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Protection by methylene blue alone or with vitamin C on gastric mucosal damage and brain histopathological changes caused by indomethacin in rats

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Abstract

The redox dye methylene blue (MethyB) exerts neuro- and gastric protective effects. In this study, the effect of MethyB and its possible modulation by vitamin C (Vit C) on the gastric and brain histopathological changes induced by indomethacin (IND) was examined. Indomethacin was subcutaneously given to rats at a dose of 30 mg/kg and at the same time rats were also treated with MethyB (30 mg/kg) alone or in combination with Vit C (100 or 200 mg/kg) intraperitoneally and euthanized 24 hr later. The number and severity of mucosal lesions were determined. Gastric mucosal and brain histopathology and histochemical staining for mucopolysaccharides in the gastric mucosa were also done. MethyB markedly reduced the number and severity of gastric mucosal lesions in indomethacin-treated rats. Histologically, IND caused extensive disruption of gastric surface epithelium, mucosal erosions and ulcers and reduced thickness of gastric mucosa. Additionally, there was complete depletion of glycoproteins in the disrupted areas in apical epithelial cells and gastric glands as shown by periodic acid Schiff staining. These changes were markedly attenuated by MethyB alone or combination with Vit C with increased mucosal thickness and restoration of gastric mucus. The histopathological changes observed in the cerebral cortex of rats treated with IND, namely spongiform changes, gliosis, dark pyknotic neurons were markedly reduced by MethyB or Vit C/MethyB co-treatment. Collectively, these results suggest a protective effect of MethyB against gastric mucosal and brain damage caused by IND.

Keywords: Methylene blue, indomethacin, vitamin C, gastric mucosa, gastric ulcer

1 Introduction

Gastroduodenal ulceration is a major health problem in terms of both morbidity and mortality [1]. Non-steroidal anti-inflammatory drugs (NSAIDs) are the most widely used drugs for their ant-inflammatory, analgesic and antipyretic effects in the treatment of rheumatoid arthritis and osteoarthritis, gouty arthritis, bursitis, tendonitis, and fever [2]. These agents act by inhibiting the enzyme cyclo-oxygenase (COX), the key enzyme responsible for the synthesis of prostaglandins [3]. The enzyme exists in two forms; COX-1 which is constitutively expressed in the gastrointestinal tract and maintains mucosal integrity through the supply of prostaglandins and COX-2 which has low expression but is induced during inflammation [4]. In the gastric mucosa, prostaglandins exert protective effects increasing mucus and bicarbonate secretion from the surface epithelial cells, inhibiting gastric acid secretion and increasing mucosal blood flow, thereby, enhancing mucosal defense against luminal acid and other injurious agents. Prostaglandins have cytoprotective properties that is their ability to protect the mucosa from noxious challenge independently of inhibiting gastric acid secretion [5]. Thus, in contrast to conventional NSAIDs, selective COX-2 inhibitors spare the gastric prostaglandins synthesis and are associated with a little gastric injury [6].

Indomethacin is a potent NSAID which inhibits both COX-1 and COX-2, depriving the gastric mucosa from cytoprotective prostaglandins [7]. The drug also decreases gastric mucosal blood flow, an important mucosal defense mechanism against H^+ back diffusion [8]. Indomethacin thus results in the development of gastric mucosal erosions and bleeding and is widely used to induce gastric mucosal damage in experimental animals [9].

Methylene blue (MethyB) is a redox dye having many applications in human diseases such as the treatment of methemoglobinemia [10], cyanide poisoning [11] and encephalopathy caused by the alkylating agent ifosfamide [12]. MethyB is a reduction-oxidation agent with potent antioxidant properties which prevents the formation of mitochondrial oxygen free radicals [13, 14]. MethyB has been shown to exert neuroprotective effects in models of Huntington's disease [15], Alzheimer disease [16],

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Parkinson's disease [17], and malathion toxicity [18]. It also displayed protective effects against gastric mucosal damage caused by Na+-taurocholate [19].
The present study was therefore designed to investigate the effect of MethyB on the development of gastric mucosal and neuronal injury caused by the NSAID indomethacin in the rat. The possible modulation by the antioxidant ascorbic acid (vitamin C) was also examined.

2 Materials and Methods

2.1 Animals

Sprague-Dawley rats of both sexes, weighing 170-180 g were used in the study. Animals were provided by the Animal House of the National Research Centre, Cairo. 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 and the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2 Drugs and chemicals

Methylene blue (Sigma, St Louis, MO, USA) and indomethacin (Kahira Pharm & Chem. IND Co., Cairo, Egypt) were used. Other chemicals and reagents were purchased from Sigma (St Louis, MO, USA). Methylene blue was dissolved in isotonic (0.9% NaCl) saline solution immediately before use. Indomethacin was dissolved in a 5% solution of sodium bicarbonate. The dose of methylene blue used was based on previous studies [19].

2.3 Experimental design

Rats were divided into five equal groups (six rats each). Indomethacin was subcutaneously given at a dose of 30 mg/kg. The effect of MethyB (30 mg/kg, *i.p.*) administered at the time of indomethacin injection alone or combined with Vit C (100 or 200 mg/kg, i.p.) was studied. Food and water were provided *ad libitum*. Rats were euthanized 24 hr after indomethacin. The brain and stomach of each rat were then quickly removed, washed with ice-cold phosphate-buffered saline (PBS, pH 7.4).

2.4 Gastric ulcerogenic studies

The stomachs were opened along the greater curvature; the stomachs were then rinsed with saline, extended on a plastic board, and examined for mucosal lesions. The number and severity of mucosal lesions were noted and lesions were scaled as described by Mózsik et al. [20]. The lesions were scaled as follows: petechial lesions = I: lesions smaller than I mm = 2; lesions between I 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.

