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Benzyl isothiocyanates modulate inflammation, oxidative stress, and apoptosis *via* Nrf2/HO-1 and NF-κB signaling pathways on indomethacin-induced gastric injury in rats

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The present study investigated the gastroprotective activity of benzyl isothiocyanates (BITC) on indomethacin (IND)-induced gastric injury in a rat model and explicated the possible involved biochemical, cellular, and molecular mechanisms. The rat model with gastric ulcers was established by a single oral dose of IND (30 mg per kg b.wt). BITC (0.75 and 1.5 mg kg^{-1}) and esomeprazole (20 mg per kg b.wt) were orally administered for 3 weeks to rats before the induction of gastric injury. Compared with the IND group, BITC could diminish both the macroscopic and microscopic pathological morphology of gastric mucosa. BITC significantly preserved the antioxidants (glutathione GSH, superoxide dismutase SOD), nitric oxide (NO), and prostaglandin E2 (PGE2) contents, while decreasing the gastric mucosal malondialdehyde (MDA), tumor necrosis factor alpha (TNFα), and myeloperoxidase (MPO) contents. Moreover, BITC remarkably upregulated the expression of nuclear factor erythroid 2-related factor 2 (Nrf2), hemoxygenase-1 (HO-1), and NAD(P)H : quinone oxidoreductase (NQO1). In addition, BITC activates the expression of heat shock protein 70 (HSP-70) and downregulated the expression of nuclear factor-κB (NF-κB) and caspase-3 to promote gastric mucosal cell survival. To the best of our knowledge, this study is the first published report to implicate the suppression of inflammation, oxidative stress, and Nrf2 signaling pathway as a potential mechanism for the gastroprotective activity of BITC.

Introduction

Gastric ulcers are one of the most prevalent digestive system disorders that affect up to 10% of the population.[1] Gastrointestinal mucosal damage occurs due to the long-term exposure of various aggressive factors including *Helicobacter pylori* (*H. pylori*), excessive alcohol consumption, hyperacidity, smoking, stress, and drugs.[1,2] Non-steroidal anti-inflammatory drugs (NSAIDs) are the second most common etiologic factor disrupting the gastric mucosal integrity after *H. pylori* infection.[2] Therefore, priority is to effectively prevent or safely treat NSAIDs-induced gastrointestinal mucosal damage.

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Oxidative stress has been strongly implicated in the pathogenesis of NSAIDs-associated gastropathy.[3] The mechanism of action of NSAIDs is based mainly on the non-selective inhibition of cyclooxygenase (COX) enzyme, thereby blocking the conversion of arachidonic acid into prostaglandins (PGEs) whose actions are detrimental to mucosal defense and healing.[4] The damaging consequences of decreased PGE production include disrupted microvascular blood flow, decreased mucus secretion, and increased gastric acid secretion with gastric mucosal injury. The subsequent aggregation of neutrophils in the injured gastric vascular endothelium leads to the overshooting of reactive oxygen/nitrogen species (ROS/RNS) production, which triggers the oxidative burden and destroys the gastric mucosa.[5,6] Moreover, it is evident that NSAIDs possess the ability to uncouple mitochondrial oxidative phosphorylation and thus inhibit ATP synthesis.[7] The uncoupling is ultimately accompanied by a series of downstream effects including the accumulation of ROS within the mitochondria, the depletion of GSH, increased superoxide anion, and the activation of pro-apoptogenic proteins, leading to apoptosis and cell death.[8,9] Therefore, the application of phytochemicals with antioxidant activity for attenuating NSAIDs-induced gastropathy has constituted a renewed interest.[10,11]

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Isothiocyanates (ITCs) are plant products that are generated by the enzymatic hydrolysis of glucosinolates.[12] Benzyl isothiocyanate (BITC) is an aromatic isothiocyanate compound derived from *Salvadora persica* roots and its oil (69.6% and 74.4%, respectively),[13,14] and also cruciferous vegetables. Similar to other ITC members, BITC has demonstrated significant pharmacological activities including anti-cancer,[15–22] anti-inflammatory, antimicrobial,[23] virucidal activity against *Herpes simplex* virus,[24] nephroprotective,[25] and antioxidative activities.[26] BITC showed anti-inflammatory activities in the rat model of joint inflammatory hyper nociception[27] and murine macrophages and mouse skin lines.[28] The antioxidant potential of BITC involves the reduction of superoxide generation and the activation of cytochrome P450 enzymes.[29] A combination of allyl ITC and wasabi leaf extract significantly ameliorated the severity of gastric erosion and petechial hemorrhage induced by *Helicobacter pylori* in humans.[30] Moreover, *S. persica* extract exhibited antiulcer activities in ethanol-induced gastric ulcer[31] and acetylsalicylic acid-induced gastric ulcer in rat models.[32] Therefore, BITC may have gastroprotective effects. However, its impact in the mitigation of IND-induced gastric injury remains unknown.

The current study aimed to investigate the gastroprotective and antiulcerogenic activities of BITC by comparing its effects to that of esomeprazole, a widely used drug for the therapeutic management of gastric ulceration along with the exploration of the underlying mechanisms using IND-induced gastric ulcer rat model.

Materials and methods

Chemicals, drugs, and diagnostic kits

All chemicals used in the study were of analytical grade. BITC (CAS number: 622-78-6, linear formula: $C_6H_5CH_2NCS$, molecular weight; 149.21) was obtained from Sigma-Aldrich. Indomethacin (IND) (Sigma Chemical Co., St Louis, MO, USA) was used for the induction of gastric injury, while esomeprazole (217087-09-7, Sigma-Aldrich, Taufkirchen, Germany) was employed as the standard gastroprotective drug. The used biochemical kits included rat myeloperoxidase (MPO) ELISA kit (HK105-02, Hycult Biotech Inc., Wayne, USA), rat tumor necrosis factor alpha (TNFα) (ELISA kits (KRC3011), Thermo Fisher Scientific, Waltham, MA, USA), prostaglandin E2 (PGE2) (ELISA kit, MBS730592, MyBioSource, San Diego, USA). Antioxidant kits: malondialdehyde (MDA); MD 25 29, reduced glutathione activity (GSH); GR 25 11, superoxide dismutase (SOD); SD 25 21, and nitric oxide (NO); NO 25 33, were purchased from Biodiagnostic Co. (Dokki, Giza, Egypt). RNeasy mini kit (Qiagen), SuperScript IV VILO reverse transcriptase kit (Invitrogen), and Luminaries Color HiGreen Low ROX qPCR Master kit (Thermo Scientific) were used for gene expression analysis.

