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RESEARCH ARTICLE

Exacerbation of diclofenac-induced gastroenterohepatic damage by concomitant exposure to sodium fluoride in rats: protective role of luteolin

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ABSTRACT

NSAID-induced gastrointestinal toxicity is associated with non-selective inhibition of cyclooxygenase (COX)-mediated synthesis of prostaglandins. Fluoride salts, known to stimulate COX-2 synthesis, have also been associated with gastrointestinal damage. The effects of fluoride treatment on NSAID toxicity are, however, yet to be clarified. This study examined the effect of sodium fluoride (NaF) on diclofenac (DIC)-induced gastroduodenal and hepatic toxicity in rats. In addition, the potential protective role of Luteolin (Lut), an antioxidant and anti-inflammatory flavonoid, in co-exposure to NaF and DIC was also investigated. Five groups of rats were treated thus: Group A (control): distilled water vehicle for 8 days; Group B: DIC (9 mg/kg) orally, twice daily from days 6 to 8; Group C: NaF (300 ppm) plus DIC for the final 3 days; Groups D and E: Luteolin at 100 mg/kg and 200 mg/kg, respectively, with concurrent NaF and DIC exposures. Rats co-treated with DIC and NaF exhibited the highest severity of dark watery diarrhea and gastroduodenal hemorrhages. NaF aggravated the DIC-induced increases in malondialdehyde (MDA), advanced oxidation protein products (AOPP), protein carbonyls (PC), H_2O_2, and nitric oxide, while inhibiting glutathione peroxidase (GPx) and glutathione S-transferase (GST) in all the tissues. In contrast, Luteolin treatment significantly attenuated the gastroduodenal and hepatic damage caused by NaF and DIC co-administration by suppressing oxidative damage and lesions in the tissues. These results show, for the first time, that NaF may enhance diclofenac-induced gastrointestinal toxicity and also suggest that Luteolin may be a promising lead for the treatment of drug-induced gastroenteropathy.

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Gastroenterohepatic damage; nonsteroidal anti-inflammatory drugs; fluoride; oxidative stress

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Introduction

Nonsteroidal anti-inflammatory drugs (NSAID) e.g., diclofenac, indomethacin, ibuprofen, etc., are widely available over-the-counter drugs for the treatment of pain, inflammation, and fever (Wallace 2016). They are, however, well known for their toxic side effects of gastrointestinal ulceration and bleeding (Chatterjee and Bandyopadhyay 2014). NSAIDs are known to block the synthesis of muco-protective prostaglandins by inhibiting the expression of both constitutive (COX-1) and inducible (COX-2) cyclooxygenase enzymes. Diclofenac (DIC) is a lipophilic NSAID used in the treatment of pain of musculoskeletal disorders, rheumatoid arthritis, and osteoarthritis (Tieppo Francio *et al*. 2017). Like most other NSAIDs, high doses of DIC present side effects of enteropathy, gastrointestinal ulceration, and bleeding amongst other effects. Studies have shown that DIC administration produces acute erosions and immediate gastroduodenal damage within hours of ingestion (Hawkins and Hanks 2000). The mechanisms of DIC toxicity has been reported to involve increased oxidative stress and inflammation, as well as mitochondrial dysfunction (Galati *et al*. 2002).

The pathogenesis of gastroenteropathies must, however, be considered from the point of view of a multifactorial etiology, rather than exposure to single compounds. This is because, in natural situations, the gastrointestinal (GI) tract may be exposed concurrently to a wide variety of agents in water, food and drugs, as well as microbes, such as *Helicobacter pylori*, all of which can potentially damage the GI mucosa. Potential interactions between different mucosal irritants may produce aggravation or alleviation of mucosal injury when present together in the GI tract (Singh *et al*. 2017). This situation poses a challenge for the clinical management of gastroenteropathies as the development of appropriate preventive or therapeutic strategies must take into account the underlying mechanisms of toxicities of the different compounds.

Fluoride is a ubiquitous environmental ingredient of drinking water, food, fluoride supplements, fluoride tablets and gels, dental prophylaxis, and some drugs (WHO 2002). Once swallowed, the gastrointestinal tract is its first site of contact, with the highest absorption taking place in the small intestine. In low concentrations, fluoride is an acknowledged pharmacological agent used in the prevention of dental caries. However, high exposure to fluoride ions may produce

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acute poisoning manifested as gastrointestinal symptoms such as nausea, abdominal pain, and vomiting (Martinez-Mier 2011, Whitford 2011). Several reports in humans, animals, and plants have demonstrated the induction of oxidative stress as a key mechanism underlying the toxic effects of fluoride exposure (Barbier *et al.* 2010). It has also been suggested that fluoride ingestion can stimulate acid production in the stomach and can also combine with hydrochloric acid to form hydrofluoric acid, which causes gastrointestinal irritation (Akyuz *et al*. 2015) Interestingly, fluoride salts have been found to induce transcriptional upregulation of COX-2 in human pulmonary epithelial cells (Ridley and Matsuoka 2009) with the resultant production of prostaglandins.

Despite the widely reported gastrointestinal toxicities due to NSAIDs and fluoride, there appears to be no information available in literature on the possible interactions, and the implications on gastrointestinal toxicity, when the two compounds are administered concurrently. Previous studies have indicated that concurrent fluoride administration with other chemicals or drugs may exacerbate toxicity to various organs. For instance, combined fluoride and nitrate intoxication was found to cause severe oxidative and nitrosative stress-dependent connective tissue degradation in rats' gastric mucosa (Akimov *et al*. 2019) Fluoride was also found to aggravate acetaminophen toxicity in liver and kidney of rats (Inkielewicz-Stepniak and Knap 2012). Certain NSAID/Fluoride periodontal patents developed for the treatment of dental caries have also raised the possibility for concurrent exposures to both NSAIDs and fluoride (Aberg *et al*. 1998). The potential impacts of such formulations on the gastrointestinal tract have, however, not been investigated.

Toward providing a complete solution against the possible multifactorial nature of chemical-induced gastroenteropathy, alternative therapies with natural products from plants have been proposed as potential lead compounds (Susheela and Bhatnagar 2002). Luteolin (3′, 4′, 5, 7-tetrahydroxyflavone) is a naturally occurring phenolic compound found in many fruits, vegetables, and medicinal plants. It functions biologically as an antioxidant, anti-inflammatory, and anticancer agent due to the presence of hydroxyl groups at the carbons at positions 5, 7, 3′, and 4′ in the flavonoid structure (Lin *et al.* 2008). Its mechanisms of cellular protection involve direct scavenging of reactive oxygen species and the modulation of the expression of antioxidant and cyto-protective genes (Zhang *et al.* 2013).

Given that the mechanisms of toxicity of DIC and fluoride are reported to involve oxidative stress and inflammation, and that Luteolin has been proven to possess significant antioxidant and anti-inflammatory properties, the present study was designed to evaluate the possible protective role of Luteolin on gastric, duodenal and hepatic tissues of rats, during concomitant exposure to DIC and sodium fluoride. Reactive oxygen species generation and the activities of some antioxidant enzymes in DIC/Fluoride co-exposure were evaluated and the possible protective role of Luteolin was examined. Gastrointestinal injury was observed macroscopically and microscopically in rats.

Materials and methods

Chemicals

Luteolin, Sodium fluoride, reduced glutathione (GSH), 1, 2-dichloro-4-nitrobenzene (CDNB), thiobarbituric acid (TBA), trichloroacetic acid (TCA), sodium hydroxide, xylenol orange, potassium hydroxide, and hydrogen peroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diclofenac sodium (Voltaren®) (2-[{2, 6-dichlorophenyl} amino] benzene acetic acid) was purchased from a reputable pharmacy in Ibadan, Nigeria. All other chemicals were of the highest purity commercially available.

