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Folic acid protects and heals gastric mucosa: role of acid output, inflammatory cytokines, angiogenic and growth factors

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Abstract

Background: Folic acid modulates gastrointestinal inflammatory disorders via a number of suggested gastroprotective mechanisms. Gastric acid, inflammation, cell proliferation and angiogenesis play significant role in gastroprotection and restoration of gastrointestinal mucosal integrity following injury. This two-section-study assessed (1) acid output, parietal cell mass, neutrophil infiltration and inflammation after 6 h pyloric ligation, and (2) healing via inflammation, mucosa cell proliferation and angiogenesis in acetic acid induced gastric ulcer in albino Wistar rats upon pre-treatment with Folic acid (FA).

Results: Folic acid significantly lessens the mucosa injury associated with pylorus ligation in a dose-dependent manner. Acid output, parietal cell mass and neutrophil infiltration reduced significantly when compared with the control group. In the acetic acid ulcer group, FA equally reduced ulcer severity ($p < 0.05$). Moreover, EGFR and Ki-67 were enhanced, while CD31 and Factor VIII were significantly enhanced only on day 10. Also, EGF and VEGF were enhanced, but TNF-α and IL-1β were suppressed in favour of IL-4 and IL-10 dose-dependently in both studies.

Conclusion: These results suggest that folic acid supplementation protects the stomach mucosa with reduced gastric acid and inflammation, and also accelerates the healing of ulcers via enhanced mucosal cell proliferation and angiogenesis.

Keywords: Folic acid, Ulcer healing, Inflammation, Mucosal proliferation, Angiogenesis, Rats

Background

Folic acid is a type of vitamin B, normally found as folate in foods such as dried beans, peas, lentils, oranges, whole wheat products etc. It functions primarily in promoting the growth of new cells in the body, entailing DNA synthesis, repair and methylation (Faris et al., 2015). It is a safe and effective supplement that may prevent isolated systolic hypertension and stroke (Wang et al., 2007), neural tube defects (Williams et al., 2015), subfertility (Mathieu d'Argent et al., 2021) and several malignancies including cancer of the colorectum, pancreas, oesophagus, stomach, cervix, breast, ovary, and neuroblastoma (Kim, 2016). Previous studies have revealed that folic acid supplementation modulates gastrointestinal inflammatory disorders via a number of suggested gastroprotective mechanisms. Folic acid possesses antisecretory and anti-oxidative properties in indomethacin induced stomach injury (Ajeigbe et al., 2011); then anti-inflammatory and antiapoptotic activities in ethanol-induced gastric ulceration (Ajeigbe et al., 2017a). Hence, it is easily hypothesized that folic acid exhibits protective activity on the stomach mucosa.

The stomach is known to play a pivotal role in the digestion of foods we eat. With the exception of rare cases, this organ can resist an outsized sort of noxious factors, including acid, refluxed bile salts and alcohol. The high resistance to these noxious factors depends

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on a number of physiological responses elicited by the mucosal lining, against the potentially harmful luminal agents, as well as to the ability of rapidly repairing the mucosal damage when it does occur (Laine et al., 2008). Hence, when the gastroprotective defense layer is compromised, ulcer develops.

Gastric ulcer is a defect in the normal gastric mucosa integrity and architecture. It extends through the muscularis mucosa into submucosa or deeper, which results when aggressive factors (endogenous, exogenous and/or infectious agent) overcome the mucosal defence mechanisms (Wallace & Granger, 1996; Tulassay & Herszenyi, 2010). Following mucosal injury, a complex repair process of tissue regeneration and angiogenesis begins in the granulation tissue after 48–72 h. The tissue regeneration involves cell migration, proliferation, re-epithelialization, and gland reconstruction while angiogenesis is the formation of new blood vessel from the pre-existing vessels, or vasculogenesis which presents new blood vessel from bone marrow-derived angiogenic precursor cells, and matrix formation, all ultimately leading to scar formation (Tarnawski, 2005a, 2005b). So, in addition to the epithelial structures re-constructing, new vessels generated are needed to supply adequate oxygen and nutrients to the healing mucosa (Guo et al., 2002). The degree of neovascularization within granulation tissue of the ulcer bed correlates strongly with the rate of ulcer healing. Meanwhile, all these processes of ulcer healing are controlled by growth factors, cytokines, hormones and transcription factors. Aside the initial pool of growth factors derived from platelets, macrophages and injured tissue, ulceration triggers cells lining the mucosa of the ulcer margin, genes encoding for growth factors like Epidermal Growth Factor (EGF), beta Fibroblast Growth Factor (bFGF), Vascular Endothelial Growth Factor (VEGF) etc. in a well synchronized spatial and temporal manner (Fagundes et al., 2020).

Despite improved hygiene and gastric anti-secretory treatment regimens, peptic ulcer still remains a serious and global health problem, which by virtue of unabated *Helicobacter pylori* infection and indiscriminate use of NSAIDs, affects approximately 50% of the World population (Akbulut et al., 2021; Eshraghian, 2014; Hooi et al., 2017; Aitila et al., 2019).

In an attempt to stem this tide and also provide for adequate gastroprotection, enhanced quality of healing with less adverse effect, several studies have suggested complementing existing therapy with dietary supplementation (Ajeigbe et al., 2017b; Jong et al., 2021; Pilar et al., 2019), and folic acid supplementation, for example, has been hypothesized to be promising in that regard due to its anti-apoptotic and anti-inflammatory properties on the stomach mucosa (Ajeigbe et al., 2017a).

In this present study, we aimed at investigating the gastroprotective effects of folic acid supplementation on pylorus ligation induced ulcer, and possible mucosal healing effects on the acetic acid ulcer model in the rat. While healing depicts balance of cell damage and repair at the ulcer site, ulcer formation is a consequence of imbalance between the attack and defence factors (Ahluwalia et al., 2014). We, therefore, measured acid output, gastric pH, juice volume and inflammatory cytokines in the pylorus ligation study, and growth and angiogenic factors in the acetic acid ulcer study. Further, the choice of animal species and ulcer models are critical to the antisecretory, anti-inflammatory and mucosa repair mechanisms in ulcerogenesis.

Methods

Animals

Seventy-five (75) healthy albino male rats of Wister strain weighing between 150 and 200 g were used for this study. The animals were obtained from the Animal House of Igbinedion University Okada, and housed under standard conditions of temperature (23 ± 2 °C), humidity (55 ± 15%) and 12 h light (7:00 am–7:00 pm). The cages were constantly kept clean in order to prevent the animals from disease. They were fed with standard commercial rat pellets and allowed free access to water ad libitum.

All studies on the animal experimentation were conducted in accordance with the Current Animal Care Regulations and Standards approved by the Institute for Laboratory Animal Research (ILAR, 1996) and protocols approved by the Animal Ethics Committee of the College of Health Sciences, Igbinedion University, Okada.

Drugs

Folic acid tablets and Omeprazole were obtained from a local Pharmacy duly registered by the Pharmacists' Council of Nigeria (PCN). Antibodies were gotten from Leica Biosystems, Nussloch. All other reagents were of analytical grade and obtained from British House, Poole, UK.

Experimental design

Pylorus ligation model protocol

Animal grouping

The animals for this ulcer studies were randomly assigned into five (5) groups of five rats each ($n = 5$) and treated as follows:

Group 1: Distilled water (1 ml/d) + 6 h Pylorus ligation.
Group 2: Omeprazole (20 mg/kg) only + 6 h Pylorus ligation.

Group 3: Folic acid (2 mg/kg/d) + 6 h Pylorus ligation.
Group 4: Folic acid (3 mg/kg/d) + 6 h Pylorus ligation.
Group 5: Overall Control; No, treatment, No Pylorus ligation.

The route of administration for folic acid is oral using metal cannula and calibrated hypodermic syringe once daily for twenty-one (21) days at the volume of 1 ml/100 g body weight. All the animals were euthanized under xylazine/ketamine.

Acid secretion studies

Gastric acid collection was done as previously described by Olaleye et al., 2008 and modified by Ajeigbe et al., 2014. The 6 h gastric juice was collected, centrifuged at 2000 rpm for 10 min. The supernatant volume and pH were recorded while total acid content was determined by titration.

Ulcer induction and determination

Six hours after the ligation of the pylorus, the animals were sacrificed and the stomachs removed to assess the mucosal injury. The stomachs were opened along the greater curvature, washed, photographed and the ulcer area determined. According to the method of Dae-Kwon Bae et al., 2011, the ulcer index was measured using the varying scores involving the number and severity of ulcers. The number and degree of erosions and ulcers were scored in 0–5 levels as shown in the Table 1.

The sum of total activity score in each group divided by the number of rats in the group was considered as mean ulcer index. Two independent individuals assisted in the scoring of ulcers. After evaluating the ulcer areas, gastric tissues were prepared for histological and biochemical analysis.

Table 1 Mucosal damage evaluation after pylorus ligation in rats (Small < 2 mm; marked ≥ 2 mm)

Ulcer score	Macroscopic damage
0.0	No lesions
0.5	Diffuse hyperemia
1.0	1–2 small erosions
1.5	3–6 small erosions
2.0	7–10 small erosions
2.5	More than 10 small erosions
3.0	1 marked erosion plus 0–4 small erosions
3.5	1 marked erosion plus 5 or more small erosions
4.0	2 marked erosions plus 0–4 small erosions
4.5	2 marked erosions plus 5 or more small erosions
5.0	3 or more marked erosions

Analysis of TNF-α, IL-1β, IL-4 and IL-10 The levels of Tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), Interleukin-6 and Interleukin-10 in stomach tissues were analysed with ELISA kit (ElabScience, USA) following the manufacturer's protocol.

Histopathological studies

According to Ogihara & Okabe, 1993, small pieces of ulcerated stomach tissues were prepared, paraffinized and sectioned for histological evaluation. Neutrophil infiltration (Haber & Lopez, 1999) and inflammatory cells infiltration assessment were later done subjectively using the scale: 0 = no infiltration; 1 = very mild infiltration; 2 = mild infiltration; 3 = moderate infiltration; 4 = marked infiltration (Trevethick et al., 1993). Parietal cell mass index was also calculated as described by Perrasso et al. (1991) as the number of cells per mm^2 multiplied by the thickness of the glandular layer.