Experimental animals and laboratory diet

This study was approved by the Institutional Animal Care and Use Committee (IACUC), Cairo University (date: 10/10/2019/ certificate code: CU III S 79 17). Adult Wistar rats of both sexes, aged (10–12 weeks) and weighing (250–300 g), were obtained from the Animal House Colony at Vac-Sera-Egypt. The animals were housed in laboratory animal housing facilities at the Faculty of Veterinary Medicine, Cairo University, Giza, Egypt and allowed to acclimatize for one week before starting the experiment and maintained under standard laboratory conditions (controlled room temperature, 22 ± 1 °C; relative humidity, 54–68%; and 12 h light/dark cycle) with free access to a well-balanced diet (vitamins mixture, 1%; minerals mixture, 4%; corn oil, 10%; sucrose, 20%; cellulose, 0.2%; casein, 10.5%; and starch, 54.3%) and water.

Experimental design

Fifty rats were randomly allocated into 5 groups of 10 rats each. Animals of groups I and II served as the normal control and the control positive (IND group) received a daily intragastric oral dose of (1 mL) distilled water using gavage for three weeks. Animals of groups III served as the standard and received a daily oral dose of aqueous solutions of esomeprazole (20 mg kg^{-1}); groups (IV,V) received daily oral doses of BITC dissolved in corn oil (0.75 and 1.5 mg per kg b.wt, respectively).[10] Gastric lesions were induced in groups II to V according to the procedure described by[33] using single oral dose of IND (30 mg per kg b.wt) suspended in distilled water. Rats were deprived of food but had free access to water 24 h prior to the induction of gastric injury.

On the twenty-second day of study, 4 h after IND treatment, the animals were sacrificed by cervical dislocation, the stomachs were immediately excised, opened along the greater curvature, washed with normal saline solution, and examined for gastric lesions following the macroscopic scoring system previously presented by[34] as follows: score 1 was assigned for haemorrhage with punctiform size (<2 mm), 0–4 in number or unilateral site. On the other hand, score 2 was given for mild haemorrhage (2–5 mm), 5–6 in number or bilateral site, and score 3 was assigned for intense haemorrhage (>5 mm) and more than 7 in number. Specimens of glandular gastric tissues were divided into two portions; one fixed in 10% formalin/saline for histopathological examination and immunohistochemical assessment, while in the other portion, gastric mucosae were scraped, weighed, and stored separately at −80 °C to be used for the evaluation of inflammatory biomarkers, antioxidants, and gene expression analysis. Gastric homogenates were prepared using appropriate buffers according to the manufacturers' protocols and supernatants were collected by its sonication, followed by centrifugation at 1500*g* for 15 minutes at 4 °C.

Estimation of gastric inflammatory biomarkers

The extent of granulocyte accumulation in the gastric mucosa was indicated by the estimation of myeloperoxidase content using MPO Rat ELISA kit.[35] In addition, tumor necrosis factor alpha (TNF-α), a marker for neutrophil infiltration, was determined using specific kits, where solid-phase sandwich ELISA was employed. The estimation of Prostaglandin E2 (PGE2) was

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performed using corresponding Competitive Rat ELISA kit, following the manufacturer instructions.

Antioxidant assays

GSH, SOD, and NO activities were estimated spectrophotometrically in gastric mucosal samples based on the methods of[36–38] using specific kits, respectively. In addition, lipid peroxidation was estimated by the determination of MDA formation, as described by ref. 39.

Histopathological studies

Specimens of the stomach were harvested from all the experimental groups and fixed in neutral buffered formalin 10% for 24 hours, washed, dehydrated, cleared, and embedded in paraffin. Paraffin blocks were sectioned for obtaining 4–5 micron thickness tissue section slides. The tissue sections were stained with haematoxylin and eosin[40] for microscopical histopathological examination (Olympus BX50, Tokyo, Japan). All recorded histopathological lesions in the stomach were scored according to ref. 41.

Immunohistochemical analysis

Caspase-3 and heat shock protein immunohistochemistry: The immunohistochemical procedure was performed according to the methods described by.[42,43] The stomach sections were deparaffinized and rehydrated. The antigenic retrieval method was performed according to ref. 44. The tissue sections were washed three times with Tris buffered saline, then incubated with one of the following primary antibodies: caspase-3, rabbit anti-caspase-3 polyclonal antibody (ab13847; Abcam, Cambridge, UK) at 1:100 dilution, and HSP-70 (Santa Cruz Biotechnology USA), overnight in a humid chamber. In the negative control slides, primary antiserum was removed with 1 mg mL^{-1} BSA (Sigma). The immunostaining was amplified and completed by Horseradish Peroxidase complex (Dako). Sections were developed and visualized using 3,3-diaminobenzidine (DAB chromogen) (Dako). The substrate system gave a brown color at the site of the target antigen. Sections were counterstained with Mayer hematoxylin and cover slipped for microscopical examination. The quantification of Caspase-3 and HSP-70 was done by measuring the area% expression from 7 randomly chosen fields per slide using an image analysis software (Image J, version 1.46a, NIH, Bethesda, MD, USA).

