. doi: 10.1111/j.1440-1681.2006.04418.x.
58. Ichikawa T, Ishihara K. Protective effects of gastric mucus. In: *Gastritis and Gastric Cancer: New Insights in Gastroprotection, Diagnosis and Treatments* (P. Tonino). Intech Open Access Publisher, London, UK. 2011, pp. 3-24.

©2019 AIMSCI Inc. All Rights Reserved.