Acetic acid model protocol

Animal grouping

The animals were divided into 5 groups with 10 rats each as follows:

Group 1: Distilled water (1 ml/d) + Acetic acid ulcer.
Group 2: Omeprazole (10 mg/kg/d) + Acetic acid ulcer.
Group 3: Folic acid (2 mg/kg/d) + Acetic acid ulcer.
Group 4: Folic acid (3 mg/kg/d) + Acetic acid ulcer.
Group 5: No treatment, No acetic acid ulcer.

The rats were treated with folic acid for 21 days before ulcer induction and continued 4 and 9 days post-induction. The route of administration for folic acid is oral using metal cannula and calibrated hypodermic syringe once daily for twenty-one (21) days at the volume of 1 ml/100 g body weight. All the animals were euthanized under xylazine/ketamine.

Ulcer induction and determination

Gastric kissing ulcers were induced by luminal application of acetic acid solution to rats (Tsukimi & Okabe, 2001). Briefly, the abdomen was opened under xylazine/ketamine anaesthesia, and the stomach was exteriorized. The anterior and posterior walls of the stomach were clamped together with a pair of forceps with a round ring (10 mm in diameter) situated between the two arms of the forceps. A 40% acetic acid solution of 0.1 ml was injected into the clamped portion through the forestomach via a 21-gauge needle. After 60 s, the acid solution

was withdrawn and the abdomen was closed. Thereafter, rats were fed a standard diet and given distilled water. They were also maintained at normal temperature and pressure.

After treatment, rats were sacrificed at day 5 or 10 after ulcer induction. The stomachs were opened along the greater curvature, washed, photographed and the ulcer area determined. The severity score was assigned according to Takagi & Okabe, 1968 (Table 2):

The sum of total activity score in each group divided by the number of rats in the group was considered as mean ulcer index. After measuring the ulcer areas, gastric tissues were excised for histopathological, enzyme-linked immunosorbent assay (ELISA) and immunohistochemical analysis.

ELISA

Tissue epidermal growth factor (EGF) and vascular endothelial cell growth factor (VEGF) were measured according to the manufacturer's instructions on the ELISA kit (ElabScience, USA). For measurements, 100 mg tissue was rinsed with PBS, homogenized in 1 ml of PBS and stored overnight at − 20 °C. After two freeze–thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 min at 5000 × g, 2–8 °C. The supernatant was removed and assayed immediately.

Immunohistochemical studies

Avidin–biotin horseradish peroxidase method was employed for immunostaining after paraffinized stomach sections has been stained with H&E (Ajeigbe et al., 2012; Wang et al., 2000). Briefly, hot citric acid was used to carry out antigen retrieval, and after peroxidase activity block with 3% hydrogen peroxide (H_2O_2) and wash in PBS, protein blocking was later achieved avidin for 15 min, and endogenous biotin in tissue were blocked using biotin for another 15 min.

Incubation followed, thereafter, with the respective diluted primary antibody EGFR, Ki-67, CD31 and Factor VIII (Leica Biosystems, Nussloch). Excess antibody were washed off with PBS and a secondary antibody (LINK) were applied on sections for 15 min. Sections were washed and the (LABEL) which is the horseradish peroxidase (HRP) were applied on the sections for 15 min. Staining was later done by immersing in chromogen and counterstained in haematoxylin. Cells with specific brown colours in the cytoplasm, cell membrane or nuclei depending on the antigenic sites are considered to be positive. The haematoxylin stained cells without any form of brown colours are scored negative.

Positive immunoreactive cells were quantified and expressed as intensity and proportion of positive cells and assigned to one of four categories under light microscope (40X objective): −, Negative; +, Very mild surface/deep expression; ++, Mild and focal (< 50%) or diffuse (> 50%) surface or deep expression; +++, Moderate focal or diffuse surface or deep expression; ++++, HIGH surface or deep expression (Cho & Kim, 1998).

Second, % Area and/or point counting of positive immunoreactive cells were estimated, as labeling index, using Image J analysis software (NIH, USA) (Rangan & Tesch, 2007).

Statistical analysis

Data are presented as mean ± SEM and subjected to one-way and two-way analysis of variance (ANOVA) and Kruskal Wallis test using the Graphpad prism version 6.0 for Windows from GraphPad software, San Diego, CA, USA. Values of $p < 0.05$ were regarded significant.

Table 2 Mucosal damage evaluation after acetic acid ulcer in rats

Ulcer score	Macroscopic damage
0	No pathological change
1	Mucosa edema and petechial haemarrhages
2	1–5 small ulcers (1–2 mm)
3	More than five small ulcers or one medium ulcer (3–4 mm)
4	Two medium ulcers or one large ulcer (more than 4 mm)
5	Perforated ulcer

Results

Development of gastric lesions in the pylorus ligation rats treated with folic acid and omeprazole

Effect of folic acid and Omeprazole on pyloric ligation induced ulcer is shown in Fig. 1. The mean ulcer severity score of the pyloric ligated rat (ulcer control) rats is 4.30 ± 0.04. On pre-treatment with Folic acid, ulcer severity lessened with a score of 2.50 ± 0.05 for 2 mg/kg and 1.90 ± 0.05 for 3 mg/kg ($p < 0.05$). Omeprazole reduced the ulcer score to 0.48 ± 0.01. Observation on the stomach of the animals that were not treated showed neither ulceration nor erosion.

Effects of varying doses of folic acid and omeprazole on gastric juice profile in pyloric ligation ulcer model

Pylorus ligation for 6 h resulted in the accumulation of gastric secretion and increase in the titratable acidity. Folic acid produced dose-dependent significant decrease in gastric juice volume and acid output while enhancing pH (Table 3). Expectedly, Omeprazole recorded significant interventionist control in the gastric juice profile.

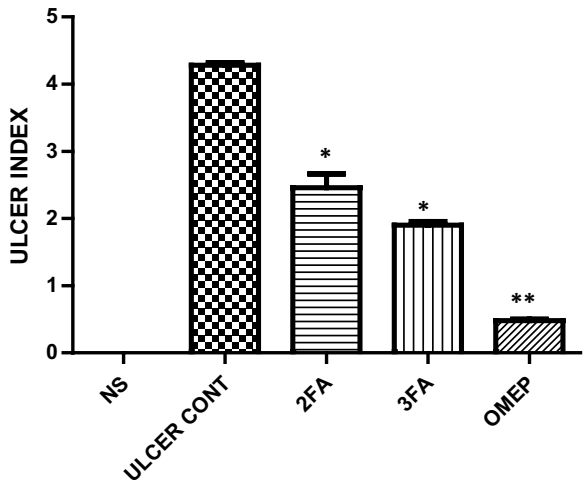

Fig. 1 Effect of graded doses of folic acid supplementation on pyloric ligation-induced ulcer in the rat. (*$p < 0.05$, **$p < 0.001$ cf ulcer control) NS = Normal stomach, ULCER CONT = Pylorus ligation Ulcer control, 2FA = 2 mg/kg Folic acid, 3FA = 3 mg/kg Folic acid, OMEP = Omeprazole

Histopathological examination: effect of Folic acid on neutrophil infiltration and parietal cell mass

Neutrophil and inflammatory cells infiltration

A significant reduction was observed in the neutrophil counts of the pyloric ligation (ulcerated) stomach pre-treated with the two doses of Folic acid (20.00 ± 1.60 cells/field; 2 mg/kg, 25.00 ± 1.40 cells/field; 3 mg/kg versus 30.00 ± 0.90 cells/field ulcer control) (Fig. 2). Pyloric ligation caused mucosal cytoarchitectural damage with pronounced inflammatory cells infiltration, which is significantly remedied by Folic acid and Omeprazole (Fig. 3).

Similarly, the inflammatory cells infiltration score reduced in the folic acid and omeprazole treated animals compared to the ulcer control rats ($p < 0.05$) (Figs. 4, 5).

Parietal cell mass

Folic acid produced a decrease in the parietal cell numbers in the gastric mucosa (11.5 ± 0.20 cells/field; 2 mg/kg, 10.5 ± 0.50 cells/field; 3 mg/kg versus 20.6 ± 0.80 cells/field ulcer control; $p < 0.05$). Omeprazole significantly reduced the parietal cell numbers (Figs. 6, 7).

Tissue cytokine levels in pyloric ligated stomach treated with folic acid and omeprazole

Tissue level of IL-4 and IL-10 were significantly decreased, while TNF-α and IL-1β were increased in the pyloric ligation group compared with the control group ($P < 0.05$). Pre-treatment with folic acid reduced both TNF-α and IL-1β but enhanced IL-4 and IL-10 ($P > 0.05$) when compared with pyloric ligation group. Omeprazole equally suppressed the increase in TNF-α and IL-1β associate with pylorus ligation (Table 4). Similar trend was observed in the acetic acid ulcer studies (Table 6).

Development of gastric lesions in the acetic acid stomach ulcer pre-treated with folic acid and omeprazole

The outcome of the severity of acetic acid induced ulceration on the stomach of experimental rats treated with folic acid and Omeprazole compared to their controls on day 5 and 10 is shown in Fig. 8. The folic acid and Omeprazole treated groups exhibited different degrees of severity. The 2 mg/kg Folic acid exhibited score of 3.5 ± 0.1, 3 mg/kg FA; 3.0 ± 0.1 and Omeprazole; 2.5 ± 0.1 on day 5, compared to the ulcer control group with a severity score of 4.5 ± 0.1.