Relative mRNA expression analysis by qRT-PCR

Total RNA was extracted from frozen gastric mucosal samples using RNeasy mini kit (Qiagen) according to the manufacturer's guidelines. Isolated RNA was treated with DNAase kit (Invitrogen) to remove any residual DNA. The integrity and quantity of the isolated RNA were assessed by agarose gel electrophoresis and a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), respectively. The first strand cDNA was synthesized using SuperScript IV VILO reverse transcriptase kit (Invitrogen). In a real-time detector, qPCR was performed with Luminaries Color HiGreen Low ROX qPCR Master kit (Thermo Scientific) according to the manufacturer's protocol. The thermal protocol of the qPCR amplification reaction was as follows: initial denaturation at 95 °C for 40 s, and 45 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 30 s, with a final extension (72 °C for 5 min) to complete the amplification procedure. The primer sequences used for Nrf2, H0-1, NQO1, and NF-κB are listed in Table 1. Each sample was analyzed with three replicates. The relative mRNA expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ method and the results are presented as fold-change with respect to the normal control mean values, normalized to β-actin.

Table 1 Primer sequence for RT-PCR

Gene		Sequence	Accession #
Nrf2	F	CACATCCAGACAGACACCAGT	XM_006234398.3
	R	CTACAAATGGGAATGTCTCTGC	
HO-1	F	ACAGGGTGACAGAAGAGGCTAA	NM_012580.2
	R	CTGTGAGGGACTCTGGTCTTTG	
NQO1	F	TCAAGAGGAGCAGAAAAAGAACAAG	NM_017000.3
	R	CTGAAAGCAAGCCAGGCAAAC	
NF-κB	F	CTGGCAGCTCTTCTCAAAGC	XM_006233360.3
	R	CCAGGTCATAGAGAGGCTCAA	

Statistical analysis

Different analytical determinations were carried out in triplicate and the results are expressed as the mean ± SD (standard deviation), where $n = 7$. Data were analyzed by the one-way analysis of variance (ANOVA) test for multiple variable comparisons between groups using SPSS version 24 package for Windows (SPSS Inc., Chicago, IL, USA). L.S.D *post hoc* test was used to check the inter-group comparison. (*) and (**) indicated the significant difference compared to the IND group at $P < 0.05$ and <0.005, respectively, whereas (#) and (##) indicated the significant differences compared to the negative control group at $P < 0.05$ and <0.005, respectively. Figures were obtained using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, California USA).

Results

Macroscopic score evaluation

The macroscopic examination of gastric mucosa revealed tissue damage evidenced by the presence of haemorrhagic lesions in the IND group compared to the normal group, which scored zero, as shown in (Fig. 1a and b). Pre-treatments with BITC reduced the IND-induced gastric lesions significantly in a dose-dependent manner such that the recorded score of BITC 1.5 mg kg^{-1} group was almost the same as that of esomeprazole, the standard gastroprotective drug (Fig. 1a and b).

Effects on gastric inflammatory biomarkers

IND induced a significant increase in gastric MPO and TNFα content, while the PGE2 concentration was significantly

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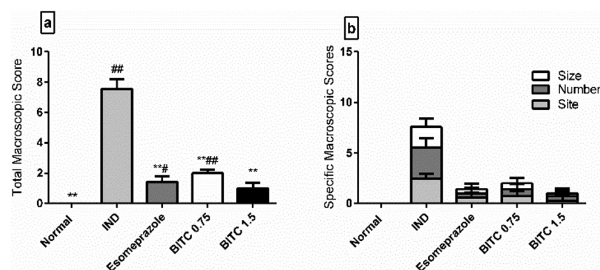

Fig. 1 Macroscopic scores of IND-induced gastric lesions (IND) observed after treatment with BITC (0.75, 1.5 mg per kg b.wt); (a) total scores, (b) macroscopic score including the evaluated parameters (haemorrhage size, number, and site). $n = 7$, mean ± SD, SD; standard deviation. (*) and (**) indicate the significant difference compared to the IND group at $P < 0.05$ and <0.005, respectively, whereas (#) and (##) indicate the significant differences compared to the negative control group at $P < 0.05$ and <0.005, respectively.

decreased in comparison with the normal group ($p < 0.005$). Pre-treatment with BITC prevented IND-induced effects on inflammatory biomarkers MPO, PGE2, and TNFα, restoring their values to near that of normal and esomeprazole groups as shown in Table 2.

Effects on oxidant/antioxidant biomarkers

The GSH, SOD, and NO activities were significantly increased in all BITC pre-treated groups up to near normal values compared to the IND group, while MDA was significantly decreased in all the BITC pre-treated groups up to near normal values compared to the IND group (Table 3).

Histopathology

The normal control group revealed normal histological architecture of the gastric mucosa and submucosa with a normal epithelial and glandular structure (Fig. 2a). On the one hand, exposure to IND caused severe gastric lesions in the form of mucosal haemorrhage, epithelial loss with the erosion of gastric mucosa, apoptosis, fragmentation of the gastric gland, heavy mucosa in submucosal inflammatory cell infiltration mainly with neutrophils, submucosal edema, and congestion of the blood vessels (Fig. 2b and c). On the other hand, the pre-treatment of rats with BITC 0.75 mg kg^{-1} and BITC 1.5 mg kg^{-1} caused marked a attenuation of these gastric lesions induced in the IND group (Fig. 2e and f). The gastric mucosa and submucosa appeared with normal lamina epithelia, a few desquamated epithelial cells, normal gastric gland, and few inflammatory cells' infiltration (Fig. 2).

Regarding the gastric lesion scorning, IND group revealed a significant elevation in mucosal loss, leucocytic infiltration, gastric haemorrhage, and total lesion score compared to the normal control group. The pre-treatment of rats with BITC 0.75 mg kg^{-1} and BITC 1.5 mg kg^{-1} revealed a significant reduction in all the estimated histopathological lesions compared to the IND-treated group (Fig. 3).

