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The dual modulatory effect of folic acid supplementation on indomethacin- induced gastropathy in the rat

Kazeem AJEIGBE[1], Eyitayo OLADEJO[1], Ben EMIKPE[2], Atamgba ASUK[3], Samuel OLALEYE[1]

Departments of [1]Physiology and [3]Biochemistry, Igbinedion University, Okada, Nigeria
Department of [2]Veterinary Pathology, University of Ibadan, Ibadan, Nigeria

Background/aims: *Folic acid modulates several disorders in humans. We investigated the effects of folic acid supplementation at varying doses on ulcer formation in the rat.* ***Materials and Methods:*** *Male Wistar rats were treated with 1 mg/kg, 2 mg/kg and 3 mg/kg diet of folic acid for 21 days. Gastric ulceration was induced by indomethacin, scored, and assayed to determine the concentration of mucus, malondialdehyde, catalase, and superoxide dismutase in homogenized samples. Normal saline- and ranitidine-treated groups served as negative and positive control, respectively.* ***Results:*** *Indomethacin caused severe damage to the glandular portion of the rats' stomachs with increase in malondialdehyde concentration and reduction in mucus, catalase and superoxide dismutase concentration ($p<0.001$). Folic acid supplementation at 2 mg/kg diet reduced significantly the formation of gastric lesions by indomethacin, while at 3 mg/kg, potentiation of the lesions was observed ($p<0.05$). Malondialdehyde concentration significantly decreased and superoxide dismutase activity increased in the 2 mg/kg folic acid pre-treated group, while 3 mg/kg folic acid significantly increased the malondialdehyde concentration and decreased both catalase and superoxide dismutase. Mucus concentration was increased in the 2 mg/kg folic acid pretreated group, but decreased in the 3 mg/kg folic acid pretreated group when compared with controls. Pre-treatment with 1 mg/kg diet of folic acid produced no significant changes. Histopathological studies underlined differences in the indomethacin-induced alterations in gastric mucosal structure following pre-treatment with a 2 mg/kg or 3 mg/kg diet of folic acid.* ***Conclusions:*** *Folic acid is gastroprotective at the basal requirement supplemental dose; high dose may be dangerous to the integrity of the stomach.*

Key words: Folic acid, indomethacin, ulcer, malondialdehyde, superoxide dismutase, catalase

Sıçanlarda indometazine bağlı gastropatide folik asid uygulamasının ikili modülatör etkisi

Giriş ve Amaç: *Folik asid insanlarda birçok hastalığı modüle etmektedir. Çeşitli dozlarda folik asid uygulamasının sıçanlarda ülser gelişimi üzerine olan etkisini araştırdık.* ***Gereç ve Yöntem:*** *Erkek Wistar sıçanlar 21 gün boyunca 1 mg/kg, 2 mg (kg ve 3 mg/kg folik asid içeren diyet ile beslendiler. İndometazin ile gastrik ülser indüklendi ve skorlandı ve ayrıca homojenize örneklerde gastrik mukus, malonildialdehid, katalaz ve süperoksid dismutaz konsantrasyonları ölçüldü. Normal salin ve ranitidin uygulanan gruplar negatif ve pozitif kontroller olarak kullanıldı.* ***Bulgular:*** *İndometazin sıçanların midesinin glandüler kısmında ağır hasara, malonildialdehid konsantrasyonunda artışa, mukusta, katalaz ve süperoksid dismutaz konsantrasyonlarında azalmaya neden oldu ($p<0.001$). Folik asit desteği (2 mg/kg) indometazine bağlı gastrik lezyonların oluşmasını azaltırken, 3 mg/kg dozda lezyon oluşumunu arttırdığı görüldü ($p<0.05$). Malonildialdehid konsantrasyonunda azalma ve süperoksid dismutazda artma 2 mg/kg grubunda gözlenirken, 3 mg/kg grubunda malonildialdehidde artış ve katalaz ve süperoksid dismutazda azalma gözlendi. Kontroller ile karşılaştırıldığında 2 mg/kg grubunda mukusta artış görülürken, 3 mg/kg grubunda azalma tespit edildi. Bunlara karşılık 1 mg/kg folik aist uygulaması ile anlamlı değişiklikler görülmedi. Histopatolojik incelemede 2 mg/kg veya 3 mg/kg folik asit ile birlikte indometazine bağlı gelişen değişikliklerin belirginleştiği görüldü.* ***Sonuç:*** *Folik asit tamamlayıcı dozda gastroprotektifken, yüksek dozlarda mide için zararlı olabilir.*