However, the severity of the ulcer for both the ulcer control group and the treated group was lower on day 10. ($p < 0.05$). The ulcer sore was 2.5 ± 0.1 for the two doses

Table 3 Effect of varying doses of folic acid on pyloric ligation induced gastric ulcer: gastric juice volume, pH and acid output

Group	Treatment	Gastric juice volume (ml/6 h)	pH	Acid output ($\times 10^4$ mmol/6 h)
1	Normal saline (1 ml/kg b.w)	3.80 ± 0.15	2.60 ± 0.01	5.20 ± 0.12
2	6 h Pyloric Ligation	6.50 ± 0.20[a]	1.45 ± 0.002[a]	8.20 ± 0.05[a]
3	2FA + 6 h Pyloric Ligation	4.00 ± 0.10[b]	2.00 ± 0.01	5.20 ± 0.03[b]
4	3FA + 6 h Pyloric Ligation	4.20 ± 0.12[b]	2.10 ± 0.02[b]	5.10 ± 0.01[b]
5	OMEP + 6 h Pyloric Ligation	3.50 ± 0.05[c]	2.90 ± 0.05[c]	4.00 ± 0.01[c]

2FA 2 mg/kg Folic acid, *3FA* 3 mg/kg Folic acid, *OMEP* omeprazole

[a] $P < 0.05$, Pyloric ligation versus control group

[b] $P < 0.05$, 2FA + 6 h Pyloric Ligation versus 6 h Pyloric Ligation

[c] $P < 0.05$, Omeprazole + 6 h Pyloric Ligation versus 6 h Pyloric Ligation only. (Mean ± SEM, $n = 5$)

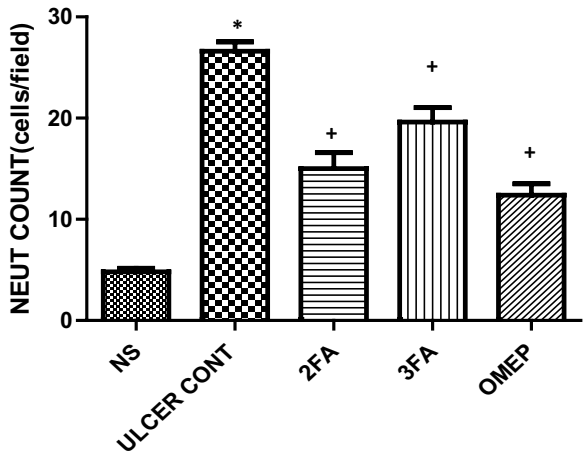

Fig. 2 Neutrophil count in the stomach of rats treated with varying doses of folic acid and Omeprazole after pyloric ligation ulcer. (*$p < 0.05$ ulcer control cf. control; $^{+}p < 0.05$ cf. ulcer control). NEUT COUNT, Neutrophil Count. NS = Normal stomach, ULCER CONT = Pylorus ligation Ulcer control, 2FA = 2 mg/kg Folic acid, 3FA = 3 mg/kg Folic acid, OMEP = Omeprazole

of folic acid treated group, and 1.5 ± 0.1 for the Omeprazole group while the ulcer control group was 3.5 ± 0.1.

Histopathological analysis of healing ulcerated stomach treated with folic acid and omeprazole

The control (Normal) stomach showed no sign of inflammation or ulcer.

Ulcer control

On Day 5, photomicrographs of gastric tissue showed severe ulceration of mucosa with associated chronic inflammation, haemorrhage and fibrosis. There was chronic inflammation of submucosa and muscularis externa. But, on Day 10, the gastric tissue showed moderate ulceration of mucosa with associated chronic inflammation and fibrosis.

Folic acid treated

On Day 5, photomicrographs of gastric tissue treated with 2 mg/kg of folic acid showed moderate ulceration of mucosa with associated chronic inflammation and fibrosis in submucosa and muscularis externa while on Day 10, moderate inflammation of mucosa, submucosa

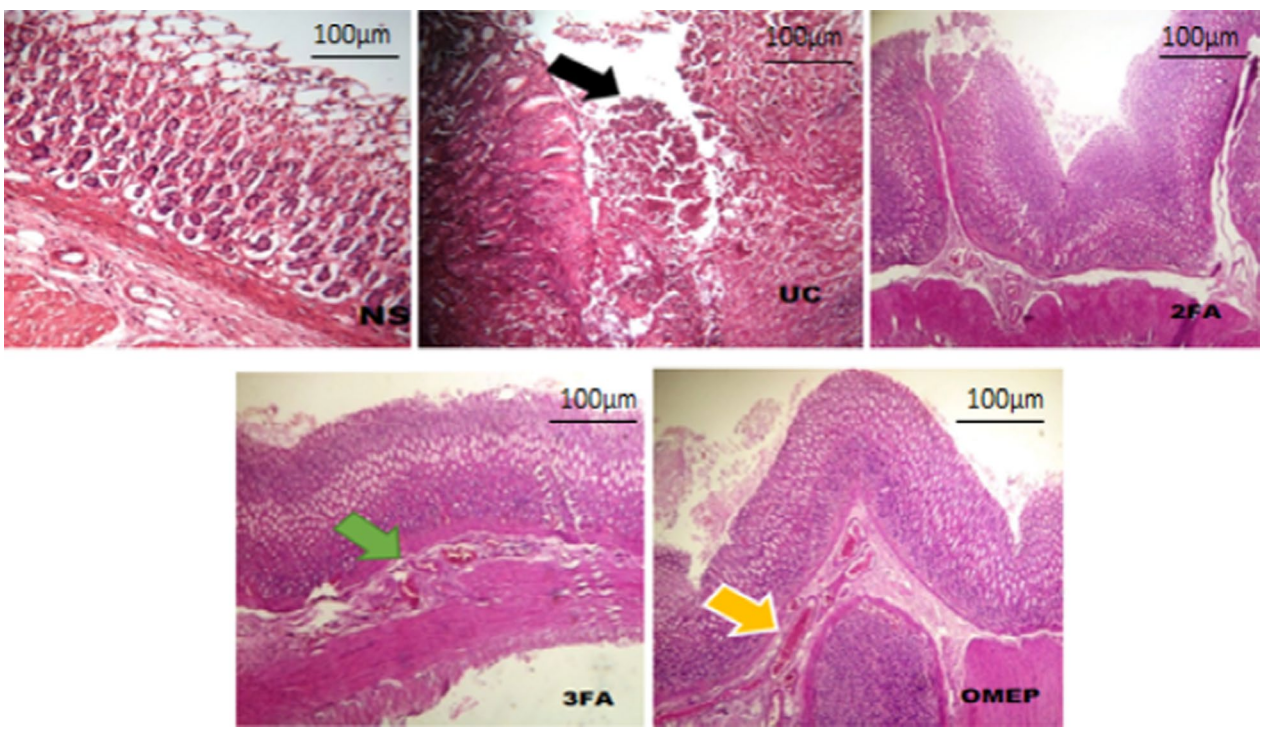

Fig. 3 Histological evaluation of rats' gastric tissues treated with varying doses of folic acid and omeprazole prior pyloric ligation, (H&E, X40). NS (overall control) = Normal mucosa and submucosa. UC (Ulcer control) = Acute inflammation of the mucosa as well as submucosa, with an appearance of cellular debris. 2FA = Mild papillary infolding, mild inflammation of mucosa and submucosa**.** 3FA = Moderate congestion of the mucosa and submucosa (green arrow), moderate inflammation of submucosa and mild erosion of the surface epithelial. OMEP = Mild congestion (orange arrow), mild infiltration of inflammatory cells and adipocytes into the submucosa

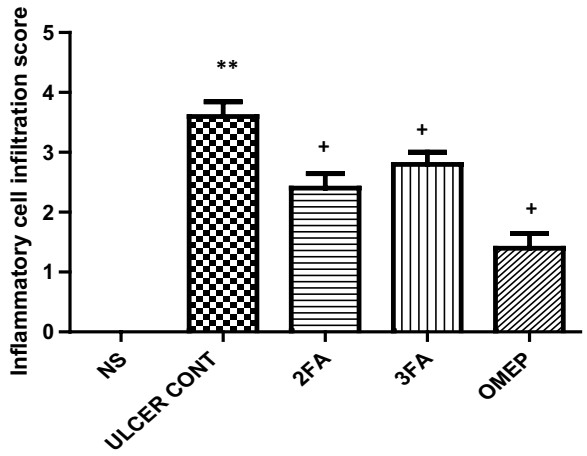

Fig. 4 Inflammatory cells infiltration in the stomach of rats treated with varying doses of folic acid and Omeprazole after pyloric ligation ulcer. (**$p < 0.001$ ulcer control cf. control; $^{+}p < 0.05$ cf. ulcer control). NEUT COUNT, Neutrophil Count. NS = Normal stomach, ULCER CONT = Pylorus ligation Ulcer control, 2FA = 2 mg/kg Folic acid, 3FA = 3 mg/kg Folic acid, OMEP = Omeprazole

and mild inflammation of muscularis externa was seen. Moreover, the gastric tissues treated with 3 mg/kg folic acid showed mild ulceration of mucosa, severe necrosis of mucosa, submucosa and muscularis externa, though there was a chronic inflammation of muscularis externa, but on Day 10, gastric tissue showed mild inflammation of mucosa.

Omeprazole

On Day 5, there was a mild ulceration of the mucosa with associated chronic inflammation and fibrosis. There was mild congestion of blood vessels in submucosa. But, on day 10, the gastric tissue showed slight ulceration of mucosa with associated chronic inflammation and fibrosis even though there is chronic inflammation of submucosa and muscularis externa (Fig. 9).

Assessment of mucous cell density (MCD) in the healing ulcerated stomach treated with folic acid and omeprazole

The mucous cell density in the ulcer untreated group was reduced by 32% and 55% on both day 5 and 10 respectively when compared with the overall control. Folic acid enhanced the MCD on day 5 (10.5 ± 0.6 cells/field, 10.5 ± 0.6 cells/field versus 8.5 ± 0.1 cells/field ulcer control) and day 10 (10.0 ± 0.2 cells/field, 12.0 ± 0.3 cells/field versus 12.5 ± 0.2 cells/μm^2 ulcer control) (Fig. 10). Omeprazole did equally enhance the MCD in the healing mucosa ($p < 0.05$) (Fig. 11).

Mucosal cell proliferation and angiogenesis analysis in the healing ulcerated stomach treated with folic acid and omeprazole

Mucosal cell proliferation

Epidermal growth factor receptor (Fig. 12) and Ki-67 (Fig. 13) were expressed in both the ulcer treated and untreated groups on day 10, evidence of proliferation on the healing mucosa. EGFR expression was very mild in the ulcer untreated group on day 5, similar to the folic acid treated groups. The omeprazole also group expressed a mild expression on day 5. Meanwhile, folic acid (3 mg/kg) and omeprazole treated groups showed high EGFR and Ki-67 expression while folic acid (2 mg/kg) and the control group remained mild (Table 5). However, the mucosal level of epidermal growth factor (EGF) showed no significance on day 5 between the folic acid treated and the ulcer control. Meanwhile on day 10, EGF level increased significantly in the folic acid treated when compared with the ulcer control (Fig. 14).