Immunohistochemical analyses of caspase-3 and heat shock protein 70

The anti-caspase-3 protein expression was localized in the cytoplasm and the nucleus of epithelial cells, parietal cells, and chief cells of the gastric gland. The normal control group showed very weak anti-caspase-3 protein expression in epi-

Table 2 Effect on inflammatory biomarkers observed after treatment with BITC (0.75, 1.5 mg per kg b.wt) on IND-induced gastropathy in rats

Group	MPO contents ng per mg tissue	PGE2 ng per g tissue	TNF-α pg per g tissue
Normal	69.2 ± 11.99**	254.0 ± 47.14**	1427.7 ± 89.03**
IND	205.6 ± 12.96##	107.0 ± 16.85##	3738.6 ± 174.52##
Esomeprazole	74.6 ± 6.67**	241.8 ± 25.53**	1329.6 ± 152.36**
BITC 0.75	84.69 ± 4.89**##	235.4 ± 17.16**	1322.7 ± 106.38**
BITC 1.5	65.2 ± 6.35**	269.2 ± 18.91**	1313.9 ± 165.37**

Mean ± SD, SD; standard deviation. (*) and (**) indicate the significant difference compared to the IND group at $P < 0.05$ and <0.005, respectively, whereas (#) and (##) indicate the significant differences compared to the negative control group at $P < 0.05$ and <0.005, respectively.

Table 3 Effect on the antioxidant activity observed after treatment with BITC (0.75, 1.5 mg per kg b.wt) on IND-induced gastropathy in rats

Group	GSH nmol per g tissue	SOD U per g tissue	NO μmol per g tissue	MDA nmol per g tissue
Normal	26.63 ± 0.621**	38.50 ± 2.768**	73.54 ± 2.292**	4.74 ± 2.656**
IND	11.54 ± 0.378##	12.71 ± 0.840##	24.13 ± 1.532##	13.16 ± 1.779##
Esomeprazole	26.64 ± 0.151**	37.03 ± 0.912**	69.64 ± 3.215**##	2.38 ± 0.837**#
BITC 0.75	26.07 ± 0.568**#	33.72 ± 3.262**##	66.74 ± 1.348**##	3.54 ± 0.696**
BITC 1.5	26.24 ± 0.465**	39.24 ± 1.421**	74.53 ± 2.201**	2.65 ± 0.184**#

Mean ± SD, SD; standard deviation. (*) and (**) indicate the significant difference compared to the IND group at $P < 0.05$ and <0.005, respectively, whereas (#) and (##) indicate the significant differences compared to the negative control group at $P < 0.05$ and <0.005, respectively.

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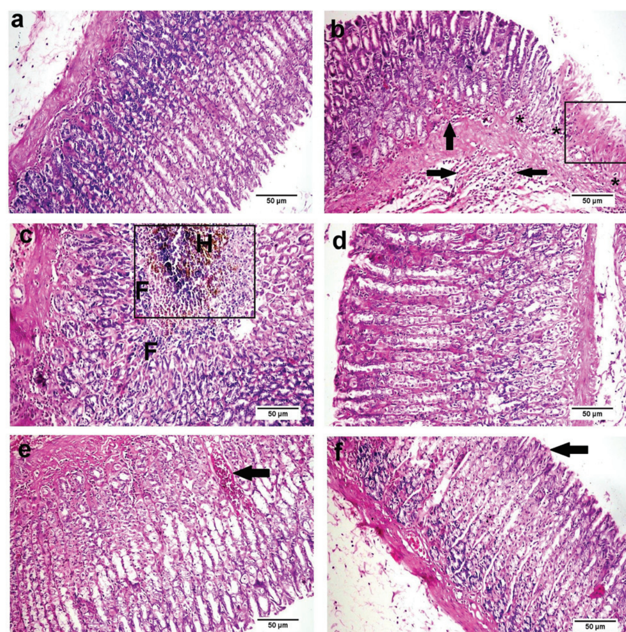

Fig. 2 Histopathological pictures of the stomach in different groups (H&E stain). (a) Normal control group showing the normal histological finding of the stomach. (b) The IND group showing mucosal loss with the mucosa (rectangle), apoptosis of the gastric gland (stars), mucosal and submucosal inflammatory cell (arrows) aggregation and submucosal edema. (c) The IND group showing mucosal haemorrhage (H), epithelial loss, and fragmentation (F) of the gastric glands (rectangle). (d) Esomeprazole (20 mg kg^{-1}) group showing normal gastric mucosa and submucosa. (e) BITC (0.75 mg kg^{-1}) showing normal gastric mucosa and submucosa with individual cell necrosis and congestion of blood vessels (arrow). (f) BITC (1.5 mg kg^{-1}) group showing intact lamina epithelia (arrow), lamina propria, and submucosa.

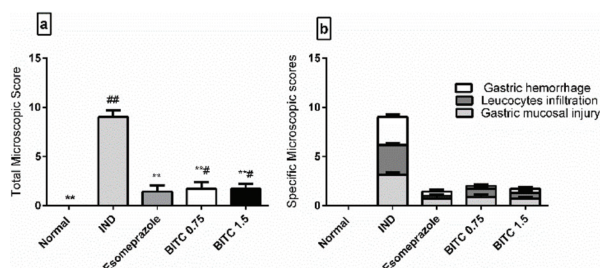

Fig. 3 Microscopic scores of IND-induced gastric lesions observed after treatment with BITC (0.75, 1.5 mg per kg b.wt); (a) total scores, (b) microscopic score including the evaluated parameters (gastric haemorrhage, leucocytes infiltration, and gastric mucosal injury). $n = 7$, mean ± SD, SD; standard deviation. (*) and (**) indicate the significant difference compared to the IND group at $P < 0.05$ and <0.005, respectively, whereas (#) and (##) indicate the significant differences compared to the negative control group at $P < 0.05$ and <0.005, respectively.

thelial cells and the gastric glands (Fig. 4a). The IND group revealed a significant elevation of anti-caspase-3 protein expression in the stomach of treated rats compared to the normal control group (Fig. 4b and c). However, rats pre-treated with BITC (0.75 mg kg^{-1}) and BITC (1.5 mg kg^{-1}) showed a significant reduction in anti-caspase-3 protein expression (Fig. 4e and f) when compared with the IND group (Fig. 6). The HSP-70 protein expression was localized in parietal and chief cells of the gastric gland. The normal control group showed a very weak immune-positive staining of HSP-70 (Fig. 5a). The IND group revealed a significant increase in the HSP-70 expression in the stomach compared to the normal control group (Fig. 5b and c). Groups pre-treated with BITC (0.75 mg kg^{-1}) and BITC (1.5 mg kg^{-1}) showed a significant elevation in HSP-70 expression in the stomach of rats (Fig. 5e and f) compared with the IND group. Fig. 6 summarizes the immunohistochemical evaluation of anti-caspase-3 protein and HSP-70 expression