Anahtar kelimeler: Folik asit, indometazin, ülser, malonil dialdehid, superoksid dismutaz, katalaz

Address for correspondence: Kazeem AJEIGBE
Igbinedion University, Department of Physiology, Okada, Nigeria
E-mail: omowoyira@yahoo.com

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INTRODUCTION

Folate is a water-soluble vitamin essential for cell replication and DNA synthesis, repair, and methylation (1). Folic acid plays an important role in the pathophysiology of several disorders in humans, including macrocytic anemia, cardiovascular diseases (2), thromboembolic processes (3), neural tube and congenital defects (4), adverse pregnancy outcomes (5), and neuropsychiatric disorders (6). Supplementation with folic acid reduces the risk of congenital heart defects, cleft palate, limb defects, urinary tract anomalies, neural tube defects, and chronic liver diseases (7-10). It is also a safe and effective supplement that may prevent isolated systolic hypertension (11) and several malignancies, including cancer of the colorectum, lungs, pancreas, esophagus, stomach, cervix, and breast (12,13), neuroblastoma, and leukemia (14,15). Some data have indicated equally that folic acid may play an important role in the chemoprevention of gastric carcinogenesis by enhancing gastric epithelial apoptosis in patients with premalignant lesions (16).

Recently, we reported from our laboratory the gastroprotective activity of folic acid supplementation at the basal requirement supplemental dose of 2 mg/kg diet against the lipid peroxidative activity of indomethacin (17). One of the most commonly used models for experimental ulceration is the oral administration of indomethacin, a non-steroidal anti-inflammatory drug (NSAID).

Indomethacin induces an injury to the gastrointestinal mucosa in experimental animals and humans, and its use is associated with a significant risk of hemorrhage, erosions, and perforation of both gastric and intestinal ulcers (18).

Though folic acid supplementation is believed to be safe and free of toxicity (19), it may demonstrate some undesirable effects, especially in some populations not targeted for the dietary fortification (15). For instance, clinical and experimental studies suggest that folate possesses dual modulatory effects on carcinogenesis, depending on the timing and intervention dose (20).

Therefore, the purpose of this study was to examine whether or not the anti-oxidative property of folic acid supplementation on gastric mucosa reported earlier is dose-dependent. This study will further descriptively and/or pharmacologically evaluate the effects of folic acid on indomethacin- induced gastropathy.

MATERIALS AND METHODS

Animals

Forty-two male albino rats of the Wistar strain weighing between 180-250 g were used for this study. The animals were obtained from the Animal House of Igbinedion University, Okada, and then separated randomly into six wire-meshed cages with seven rats each, where they were kept for four weeks before the commencement of the experiment. The animals were housed under standard conditions of temperature (23±2°C), humidity (55±15%), and 12-hour (h) light (7:00 am - 7:00 pm). The cages were cleaned constantly in order to protect the animals from disease. They were fed with standard commercial rat pellets (Ladokun Feeds Limited, Nigeria) and allowed water *ad libitum*.

Drugs

Folic acid tablets and indomethacin were obtained from a local pharmacy duly registered by the Pharmacists' Council of Nigeria (PCN). All other reagents were of analytical grade and obtained from British Drug Houses, Poole, UK.