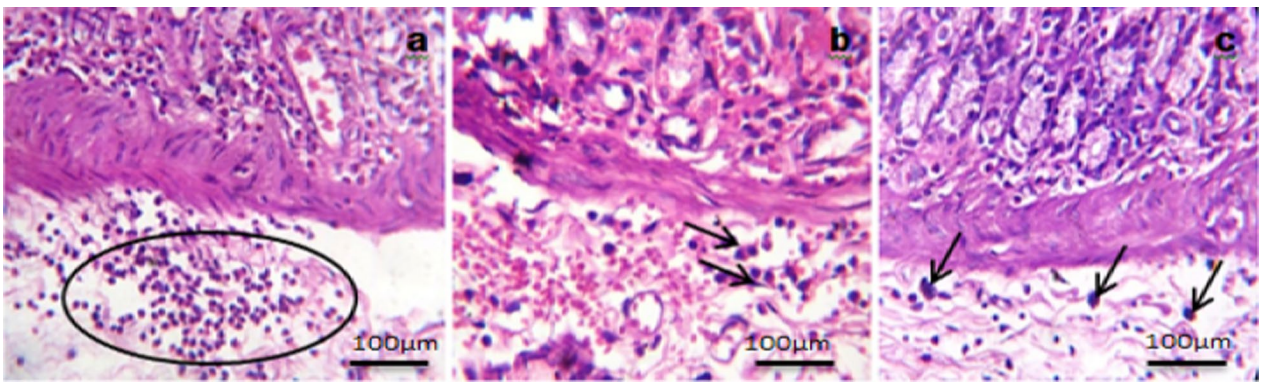

Fig. 5 Representative slides for evaluation of inflammatory cells infiltration in the pyloric ligated stomach ulcer pretreated with folic acid and omeprazole. (H&E, X100) **a** Pyloric ligated stomach with no treatment. **b** Pyloric ligated stomach treated with Folic acid. **c** Pyloric ligated stomach treated with Omeprazole. Black arrow and circled area: Inflammatory cells

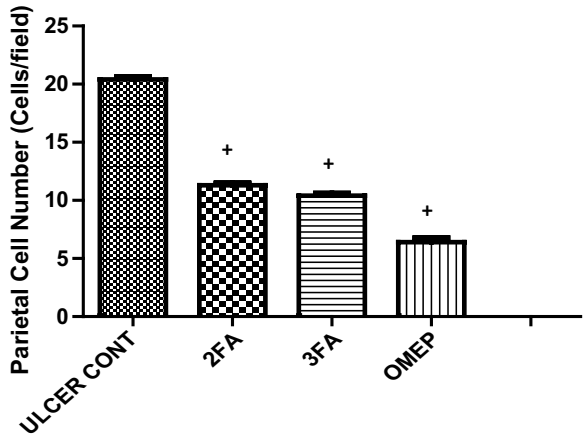

Fig. 6 Parietal cell numbers in the stomach mucosa of rats treated with varying doses of folic acid and Omeprazole after pyloric ligation ulcer. (**$p < 0.001$ ulcer control cf. control; $^{+}p < 0.05$ cf. ulcer control). ULCER CONT = Pylorus ligation Ulcer control, 2FA = 2 mg/kg Folic acid, 3FA = 3 mg/kg Folic acid, OMEP = Omeprazole

The EGFR labeling index was (45 ± 1.0%, 48 ± 1.2%, and 50 ± 1.2% vs. 35 ± 1.5% ulcer control) on day 5, and (60 ± 1.5%, 65 ± 1.0%, 72 ± 1.0% vs. 55 ± 1.0%) on day 10 for FA (2 mg/kg), FA (3 mg/kg) and Omeprazole groups respectively (Fig. 15). Also, the Ki-67 labelling index was (25 ± 0.0%, 30 ± 0.0%, and 36 ± 1.5% vs. 15 ± 1.2%) on day 5, and (50 ± 3.5, 55 ± 3.5, 62 ± 0.0 vs. 35 ± 2.5% ulcer control) on day 10 for FA (2 mg/kg), FA (3 mg/kg) and Omeprazole groups respectively (Fig. 16).

Angiogenesis

A moderate expression of CD 31 (Fig. 17) and Factor VIII (Fig. 18) were observed in folic acid (3 mg/kg) and Omeprazole treated groups on day 10, suggestive of formation or re-establishment of microvascular network in the granulation tissue. The expression in the folic acid and Omeprazole treated group on day 5 was very mild. There was equally very mild expression of CD 31 on the untreated group on day 5 (Table 5) while the mucosal level of vascular endothelial growth factor (VEGF) showed no significance on day 5 between the folic acid treated and the ulcer control. However, VEGF level increased significantly in the folic acid treated when compared with the ulcer control on day 10 (Fig. 19).

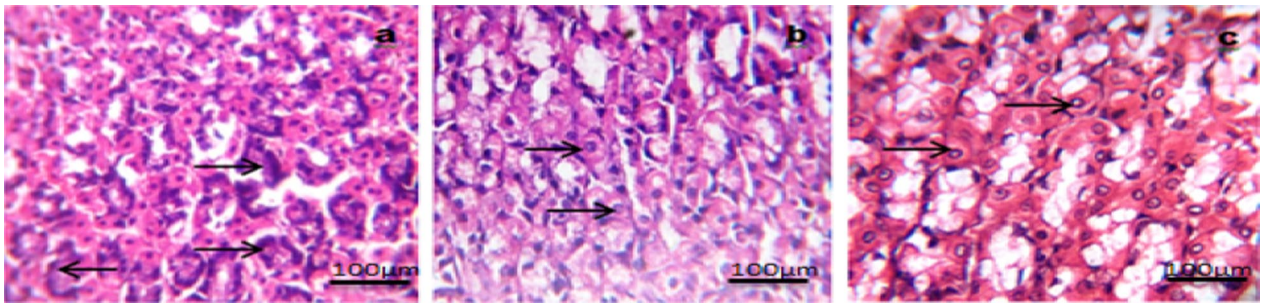

Fig. 7 Representative slides for evaluation of parietal cell numbers in the pyloric ligated stomach ulcer pretreated with folic acid and omeprazole. (H&E, X400). **a** Pyloric ligated stomach with no treatment. **b** Pyloric ligated stomach treated with Folic acid. **c** Pyloric ligated stomach treated with Omeprazole. Black arrow: parietal cells

Table 4 Effect of varying doses of folic acid on pyloric ligation induced gastric ulcer: pro-inflammatory and anti-inflammatory cytokines

Group	Treatment	TNF-α (pg/mg protein)	IL-1β (pg/mg protein)	IL-4 (pg/mg protein)	IL-10 (pg/mg protein)
1	Normal saline (1 ml/kg b.w)	15.5 ± 3.5	10.0 ± 3.5	50.5 ± 5.0	70.5 ± 5.0
2	6 h Pyloric Ligation	54.5 ± 5.0[a]	45.5 ± 5.0[a]	15.7 ± 2.0[a]	45.5 ± 5.0[a]
3	2FA + 6 h Pyloric Ligation	30.7 ± 2.5[b]	25.0 ± 2.5[b]	25.6 ± 1.5[b]	55.5 ± 2.0[b]
4	3FA + 6 h Pyloric Ligation	28.0 ± 5.0[b]	25.0 ± 5.0[b]	30.5 ± 4.5[b]	60.0 ± 2.5[c]
5	OMEP + 6 h Pyloric Ligation	25.0 ± 2.0[c]	20.0 ± 2.0[c]	40.5 ± 1.0[c]	65.9 ± 4.0[d]

2FA 2 mg/kg Folic acid, *3FA* 3 mg/kg Folic acid, *OMEP* omeprazole

[a] $P < 0.05$, Pyloric ligation versus control group

[b] $P < 0.05$, 2FA + 6 h Pyloric Ligation versus 6 h Pyloric Ligation

[c] $P < 0.05$, Omeprazole + 6 h Pyloric Ligation versus 6 h Pyloric Ligation only. (Mean ± SEM, $n = 5$)

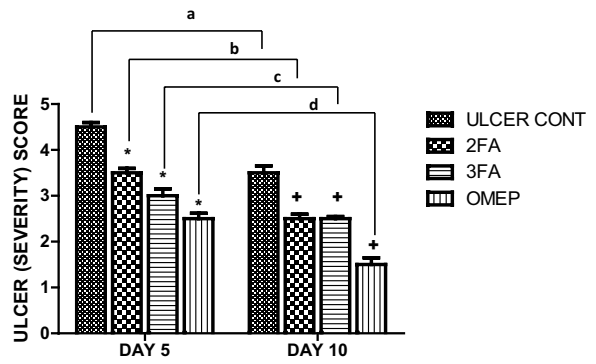

Fig. 8 Effect of folic acid (2 mg/kg), folic acid (3 mg/kg), and Omeprazole on acetic acid induced gastric ulcer: ulcer (severity) score ($*p < 0.05$ cf. ulcer control, day 5; $+p < 0.05$ cf. ulcer control, day 10; a–d $p < 0.05$ cf. each group on day 10. ULCER CONT = Acetic acid Ulcer control, 2FA = 2 mg/kg Folic acid, 3FA = 3 mg/kg Folic acid, OMEP = Omeprazole

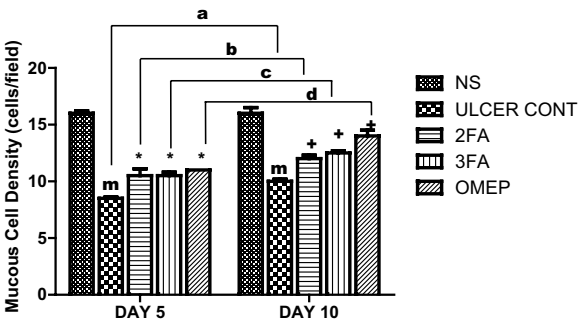

Fig. 10 Effect of folic acid and Omeprazole on mucous cell density during healing of acetic acid induced gastric ulcer. ($*^{,+}p < 0.05$ cf ulcer control; a–d $p < 0.05$ cf. each group on day 10 $^{m}p < 0.05$ cf NS). ULCER CONT = Acetic acid Ulcer control, 2FA = 2 mg/kg Folic acid, 3FA = 3 mg/kg Folic acid, OMEP = Omeprazole