Animals and dosage regimen

Thirty-five male Wistar rats, about 10–12 weeks old, weighing 110–170 g were used for the experiments. The rats were obtained from the Experimental Animal Unit of the Faculty of Veterinary Medicine, University of Ibadan, Nigeria. They were housed and allowed to acclimatize for a week in a well-ventilated animal house with free access to standard rat chow and clean tap water, while a 12-h light, 12-h dark cycle was maintained. All animals were handled in accordance with guidelines outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institute of Health (PHS 1996). The study was conducted following guidelines approved by the Animal Care and Use Research Ethics Committee (ACUREC) of the University of Ibadan.

The animals were randomly assigned to five groups ($n = 7$ per group) and were treated based on a previously published NSAID gastroenteropathy model (Singh *et al*. 2017) which was slightly adjusted because of the occurrence of death of animals during the pilot study. Unlike the model described by Singh *et al.* (2017) where DIC was administered for 5 days, DIC was administered only for 3 days because of the observation of death of rats from the fourth day of DIC administration during the pilot study. The experimental groups were as follows:

Group A (Vehicle): rats treated with distilled water for 8 days
Group B (DIC group): rats treated with DIC (9 mg/kg b.w.) orally, twice daily for the final 3 days
Group C (DIC + NaF group): rats treated with NaF (300 ppm) in drinking water for 8 days plus DIC (9 mg/kg b. w.) orally, twice daily for the final 3 days.
Group D (DIC + NaF + Lut1): rats treated with NaF (300 ppm) in drinking water for 8 days, Luteolin (100 mg/kg b.w.) by oral gavage once daily for 8 days and DIC (9 mg/kg b. w.) orally, twice daily for the final 3 days.
Group E (DIC + NaF + Lut2): rats treated with NaF (300 ppm) in drinking water for 8 days, Luteolin (200 mg/kg b.w.) by oral gavage once daily for 8 days and DIC (9 mg/kg b. w.) orally, twice daily for the final 3 days.

The selected dosages of DIC (Singh *et al.* 2017) NaF and Lut (Oyagbemi *et al*. 2018) were based on previously published studies and all compounds were dissolved in pure

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distilled water for administration to the rats. The rats were fasted from the last administration but were allowed free access to water prior to termination of the experiment. About 24 hours after the last administration, the rats were anesthetized with diethyl ether, blood samples were withdrawn from the retro-orbital plexus and the rats were euthanized by cervical dislocation.

Body and organ weight changes

The body weights of the rats were measured before the start of the experiment, every other day for necessary dosage adjustments and then prior to euthanasia. The initial and final weights were necessary to monitor the changes in body weight that may result from administration of the compounds. The percentage change (gain or loss) in body weight was calculated with the formula: (Final weight − Initial weight)/initial weight multiplied by 100.

Following euthanasia, the abdomen was opened and the liver, stomach, and duodenum were removed. The stomach was opened along the greater curvature; the duodenum was opened longitudinally along the entire length, while the entire liver was also collected. All tissues were rinsed in cold physiological saline solution (0.9% NaCl), after which the adherent fluid was blotted on dry filter paper. The absolute wet weights of the organs were recorded for all the rats and the relative organ weights in all the rats were calculated using the formula: (Absolute organ weight/body weight) × 100. Small portions of the organs were immediately transferred to 10% phosphate-buffered formalin for histopathological examination. The remaining portions were reserved for biochemical assessments.

Macroscopic features of gastrointestinal toxicity

The rats were observed throughout the experiment for evidences of gastrointestinal toxicity and other clinical features including stool consistency, reduced appetite, etc. Stool consistency was scored on the 7th day as 0 for normal feces (firm and dry); 1 for pasty feces; 2 for thick and fluid-like feces and 4 for watery diarrhea (Sun *et al*. 2019), by a pathologist who was blind to the treatment conditions. Following euthanasia, the gross appearance of the gastric and duodenal mucosa was also captured with a digital camera. The macroscopic scoring of gastric and duodenal lesions was based on evaluation of two observable parameters of mucosal damage, as described by Simoes *et al*. (2019), namely (a) Hemorrhage size, including score 1 (Punctiform, <2 mm); score 2 (mild, 2–5 mm) or score 3 (Intense, >5 mm), and (b) Hemorrhage number, including score 1 (0–4); score 2 (5–6) or score 3 (≥ 7). Sections with no observable lesions were assigned a score of 0.

Estimation of oxidative stress markers and activity of antioxidant enzymes

Following euthanasia of the experimental animals, the stomach, duodenum and liver samples collected for the estimation of biochemical parameters were homogenized in 100 mM potassium phosphate buffer (pH 7.4) and the resulting homogenate was centrifuged at 10 000 × *g* for 15 min at 4 °C in a cold centrifuge. The supernatant obtained was subsequently used for the biochemical assays of oxidative stress and antioxidant markers. Protein concentration was measured by the Biuret method according to the method described by Gornal *et al*. (Gornal *et al*. 1949). Hydrogen peroxide (H_2O_2) concentration was determined spectrophotometrically at 560 nm using the method of Wolff (1994). Nitric oxide (NO) level, measured as the content of nitrites in the tissues, was measured according to the method described by Olaleye (2007). The result was expressed as μmol of nitrites/litre. The tissue content of Malondialdehyde (MDA) was used as an index of lipid peroxidation and this was estimated using the method of Varshney and Kale (1990). MDA content was quantified with a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\,cm^{-1}$. The content of advanced oxidation protein products (AOPP) in the tissues was determined following Kayali et al.'s (2006) model. Protein carbonyl (PC) content in the tissues was measured using the method of Reznick and Packer (1994). Glutathione peroxidase (GPx) activity was determined using the method of Rotruck *et al.* (1973). Glutathione S-transferase (GST) activity was estimated by the method of Habig (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. Superoxide dismutase (SOD) activity was determined according to the method described by Misra and Fridovich (1972) with slight modifications by Oyagbemi *et al*. (2015). The assay was based on the inhibition of the auto-oxidation of epinephrine at pH 7.2 at 30 °C. Briefly, 100 mg of epinephrine was dissolved in 100 mL distilled water and acidified with 0.5 mL concentrated hydrochloric acid. Then, 0.01 mL of each sample was added to 2.5 mL of 0.05 mol/L carbonate buffer (pH 10.2), followed by the addition of 0.3 mL of 0.3 mmol/L epinephrine. The increase in absorbance at 480 nm was monitored every 30 s for 150 s. One unit of SOD activity represents the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adrenochrome in 1 min. Tissue content of reduced glutathione (GSH) was measured by the method of Beutler *et al.* (1963). The protein thiol contents were determined using Ellman's (1959) template.

Histopathological studies

Following euthanasia of the experimental animals, small portions of the stomach, duodenum, and liver were immediately transferred to 10% phosphate-buffered formalin for histopathological examination. For this purpose, they were embedded in paraffin wax, and sections of 5–6 mm in thickness were made and thereafter stained with hematoxylin and eosin for histopathological examination according to the method described by Drury *et al.* (1976). Thereafter, the sections were examined by a blinded pathologist with light microscopy. The histological damage in the gastric sections was determined using the two observable criteria representing mucosal damage, according to Simoes *et al.* (2019) namely: (a) Depth of mucosal erosion, where score

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1 = erosion of *one-third* of total mucosal depth; score 2 = erosion of two-thirds of total mucosal depth and score 3 = erosion of total mucosa; and (b) Presence of hemorrhagic lesions, where score 1 = focal, score 2 = mild and score 3 = severe. Absence of lesions was recorded as 0. A total of five sections were examined and computed for each examined slide.

For the duodenal sections, four observable criteria were studied, based on methods described by Khayyal *et al.* (2019), namely: (a) Focal necrosis and fusion of villi, (b) Mucosal and submucosal edema, (c) Congestion of blood vessels, and (d) Activation of glands. The severity of damage for each parameter was evaluated as follows: 0 = normal; 1 = mild; 2 = moderate and 3 = severe.