2.5 Histopathological studies

The brain and stomach of different groups were fixed in 10% formol saline 5 µm thick paraffin sections were stained with haematoxlin and eosin and investigated by light microscope. Morphometric measurement of the mean value of mucosa was carried out by image analysis.

2.6 Histochemical and morphometric analysis of polysaccharides

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 mucus production and to note the changes in both acidic and basic glycoproteins for histochemical investigation [21]. The stained sections were examined using Image Analyzer (Leica Qwin 500, Cambridge, England) for detection of the PAS intensity. Images were captured with a 10X magnification objective. Five fields were randomly selected from those that did not include large no 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, by 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 grayscale image is a color mode that displays image using 256 shades of gray, referred to as 8-bit grayscale 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, ranging from 0 (black) to 255 (total white). The final PAS intensity was calculated according to the formula:
f = 255 – i where f = final PAS intensity, i = mean PAS intensity obtained from the software; i ranges from 0 (zero = deep brown, highest expression), to 255 (total white) [22].

2.7 Statistical analysis

Values are presented as means ± SE. Results of 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 histological study were analyzed using one-way ANOVA followed by Duncan's multiple range test. Statistical analysis of results was done using SPSS software (SAS Institute Inc., Cary, NC). A probability value of less than 0.05 was considered statistically significant.

3 Results

3.1 Gastric mucosal lesions

MethyB (30 mg/kg) given at the time of IND administration prevented the development of gastric mucosal lesions in a

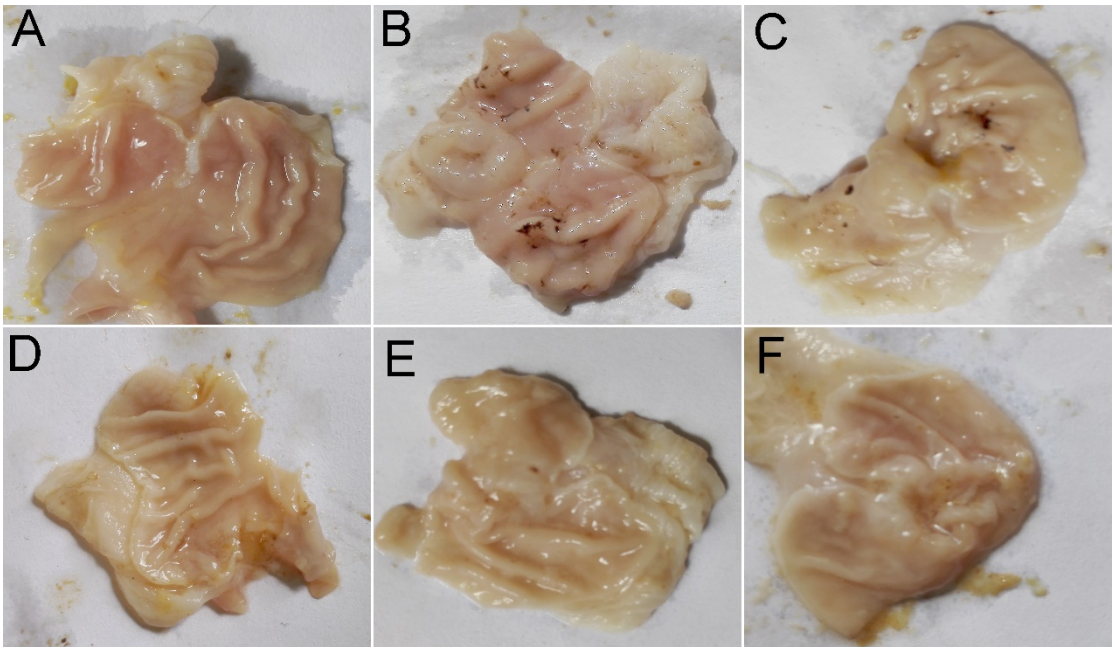

Figure 1. Gross appearance of rat gastric mucosa treated with (A) Saline. (B & C) IND alone. (D) IND + MethyB 30 mg/kg. (E) IND + MethyB + Vit C 100 mg/kg. (F) IND + MethyB + Vit C 200 mg/kg.

Table 1. Effect of methylene blue (MethyB) on the indomethcin-induced gastric mucosal lesions

Treatment	Number of lesions/rat	Severity of lesions/rat
Saline	0.00 ± 0.00	0.00 ± 0.00
IND	4.28 ± 0.36	4.40 ± 0.28
IND + MethyB 30 mg/kg	0.33 ± 0.19* (-92.3%)	0.33 ± 0.19* (92.5%)
IND + MethyB + Vit C 100 mg/kg	0.50 ± 0.20^{*+} (-88.3%)	0.50 ± 0.20^{*+} (-88.6%)
IND + MethyB + Vit C 200 mg/kg	0.50 ± 0.20^{*+} (-88.3%)	0.67 ± 0.30$^{*+\#}$ (-83.8%)

Values are means ± SEM (n = 6) and percent inhibition (%) compared to the IND control group. $^{*}p < 0.05$ compared to the saline control group. $^{+}p < 0.05$ compared to the IND control.

dose-dependent manner. The number of lesions caused by IND was reduced by 92.3% from a control value of 4.28 ± 0.36 to 0.33 ± 0.19. The severity of lesions was also decreased by 92.5% from a control value of 4.40 ± 0.28 to 0.33 ± 0.19. The co-administration of MethyB and Vit C to IND-treated rats decreased the number and severity of gastric lesions by 88.3% and 88.6% (Table 1). Figure 1 shows the gross appearance of rat gastric mucosa of different treated groups.