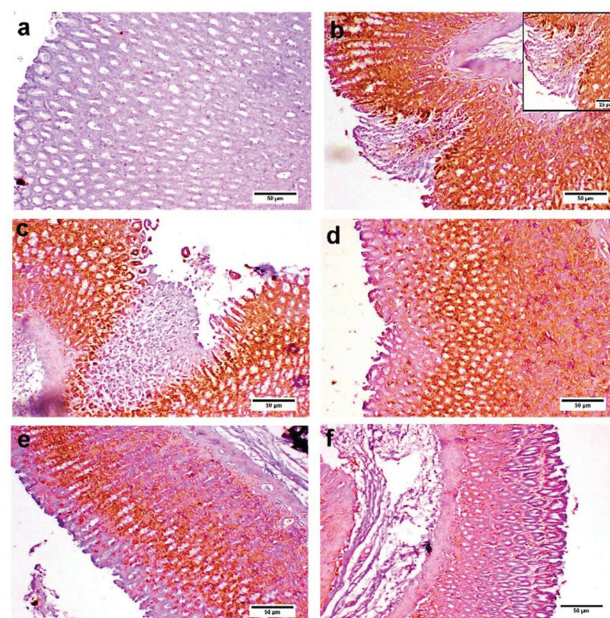

Fig. 4 Immunohistochemical analysis of caspase-3 expression in the stomach. (a) Normal control group showing very weak immune-positive reaction (arrow). (b and c) IND group showing strong immune-positive staining in epithelial cells, parietal cells, and chief cells of the gastric gland; inserted picture with higher magnification power showing strong immune-positive staining (arrow) in the cells around the area of mucosal loss. (d) Esomeprazole (20 mg kg^{-1}) group showing moderate immune-positive reaction (arrow). (e) BITC (0.75 mg kg^{-1}) showing moderate immune-positive reaction (arrow). (f) BITC (1.5 mg kg^{-1}) group showing weak immune-positive staining.

Effects on mucosal Nrf2, HO-1, NOQ1, and NF-κB mRNA expression

To further explore the mechanism underlying the gastroprotective effect, the expression of Nrf2 signaling related genes and NF-κB was examined by qRT-PCR. Our results showed that the expression levels of Nrf2 and its downstream genes HO-1 and NQO1 were remarkably lowered in the gastric mucosa of the model group ($p < 0.005$) compared to the normal group (Fig. 7), whereas there was a significant upregulation in the mRNA expression of Nrf2, HO-1, and NQO1 in groups treated with BITC 0.75 mg kg^{-1} and BITC 1.5 mg kg^{-1}, as compared to those of the model group. On the other hand, rats subjected to IND administration exhibited a significant increase in the

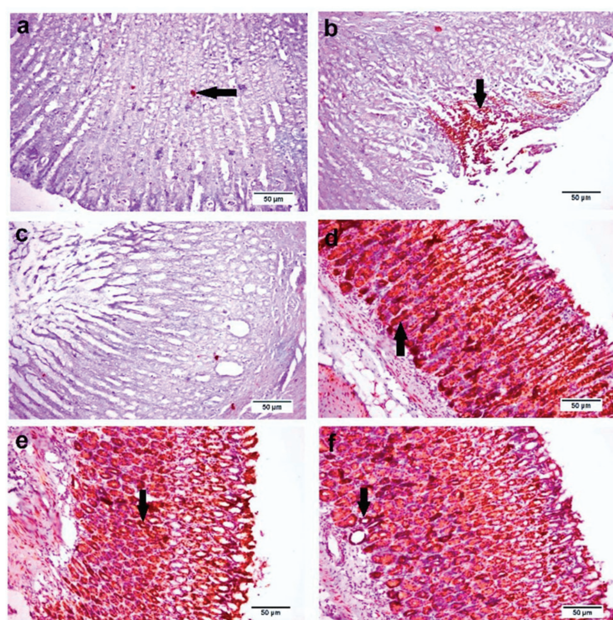

Fig. 5 Immunohistochemical analysis of HSP-70 expression in the stomach. (a) Normal control group showing very weak immune-positive reaction (arrow). (b and c) IND group showing slight immune-positive staining (arrow). (d) Esomeprazole (20 mg kg^{-1}), (e) BITC (0.75 mg kg^{-1}), and (f) BITC (1.5 mg kg^{-1}) groups showing strong immune-positive staining in parietal cells and the chief cells of the gastric gland (arrow).

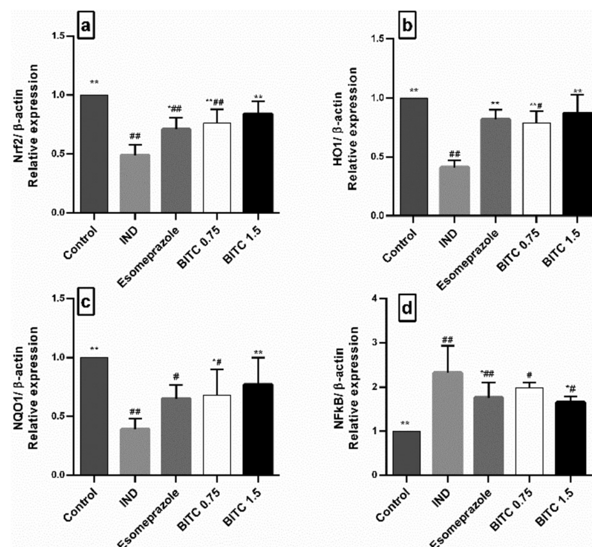

Fig. 7 Effect of BITC (0.75, 1.5 mg per kg b.wt) on the gastric mucosal mRNA expression of Nrf2 (a), HO-1 (b), NOQ1 (c), and NF-κB (d) of IND-induced gastric injury in rats. The relative gene expression is represented as fold changes over that of the normal control after normalization to β-actin using the $2^{-\Delta\Delta Ct}$ method. Mean ± SD, SD; standard deviation. *(*) and (**) indicate the significant difference compared to the IND group at $P < 0.05$ and <0.005, respectively, whereas (#) and (##) indicate the significant differences compared to the negative control group at $P < 0.05$ and <0.005, respectively.