Experimental Design

Grouping

The animals were divided into six groups of seven rats each.

Group One: Animals were treated with distilled water. They served as the overall control group.

Group Two: Animals were treated with indomethacin (25 mg/kg) after 24-h fasting. This group served as the treated control.

Group Three: Animals were treated with 1 mg/kg of folic acid for three weeks before indomethacin (25 mg/kg) administration.

Group Four: Animals were treated with 2 mg/kg of folic acid for three weeks before indomethacin (25 mg/kg) administration.

Group Five: Animals were treated with 3 mg/kg of folic acid for three weeks before indomethacin (25 mg/kg) administration.

Group Six: Animals received ranitidine (4 mg/kg) prior to indomethacin administration. This group served as the positive control group.

The route of administration for both folic acid and indomethacin is oral. All the animals were sacrificed under sodium pentobarbitone anesthesia. The Central Animal Facility/Ethics Committee of Igbi-

nedion University, Okada approved the experimental protocols.

Ulcer Induction and Index Determination

Four hours after the oral administration of indomethacin, the stomachs were opened along the greater curvature, washed in normal saline to remove debris and pinned on a cork mat for ulcer scoring. This was done by locating the wounds in the glandular region under a simple microscope. The length (mm) of all the elongated black-red lines parallel to the long axis of the stomach in the mucosa was measured. The index of ulceration was calculated as the total lesion lengths divided by the number in each group (21).

Biochemical Analysis

Determination of Malondialdehyde (MDA): The assay method of Hunter et al. (22), modified by Gutteridge and Wilkins (23), was adopted. MDA, a product of lipid peroxidation, when heated with 2-thiobarbituric acid (TBA) under acid conditions forms a pink-colored product that has a maximum absorbance of 532 nm. The stomach homogenate was supplemented with I g of TBA in 100 ml of 0.2% NaOH and 3 ml of glacial acetic acid, thoroughly mixed and incubated in a boiling water bath for 15 minutes (min), then allowed to cool, after which they were centrifuged. Absorbance was read at 532 nm and the results expressed as nanomoles MDA/mg wet tissue.

Determination of Catalase (CAT) Activity: Activity of CAT in the gastric mucosa was determined according to the procedure of Sinha (24). This method is based on the reduction of dichromate in acetic acid to chromic acetate when heated in the presence of H_2O_2, with the formation of perchromic acid as an unstable intermediate. The chromic acetate so produced is measured. Absorbance was read at 480 nm within 30-60 seconds against distilled water.

Determination of Superoxide Dismutase (SOD) Activity: A method originally described by Misra and Fridovich (25) as reported by Magwere et al. (26) was employed. The homogenate was supplemented with 2.5 ml of carbonate buffer, followed by equilibration at room temperature; 0.3 ml of 0.3 nm adrenaline solution was then added to the reference and the test solution, followed by mixing and reading of absorbance at 420 nm.

Determination of Gastric Mucus: Adherent gastric glandular mucus was measured by the method of Corne et al. (27). The excised stomach was soaked for 2 h in 0.1% Alcian blue dissolved in buffer solution containing 0.1M sucrose and 0.05M sodium acetate (pH adjusted to 5.8 with hydrochloric acid). After washing the stomach twice in 0.25 M sucrose (15 and 45 min), the dye complexed with mucus was eluted by immersion in 10 ml aliquots of 0.5 m MgCl2 for 2 h. The resulting blue solution was shaken with equal volumes of diethyl ether, and optical density of the aqueous phase was measured at 605 nm using a spectrophotometer.

Using a standard curve, the absorbance of each solution was then used to calculate the various concentrations of the dye and the weight of the dye (expressed in mg). The weight of the dye was then expressed over the weight of the stomach.

Histopathological Studies

Histological examination was done by fixing both the normal and ulcerated stomachs of the rats in 10% formalin, which were then processed and embedded in paraffin wax. Tissue blocks were sectioned 5 µm thick and stained with hematoxylin and eosin (H&E).