3.2 Stomach histopathology

H & E sections from saline-treated rats showed normal gastric mucosa studded with many gastric pits. The gastric mucosa is composed of three layers; epithelium, lamina propria containing fundic (gastric) glands and muscularis

Table 2. Thickness of gastric mucosa of different treated groups

Groups	Gastric mucosal thickness (µm)
Saline	105.21 ± 1.23
IND	39.69 ± 0.35*
IND + MethyB 30 mg/kg	72.51 ± 0.83**
IND + MethyB + Vit C 100 mg/kg	71.22 ± 1.09**
IND + MethyB + Vit C 200 mg/kg	65.46 ± 1.4**

All data are presented as mean ± SEM; *Significant decrease at $p < 0.05$ as compared with the saline group; ** Significant increase at $p < 0.05$ as compared with the IND only group

Table 3. Grey level of mucus in gastric mucosa of treated groups

Groups	Grey level of mucus
Saline	161.9 ± 3.1
IND	84.55 ± 2.1*
IND + MethyB 30 mg/kg	159.85 ± 5.4$^{** NS}$
IND + MethyB + Vit C 100 mg/kg	126.8 ± 5.7**
IND + MethyB + Vit C 200 mg/kg	155.9 ± 4.9$^{** NS}$

All data are presented as mean ± SEM; *Significant decrease at $p < 0.05$ as compared with saline group; ** Significant increase at $p < 0.05$ as compared with the IND only group; ^{NS} No significant decrease at $p < 0.05$ as compared with saline group

mucosa (Fig. 2A). The gastric mucosa in rats treated with indomethacin only revealed extensive disruption of the surface epithelium. Some glands were fading of the lining epithelium of gastric gland. Erosion and ulcer were also seen (Figs. 2B, C). Sections of gastric mucosa from rats treated with indomethacin and MethyB showed that gastric

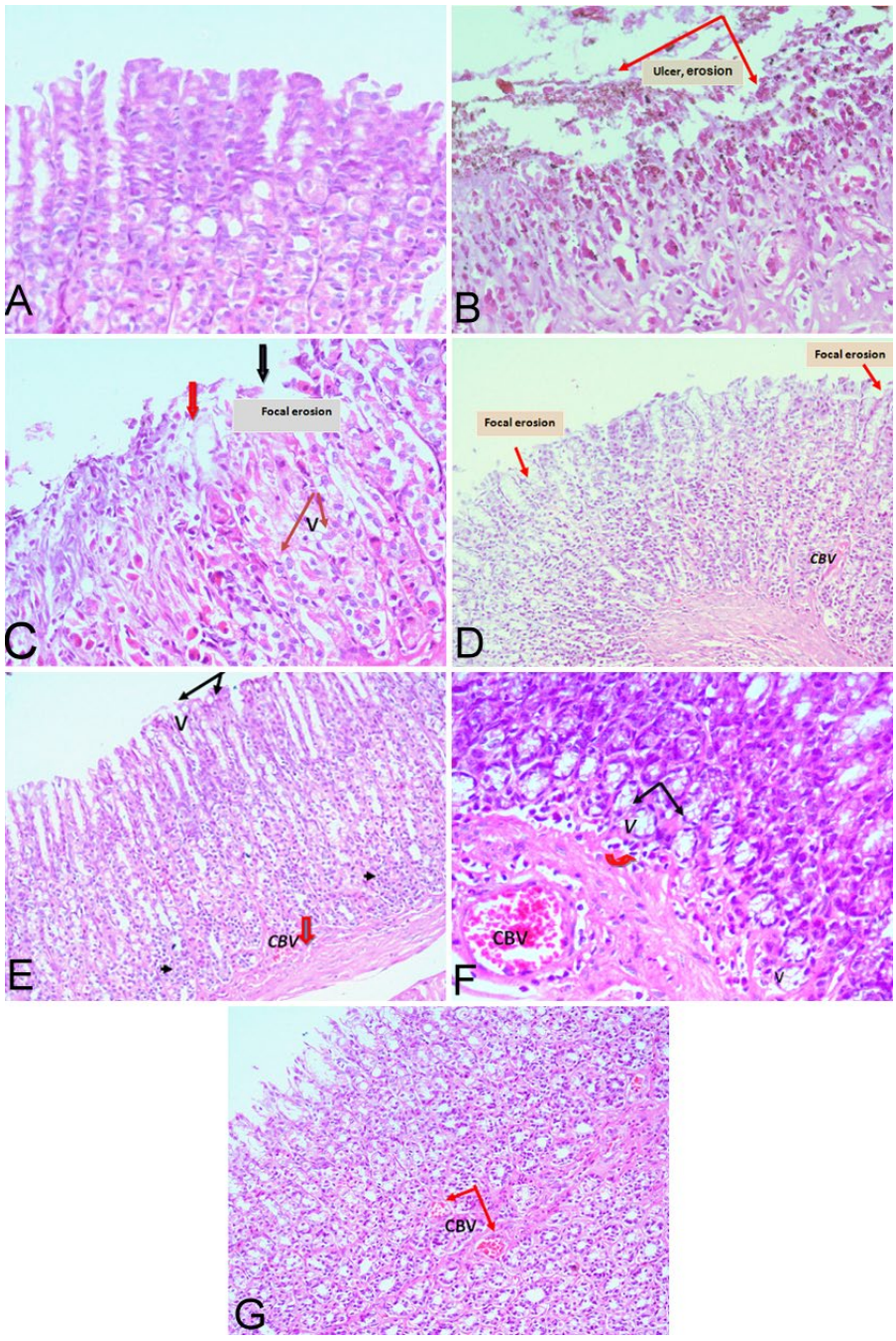

Figure 2. Sections of rat stomach after treatment with: (**A)** Saline: normal histological structure of gastric glands (H & E x 100). (**B)** IND only: ulcer and extensive disruption of the surface epithelium (H & E x400). (**C)** IND: focal erosion (red arrow) and vacuolation of gland cells (yellow arrow). Some glands with fading the lining epithelium (black arrow) (H & E x400). (**D)** IND + MethyB: some changes in the form of discontinuity in the lining epithelium (red arrow) and congestion of blood vessels in the mucosal layer (yellow arrow) (Hx&Ex200). (**E)** IND + MethyB + Vit C 100 mg/kg: stomach tissue appeared normal although vacuolation in lining of some cells of gland (yellow arrow) and congestion of blood vessels (red arrow) and few of Inflammatory infiltrate (black arrow) in mucosal layer (H & E x 200). (**F)** another filed of previous group: congestion of submucosal layer (H & E x 400). (**G)** IND + MethyB + Vit C 200 mg/kg: most of gastric gland appeared normal, congestion of some capillaries with extravasations of blood could be observed (arrow) (H & E x100).