For liver sections, scoring of hepatic lesions was based on evaluation of the following observable parameters: (a) Necrosis (b) Atrophy of hepatic cords (c) Fibrosis, and (d) Inflammation. The severity of the lesions was assigned scores as follows: 0 = absent; 1 = mild; 2 = moderate, 3 = severe, based on scoring criteria described by Vetelainen *et al.* (2006).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (Version 7.00). Data from biochemical assays were expressed as mean ± standard deviation and analyzed using one way Analysis of Variance (ANOVA) followed by the Tukey's *post hoc* test for multiple comparisons among the groups in cases where F was significant. Differences in mean values were considered statistically significant at p values <0.05. Five samples each of the stomach and duodenum from each group were examined and scored macroscopically. Damage severity was scored with values ranging from 0 to 6. The data was analyzed using Microsoft Excel and presented as stacked bar charts.

Results

Body and organ weight changes in rats co-exposed to diclofenac and sodium fluoride and treated with luteolin

The average weight of rats in the control group increased significantly by 8.83% in the course of the experiment while the body weight of the rats in all the other groups declined (Table 1). The largest decline in body weight was observed in the DIC + NaF group, with an average loss of −19.19% during the experimental period, which was significantly higher compared to an average loss of only −2.01% recorded in rats receiving diclofenac alone. Body weight losses in rats treated with Luteolin at 100 mg/kg (−17.79%) or 200 mg/kg (−15.89%) were only slightly lower than that of the DIC + NaF group.

Despite the observed reduction in body weights, there were significant ($p < 0.05$) increases in the relative weights of the stomach, duodenum, and liver of rats exposed to diclofenac and/or sodium fluoride when compared with the control. Luteolin administration, however, resulted in significant ($p < 0.05$) amelioration of the organ weight changes, especially in the duodenum and liver.

Macroscopic presentation of diclofenac and sodium fluoride-induced toxicity

No mortality was observed in all the groups up till the end of the final day of administration of the different compounds. However, rats in all other groups apart from the control exhibited reduced appetite which was more severe in the DIC and DIC + NaF groups. These latter groups of rats presented with varying degrees of dark, watery diarrhea with matted feces around the anal region. The DIC and DIC + NaF groups presented with much higher intensity ($p < 0.05$) of watery diarrhea, compared with the other groups, as indicated by the diarrhea severity scores (Figure 1(a)). Rats pretreated with Luteolin, however, had significantly lower ($p < 0.05$) diarrhea scores, compared with the DIC + NaF group. The macroscopic appearance of representative samples of gastric and duodenal mucosa following euthanasia showed varying degrees of hemorrhages with the highest severity of hemorrhages also observed in the DIC + NaF group (Figure 1(b,c)). Total mean scores of hemorrhagic lesions in the stomach samples from the DIC + NaF group was 6.00, while samples from the DIC group had a mean score of 4.60, showing an aggravation of hemorrhagic lesions in the DIC + NaF group. Luteolin treatment, however, reduced the intensity of bleeding in the stomach and duodenal mucosa, with mean scores of 2.00 and 1.20 with Lut1 (100 mg/kg) and Lut 2 (200 mg/kg), respectively. No hemorrhagic lesions were found in the stomach samples from the control group (Figure 1(b)).

In the duodenum, total mean hemorrhagic scores were 3.80 and 5.40 for the DIC and DIC + NaF groups, respectively. In contrast, no hemorrhage was detected in duodenal samples from the control group, as well as the groups treated with Luteolin (Figure 1(c)).

Table 1. Effect of Luteolin on body and organ weight changes in rats co-administered with diclofenac and sodium fluoride.

Treatment groups	Initial weight (g)	Final weight (g)	Percentage weight change (%)	Relative stomach weight (%)	Relative Duodenum weight (%)	Relative liver weight (%)
Control	175.50 ± 35.34	191.00 ± 10.86	8.83	0.58 ± 0.03	3.08 ± 0.62	3.69 ± 0.35
DIC only	162.75 ± 14.36	159.50 ± 12.15	−2.01	0.74 ± 0.05[a]	3.19 ± 0.55	4.51 ± 0.33[a]
DIC + NaF	165.00 ± 30.10	133.33 ± 19.41	−19.19	0.78 ± 0.10[a]	4.47 ± 0.33[a,b]	4.72 ± 0.36[a]
DIC + NaF + Lut1	160.25 ± 12.20	131.75 ± 7.89	−17.79	0.85 ± 0.06[a]	4.30 ± 0.65[a]	4.49 ± 0.26[a]
DIC + NaF + Lut2	167.29 ± 22.76	140.71 ± 0.13	−15.89	0.84 ± 0.13[a]	3.71 ± 0.75[c]	4.12 ± 0.31[a,c]

DIC: diclofenac; NaF: sodium fluoride; Lut1: luteolin at 100 mg/kg; Lut2: luteolin at 200 mg/kg. [a]Values differ significantly from control ($p < 0.05$). [b]Values differ significantly from DIC alone ($p < 0.05$). [c]Values differ significantly from DIC + NaF.

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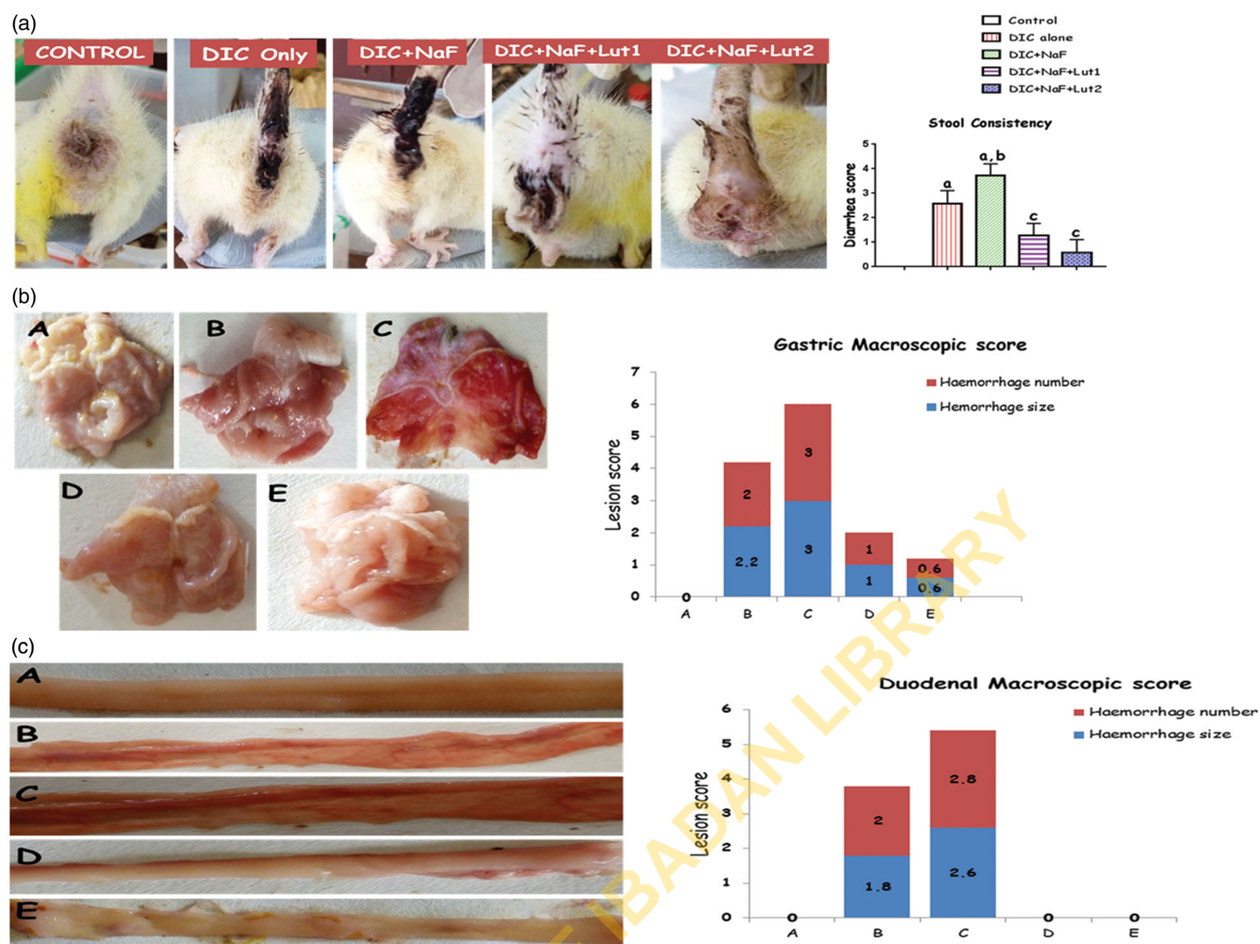

Figure 1. Stool consistency scores (a), gastric mucosa (b), and duodenal mucosa (c) of rats co-exposed to diclofenac and sodium fluoride and treated with luteolin. DIC: diclofenac; NaF: sodium fluoride; Lut1: luteolin at 100 mg/kg; Lut2: luteolin at 200 mg/kg. [a]Values differ significantly from control ($p < 0.05$). [b]Values differ significantly from DIC alone ($p < 0.05$). [c]Values differ significantly from DIC + NaF.