Statistical Analysis

Data was expressed as mean ± SEM (Standard Error of Means of 7 observations) and analyzed by application of the Statistical Package for the Social Sciences (SPSS) version 15. The Student's t-test was applied and p-values were determined. Differences were considered significant at $p<0.05$.

RESULTS

Development of Gastric Lesions

Indomethacin caused severe damage to the stomachs of the rats ($p<0.05$) (Table 1).

Pre-treatment of rats with 1 mg/kg diet of folic acid produced no significant change in the formation of ulcers by indomethacin. For animals treated with a 2 mg/kg diet of folic acid before ulcer induction by indomethacin, there was a reduction in the status of ulceration when compared with the ulcer control group ($p<0.05$). However, there was an increase in the status of the ulcer formed in the animals pre-treated with a 3 mg/kg diet of folic acid when compared with the control group ($p<0.05$) (Table 1).

Gastric Mucus Concentration

Gastric mucus was depleted significantly in the indomethacin-treated group when compared with

Table 1. Effect of different doses of folic acid on gastric mucosal injury and mucus content

Groups	Treatment	Gastric Mucus (mg/g) (Mean±SEM)	Ulcer Score (mm) (Mean±SEM)
I	Distilled water	35.50±0.90	0.00
II.	Indo only (25 mg/kg)	30.25±0.25^{+}	26.80±4.59^{++}
III.	1 mg/kg folic acid + Indo (25 mg/kg)	26.10±0.20^{a}	27.40±5.36
IV.	2 mg/kg folic acid + Indo (25 mg/kg)	34.50±0.30^{b}	20.60±4.06^{b}
V.	3 mg/kg folic acid + Indo (25 mg/kg)	24.50±0.60^{c}	35.20±4.14^{c}
VI.	Ranitidine (4 mg/kg) + Indo (25 mg/kg)	33.25±0.10^{d}	13.55±2.10^{d}

Indomethacin: Highly significant from distilled water, $^{++}$p<0.001, significant $^{+}$p<0.05. Folic acid and ranitidine: Significant from indomethacin-treated, ^{a,b,c,d,} p<0.05.

the normal saline group. Pre-treatment with 1 mg/kg and 3 mg/kg folic acid led to further decrease in the mucus concentration while 2 mg/kg caused a marked increase (p<0.05) (Table 1).

Lipid Peroxidation and Anti-Oxidative Enzymes

Figure 1 [a, b and c] shows the lipid peroxidation (MDA) and CAT and SOD activities in both the normal and ulcerated gastric mucosa. Lipid peroxidation is measured as the amount of thiobarbituric acid reactive substances (TBARs) in the gastric mucosa, and the results were expressed as MDA formed using an extinction coefficient of 1.56 X 10^{5}/Mcm. Indomethacin produced lipid peroxidation in the normal mucosa (p<0.05). In the same vein, the SOD activities reduced significantly (p<0.05) while the CAT activity remained unchanged (p>0.05). Pre-treatment with a 1 mg/kg diet of folic acid caused no significant change in MDA concentration or CAT and SOD activities when compared with the treated controls. Pre-treatment with a 2 mg/kg diet of folic acid reduced the MDA concentration and increased the SOD activity (p<0.05), with no significant change in CAT activity. Pre-treatment with a 3 mg/kg diet of folic acid increased MDA concentration and decreased CAT and SOD activities when compared with the treated controls (p<0.05).

Although 2 mg/kg of folic acid inhibited the development of ulcer by 23.1%, ranitidine afforded a 51.9% protection on the mucosa, with an increase in the mucus production (p<0.05) (Table 1). In the same vein, it reduces MDA concentration and increased the activities of both CAT and SOD (p<0.05).