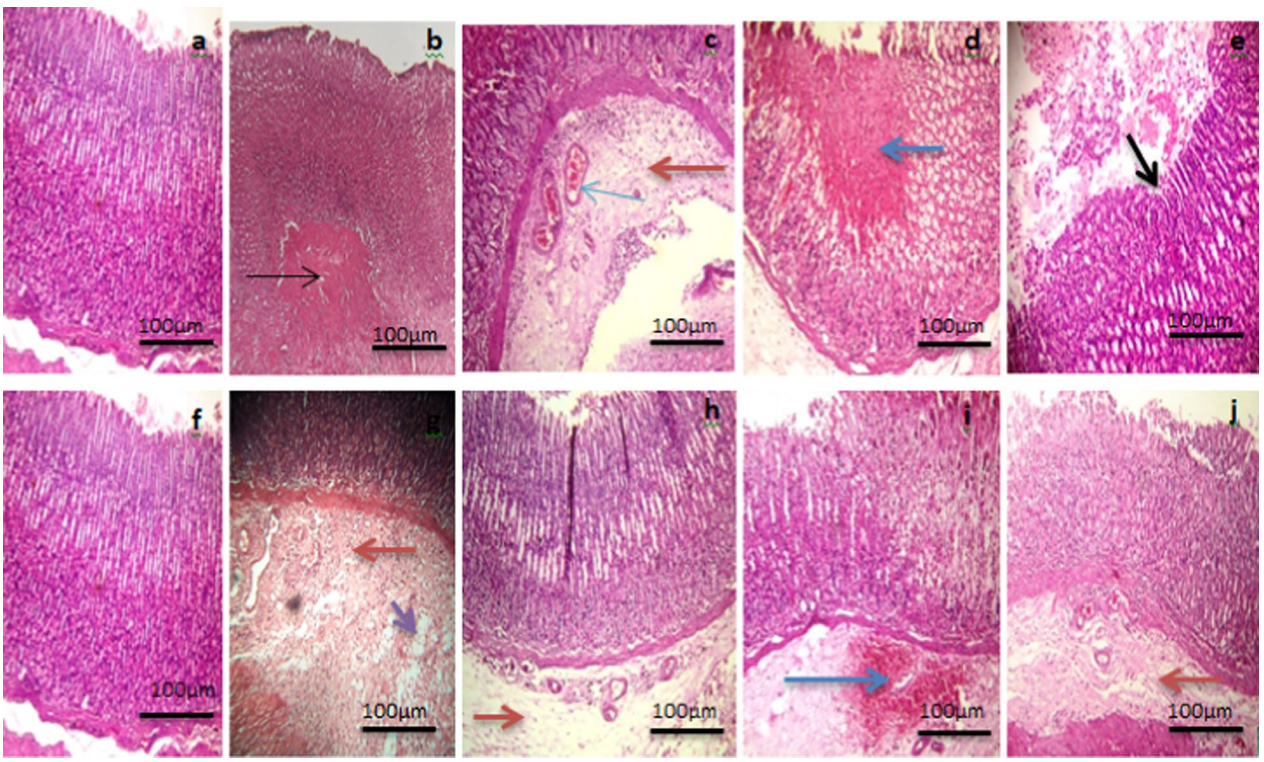

Fig. 9 Effects of folic acid and Omeprazole on the cyto-architectural alteration induced by acetic acid in the rat's stomach at day 5 and 10. **a** Normal stomach: No significant lesion, surface epithelial is intact. **b** Acetic acid treated (day 5): Severe ulceration involving the mucosa and submucosa with the production of cellular debris, there is replacement of the submucosa by connective tissue fibers. (Thin black arrow: fibrosis). **c** folic acid (2 mg/kg) treated (day 5): Mild congestion of the mucosa (thin blue arrow), severe oedema (orange arrow) of the submucosa. **d** Folic acid (3 mg/kg) treated (day 5): Moderate haemorrhage in the mucosa (blue arrow). **e** Omeprazole treated (day 5): Mild erosion of surface epithelial (black arrow) and mild inflammation of mucosa and submucosa. **f** Normal stomach (day 10): No significant lesion, surface epithelial is intact. **g** Acetic acid treated (day 10): Moderate ulcer, moderate infiltration of inflammatory cells within the mucosa, lamina propia and submucosa layer (Orange arrow: edema; Purple arrow: adipocytes). **h** Folic acid (2 mg/kg) treated (day 10): Mild oedema (orange arrow), mild inflammation of mucosa and submucosa. **i** Folic acid (3 mg/kg) treated (day 10): Moderate haemorrhage (blue arrow) of submucosa with mild inflammation of mucosa and submucosa. **j** Omeprazole treated (day 10): Slight oedema and slight inflammation of mucosa and submucosa (orange arrow). Surface epithelial is moderately preserved. (H & E, X100)

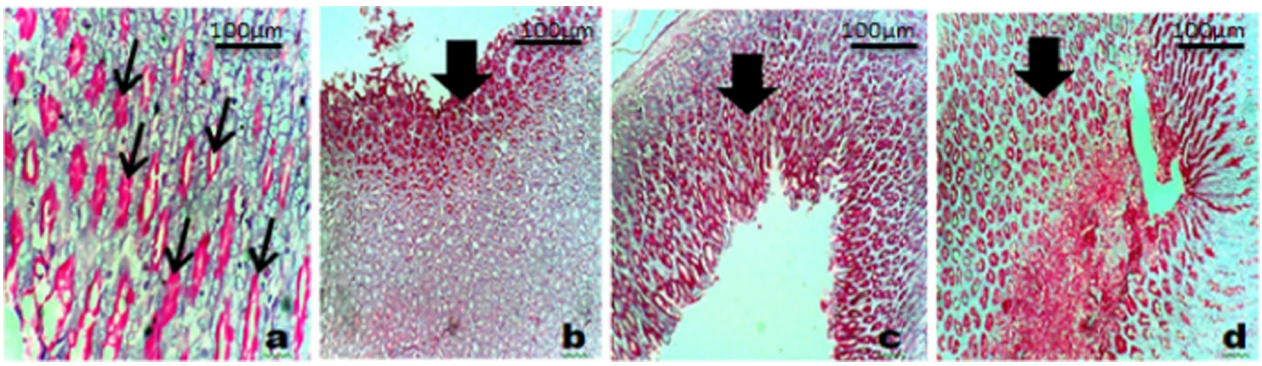

Fig. 11 Representative slides for evaluation of mucous cell density in the acetic acid induced stomach ulcer pretreated with folic acid and omeprazole. (PAS, X100). **a** Normal stomach. **b** Acetic acid stomach ulcer untreated. **c** Acetic acid stomach ulcer treated with Folic acid. **d** Acetic acid stomach ulcer treated with Omeprazole. Black (medium and large) arrow: Purple-red stained mucous cells

The CD31 labelling index was (16 ± 1.1%, 14 ± 1.5%, 17 ± 1.0% vs. 15 ± 1.5%) on day 5, and (52 ± 1.0%, 50 ± 1.5%, 55 ± 0.0% vs. 40 ± 1.0%) on day 10 for FA (2 mg/kg), FA (3 mg/kg) and Omeprazole groups respectively (Fig. 15). The Factor VIII labelling index was (24 ± 1.2%, 28 ± 1.2%, 29 ± 1.1% vs. 25 ± 1.0%) on day 5, and (60 ± 1.5%, 60 ± 1.5%, 65 ± 1.7% vs. 45 ± 1.0%) on day 10 for FA (2 mg/kg), FA (3 mg/kg) and Omeprazole groups respectively (Fig. 20).

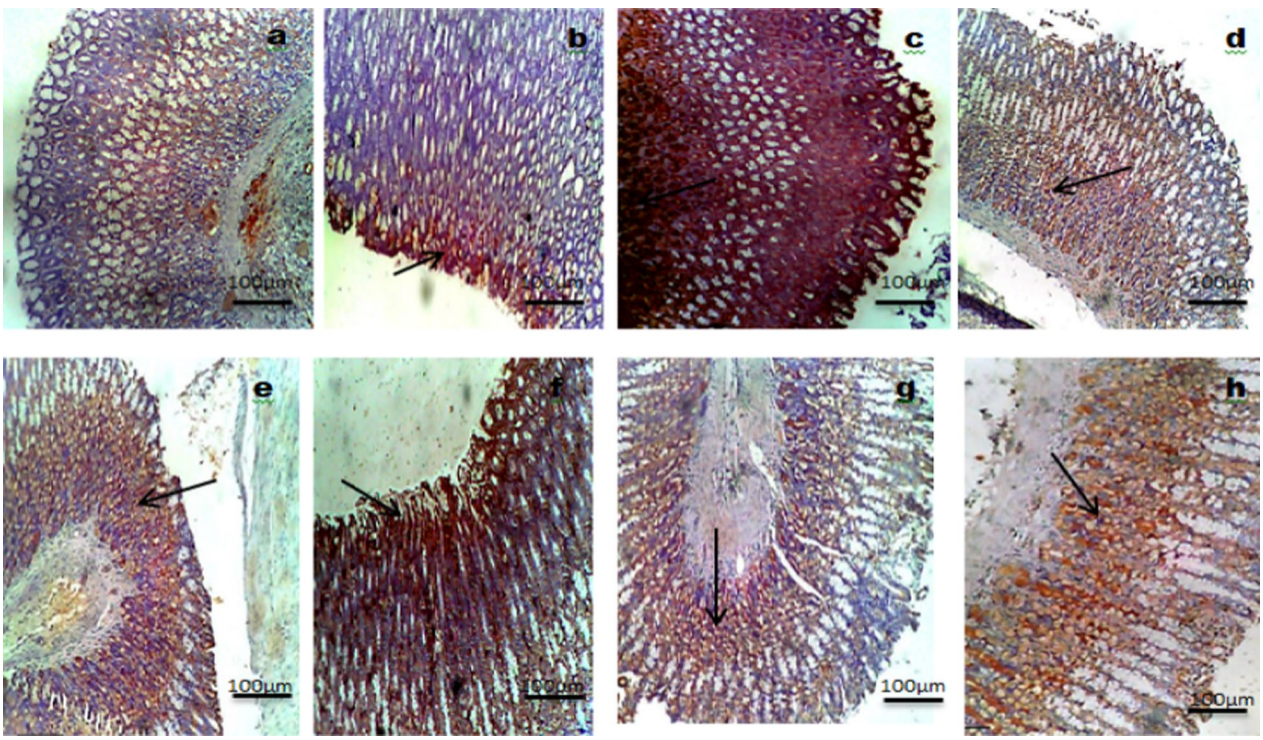

Fig. 12 IHC slides for evaluation of EGFR positive immunoreactive cells during healing of acetic acid stomach ulcer pretreated with folic acid and omeprazole. (H&E, X100). **a** Acetic acid stomach ulcer untreated (Day 5). **b** Acetic acid stomach ulcer treated with 2 mg/kg Folic acid (Day 5). **c** Acetic acid stomach ulcer treated with 3 mg/kg Folic acid (Day 5). **d** Acetic acid stomach ulcer treated with Omeprazole (Day 5). **e** Acetic acid stomach ulcer untreated (Day 10). **f** Acetic acid stomach ulcer treated with 2 mg/kg Folic acid (Day 10). **g** Acetic acid stomach ulcer treated with 3 mg/kg Folic acid (Day 10). **h** Acetic acid stomach ulcer treated with Omeprazole (Day 10). Black arrow: Point of positivity