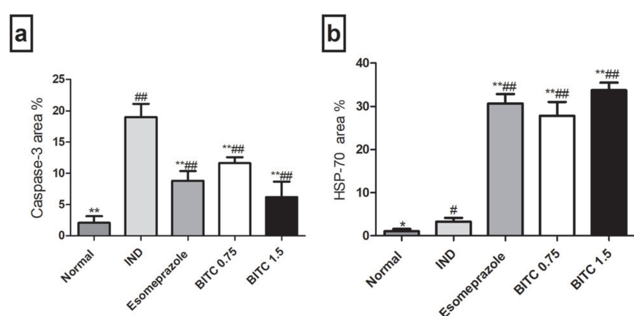

Fig. 6 Morphometrical analysis of caspase-3 and HSP-70 in the stomach of different experimental groups. (a) Caspase-3 area % (b) HSP-70 area%. $n = 7$, mean ± SD, SD; standard deviation. (*) and (**) indicate the significant difference compared to the IND group at $P < 0.05$ and <0.005, respectively, whereas (#) and (##) indicate the significant differences compared to the negative control group at $P < 0.05$ and <0.005, respectively.

mRNA expression level of NF-κB compared to the normal rats ($p < 0.005$). Treatment with BITC 0.5 mg kg^{-1} and esomeprazole also significantly down-regulated the mRNA levels of the NF-κB gene (Fig. 7).

Discussion

Considering the high NSAIDs consumption as one of the most frequently prescribed class of medications worldwide, considerable attention has been focused on their adverse effects, particularly in the upper and lower gastrointestinal tract, and in developing preventive strategies.[2,5] BITC is a natural organosulfur compound present in cruciferous vegetables and has exhibited antioxidant, anti-inflammatory, and cytoprotective activity in several studies.[13,22] To the best of our knowledge, this study is the first to evaluate the gastroprotective potential of BITC against IND-induced gastric injury in rats. The main findings in our study were that BITC could dose-dependently improve the IND-induced: (1) acute mucosal injury (macroscopic and microscopic lesion score and histological improvement), (2) inflammatory response (reduction of MPO and TNF-α levels, increased PGE2 content, and reduced inflammatory cells infiltration in the gastric mucosa), and (3) oxidative stress and apoptotic damage (increased NO, GSH content, and SOD activity, reduced mucosal MDA and downregulated caspase-3 expression). Simultaneously, BITC administration modulated the transcriptional expression of Nrf2-dependent genes and the NF-κB gene. Moreover, the gastroprotective effects of BITC in all the investigated parameters was comparable to that of esomeprazole, a widely used standard drug.

In this research, an IND (30 mg kg^{-1})-induced gastric ulceration model was established. IND has become the first-choice for the induction of the experimental ulcer model due to its higher ulcerogenic potential over other non-steroidal anti-inflammatory drugs (NSAIDs).[45] IND causes gastrointestinal injuries *via* several mechanisms including the inhibition of PGE2 synthesis, reduction of bicarbonate release, which dis-

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rupts the mucus barrier, and the induction of cytotoxicity.[46] These cytotoxic effects contribute to the recruitment of reactive oxygen species (ROS)-releasing leukocytes and inflammatory cytokines with subsequently reduced gastric blood flow,[47] all of which may contribute to gastric cell apoptosis.[48,49]

Human dietary exposure levels of BITC (8.2–11.4 mg kg^{-1} day^{-1}) are much lower than its toxic doses in rodents.[50,51] Furthermore, the lowest previously used doses were used after conversion to rat doses (0.75 and 1.5 mg kg^{-1}).[52] The observed multiple haemorrhagic macroscopic lesions in the IND group in agreement with that in the previous reports[34,46,49] were significantly prevented by BITC (0.75, 1.5 mg per kg b.wt) pre-treatment, as shown by the lesion's total score calculated from the site, number, and size of lesions (Fig. 1a and b). Furthermore, the observed macroscopic scores of BITC 1.5 group were quite similar or even slightly better compared to that of the standard group (esomeprazole). In accordance, this was supported by our histopathological microscopic scoring (Fig. 3). Moreover, similar effects were previously recorded for BITC enriched plants as *Salvadora persica* aqueous extract and methanolic extract of *Carica papaya* L. seeds (MECP).[31,53]

Considerable evidence highlights the implication of oxidative stress in the pathophysiology of gastric ulceration.[54,55] Oxidative stress arises due to an imbalance between ROS production and the cellular antioxidant defense system. In the ulcerated tissues, the infiltrating neutrophils excessively produce superoxide radical anions (O^{2-}), which reacts with lipids, causing lipid peroxidation.[56] The gastroprotective role of NO has been well defined against ethanol, acetic acid, and NSAIDs induced gastrointestinal toxicity, where NO has proved to promote gastric healing by maintaining gastric mucosal integrity and inhibiting leukocyte adherence to the endothelium, which could be correlated to its effect on sucralfate and the gastric vasodilatory effect of PGE2.[57–59] The results of the current study demonstrated IND administration associated with a marked impairment of the redox balance, as evident by the increased lipid peroxidation (MDA), while NO, GSH, and SOD contents were decreased. These findings were consistent with those of the previous studies.[46,49,60–62] Both doses of BITC (0.75 and 1.5 mg kg^{-1}) and esomeprazole pre-treatment significantly inhibited lipid peroxidation and restored the depleted NO, GSH, and SOD activity in the ulcerated mucosa (Table 3). These data revealed that BITC mitigated IND-induced gastric injury due to its antioxidant properties, confirming the findings of the previous studies in that regard, which reported that BITC is able to suppress O_2^- radical generation within inflammatory leukocytes and MDA in cisplatin-intoxicated mice. On the other hand, BITC upregulated the antioxidant enzyme expression such as glutathione peroxidase.[25,29]