glands still suffered from some pathological changes in the form of discontinuity in the lining epithelium with exudates in the lumen of submucosa and congestion of blood vessels in the mucosal layer (Fig. 2D). Rats treated with indomethacin along with Vit C 100 mg/kg and MethyB showed some improvement in the histological changes as indicated by the absence of erosion or gastric ulcer. In some rats, vacuolar degeneration in the lining epithelium of gastric gland, mild inflammatory infiltrate in mucosal and marked vascular congestion in submucosal layer were seen (Figs. 2E, F). The stomachs of rats treated with indomethacin along with Vit C 200 mg/kg and MethyB showed no disruption to the surface epithelium but edema and congestion of some capillaries with extravasation of blood and congestion of blood vessel in mucosal layer (Fig. 2G).

3.3 Gastric mucosal thickness

In saline-treated rats, the mean value of the thickness of stomach mucosa was 105.21 ± 1.23 µm (Table 2). The gastric mucosa of rats treated with indomethacin only, had

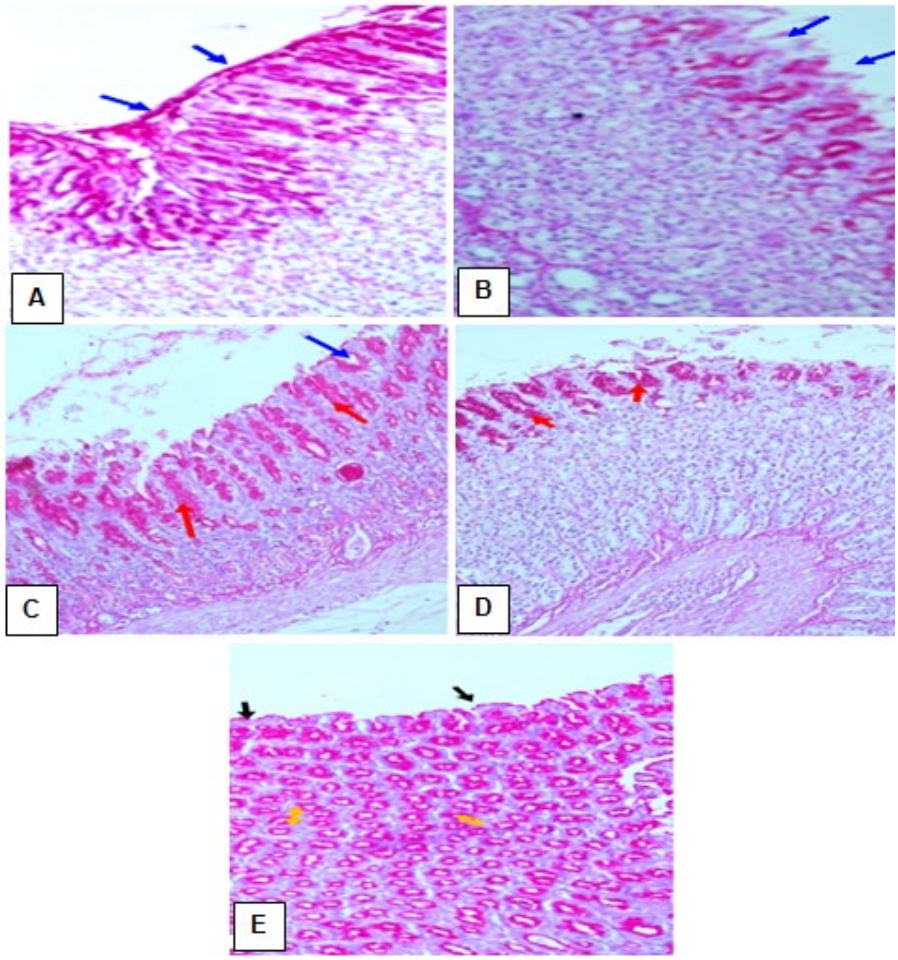

Figure 4. Section of the stomach stained with periodic acid Schiff (**A)** Saline shows normal distribution of mucus, the magenta color appear in the apical epithelial cells and the gastric pits. (**B)** IND only shows decrease in PAS +ve materials in apical epithelial cells and in gastric gland. (**C)** IND + MethyB shows intense PAS stain noted as a bright-magenta to the mucus in some area of epithelial cells (blue arrow), cells lining the gastric pits due to the carbohydrate-rich, viscous mucus they secrete (red arrow). (**D)** IND + MethyB + Vit C 100 mg/kg shows moderate increase in PAS stain in gastric pits. (**F)** IND + MethyB + Vit C 200 mg/kg shows increase in the PAS stain (magenta color) of gastric mucosa in comparison with group treated with indomethcin only indicating increase in glycoprotein content of gastric mucosa (PAS stain x400)

thickness mean value of 39.69 ± 0.35 µm. This indicated a significant decrease in the thickness as compared with the saline control value and the percentage of decrease was -62.27% (Table 2). Rats treated with indomethacin along with MethyB, Vit C 100 mg/kg and MethyB, and Vit C 200 mg/kg and MethyB showed significant increase in the

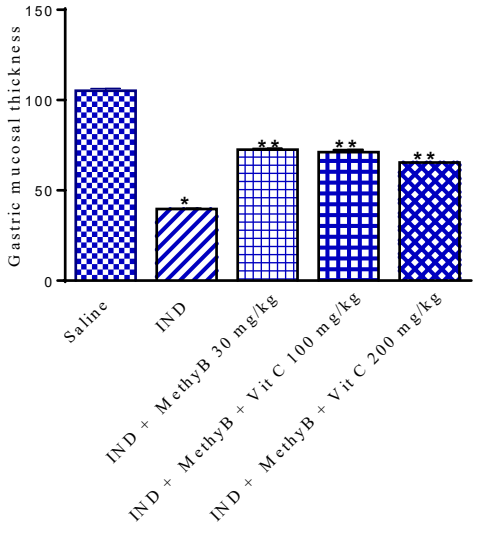

Figure 3. Thickness of stomach mucosa in different groups. All data are presented as mean ± SEM (µm); *: $p < 0.05$ vs. saline group. **: $p < 0.05$ vs. IND control group.