DISCUSSION

The findings of the present study confirmed that folate, an important factor in the *de novo* synthesis of purines and thymidine, DNA stability, and apoptosis (28), attenuated the development of gastric ulcer only at the basal requirement dose. The molecular basis for the gastrointestinal toxicity of NSAIDs is widely believed to be their inhibitory activity against cyclooxygenase, which causes them to block the production of prostaglandins and their therapeutic actions. Suppression of prostaglandin synthesis is associated with reduction of gastric mucosal blood flow, disturbance in microcirculation, and decrease in mucus secretion, lipid peroxidation, and neutrophil activation, which are involved in the pathogenesis of gastrointestinal mucosal disorders (29,30).

The effects of folic acid dietary manipulation have been extensively studied in experimental models of cancer and cardiovascular disease (15), but there is still a relative paucity of data regarding the effects of folic acid supplementation on gastrointestinal inflammatory disorders.

Gastrointestinal wall integrity is known to be controlled by two opposing forces: aggressive and defensive (27). The aggressive force encompasses the increase in acid output and subsequent lipid peroxidation, which is a result of the reaction between oxyradicals and the polyunsaturated fatty acids. The defensive are gastroprotective and involve the

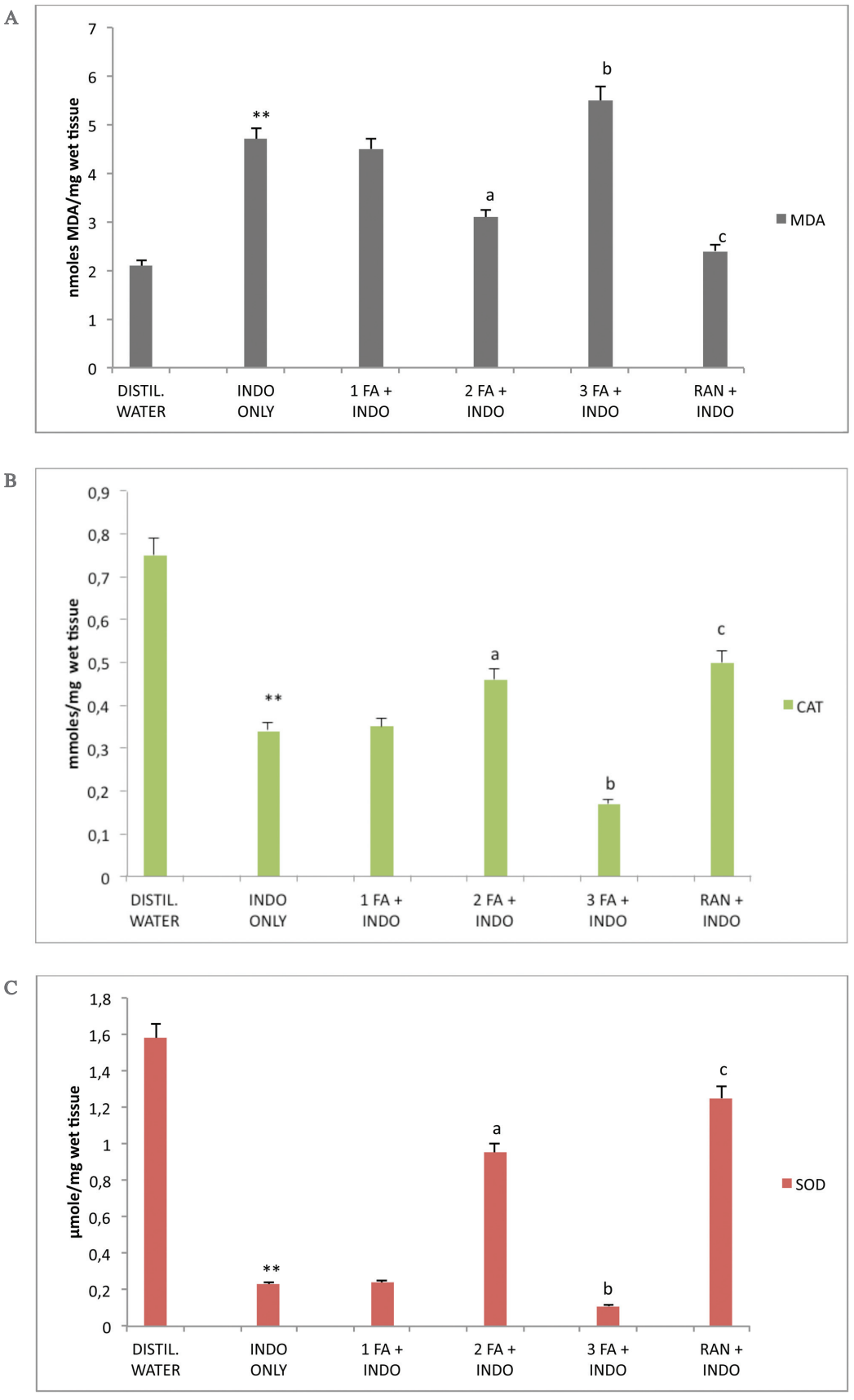

Figure 1 A, B, C. Effect of folic acid on MDA **(1A)** concentration, CAT **(1B)** and SOD **(1C)** activities in gastric mucosa. Vertical bars represent mean±SEM of seven rat, each. **P<0.001 (c.f Distilled water), [a,b,c]p<0.05 (c.f Indo only).

anti-oxidative enzymes: SOD, which catalyzes the dismutation of superoxide radical anion (O_2^-) into less noxious hydrogen peroxide (H_2O_2), and CAT or glutathione peroxidase that inactivate H_2O_2 to water (31). Indomethacin has been known to cause lipid peroxidation (32,33) with depletion of endogenous antioxidant. In the present study, this is confirmed by the decrease in the activities of both CAT and SOD with the concomitant increase in MDA concentration in the homogenized gastric mucosal samples after indomethacin administration. Depletion of the antioxidant reserve and mucus in the gastric mucosa is an important factor in the pathogenesis of