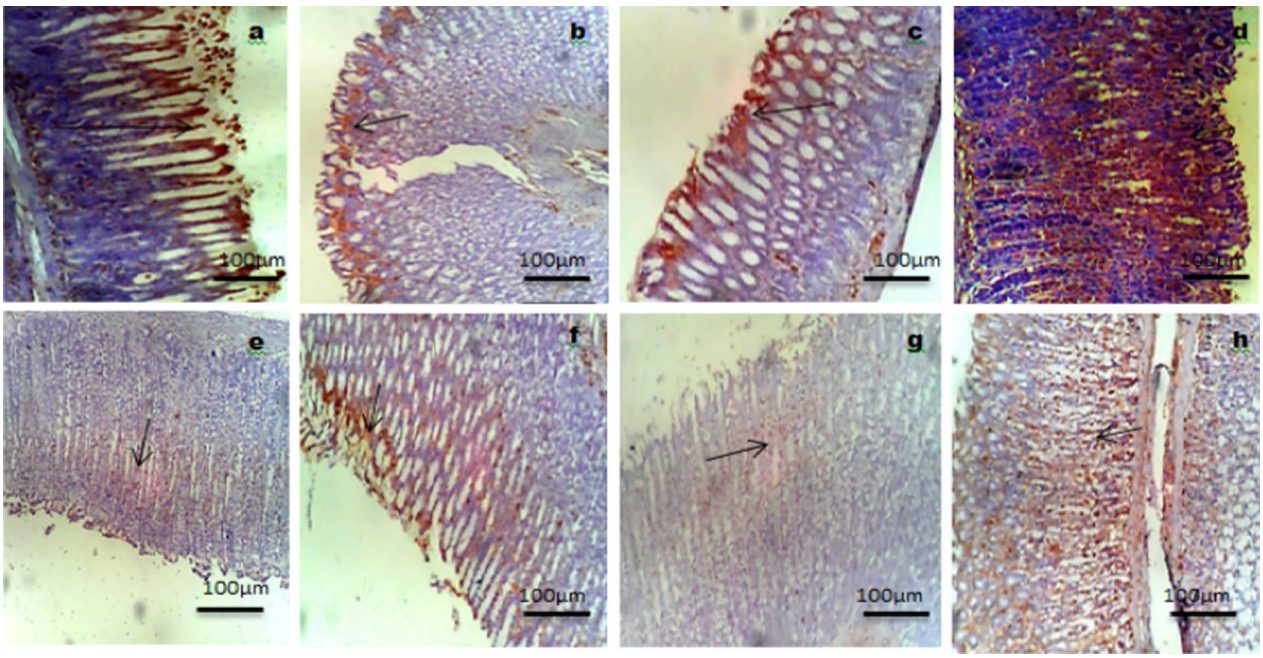

Fig. 13 Representative IHC slides for evaluation of Ki-67 positive immunoreactive cells during healing of acetic acid stomach ulcer pretreated with folic acid and omeprazole. (H&E, X100). **a** Acetic acid stomach ulcer untreated (Day 5). **b** Acetic acid stomach ulcer treated with 2 mg/kg Folic acid (Day 5). **c** Acetic acid stomach ulcer treated with 3 mg/kg Folic acid (Day 5). **d** Acetic acid stomach ulcer treated with Omeprazole (Day 5). **e** Acetic acid stomach ulcer untreated (Day 10). **f** Acetic acid stomach ulcer treated with 2 mg/kg Folic acid (Day 10). **g** Acetic acid stomach ulcer treated with 3 mg/kg Folic acid (Day 10). **h** Acetic acid stomach ulcer treated with Omeprazole (Day 10). Black arrow: Point of positivity

Table 5 Intensity of the immunoreactive EGFR, Ki-67, CD31 and Factor VIII positive cells in the ulcerated stomach treated with Folic acid (FA) and Omeprazole (OMEP)

	Day 5				Day 10			
	UC	FA (2 mg/kg)	FA (3 mg/kg)	OMEP	UC	FA (2 mg/kg)	FA (3 mg/kg)	OMEP
EGFR	+	+	+	++	+	++	+++	+++
Ki-67	+	+	+	++	++	++	+++	+++
CD31	+	+	+	+	++	++	+++	+++
Factor VIII	+	+	+	+	+	+	+++	+++

UC acetic acid ulcer control, *2FA* 2 mg/kg Folic acid, *3FA* 3 mg/kg Folic acid, *OMEP* omeprazole

−, No expression

+, Very mild surface/deep expression

++, Mild and focal (< 50%) or diffuse (> 50%) surface or deep expression

+++, Moderate focal or diffuse surface or deep expression

Discussion

In this present study, the gastroprotective and ulcer healing effect of folic acid in the rat was examined. The results of this study underscore the earlier reported protective tendencies of folic acid on gastric mucosa injury induced by either indomethacin (Ajeigbe et al., 2011) or ethanol (Ajeigbe et al., 2017a), as it exhibits not only antisecretory properties in the pyloric ligation ulcer model but also enhances gastric mucosal healing in the rat via improved cell proliferation and angiogenesis (Fig. 21, Table 6).

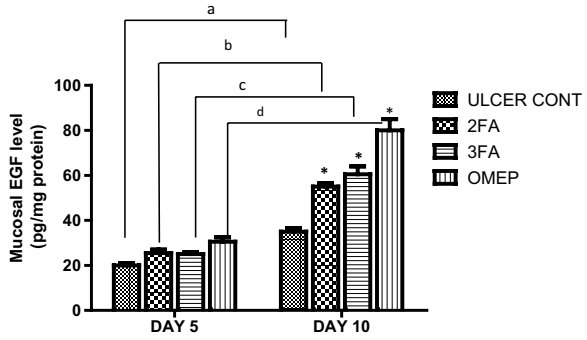

Fig. 14 Effect of folic acid supplementation on epidermal growth factor (EGF) levels in healing ulcerated gastric mucosa. (*$p < 0.05$ cf ulcer control; a–d $p < 0.05$ cf. each group on day 10). ULCER CONT = Acetic acid Ulcer control, 2FA = 2 mg/kg Folic acid, 3FA = 3 mg/kg Folic acid, OMEP = Omeprazole

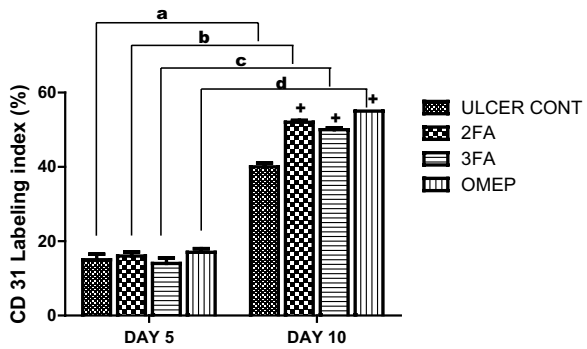

Fig. 15 Labelling index of CD31 in healing ulcerated stomach treated with folic acid and Omeprazole (*$p < 0.05$ cf ulcer control; a–d $p < 0.05$ cf. each group on day 10). ULCER CONT = Acetic acid Ulcer control, 2FA = 2 mg/kg Folic acid, 3FA = 3 mg/kg Folic acid, OMEP = Omeprazole

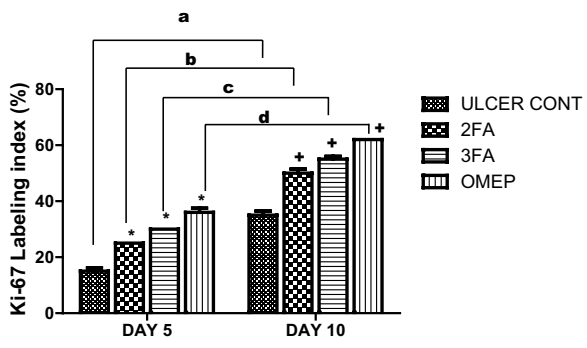

Fig. 16 Labelling index of Ki-67 in healing ulcerated stomach treated with folic acid and Omeprazole (*$p < 0.05$ cf ulcer control; a–d $p < 0.05$ cf. each group on day 10). ULCER CONT = Acetic acid Ulcer control, 2FA = 2 mg/kg Folic acid, 3FA = 3 mg/kg Folic acid, OMEP = Omeprazole

Gastric ulcer is characterized by erosion of the mucosa surface of the stomach. It is a form of peptic ulcer whose development remains centred on the algebraic sum of the two groups of forces acting upon the gastrointestinal mucosa; the first is defensive while the other is aggressive (Hollander & Harlan, 1973; Lawande et al., 2012). Hence, gastrointestinal wall integrity and homeostasis are delicately maintained by the balance between the two opposing forces. Asides other aggressive factors like *Helicobacter pylori* infection, smoking, alcohol, stress etc., gastric acid, and subsequent lipid peroxidation have been consistently shown as a strong attack factor in the gastric mucosa (Guo et al., 2005). The gastric (hydrochloric) acid which is produced by the parietal cells in the stomach is finely regulated by overlapping neural, hormonal, paracrine pathways (Yao & Forte, 2003), and controlling acid output is an important factor in anti-ulcerogenesis (Schmassmann, 1998). We observed reduced gastric juice volume, enhanced pH with a corresponding attenuated titratable acid content in the folic acid and omeprazole treated upon pylorus ligation, supported by reduction in parietal cell numbers in the gastric mucosa. This is in line with our earlier report that folic acid, using continuous perfusion method, decreases both basal and histamine stimulated acid secretory rates. Parietal cell mass examination was done as a corroborating study since gastric acid secretion is known to be linearly related with the parietal cell mass, and its attacking effect on the mucosa to be inversely related with the mucus cell population (Brunton et al., 2005). This may explain the attenuation of acid output by the parietal cells.