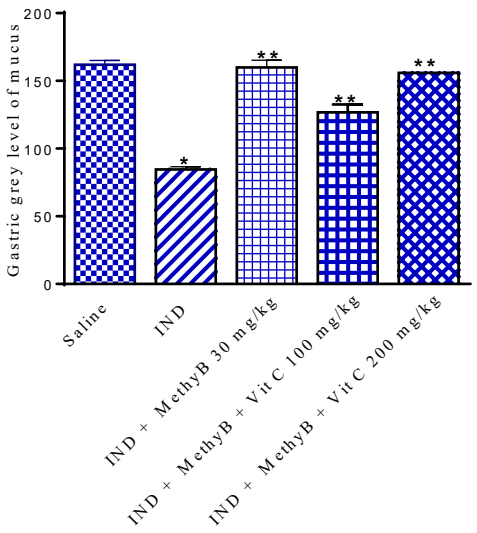

Figure 5. Grey level of mucus in stomach mucosa of different groups. *$p < 0.05$ vs. saline group. **$p < 0.05$ vs IND control group.

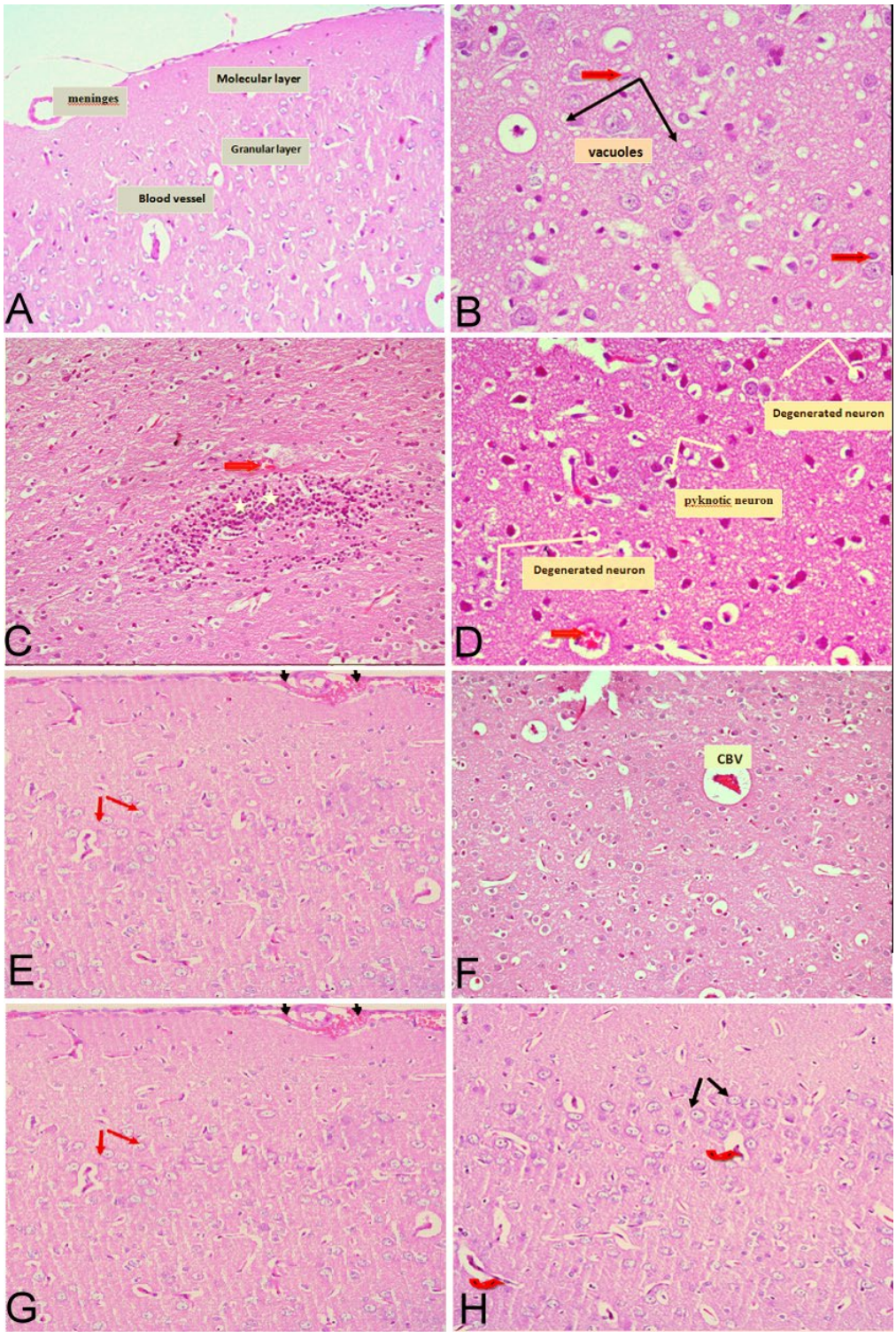

Figure 6. Sections in the cerebral cortex. (**A)** Saline: normal histological structure (H & E x 400). (**B)** IND only: spongiform change that characterized by diffuse or focally clustered small round or oval vacuoles (orange arrow) in the neuropil with gliosis. The granular cells have large vesicular nuclei with a well-defined nucleolus (H & E x 400). (**C)** IND only: dark pyknotic neurons (black arrows), few degenerated neurons (yellow arrow) and congested cerebral blood vessel (red arrow) (H & E x 200). (**D)** IND only: aggregates of inflammatory cells (star). **(E)** IND + MethyB: improvement in pathological changes although some blood vessels show congestion (arrow) (H & E 200). (**F)** IND + MethyB + Vit C 100 mg/kg: normal appearance of granulae cells (black arrow) although cerebral blood vessel (red arrow) (H & E X 200). (**G)** IND + MethyB + Vit C 200 mg/kg: normal appearance of granulae cells (red arrow) but hemorrhage in meninges above the surface was observed (black arrow) (H & E x 200).