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Luteolin reversed diclofenac- and sodium fluoride-induced changes in hydrogen peroxide and nitric oxide levels in gastric, duodenal and hepatic tissues of rats

The effects of Luteolin on hydrogen peroxide and nitric oxide levels in the stomach, duodenum, and liver of rats treated with diclofenac and sodium fluoride are presented in Figure 2. Exposure to DIC alone led to significant ($p < 0.05$) increase in gastric H_2O_2 levels, when compared with control, while the duodenal and hepatic H_2O_2 levels were unaltered. Co-treatment of DIC with NaF resulted in significant ($p < 0.05$) rise in duodenal H_2O_2 levels, when compared with the DIC group. Nevertheless, Luteolin treatment significantly ($p < 0.05$) reduced H_2O_2 production in the stomach, duodenum, and liver, when compared with the DIC + NaF group.

Nitric oxide (NO) levels in the duodenum of rats treated with DIC alone were significantly ($p < 0.05$) higher than the control, while no changes were recorded in stomach and liver NO levels. The largest enhancement of NO production was observed in the DIC + NaF group in which gastric and duodenal NO values were significantly higher than those of the control and DIC groups. Hepatic NO levels remain unchanged in the rats from the DIC and DIC + NaF groups. Luteolin administration however resulted in significant ($p < 0.05$) reduction in nitric oxide levels in the duodenum and liver.

Luteolin inhibited diclofenac- and sodium fluoride-induced production of lipid and protein oxidation products in gastric, duodenal and hepatic tissues of rats

The data presented in Figure 3 shows the effects of Luteolin on some products of lipid and protein oxidation, namely malondialdehyde (MDA), advanced oxidation protein products (AOPP), and protein carbonyls (PC) in the stomach, duodenum, and liver of rats co-exposed to diclofenac and sodium fluoride. Exposure to DIC alone caused significant ($p < 0.05$) increase in MDA levels in the stomach, duodenum, and liver, compared to the control. These changes were significantly ($p < 0.05$) exacerbated in the stomach and duodenum of rats in the DIC + NaF group, which showed further increase in MDA levels compared to the control and DIC groups. However, Luteolin pretreatment produced significant ($p < 0.05$) attenuation of MDA levels in all the tissues, when compared with the DIC + NaF group.

In similar fashion, administration of DIC alone caused significant ($p < 0.05$) increase in AOPP levels in the stomach, duodenum, and liver, when compared to the control. Maximum AOPP levels were again observed in the DIC + NaF group, which showed significantly ($p < 0.05$) higher AOPP levels in the stomach and duodenum, compared to the control and DIC groups and again suggesting an exacerbation of

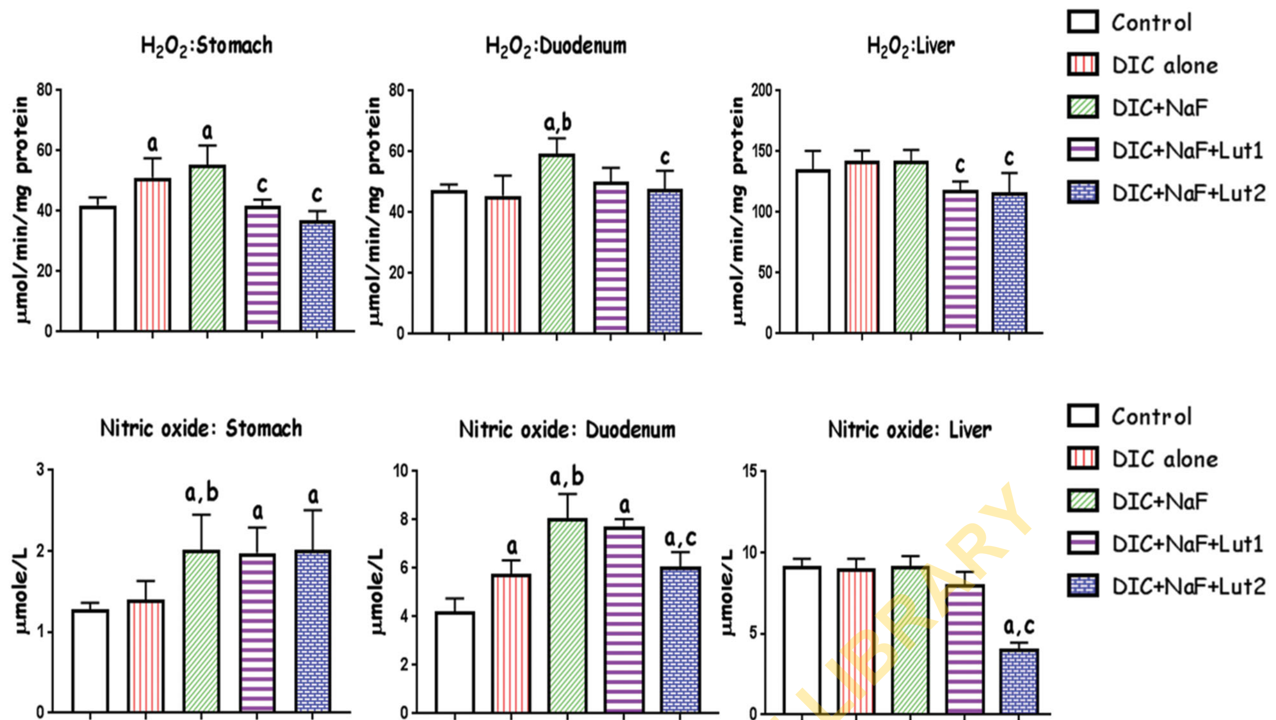

Figure 2. Effects of luteolin on hydrogen peroxide and nitric oxide levels in gastric, duodenal, and hepatic tissues of rats co-administered with diclofenac and sodium fluoride. DIC: diclofenac; NaF: sodium fluoride; Lut1: luteolin at 100 mg/kg; Lut2: luteolin at 200 mg/kg. [a]Values differ significantly from control ($p < 0.05$). [b]Values differ significantly from DIC alone ($p < 0.05$). [c]Values differ significantly from DIC + NaF.

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Figure 3. Effects of Luteolin on levels of malondialdehyde (MDA), advanced oxidation protein products (AOPP) and protein carbonyls in gastric, duodenal and hepatic tissues of rats co-administered with diclofenac and sodium fluoride. DIC: diclofenac; NaF: sodium fluoride; Lut1: luteolin at 100 mg/kg; Lut2: luteolin at 200 mg/kg. [a]Values differ significantly from control ($p < 0.05$). [b]Values differ significantly from DIC alone ($p < 0.05$). [c]Values differ significantly from DIC + NaF.

oxidative damage in these tissues. Treatment with Luteolin significantly ($p < 0.05$) prevented the increased production of AOPP induced by DIC and NaF in all the tissues examined in this study.

Exposure of rats to DIC alone caused significant ($p < 0.05$) increase in protein carbonyl levels only in duodenal tissues, when compared with the control, while no significant differences were observed in the gastric and hepatic protein carbonyl levels. Additionally, it was evident from the results that co-treatment of DIC with NaF resulted in further significant ($p < 0.05$) increase in gastric, duodenal, and hepatic protein carbonyl levels, compared with the DIC group. However, Luteolin treatment significantly ($p < 0.05$) reduced protein carbonyl levels in the stomach, duodenum, and liver when compared with the DIC + NaF group.