To further excavate the mechanism underlying the potential of BITC in resisting oxidative injuries, our research was mainly focused on the Nrf2 signaling pathway as a major antioxidant defense mechanism against oxidative stress.[63,64] Nrf2 is a redox-sensitive transcriptional factor that regulates the expression of genes encoding various antioxidant and detoxifying enzymes. Under normal conditions, Nrf2 localizes to the cytoplasm and interacts with the Kelch-like erythroid cell-derived protein with CNC homology-associated protein-1 (Keap1). During cellular stress, Nrf2 undergoes dissociation from Keap1 and translocation into the nucleus, where it binds the antioxidant response element (ARE) in the promoter region of its downstream antioxidant genes, leading to their transcription.[65] The beneficial role of Nrf2 has been demonstrated in a variety of oxidative stress-related gastrointestinal injuries;[54,66] therefore, Nrf2 signaling activation has been considered as a potential therapeutic approach to protect the GI mucosa.[66] HO-1 is a stress-inducible phase II anti-oxidative enzyme catalyzing the rate-limiting step in heme degradation.[67,68] The regulatory regions of the *HMOX1* gene contains ARE as a dominant sequence motif. In consequence, Nrf2 exerts a pivotal role in *HMOX1 regulation* by activating its expression.[63] HO-1 exerts its cytoprotective effects through the degradation of the highly deleterious heme, and in turn maintains the cellular antioxidant capacity. Moreover, carbon monoxide derived from HO-1 enhances the ulcer healing process through vasodilatation and improving the gastric blood flow at the margin of the ulceration lesion.[56] In addition, HO-1 has been reported to protect cells against apoptosis. NQO1 is another ARE genes that acts side by side with HO-1 to maintain the cellular redox state in response to oxidative damage and inflammatory stimuli.[69] NQO1 is an inducible antioxidant flavoprotein that catalyzes the reduction of various reactive species with a direct superoxide scavenging potential.[70] Both HO-1 and NQO1 are prominently involved in the mechanism of antioxidant therapies of inflammatory bowel disorders.[55]

Emerging evidence reports that BITC induces the expression of detoxifying enzymes *via* the activation of the Nrf2/ARE signaling pathway. Previous studies have stated that BITC suppresses oxidative damage through the upregulation of the Nrf2-dependent phase II genes including HO-1, and glutathione S-transferase. Thus, Nrf2 knockdown abrogates BITC enhancement of antioxidant enzymes and subsequently reverses BITC protection against obesity- and acetaldehyde-induced cytotoxic damage.[71,72] Our findings showed that Nrf2, HO-1, and NQO1 expressions were downregulated in the gastric mucosa of rats in response to IND-induced injury (Fig. 7a–c). BITC pre-treatment effectively prevented Nrf2 downregulation induced by IND. Furthermore, the mRNA expression levels of Nrf2-downstream genes (HO-1 and NQO1) were also augmented in BITC-treated groups compared to the IND group. In accordance with multiple studies suggesting that the activation of Nrf2/ARE pathway ameliorates gastric ulceration by stimulating the anti-oxidative state,[54,73] the current study showed that the induction of Nrf2 with the co-ordinated action of both detoxification (HO-1, NQO1) and anti-oxidant enzymes (SOD) could mediate the anti-oxidative effects of BITC against IND-induced gastric mucosal oxidative damage. In the same context, the most recorded attribute of sulforaphane, a hydrolysis product of isothiocyanate precursor in plant cells, in both NSAID-induced and *H. pylori* gastrointestinal injuries, is its nutrigenomic effects by inducing signifi-

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cant changes in the Nrf2 target genes.[64] Another mechanism for Nrf2-mediated protection against oxidative stress is its ability to mitigate the inflammatory response through the inhibition of proinflammatory signaling.[66,74]

Based on our findings, IND intake triggered sever inflammatory response in the gastric mucosa, as indicated by the upregulation of NF-κB mRNA expression and increase in its downstream signals, such as TNF-α. The accumulation of TNF-α inflammatory cytokines leads to the recruitment of neutrophils at the injured tissues, as evidenced by the increased MPO, the neutrophil infiltration index marker (Table 2). These results are in line with previous studies reporting that IND induces proinflammatory damage in the gastrointestinal mucosa. Furthermore, TNF-α has been previously reported to increase the severity of gastric inflammation by reducing PGE2 synthesis and disrupting the gastric mucosal barrier.[25,35,49] Administration of BITC (0.7, 1.5 mg kg^{-1}) and esomeprazole groups is associated with a significant decline in the MPO concentration and restores PGE2 and TNF-α levels in the gastric mucosa (Table 2). Similar observations that BITC reduces the MPO in the inflamed region in mouse skin punches and TNF-α in macrophages have been recorded in former investigations.[22,28,29,75] Consistently, *S. persica* extracts were previously reported to reduce the secretion of inflammatory mediators, interleukin-1β, IL-6, and TNF-α in paw edema.[76] These effects could be associated with the gastroprotective activity of BITC by reducing neutrophil infiltration.[49]

The findings of the histopathological examination confirmed the results of biochemical assessments. The observed mucosal haemorrhage, erosion of gastric mucosa, apoptosis, and fragmentation of the gastric gland indicate gastric mucosal damage and account for the recorded deteriorations in the antioxidant and generated oxidative stress. Similarly, excessive leukocytic infiltration goes in parallel with the elevated mucosal tissue levels of TNF-α and MPO and both the macroscopic and microscopic scoring of gastric ulceration (Fig. 1 & 3). In contrast, BITC pre-treated groups exhibited significant amelioration of all the pathological alterations (Fig. 1–3).