thickness of stomach mucosa as compared with the indomethacin only group. The percentages of increase were 82.7%, 70.4% and 64.9%, respectively (Table 2, Fig. 3).

3.4 Histochemical and quantitative analysis of polysaccharides

PAS stained gastric mucosal sections from saline-treated rats showed normal distribution of mucus. The magenta

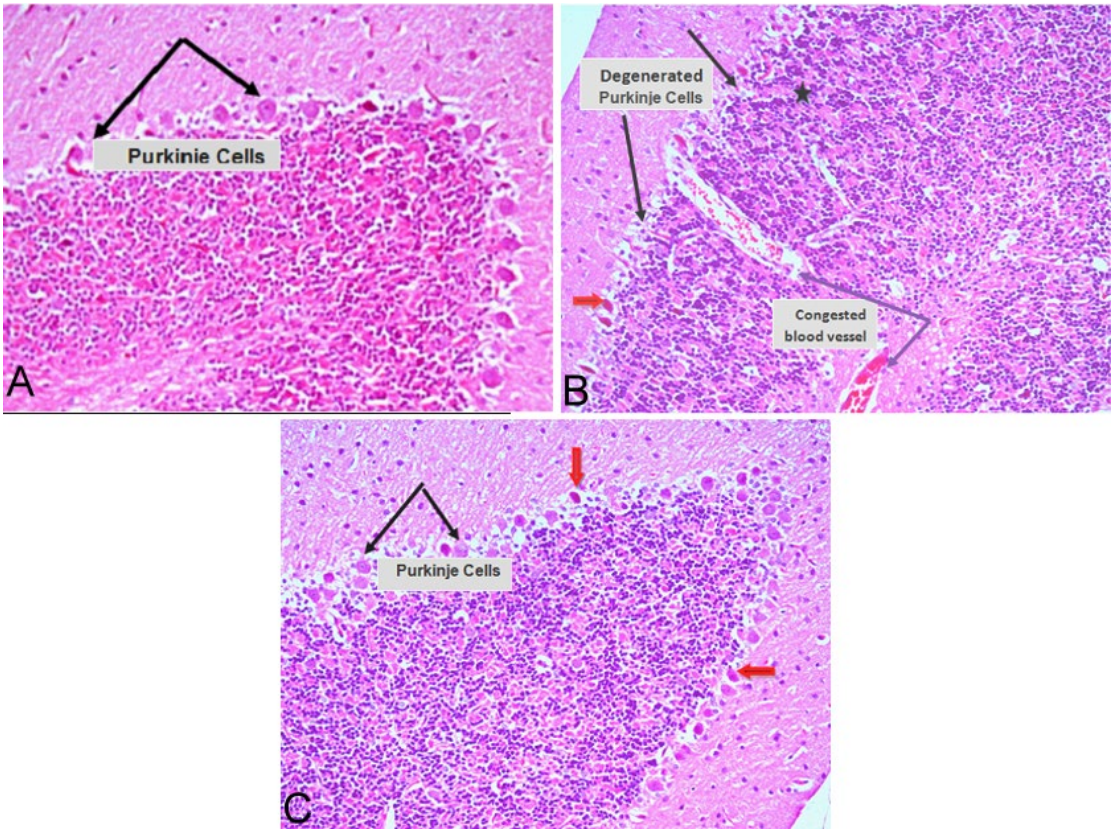

Figure 7. Sections in the cerebellum. (**A)** Saline: histological structure. (B**)** IND only: most of Purkinje cells are lost and leaving empty space and some cells are degenerated. Granular cells show several apoptotic cells and vascular congestion were seen. (C) IND + MethyB + Vit C 200 mg/kg: most of Purkinje cells return to normal appearance but few still degenerated (HX&Ex400).

color in the apical epithelial cells shows glycoprotein accumulation in the gastric glands (Fig. 4A). Indomethacin induced complete depletion in the disrupted areas. Other areas with thin layer of mucin showed mild depletion of PAS stained granules, denoting degeneration of surface mucous cells (Fig. 4B). Grey level of PAS stain of this group indicated marked and significant decrease as compared with the saline control group (Table 3, Fig. 4B). Rats treated with indomethacin along with MethyB, Vit C 100 mg/kg and MethyB showed mucin secretion in some areas of the lesion. Mucin, however, was still decreased in the surface, while in the healthy zones mucin secretion was preserved and even increased (Fig. 4 C, D). The group treated with indomethacin along with Vit C 200 mg/kg and MethyB exhibited very intense distinct PAS-positive reaction in normal gastric mucosa with thick surface mucin layer and intact surface (Fig. 3E). Significant increase in grey of PAS stain was found in these previous groups as compared with group treated with indomethacin only group (Table 3, Fig. 5).