Neutrophil infiltration plays a crucial role in inflammation and is not only a major cause of tissue damage (Sies, 1991) but normal tissue repair as they produce bioactive substances capable of accelerating tissue damage, including oxygen radicals (Shandall et al., 1986; Wikberg et al., 2017), digestive enzymes and pro inflammatory cytokines (Dovi et al., 2004). Several lines of evidence have implicated circulating activated and infiltrating leukocytes in ulcerogenesis due to reduced gastric mucosal blood flow and microvascular dysfunction (Alzoghaibi, 2005). We observed presently, a substantial neutrophil infiltration in the pyloric ligation untreated group which was significantly attenuated in the folic acid and omeprazole. Moreover, resultant pro-and anti-inflammatory cytokine imbalance from the neutrophil infiltration was mitigated by folic acid treatment. For both studies, pre-treatment with folic acid and omeprazole showed a reduction in the production of pro-inflammatory cytokines (TNF-α and IL-1β), in favour of elevated anti-inflammatory cytokines (IL-4 and IL-10). This clearly lends credence to our previous findings from our laboratory on the

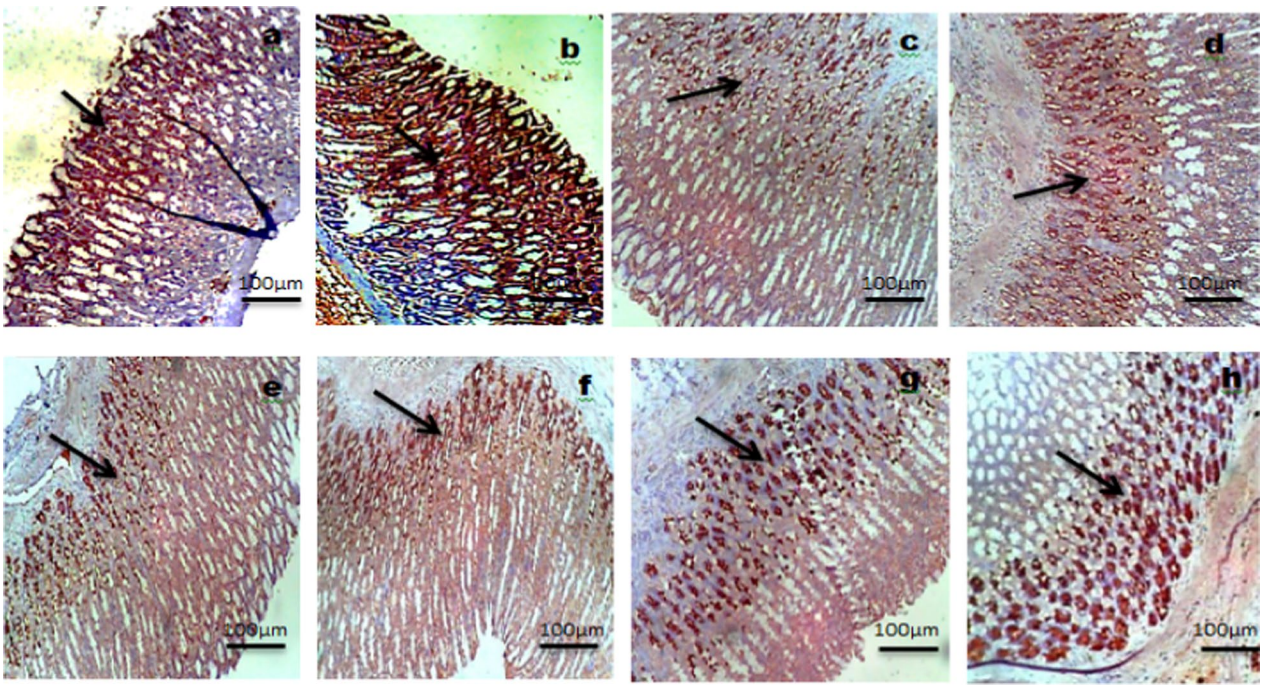

Fig. 17 IHC slides for evaluation of CD31 positive immunoreactive cells during healing of acetic acid stomach ulcer pretreated with folic acid and omeprazole. (H&E, X100). **a** Acetic acid stomach ulcer untreated (Day 5). **b** Acetic acid stomach ulcer treated with 2 mg/kg Folic acid (Day 5). **c** Acetic acid stomach ulcer treated with 3 mg/kg Folic acid (Day 5). **d** Acetic acid stomach ulcer treated with Omeprazole (Day 5). **e** Acetic acid stomach ulcer untreated (Day 10). **f** Acetic acid stomach ulcer treated with 2 mg/kg Folic acid (Day 10). **g** Acetic acid stomach ulcer treated with 3 mg/kg Folic acid (Day 10). h = Acetic acid stomach ulcer treated with Omeprazole (Day 10). Black arrow: Point of positivity

anti-inflammatory effects of folic acid in the exhibition of gastroprotective properties.

In the healing study, the luminal application of acetic acid on the gastric mucosa of rats caused characteristic gastric ulcers in the stomach. Acetic acid induced gastric ulcer has been a good choice of chronic ulcer model for evaluation of healing process because it resembles human ulcers in terms of pathology and healing process (Musumba et al., 2009). In addition, it responds well to various antiulcer drugs (proton-pump inhibitors, sucralfate) and herbs (Amagase & Okabe, 1999; Okabe & Amagase, 2005). It induces chronic ulcer mainly due to an increased volume of luminal acid and mucosal necrosis (Al Mofleh, 2010) which undergoes healing through day 7–10 as observed in this study. Subsequently, several natural protective mechanisms are activated in the system, which brings about restitution towards integrity (Amagase, 2003). In rats, the degree of angiogenesis (new vessel formation) and cell proliferation within the ulcer bed correlates strongly with the extent and speed of ulcer healing. The morphological observation of the mucosa of rats treated with folic acid indicated healing action on the acetic acid induced gastric ulcer. Furthermore, the rate of healing of the ulcer was found to be maximum on day 10 in the treated animals, as compared with the ulcer control group. It would be understandable, since the body's own defensive mechanism would be expected to play its role for the natural recovery of the animals from day 5 upward.

The acetic acid induced tissue necrosis and cell damage was decreased by folic acid administration on day 5 and 10. An increase in the expression of EGFR, Ki-67, CD 31 and Factor VIII was observed significantly on day 10, compared to day 5, which can be regarded as evidence of healing in the gastric mucosa, together with the enhanced mucosal level of EGF and VEGF.

It is well known that Epithelial Growth Factor (EGF) plays an important role in promoting gastric epithelial cell migration, proliferation and differentiation into the granulation tissue, to cover defect created by the mucosal injury. The elaboration of epithelial growth factor was assessed by measuring the mucosal level and the degree of expression of epithelial growth factor receptor (EGFR). EGFR is a good marker for the assessment of gastric epithelial cell proliferation activity, because its

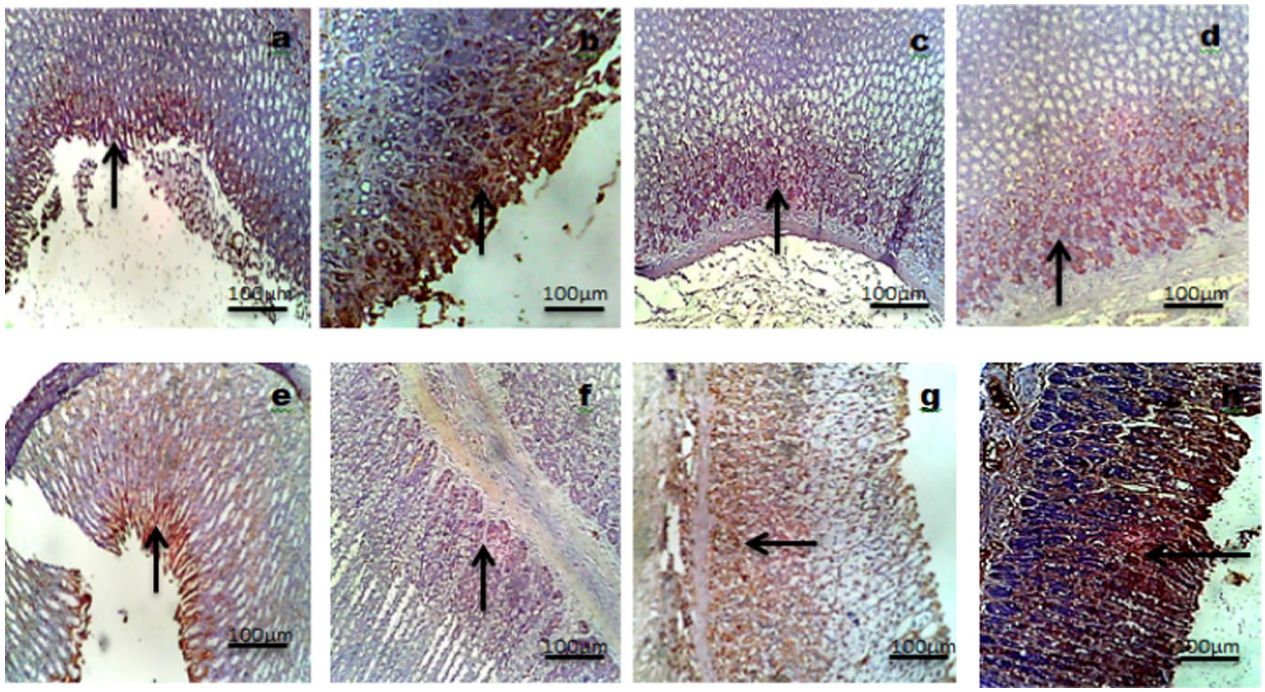

Fig. 18 IHC slides for evaluation of Factor VIII positive immunoreactive cells during healing of acetic acid stomach ulcer pretreated with folic acid and omeprazole. (H&E, X100). **a** Acetic acid stomach ulcer untreated (Day 5). **b** Acetic acid stomach ulcer treated with 2 mg/kg Folic acid (Day 5). **c** Acetic acid stomach ulcer treated with 3 mg/kg Folic acid (Day 5). **d** Acetic acid stomach ulcer treated with Omeprazole (Day 5). **e** Acetic acid stomach ulcer untreated (Day 10). **f** Acetic acid stomach ulcer treated with 2 mg/kg Folic acid (Day 10). **g** Acetic acid stomach ulcer treated with 3 mg/kg Folic acid (Day 10). **h** Acetic acid stomach ulcer treated with Omeprazole (Day 10). Black arrow: Point of positivity