Luteolin prevented diclofenac- and sodium fluoride-induced decrease in antioxidant enzyme activities in gastric, duodenal and hepatic tissues of rats

The data presented in Figure 4 depicts the effects of Luteolin on the activities of antioxidant enzymes; glutathione peroxidase (GPx), glutathione S-transferase (GST) and superoxide dismutase (SOD) in the stomach, duodenum and liver of rats co-exposed to diclofenac and sodium fluoride. When compared with the control, rats treated with DIC alone did not produce any treatment-related changes in GPx activity in the stomach, duodenum, and liver. However, co-administration with NaF resulted in significant ($p < 0.05$) reduction in GPx activity in the stomach and duodenum when compared with both control and DIC groups. Treatment with Luteolin, however, reversed the effects of DIC and NaF by significantly ($p < 0.05$) enhancing the activity of GPx in the stomach, duodenum, and liver.

The activity of GST significantly ($P < 0.05$) decreased in the stomach, duodenum, and liver of rats given DIC alone as against the control group. Further significant ($P < 0.05$) reductions in gastric and duodenal GPx activity were also noticed in the rats co-administered with NaF, when compared with the control and DIC groups, again providing evidence of an exacerbation of oxidative injury in these tissues. Luteolin treatment, however, reversed these effects by significantly ($p < 0.05$) preventing the DIC and NaF-induced reduction in GST activity in all the tissues, when compared with the DIC + NaF group.

Contrary to the effects observed for GST activity, administration of DIC alone produced significant ($p < 0.05$) increases in SOD activity in the stomach, duodenum, and liver, when

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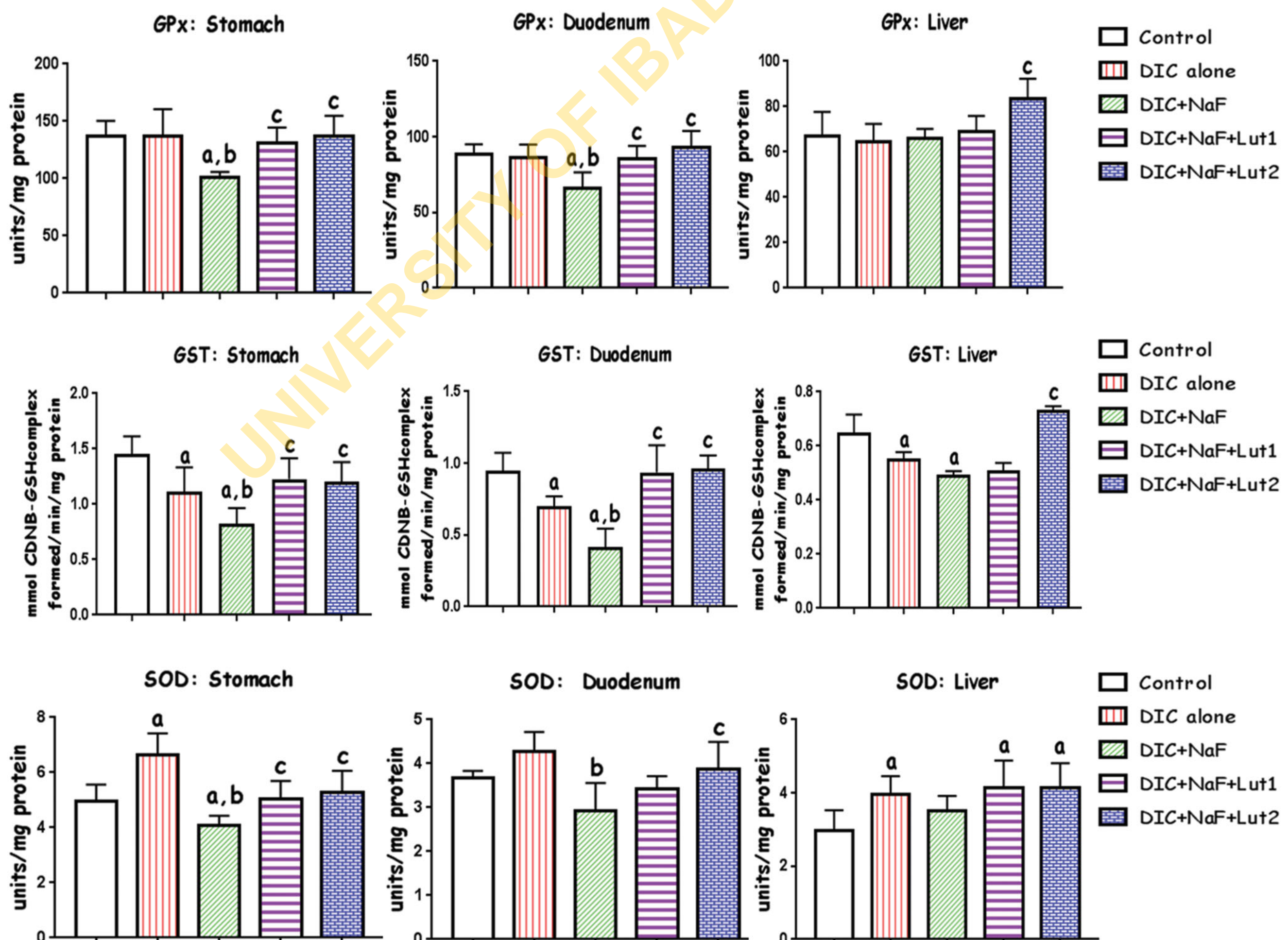

Figure 4. Effects of luteolin on the activities of glutathione peroxidase (GPx), glutatathione S-transferase (GST), and superoxide dismutase (SOD) in gastric, duodenal and hepatic tissues of rats co-administered with diclofenac and sodium fluoride. DIC: diclofenac; NaF: sodium fluoride; Lut1: luteolin at 100 mg/kg; Lut2: luteolin at 200 mg/kg. [a]Values differ significantly from control ($p < 0.05$). [b]Values differ significantly from DIC alone ($p < 0.05$). [c]Values differ significantly from DIC + NaF.

compared with the control. However, co-administration with sodium fluoride significantly ($p < 0.05$) reduced SOD activity in the stomach and duodenum when compared with the control and DIC groups. Luteolin treatment, however, provided significant ($p < 0.05$) enhancement of SOD activity in all the tissues investigated compared to the DIC + NaF group, as well as the control group.

Luteolin improved diclofenac- and sodium fluoride-induced depletion in reduced glutathione (GSH) and protein thiols in gastric, duodenal and hepatic tissues of rats

The effects of Luteolin on thiol antioxidants in the stomach, duodenum, and liver of rats co-exposed to diclofenac and sodium fluoride are presented in Figure 5. The results indicated that DIC treatment induced significant ($p < 0.05$) reduction in gastric GSH levels compared to the control rats, while the levels remained unchanged in the duodenum and liver. GSH levels in the DIC + NaF group did not differ significantly compared to the control or the DIC group. However, Luteolin treatment induced significant ($p < 0.05$) increases in GSH levels in the stomach, duodenum, and liver compared to the control and DIC + NaF groups.

Levels of protein thiols were significantly ($p < 0.05$) reduced with diclofenac administration in both the stomach and liver, when compared with the control. Similar observations were also recorded for rats treated with a combination of DIC and NaF. Luteolin administration, however, significantly ($p < 0.05$) prevented the DIC and NaF-induced decline in protein thiol levels, especially in the stomach and duodenum. Protein thiol levels in the liver remained lower than those of control rats, despite Luteolin administration.