Under different circumstances of oxidative stress, excessive ROS activate the transcription factor-κB (NF-κB), which acts as a key transcriptional activator for an array of inflammatory and apoptotic genes.[77] NF-κB is held in the cytosol as an inactive dimer by binding to its inhibitor, the IκB protein. Upon stimulation with either TNFα or IL-1β, IκBs phosphorylation occurs, followed by ubiquitination and degradation, which allow the NF-κB to translocate into the nucleus to initiate the transcription of its target genes.[78] It has been reported that IND activates NF-κB, which subsequently induces proinflammatory gene expression.[79] In our experimental data, IND significantly upregulated the expression of NF-κB in the gastric mucosa. BITC pre-treatment significantly downregulated the NF-κB transcript level. We, therefore, can suggest that BITC indirectly suppresses NF-κB activation through the inhibition of oxidative stress.[80] Notably, the observed BITC reduction of gastric TNF-α is likely a secondary effect due to the downregulation of its upstream NF-κB.[81] Herein, BITC attenuated the IND-induced inflammatory events by downregulating the mRNA expression of NF-κB along with the proinflammatory TNF-α. These multipronged effects affirm the potent anti-inflammatory actions of BITC, which are in line with the previous reports.[25,82,83]

The results of the present study revealed a significant upregulation of caspase-3 protein expression in the mucosal cells of IND-treated rats, indicating accelerated apoptotic cell death, which represents a characteristic pathological event in IND-induced ulceration.[41,84] Excessive TNF-α and ROS overproduced by the infiltrating inflammatory cells in the injured mucosa have been postulated to play a pivotal role in IND-induced gastric mucosal apoptosis. In addition, indomethacin induced structural and functional impairment of the mitochondria by the generation of intramitochondrial ROS and mitochondrial oxidative stress is common for cell damage.[85] The activation of mitochondrial death pathway contributes significantly in the apoptotic death of gastric mucosal cells by NSAIDs.[86–88] Bax and Caspase-3 were linked to apoptosis during mitochondrial damage and play a pivotal role in the disruption of stomach mucosal integrity. The accumulation of oxygen-derived free radicals in areas of gastric mucosal damage might surpass the antioxidant defense system starting the intrinsic pathway of apoptosis and activating Bax, leading to the release of other pro-apoptotic factors to the cytoplasm, stimulating the formation of protostomes, and at the end, activating Caspase-3, the executor of apoptosis, thus causing programmed cell death.[89,90]

A crosstalk between Nrf2 and NF-κB has been postulated to play a pivotal role in the regulation of cellular survival through the activation of caspase-3.[91,92] The findings of the current study support these evidences since BITC modulated the expression of Nrf2 and NF-κB in mucosal cells and therefore, might inhibit cell apoptosis by decreasing the caspase-3 expression, suggesting that BITC might inhibit the apoptosis of gastric mucosal cells, thus providing an anti-apoptosis approach against gastric mucosal damage.

Heat shock proteins (HSPs) are stress proteins that maintain cellular homeostasis against different stress factors.[93] HSP-70 is the major inducible HSPs that is thought to act as a self-protective defense factor against a variety of inflammatory and infectious diseases.[94] HSP-70 synthesis in the macrophages is stimulated in response to various stimuli such as ROS, nitric oxide, lipid mediators, and bacterial toxins.[95–97] Accumulating evidence suggested that the mucosal protection of HSP-70 occurs *via* the inhibition of the inducible nitric oxide synthase (iNOS) and inflammatory mediators such as TNF-α, and IL-1 production in the macrophages,[97,98] preserving the normal protein structures and eliminating the damaged proteins.[99,100] In the current study, BITC pretreated groups (0.75 and 1.5 mg kg^{-1}) exhibited increased HSP-70 expression levels compared to the IND group (Fig. 7). These findings suggest that BITC enhances Hsp70 production, which may protect the gastric mucosal cells by suppressing the IND-induced release of inflammatory cytokines from the macro-

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phages.[101] Moreover, HSP-70 upregulation has been reported to protect cell by the inhibition of NF-κB and modulation of NF-κB mediated pro-inflammatory cytokines.[102]

Our findings are in line with that of the previous studies in that the coordinated regulation of Nrf2 and NF-κB may play a crucial role in gastroprotection against oxidative stress-related gastric injury.[103,104] However, it is difficult to draw a solid conclusion on whether BITC gastroprotective effect is mediated by the regulation of Nrf2 and NF-κB signaling pathways. Therefore, further investigation is needed to confirm the clear mechanism of BITC using Nrf2- and NF-κB-knockout animal models.

Conclusions

In conclusion, this investigation substantiates the potent gastroprotective effect of BITC against IND-induced gastric injury with comparable efficacy to esomeprazole. BITC gastroprotection is mediated, at least in part, through its antioxidant, anti-inflammatory, and anti-apoptotic mechanisms. Based on the results, this study suggested that both Nrf2/HO-1 and NF-κB signaling cascades may be involved in mediating BITC gastroprotective activity. Further studies are warranted to clarify other mechanisms that may underlie the anti-ulcerogenic effects of BITC. Pre-clinical studies are needed to evaluate the applicability of BITC as a therapeutic agent against IND-induced gastric ulceration.

Abbreviations

BITC	Benzyl isothiocyanates
GSH	Glutathione
HO-1	Heme oxygenase-1
IND	Indomethacin
MDA	Mucosal lipid-peroxide
MPO	Myeloperoxidase
NF-κB	Nuclear factor kappa-B
NO	Nitric oxide
NQO1	NAD(P)H dehydrogenase [quinone] 1
Nrf2	Nuclear factor-E2-related factor-2
NSAIDs	Non-steroidal anti-inflammatory drugs
PGE2	Prostaglandin E2
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TNF-α	Tumor necrosis factor alpha

Author contributions

Conceptualization: Shymaa A. El Badawy, Hanan A. Ogaly, Reham M. Abd-Elsalam, Asmaa. A. Azouz; methodology, validation, formal analysis, investigation, data curation, and writing the original draft preparation: all authors; writing, review and editing; Hanan A. Ogaly, Shymaa A. El Badawy.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Institutional Animal Care and Use Committee (IACUC), Cairo University (date: 10/10/2019/certificate code: CU III S 79 17).

Conflicts of interest

There are no conflicts to declare.

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