3.5 Brain histopathology

H & E stained sections of brain tissue from saline-treated rats showed the normal cortical morphology and neuronal structure (Fig. 6A). Histopathological examination of cerebral cortex of rats treated with indomethcin only showed spongiform changes characterized by diffuse or focally clustered small round or oval vacuoles with identifiable gliosis, dark pyknotic neurons, granular cells having large vesicular nuclei with a well-defined nucleolus and aggregates of inflammatory cells (leukocytes infiltration) (Figs. 6B, C, D). Sections from the cerebral cortex of rats treated with indomethacin along with MethyB showed improvement in pathological changes although dilatation and congestion of some blood capillaries were noticed (Fig. 6E). Sections from rats treated with indomethacin along with Vit C 100 mg/kg and MethyB showed normal appearance of granulae cells (Fig. 6F). On the other hand, rats treated with indomethacin along with Vit C 200 mg/kg and MethyB showed normal appearance of granulae cells but hemorrhage in meninges above the surface was observed (Fig. 6G). Fig. 7A shows normal cerebellum. In rats treated with indomethacin, most of Purkinje cells are lost, leaving empty space and few cells are degenerated. Granular cells show several apoptotic cells and vascular congestion were seen (Fig.7B). Microscopic examination of rats treated with indomethacin along with MethyB + Vit C 200 mg/kg shows most of Purkinje cells appearing normal but few still degenerated (Fig. 7C).

4 Discussion

In the present work, the administration of indomethacin caused extensive disruption of the surface epithelium. Erosion and gastric ulcer were also seen. The results are in agreement with those of Shalaby et al. [23] who reported areas of focal degeneration in the gastric mucosa that becomes eroded and ulcerate following indomethacin

injection in rats. Other researchers also found massive necrosis, epithelial sloughing, and superficial ulcers after indomethacin administration. The histopathological findings in our study demonstrated that the administration of indomethacin resulted in the vacuolation of gastric glands, some glands were fading of the lining epithelium [24, 25]. These observations are in agreement with those of Shalaby et al. [23] who demonstrated that the indomethacin induced gastric injuries are on the form of vacuolations of the gastric gland cells and degeneration, with disappearance of their nuclei and cytoplasm. In line, Palacios-Espinosa [26] stated that the indomethacin induced some changes in gastric glands, pyknotic nuclei and partial cells appeared vacuolated. According to El-Moselhy et al. [27] treatment with indomethacin caused necrosis and loss of epithelial cells, submucosal edema, marked inflammatory cells and congested blood vessel. Our results also revealed depletion in PAS stain, thereby indicating degeneration of mucous secreting cells. These finding are in agreement with Ribeiro et al. [28] and Shalaby et al. [23] who noticed depletion of the PAS stained granules together with a relatively thin mucous coat over the surface of the gastric in rats treated with indomethacin.

The integrity of the gastric mucosa is kept owing to a number of defensive factors including mucus secretion, gastro-protective prostaglandins synthesis, bicarbonate production, and normal tissue microcirculation. The pathogenesis of gastric ulcer is largely thought to be due to imbalance between these protective mechanisms and aggressive factors such as excessive secretion of gastric acid, alcohol intake, bile salts, abnormal motility, infection with Helicobacter pylori, and NSAIDs [29]. In a previous study, Barnett et al. [24] demonstrated that NSAIDs induce gastric ulcers and bleeding by impairing the restitution process and inactivating several growth factors that are important in the mucosal defense and repair. Moreover, Godessart et al. [30] reported that NSAIDs injured the gastric mucosa as a result of the action of the drug on the cyclo-oxygenase enzyme. This was in accordance with the findings of Kimmey [31] who reported that the NSAIDs induced gastric mucosal injury also results from direct topical injury and reduced mucosal resistance due to indirect or systemic inhibition of mucosal prostaglandin synthesis. The indomethacin-induced gastric ulcer has been explained by Oluwabunmi et al. [32] who postulated that the indomethacin induced gastric ulcer is due to increasing gastric acidity. Decreased mucosal prostaglandin levels results in impaired gastro-protection and increased gastric acid secretion which are important events in the etiology of mucosal ulceration. In addition, the increase acid secretion decreases the process of restitution and ulcer healing via altering angiogenesis. Also Musumba et al. [33] and Sabiu et al. [34] found that the severity of gastric ulcer is due to abnormal elevation of gastric juice by indomethacin. This effect may be attributed to either free radicals formation or inhibition of prostaglandins synthesis. Reactive oxygen species have been implicated in the etiology of indomethacin-induced gastric mucosal damage [35].

Our histological studies also examined the changes in brain tissue after indomethacin injection. The present data shows the occurrence of spongiform changes characterized by small or oval sized vacuoles in the cerebral cortex. Vacuoles in brain parenchyma are a common histological finding that can result from several potential mechanisms associated with excitotoxicity, prion diseases, and spongiform encephalopathies [36]. Strain and Tasker [37] stated that neuronal injury leading to cell death and disintegration is a mean by which classical excitotoxic molecules may produce spaces in brain domains with vulnerable neuron populations, especially the cerebral cortex, cerebellum, and hippocampus. According to Garman [38], vacuoles caused by neurotoxins or infection are associated with neuronal degeneration. Vacuoles may develop by accumulation of fluid within astrocytes. Parenchymal vacuolation (or spongiform degeneration) is a chronic neuron infection with prion particles [39]. Also Fan et al. [40] using transmission electron microscopy detected that vacuoles may be identified as swollen, perivascular glial cell; the affected cells are astrocytes and that vacuoles represent swollen astrocytic processes. Gandin et al. [41] and Wasik et al [42] found that the activity of toxin-formed channels leads to cellular osmotic stress, which activates p38 MAP kinase controlling the triggering of several defense responses including the unfolded protein response; and its long-term induction can trigger vacuolization of endoplasmic reticulum components.