Luteolin ameliorated diclofenac- and sodium fluoride-induced histopathological changes in gastric, duodenal and hepatic tissues of rats

The histopathological changes observed with light microscopy in the stomach, duodenum, and liver sections from the rats are presented in Figure 6. The sections from the control rats appeared structurally normal with no observable lesions (Figure 6(a1,b1,c1)). However, there were obvious pathological features observed in the stomach (Figure 6(a2,a3)); duodenum (Figure 6(b2,b3)) and liver (Figure 6(c2,c3)) of rats treated with either diclofenac alone or diclofenac combined with sodium fluoride. Rats in the DIC group showed extensive erosion of the gastric epithelium, focal necrosis of the hepatocytes and atrophy of hepatic cords, as well as edema and fusion of villi in the duodenum. These changes were more severe in the DIC + NaF group, further lending evidence to an exacerbation of diclofenac-induced damage by co-exposure to sodium fluoride. Rats treated with Luteolin (Figure 6(a4,a5,b4,b5,c4,c5)) presented with milder pathologies, and in most cases, Luteolin treatment abolished the occurrence of these pathologies. Sections from rats treated with the higher dose of Luteolin appeared similar to those from control rats. Detailed analysis of the semiquantitative data obtained from scoring of different parameters of tissue damage is presented in Table 2. In the stomach samples, the depth of mucosal erosions was significantly ($p < 0.05$) higher in the DIC + NaF group (2.60 ± 0.55) than the DIC group (1.80 ± 0.45), while hemorrhages occurred with significantly

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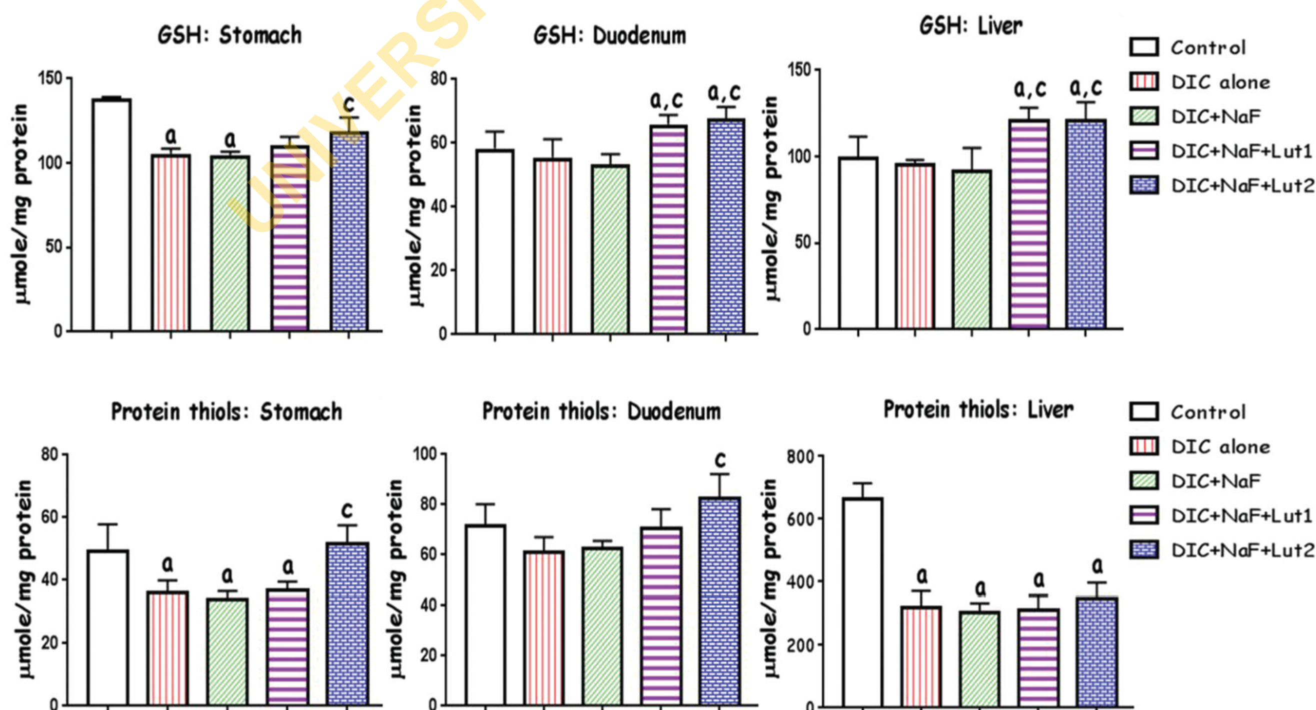

Figure 5. Effects of luteolin on reduced glutathione (GSH) and protein thiol levels in gastric, duodenal, and hepatic tissues of rats co-administered with diclofenac and sodium fluoride. DIC: diclofenac; NaF: sodium fluoride; Lut1: luteolin at 100 mg/kg; Lut2: luteolin at 200 mg/kg. [a]Values differ significantly from control ($p < 0.05$). [b]Values differ significantly from DIC alone ($p < 0.05$). [c]Values differ significantly from DIC + NaF.