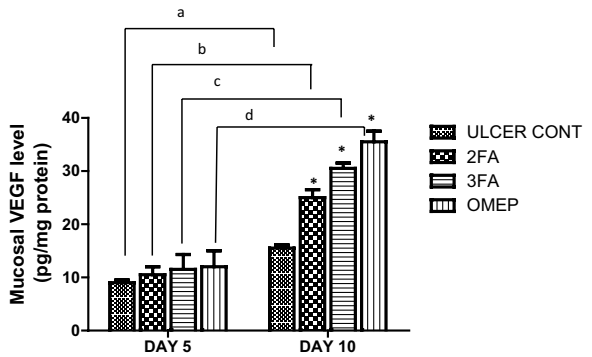

Fig. 19 Effect of folic acid supplementation on vascular endothelial growth factor (VEGF) levels in healing ulcerated gastric mucosa. (*$p < 0.05$ cf ulcer control; a–d $p < 0.05$ cf. each group on day 10). ULCER CONT = Acetic acid Ulcer control, 2FA = 2 mg/kg Folic acid, 3FA = 3 mg/kg Folic acid, OMEP = Omeprazole

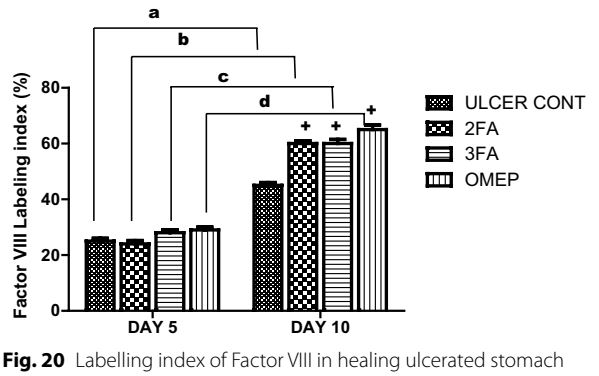

Fig. 20 Labelling index of Factor VIII in healing ulcerated stomach treated with folic acid and Omeprazole (*$p < 0.05$ cf ulcer control; a–d $p < 0.05$ cf. each group on day 10). ULCER CONT = Acetic acid Ulcer control, 2FA = 2 mg/kg Folic acid, 3FA = 3 mg/kg Folic acid, OMEP = Omeprazole

presence on the cell clearly signify that they are the targets for the proliferation stimulating action of EGF (Slomiany et al., 1998; Tarnawski, 2005a, 2005b). Similarly, Ki-67 expression has also been associated with gastric epithelial cell function. The protein is present during all active phases of the cell cycle (G(1), S, G(2) and mitosis), but absent in the resting cells G(0), making it an excellent

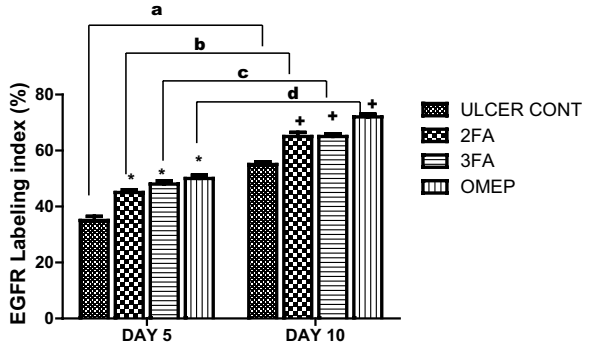

Fig. 21 Labelling index of EGFR in healing ulcerated stomach treated with folic acid and Omeprazole (*$p < 0.05$ cf ulcer control; a–d $p < 0.05$ cf. each group on day 10). ULCER CONT = Acetic acid Ulcer control, 2FA = 2 mg/kg Folic acid, 3FA = 3 mg/kg Folic acid, OMEP = Omeprazole

marker for determining gastric epithelial cell proliferation (Scholzen & Gerdes, 2000). High expression of EGFR and Ki-67 on day 10 in the folic acid treated group, shows that proliferation and epithelial cell migration occurred, and hence ulcer healing.

CD 31 (Cluster of Differentiation 31), also known as platelet- endothelial cell adhesion molecule-1 (PECAM-1), is an endothelial marker, expressed by platelets and megakaryocytes on the membrane of the endothelial cells. Together with VEGF, they play quite important role associated with participating in cell adhesion and angiogenesis (Ma et al., 2002). The expression of CD 31 and Factor VIII in the folic acid treated group at a very high concentration on day 10 indicates re-establishment of through the microvascular network through the process of angiogenesis, which is an evidence of ulcer healing.

The findings of the present study show that folic acid, an important factor in the de novo synthesis of purines, thymidine, deoxyribonucleic acid (DNA) (Huang et al., 1999), was able to attenuate the development of gastric ulcer, and as a result enhanced its healing. As a result of its ability to produce and maintain new cells, further apoptosis in the gastric mucosa may have been halted through reduced inflammation, thus enhancing the expression of EGFR, and thus ulcer healing on day 10.

Furthermore, the attacking effect of aggressive agents on the mucosa inversely relate with the mucous cell population that is, the higher the mucous cell density the stronger the defensive mechanism of the stomach (Brunton et al., 2005). The reduced mucous cell density in the acetic acid ulcer untreated group was improved in the folic acid and omeprazole treated. This might have probably fortified and aided the mucosa, in no small measure, towards healing.

Conclusions

From the results of this study, we concluded that folic acid protects the stomach against pylorus ligation ulcer via reduced acid output and inflammation (lessened neutrophil and inflammatory cells infiltration, TNF-α and IL-1β but enhanced IL-4 and IL-10). Furthermore, folic acid promotes the healing of acetic-acid induced gastric ulceration through enhanced epithelial cell proliferation (EGF, EGFR and Ki-67), and angiogenesis (VEGF, CD31 and Factor VIII) while inflammation is equally suppressed (TNF-α and IL-1β attenuated in favour of enhanced IL-4 and IL-10). In both studies, histological examination revealed an improved gastric cyto-architecture in terms of inflammation, erosion or congestion of mucosa and submucosa upon treatment with folic acid. Folic acid exhibits anti-secretory, anti-oxidative, anti-inflammatory properties in its gastroprotective activity, and pro-proliferative and pro-angiogenic mechanism in mucosal healing.

Table 6 Tissue cytokines level in the acetic acid ulcerated stomach treated with Folic acid (FA) and Omeprazole (OMEP)

	Day 5				Day 10			
	UC	2FA	3FA	OMEP	UC	2FA	3FA	OMEP
TNF-α	75.5 ± 5.0	60.5 ± 3.0[a]	59.5 ± 5.0[a]	50.0 ± 2.0[a]	40.5 ± 2.0	30.2 ± 1.0[a]	30.5 ± 1.0[a]	20.6 ± 1.5[a]
IL-1β	80.4. ± 1.0	70.2 ± 1.5[b]	60.5 ± 1.2[b]	55.0 ± 1.0[b]	50.3 ± 1.2	28.5 ± 1.4[b]	27.5 ± 2.0[b]	15.5 ± 1.1[b]
IL-4	10.5. ± 1.0	25.7 ± 2.0[c]	30.8 ± 2.5[c]	35.0. ± 1.0[c]	20.0 ± 1.3	30.5 ± 0.5[c]	38.5 ± 1.5[c]	40.5 ± 0.8[c]
IL-10	30.0. ± 1.0[d]	50.9. ± 2.5[d]	50.0. ± 1.5[d]	57.5. ± 1.5[d]	50.5 ± 1.0	67.5 ± 0.5[d]	70.5 ± 0.4[d]	80.0 ± 1.2[d]

UC acetic acid ulcer control, *2FA* 2 mg/kg Folic acid, *3FA* 3 mg/kg Folic acid, *OMEP* omeprazole

[*a,b,c,d] $p < 0.05$ cf. ulcer control each group on day 5 and 10

Abbreviations
FA: Folic acid; 2FA: Folic acid (2 mg/kg); 3FA: Folic acid (3 mg/kg); EGFR: Epidermal growth factor receptor; EGF: Epidermal growth factor; Ki-67: Marker of proliferation KI67; CD31: Cluster of Differentiation 31; TNF-α: Tumor necrosis factor alpha; IL-1β: Interleukin 1beta; IL-4: Interleukin-4; IL-10: Interleukin-10; ml: Milliter; ml/d: Milliter/day; mg/kg: Milligram/kilogram; mg/kg/d: Milligram/kilogram/day; ELISA: Enzyme linked immunosorbent assay; PBS: Phosphate buffered saline; HRP: Horseradish peroxidase; ANOVA: Analysis of variance; MCD: Mucous cell density; pg/mg: Picogram/microgram; mmol: Millimole; OMEP: Omeprazole.

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Authors' contributions
KA* contributed to the design and conception of the study, analysis, and interpretation of data and drafted the manuscript. AO also contributed to the design and conception of the study and preparation of materials required for the study. KA, SS and OA carried out the experimentation under the supervision of KA* and AO. All authors read and approved the manuscript.

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Availability of data and materials
Upon request, the analyzed datasets garnered from bench can be made available by the corresponding author.

Declarations

Ethical approval and consent to participate
All studies on the animal experimentation were conducted in accordance with the Current Animal Care Regulations and Standards approved by the Institute for Laboratory Animal Research (ILAR, 1996), and protocols approved in 2017 by the Animal Research and Ethics Committee of the College of Health Sciences, Igbinedion University, Okada. Reference number is not applicable. The animals were housed under standard conditions of temperature (23 ± 2 °C), humidity (55 ± 15%) and 12 h light (7:00 am–7:00 pm). The cages were constantly kept clean in order to prevent the animals from disease and the care and handling were done by a professional animal house attendant under the supervision of the authors. They were fed with standard commercial rat pellets and allowed free access to water ad libitum.

Consent for publication
Not applicable.

Competing interests
The authors declare that this article has no conflict of interest.

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