Inflammatory cells are very important in the response to injury in all tissues [4]. In the present work the cerebral cortex of rats treated with indomethacin exhibited aggregates of inflammatory cells in brain. According to Zang et al. [43] brain inflammation has been implicated in the development of brain edema and brain damage in ischemia and trauma. Mechanisms involved in leukocyte infiltration in the blood-brain barrier are still obscure. However, Grau et al. [44] and Takahashi et al. [45] reported that indomethacin might exert effects at a molecular level by blocking the activation of cyclooxygenase enzymes by reactive oxygen species. Indomethacin inhibits COX-1 and COX-2, which are involved in the generation of reactive oxygen species, and also modulates signaling networks involved in inflammation such as nuclear factor κB [46]. In the present work, the treatment of rats with indomethacin resulted in loss of most of Purinkje cells, leaving empty and few degenerated cells. Purkinje cell pathology is a common finding in of inherited and acquired cerebellar disorders, with the degree of Purkinje cell injury dependent on the underlying aetiology [47].

The pathological changes in the brain and stomach due to indomethacin in present work may be due to the accumulation of free radicals. Somasundaram et al. [48] reported that NSAIDs induce the accumulation of oxygen free radicals secondary to reduction of mucosal blood flow. Other authors attributed the deleterious effects of indomethacin to its direct action on mucosal cells such as Ohkawara et al. [49] who postulated that the ability of the indomethacin to cause epithelial damage may be due to part to its accumulation in these cells because of the phenomenon of ion trapping free radicals secondary to reduction of mucosal blood flow. Moreover, Pohle et al. [50] revealed that indomethacin induces impairment of the

tight junction complex morphology and permeability between viable gastric mucosal epithelial cells which might be a major contributing factor in the etiology of stomach lesions. In a previous study, Nam et al. [51] reported that the mechanism of indomethacin induced damage in gastric mucosa is due to increase reactive oxygen species which is critical role in gastric ulceration.

MethyB has been used in human and veterinary medicine for a number of therapeutic and diagnostic procedures including the use as a stain in neuro-anatomy and bacteriology, as a redox coloring agent in biochemical studies, as a targeting agent for various types of cancer such as melanoma and lung cancer, and as an antiseptic compound [52]. MethyB increases acetylcholine concentrations by inhibiting the function of acetylcholinesterase (AChE), an enzyme that hydrolyzes acetylcholine. In fact, MB shares this action mechanism with other AChE inhibitors presently used as long-term symptomatic treatment in patients with Alzheimer's disease [53, 54]. The effect of MethyB on mitochondrial functions have also been applied in ischemic and hypoxic conditions. MethyB shows significant neuroprotective effects in ischemia-reperfusion models of the brain [55] and the spinal cord [56]. In addition, MethyB can prevent nitric oxide (NO) effects by inhibiting soluble guanylate cyclase, NO, endothelial NO synthase and inducible nitric oxide synthase (iNOS) [57, 58].

Our results show that the treatment of rats with indomethacin and MethyB caused improvements in the pathological changes induced by indomethacin in the stomach and brain and increases PAS staining in comparison with rats treated with only indomethacin. These finding do not agree with Shah et al. [59] who reported an ulcerogenic action of MethyB. A previously published study, however, indicated a protective action for MethyB against gastric ulcerations caused by bile salt in rats [19]. According to Swerdlow [60], MethyB is known to possess neuroprotective properties that reduce protein aggregates, augment the antioxidant response, and enhance mitochondrial function. Similarly, MB was previously shown to increase mitochondrial oxygen consumption and restore memory retention in metabolically impaired rats by increasing brain cytochrome c oxidase activity [61]. MethyB can shift electrons in the mitochondrial electron transfer chain directly from NADH to cytochrome c, increasing the activity of complex IV, can attenuate superoxide production and strongly promoting mitochondrial activity while mitigating oxidative stress [62, 63]. Thus, MB has been established as an antioxidant that increases cell metabolism through enhancing mitochondrial activity at the cytochrome oxidase complex [64]. MB competitively races against flavoenzymes such as xanthine oxidase to transfer oxygen and prevent oxygen radicals such as superoxides being produced [58].

Our finding shows improvement in pathological changes in stomach and brain especially in rats treated with MethyB in combination with vitamin C at dose level of 200 mg/kg. Reactive oxygen species have been implicated in the etiology of indomethacin-induced gastric mucosal damage [65]. The treatment of rats with of indomethacin induced a significant decrease in the levels of superoxide dismutase, glutathione peroxidase, glutathione S-transferase and glutathione, and an increase in the lipid peroxidation level [66]. Studies indicated that ascorbic acid (vitamin C) caused protective effects against oxidative gastric mucosal damage induced by indomethacin. The changes in the levels of myeloperoxidase, antioxidant system enzymes (glutathione S-transferase, superoxide dismutase, glutathione reductase, catalase, glutathione peroxidase), lipid peroxidation and glutathione, as markers for ulceration process improved following oral administration of ascorbic acid [35, 67]. Coinciding with Miura et al. [68] found that the administration of ascorbic acid reversed the decrease in catalase, glutathione reductase and the increase in myeloperoxidase activities and in lipid peroxidation level by indomethacin in tissues. The gastroprotective properties of ascorbic acid therefore is related to its positive effects on the antioxidant system and myeloperoxidase activity in indomethacin-induced gastric in rats. Other researchers showed that the level of glutathione peroxidase in rat stomach tissues was significantly reduced by administration of indomethacin while administering ascorbic acid (100, 200, and 400 mg/kg) significantly increased the glutathione peroxidase level [35]. Ascorbic acid may be seen as a chemopreventive agent which increases the glutathione S-transferase enzyme. Glutathione and glutathione-bound enzymes in tissues especially glutathione peroxidase and glutathione S-transferase, have been proposed as potential chemo preventive agents for their antioxidant and detoxification properties [69].

5 Conclusion

In summary, the present study indicates that MethyB administered alone or in combination with Vit C protected against gastric mucosal damage and brain neuronal injury caused by indomethacin. This action of MethyB is likely to involve a decrease in oxidative stress.

Conflicts of interest

The authors declare that there are no potential conflicts of interest.

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