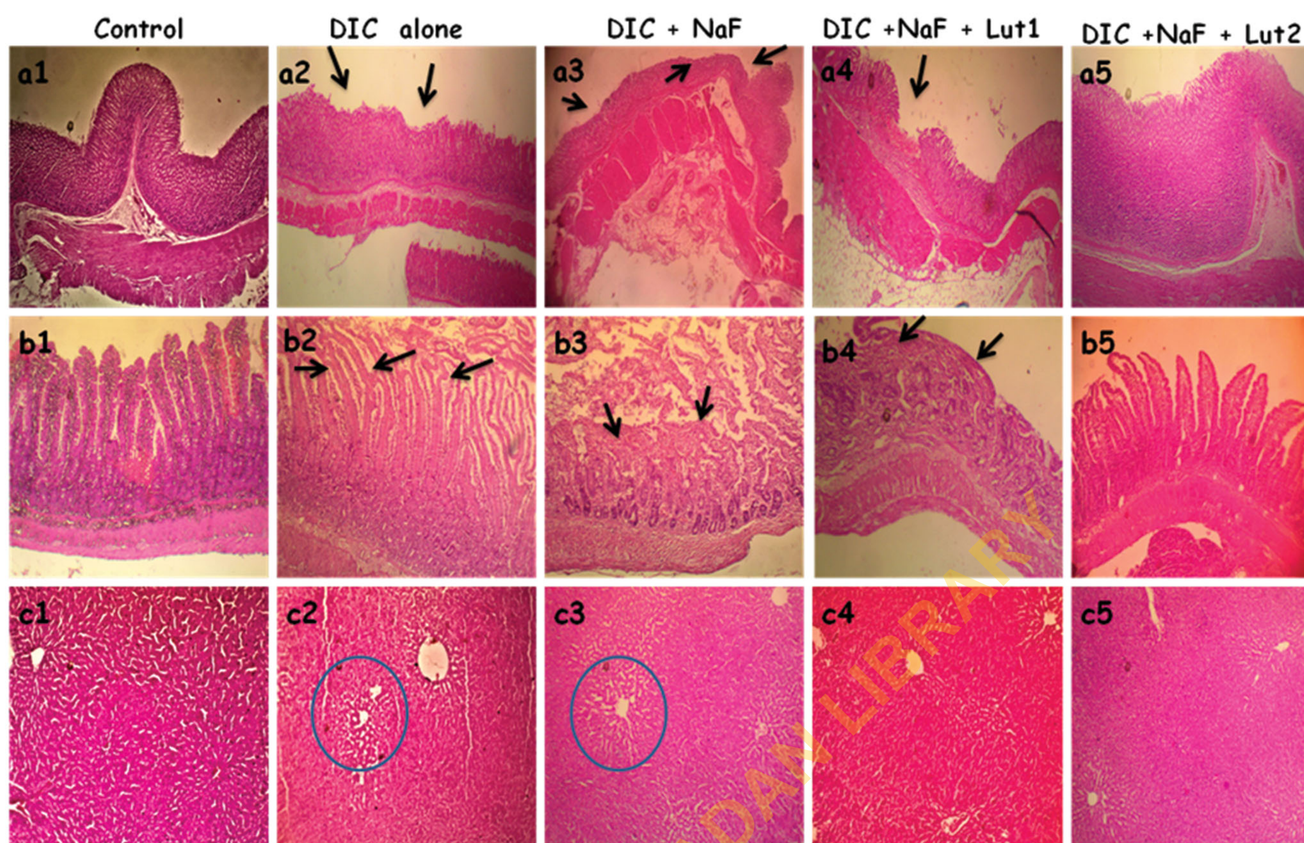

Figure 6. Representative photomicrographs of the stomach sections (a1–a5) (magnification: ×60), duodenum (b1–b5) (magnification: ×150 and liver (c1–c5) (magnification: ×150) of experimental rats. Hematoxylin and eosin staining showed normal architecture with no lesions in the stomach, duodenum, and liver of control rats (a1, b1, and c1, respectively). Diclofenac treatment produced erosions of the gastric epithelium (a2), edema of the duodenal villi (b2), and mild atrophy of the hepatic cords (c2) (circles). In rats co-exposed to diclofenac and sodium fluoride, there was more severe erosion of the gastric epithelium (a3), necrosis and fusion of the duodenal villi (b3), with atrophy of the centri-lobular and mid zonal hepatic cords (c3). These lesions (black arrows) occurred to lesser degrees in the rats treated with Luteolin at 100 mg/kg (d1, d2, and d3), while they were virtually non-existent in those treated with Luteolin at 200 mg/kg (e1, e2, and e3). DIC: diclofenac; NaF: sodium fluoride; Lut: luteolin.

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($p < 0.05$) higher severity in the DIC + NaF group (2.80 ± 0.45) compared with the DIC group (2.00 ± 0.00). Stomach samples from the control and luteolin-treated groups did not present with observable erosions or hemorrhages.

In the duodenum, severity scores for mucosal and submucosal edema were significantly ($p < 0.05$) higher in the DIC + NaF group (2.80 ± 0.45) compared to the DIC group (2.00 ± 0.00). There were, however, no statistically significant differences in the severity of other lesions, namely necrosis, congestion of blood vessels and glandular activation between the DIC and DIC + NaF groups. No lesions were observed in the control group as well as the group treated with Lut2 (200 mg/kg).

Hepatic lesion scores recorded in the DIC and DIC + NaF groups presented similar values, although these values were significantly ($p < 0.05$) higher than those of the control and Luteolin-treated groups.

Discussion

NSAIDs are widely used in the treatment of pain and inflammation. These drugs are associated with a series of side effects which predominantly affect the digestive tract. Examples of such are gastroduodenal hemorrhagic lesions, changes in intestinal permeability, and visceral organ perforations (Graham 1990). The clinical management of NSAID-induced enteropathic damage is reportedly worsened by concomitant administration of other drugs, including gastroprotective anti-secretory drugs such as ranitidine and omeprazole, both of which have been found to cause significant exacerbation of NSAID-induced enteropathic lesions (Wallace *et al.* 2011). Furthermore, recent reports have also indicated that NSAID-induced gastrointestinal toxicity may also be modified by the simultaneous presence of other chemicals within the gastrointestinal lumen (Goldstein and Cryer 2015). These include NSAID/fluoride combinations frequently encountered in oral medicaments used for treatment of gingivitis, as toothpastes and in other pharmaceutical preparations, including fluorinated NSAIDs, such as flurbiprofen (Aberg *et al.* 1998, Holzer *et al.* 2001). Concomitant exposure to drug and chemical combinations in these instances may create situations of gastroenteropathic damage of multifactorial etiology and necessitate the development of adjunct strategies with the potential to provide complete solution to GI toxicity. In this study, therefore, it was hypothesized that (i) diclofenac-induced gastrointestinal toxicity may be modulated when administered concomitantly with sodium fluoride, a ubiquitous element found naturally in food and water, and (ii) treatment with Luteolin, a well reported antioxidant and anti-inflammatory flavonoid, may potentially protect against

Table 2. Effect of luteolin on histological features of the stomach, duodenum, and liver of rats treated with diclofenac and sodium fluoride.

Scoring parameters	Control	DIC only	DIC + NaF	DIC + NaF + Lut1	DIC + NaF + Lut2
Stomach					
1. Depth of mucosal erosion	0.00 ± 0.00	1.80 ± 0.45[a]	2.60 ± 0.55[a,b]	0.00 ± 0.00[c]	0.00 ± 0.00[c]
2. Presence of hemorrhage	0.00 ± 0.00	2.00 ± 0.00[a]	2.80 ± 0.45[a,b]	0.00 ± 0.00[c]	0.00 ± 0.00[c]
Duodenum					
1. Necrosis and fusion of villi	0.00 ± 0.00	1.60 ± 0.55[a]	1.60 ± 0.55[a]	1.40 ± 0.55	0.00 ± 0.00[c]
2. Mucosal/submucosal edema	0.00 ± 0.00	2.00 ± 0.00[a]	2.80 ± 0.45[a,b]	1.80 ± 0.45[c]	0.00 ± 0.00[c]
3. Congestion of blood vessels	0.00 ± 0.00	1.60 ± 0.55[a]	1.60 ± 0.55[a]	0.80 ± 0.45[c]	0.00 ± 0.00[c]
4. Activation of glands	0.00 ± 0.00	1.80 ± 0.45[a]	2.00 ± 0.00[a]	1.60 ± 0.55	0.00 ± 0.00[c]
Liver					
1. Necrosis	0.00 ± 0.00	1.20 ± 0.45[a]	1.20 ± 0.45[a]	0.00 ± 0.00[c]	0.00 ± 0.00[c]
2. Atrophy of hepatic cords	0.00 ± 0.00	1.80 ± 0.45[a]	2.00 ± 0.00[a]	0.00 ± 0.00[c]	0.00 ± 0.00[c]
3. Sinusoidal dilatation	0.00 ± 0.00	1.80 ± 0.45[a]	2.00 ± 0.00[a]	0.00 ± 0.00[c]	0.00 ± 0.00[c]
4. Congestion of hepatic vessels	0.00 ± 0.00	0.60 ± 0.55[a]	0.60 ± 0.55[a]	0.00 ± 0.00[c]	0.00 ± 0.00[c]

Values are expressed as mean ± standard deviation. DIC: diclofenac; NaF: sodium fluoride; Lut1: luteolin at 100 mg/kg; Lut2: luteolin at 200 mg/kg. [a]Values differ significantly from control ($p < 0.05$). [b]Values differ significantly from DIC alone ($p < 0.05$). [c]Values differ significantly from DIC + NaF.

possible gastroenetrohepatic damage caused by co-administration of DIC with NaF.

Administration of DIC alone for 3 days resulted in observable gastroenteropathic damage with accompanying signs of watery diarrhea, loss of appetite, dullness, and loss of body weight. However, the effects produced by DIC were significantly exacerbated when rats were initially pre-exposed to sodium fluoride for 5 days, with further co-exposure to diclofenac and sodium fluoride for another 3 days. Interestingly, treatment of rats with Luteolin resulted in significant attenuation of diclofenac/sodium fluoride–induced gastroenterohepatic damage. This study thus demonstrates for the first time, the aggravation of diclofenac-induced gastrointestinal damage by sodium fluoride, as well as the efficacy of Luteolin in attenuating the combined toxicity of DIC and NaF. Several studies have provided evidence that co-administration of fluoride with other chemicals or drugs resulted in a potentiation of toxicity to various organs (Inkielewicz-Stepniak and Knap 2012, Arab-Nozari *et al*. 2020), which lends support to the findings in the present study.