peptic ulceration. Hence, increase in the SOD activity and mucus concentration observed in the group of ulcerated animals pretreated with 2 mg/kg folic acid portend somewhat the gastroprotective tendencies of folate (at this dose), because they are scavengers that mop up and resist free radicals predisposing the stomach to inflammation. Moreover, this is underscored by the decrease in the MDA concentration observed in this group of animals, which agrees with the mild severity of the wound in the glandular portion of the stomach when viewed macromorphologically and assessed histopathologically. The implication of this could mean that folate inhibits the lipid peroxidation activity of indomethacin. These may not be in dissension with the earlier reports of Cao et al. (16), which demonstrated that both the epithelial apoptosis rate and the tumor suppressor p53 expression in gastric mucosa were significantly increased, while the expression of Bcl-2 oncogene protein decreased after folic acid treatment in patients with premalignant gastric lesions.

However, ulcerated animals pre-treated with 1 mg/kg diet of folic acid showed no remarkable difference either macromophologically or biochemically. It may be suggested, therefore, that antioxidant or gastroprotective tendencies of folate may be found wanting when administered at a subnormal dose.

Animals pre-treated with a 3 mg/kg diet of folic acid show an increase in the MDA concentration with reduced activities of both CAT and SOD, supported by more severe wounds in the glandular portion of the stomach.

Folic acid has been shown to possess a dual modulatory role, depending on the dose and timing of intervention in disease states (13), which is critical in providing safe and effective chemopreventiion. Marsillach et al. (34) demonstrated that moderately high folic acid supplementation exacerbates experimentally induced liver fibrosis in rats. This could be interpreted to indicate the fact that under certain clinical conditions, (moderately) high folic acid supplementation can have undesirable effects.

Conclusively, folic acid supplementation is ameliorative on indomethacin-induced gastric ulceration at the basal requirement supplemental level. While low folic acid may have no effect, high folic acid potentiates gastric ulceration.

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