Body and organ weight changes have been used as sensitive indicators of the effect of exposure to test compounds (Bailey *et al.* 2004). Over the duration of the present study, it was observed that rats exposed to DIC alone or in combination with NaF showed significant reduction in body weight, irrespective of Luteolin treatment. Only the rats in the control group did not suffer weight loss. This decrease in body weight may be explained on the basis of the observed loss of appetite and decline in food consumption (data on food consumption not recorded) which is commonly associated with chemical exposure (Klaassen 2001). Reduced body weight has also been associated with decreased muscle mass and cachexia caused by oxidative stress (Yeh *et al*. 2009, Patra *et al*. 2011). In a similar study, Singh *et al.* (2017) also recorded body weight reduction in rats co-treated with diclofenac and ranitidine. Interestingly, the reduction in body weight obtained in this study was accompanied by increase in the relative weights of the stomach, duodenum, and liver, suggesting that loss of body weight might be due to depletion of other tissues, such as skeletal muscles (Londhe and Guttridge 2015). Our findings on body and organ weight changes corroborated with observations by Anfal and Samir (2017), who reported that NaF treatment at a dose 400 ppm caused a decrease in body weight and an increase in relative liver weight compared to the control rats. The observed increase in organ weights appears to be consistent with lesions of inflammation and edema observed in the histopathological examination of the tissues.

The frequently reported toxic effects of NSAID administration include the occurrence of subepithelial hemorrhages, erosions, and ulcerations commonly observed in the upper GIT, although current evidence suggests that there is equally increased risk of lower gastrointestinal bleeding and perforation to a similar extent as seen in the upper GIT (Sostres *et al*. 2013). In the present study, DIC-treated rats presented the highest severity of gastrointestinal lesions in the DIC + NaF group which showed significantly higher scores of diarrhea and mucosal hemorrhages compared to the rats treated with DIC alone. These macroscopic lesions provided the first evidence of the likely exacerbation of DIC toxicity by NaF, which were also corroborated by histopathological observations. Histologic lesions such as necrosis and erosions of the gastric epithelium, as well as necrosis, swelling, erosion, and fusion of duodenal villi were observed to varying degrees and correlated with the observed macroscopic features of gastroenteropathic damage. These lesions were, however, more pronounced in the DIC + NaF group compared to the DIC group. Gastroduodenal lesions (e.g., acute hemorrhages and erosions,) similar to those found in NSAID-treated individuals have been reported in patients exposed to toxic levels of fluoride (Müller *et al.* 1992, Das *et al.* 1994). Based on the findings from this study, the possibility of an aggravation of upper gastrointestinal tract lesions by co-exposure to DIC and NaF appears to be a straightforward proposition. However, the mechanisms involved in such potentiation of toxicity require further studies. Treatment with Luteolin produced significant attenuation of both macroscopic and microscopic lesions of gastroenterohepatic damage by reducing hemorrhages, mucosal erosions, and cellular necrosis.

The pathogenesis of tissue damage induced by either DIC or NaF has been linked to increased generation of reactive oxygen species and oxidative stress (Galati *et al.* 2002, Inkielewicz-Stepniak and Knap 2012). However, tissues are normally equipped with antioxidant defense systems that offer protection against oxidative cell injury and enhance survival of cells by mopping up free radicals and reactive oxygen species during exposure to xenobiotics. In order to

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understand the oxidant/antioxidant status of the tissues investigated in this study, their respective postmitochondrial fractions were assayed to identify levels of (a) MDA, a product of lipid peroxidation; (b) AOPP and protein carbonyls as markers of protein oxidation, and (c) H_2O_2 and nitric oxide as reactive oxygen and nitrogen species, respectively. In addition, the activities of critical antioxidant enzymes, GPx, GST and SOD, as well as levels of GSH and protein thiols were also measured.

There was significant elevation in MDA and AOPP in all the three tissues following administration of DIC alone, while H_2O_2 and nitric oxide levels were significantly elevated in the stomach and duodenum. Levels of these parameters were further elevated in rats co-exposed to DIC and NaF. These observations point to a state of oxidative/nitrosative stress in the tissues following diclofenac treatment, which was aggravated with its co-treatment with sodium fluoride. These findings are consistent with previous observations by Inkielewicz-Stepniak and Knap (2012), who also reported that combined exposure to fluoride and acetaminophen resulted in enhancement of oxidative/nitrosative stress in the kidneys of rats, compared to separate treatments with either compound.

Moreover, measurements of the activities of antioxidant enzymes in the present study further confirmed the induction of oxidative stress in the tissues following exposure to diclofenac and sodium fluoride. In the stomach and duodenum, significant reduction in GPx and GST activities was observed when rats were co-exposed to DIC and NaF compared to when treated with DIC alone. In addition, there were reductions in GSH and protein thiol levels in virtually all the tissues, when rats were exposed to DIC and NaF. Interestingly, significant increase was found in SOD activity in the stomach and duodenum of rats exposed to DIC alone, as against those in the control, and this point to an induction of SOD activity in these rats. Curcelli *et al.* (2008) had previously observed such beneficial effects of DIC therapy on antioxidant enzymes, including SOD in rats. The report indicated that the improvement of antioxidant activity by DIC might occur via enzyme activation by increasing substrate linkage affinity to the enzyme-catalytic site.

In this study, administration of Luteolin mediated reversal of most of the macroscopic features and alterations in oxidative stress parameters induced by DIC and NaF co-administration. Rats treated with Luteolin had significantly improved diarrhea scores, with stool consistency similar to that of the control rats. In addition, there was much reduction in the incidence of hemorrhagic lesions in the gastric and intestinal mucosa. These improvements were likely due to the antioxidant effects of Luteolin, which brought about significant reductions in the levels of MDA, AOPP, Protein carbonyls, H_2O_2, and nitric oxide in the various tissues. Furthermore, Luteolin administration led to improvements in the activities of GPx, GST, and SOD, while also increasing GSH and protein thiol levels. Several reports in literature have provided evidence for the protective role of Luteolin against chemical and drug-induced hepatotoxicity (Liu *et al.* 2014, Zhang *et al.* 2017), while reports of its effects on gastric and intestinal tissues appear to be relatively scarce. Proposed molecular mechanisms of Luteolin's protection in hepatic tissues include the upregulation of Nrf-2 and decreased expression of NF-κB (Zhang *et al.* 2017), with the overall effect of decreasing oxidative stress. Similar mechanisms may, therefore, be involved in the protection of gastric and duodenal tissues against DIC and NaF as obtained in this study.

Notably, data from this study showed that nitric oxide levels in the stomach and duodenum remained higher than control values in the rats treated with Luteolin, suggesting the possibility of induction of nitric oxide synthesis in the tissues. Luteolin has been found to increase phosphorylation of endothelial nitric oxide synthase in isolated rat aortic rings with subsequent increase in NO production and vaso-relaxation (Si *et al.* 2014). This effect may be beneficial in improving GI function as nitric oxide has been shown to exhibit protective functions by maintaining gastric mucosal integrity, inhibiting endothelial leukocyte adherence, and repairing NSAID-induced damage (Hossain *et al.* 2012).

Taken together, exposure of rats to diclofenac resulted in significant gastroenterohepatic damage, as evidenced by the macroscopic and microscopic lesions seen as well as the results of the examination of the rats, in accordance with the biochemical parameters of oxidative stress, particularly in the gastric and duodenal tissues, and to a lesser extent, in the liver. An important finding in this study was that these alterations were significantly exacerbated in rats that were treated concomitantly with sodium fluoride. The data from this study suggests that formulations combining the concurrent use of these compounds must be administered with caution, because of potential interactions that may aggravate gastrointestinal damage. The gross and histologic manifestations of gastrointestinal injury may be partly explained by the alterations observed in the oxidative status of the tissues. However, there is a need for future studies to investigate underlying molecular mechanisms involved in the process. The flavonoid, Luteolin, was studied and its protective influence against diclofenac and sodium fluoride-induced injury was documented. This influence may be attributed to its antioxidant and/or anti-inflammatory activities. Based on the findings of the study, Luteolin may thus be considered an important agent for the amelioration of gastrointestinal and hepatic damage elicited by combined administration of gastrointestinal toxicants such as diclofenac and sodium fluoride.

Limitation of study

The study approach adopted in this study was based on guidance by our Animal Ethics Committee limiting the use of rats to the minimum numbers required to sufficiently address the primary objectives as outlined in this study. It may, however, be important for future studies to assess the effects of separate exposures to NaF or Luteolin on the tissues under investigation.

Ethical approval

All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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Disclosure statement

The authors declare no conflict of interest.

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